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Preservation and evolution of organic matter during experimental fossilisation of the hyperthermophilic Archaea *Methanocaldococcus jannaschii*

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Short title

Preservation of organic matter during experimental fossilisation

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Archaea, Methanocaldococcus jannaschii, fossilisation, amino acids, monosaccharides, fatty acids

Abstract

Identification of the earliest traces of life is made difficult by the scarcity of the preserved microbial remains and by the alteration and potential contamination of the organic matter (OM) content of rocks. These factors can confuse interpretations of the biogenicity and syngenicity of fossilised structures and organic molecules found in ancient rocks. In order to improve our knowledge of the fossilisation processes and their effects at the molecular level, we made a preliminary study of the fate of OM during experimental fossilisation. Changes in the composition and quantity of amino acids, monosaccharides and fatty acids were followed with HPLC, GC and GC-MS analyses during one year of silicification of the hyperthermophilic Archaea *Methanocaldococcus jannaschii*. Although the cells themselves did not fossilise and the accompanying extracellular polymeric substances (EPS) did, our analyses showed that the OM initially present in both cells and EPS was uniformly preserved in the precipitated silica, with amino acids and fatty acids being the best preserved compounds. This study thus completes previous data obtained by electron microscopy investigations of simulated microbial fossilisation and can help better identification and interpretation of microbial biosignatures in both ancient rocks and in recent hydrothermal formations and sediments.

Introduction

Traces of ancient life in rocks occur as fossilised remains of microorganisms, their communities and biostructures, as degraded organic compounds derived from original biological components, or as isotopic signatures (e.g. Brocks and Pearson 2005 and references therein; Westall 2011; Westall and Cavalazzi 2011). When it comes to investigations of the most ancient traces of life, the search is complicated by the limited preservation of ancient rocks in terms of quantity and metamorphic grade. The oldest known sedimentary rocks occur in the Barberton Greenstone Belt (South Africa, 3.2-3.5 Ga), the Pilbara Craton (NW Australia, 3.3-3.5 Ga) and the Isua and Akilia Greenstone Belts (Greenland, 3.7-3.8 Ga). Only those in Barberton and the Pilbara are of low enough metamorphic grade to be useful for in depth investigation of biosignatures. The oldest traces of life identified in these rocks (3.5-3.8 Ga) comprise silicified microfossils in the form of colonies, microbial mats and stromatolites that probably included chemotrophic and possibly anoxygenic photosynthetic microorganisms (Walsh 1992, 2004; Tice and Lowe 2004, 2007; Allwood et al. 2006, 2009; Westall and Southam 2006; Westall et al. 2006a,b, 2011; Westall 2011). However, due to the

degradation sustained by these biosignatures and the possibility of a contamination by non-syngenetic OM (Brocks 2011), numerous debates have arisen concerning the biogenicity of observed structures (e.g. Apex Chert: Schopf (1993) and Brasier et al. (2002); Isua: Pflug and Jaeschke-Boyer et al. (1979) and Westall and Folk (2003), Mojzsis et al. (1996) and van Zuilen et al. (2002)). These debates have highlighted the difficulty of identifying physical structures as well as molecules preserved in ancient rocks as traces of life, and our lack of knowledge concerning the fossilisation processes that lead to the long-term preservation of biosignatures and, especially, of their most fragile cellular/organic components.

This article is part of a broader study of the preservation of the kinds of microorganisms that could have lived under the extreme environmental conditions of the early Earth, such as the deep-sea hyperthermophilic methanogenic Archaea *Methanocaldococcus jannaschii* (Jones et al. 1983), through experimental fossilisation. Previous experiments to fossilise microorganisms had been successfully applied to a wide range of species, including Cyanobacteria (Oehler and Schopf 1971; Oehler 1976; Francis et al. 1978; Phoenix et al. 2000; Benning et al. 2004a, 2004b), diverse marine microbes (Westall et al. 1995), Gram-positive (Ferris et al. 1988; Westall 1997) and -negative Bacteria (Birnbaum et al. 1989; Westall et al. 1995 ; Westall 1997; Toporski et al. 2002; Lalonde et al. 2005), and Archaea (Orange et al. 2009, 2011). They provided important information for the understanding of the fossilisation processes. In particular, in two previous studies (Orange et al. 2009, 2011) we investigated the mechanisms of fossilisation of Archaea in saturated silica solutions and the preservation of morphological structures by electron microscopy monitoring. In one species, most of the cells of *M. jannaschii* lysed naturally within a week after the beginning of the experiment despite the rapid formation of a silica precipitate (Orange et al. 2009). Only a few significantly deformed cells and cell remains were still present after 1 month of fossilisation and they lost all their recognisable features as the fossilisation continued over the period of a year. However, a significant amount of extracellular polymeric substances (EPS) was fossilised and preserved. This study is the first reported case of an experimental fossilisation that did not lead to the preservation of the cells and it underlines the importance of heterogeneous cell preservation in the rock record.

In this particular context, we wanted to investigate the processes that take place during the experimental fossilisation of *M. jannaschii* in more detail through the monitoring of progressive changes in the OM. The particular unfolding of this fossilisation seemed to us more interesting and worthwhile to obtain information on the changes in the OM compositions during fossilisation, since the actual physical preservation of cells in the rock record is less common than the preservation of the organic remnants of the cells. More precisely, our objectives were to verify the impact of silicification at the molecular level, through a year-long monitoring of the most common and most labile organic compounds of *M. jannaschii* cells and EPS (amino-acids, monosaccharides, fatty acids) and to provide a first insight into the preservation of the OM during silicification at the molecular level. This was particularly important given that, in this case, these biological compounds were not directly protected by efficient fossilisation of the cellular structures.

Materials and Methods

Cell growth

Pure cultures of *M. jannaschii* were provided by the Laboratoire de Microbiologie des Environnements Extrêmes, Plouzané, France. *M. jannaschii* was cultured in an autotrophic medium. The medium contained (per litre of distilled water) 25 g of NaCl, 3 g of MgCl₂, 1 g of NH₄Cl, 0.15 g of CaCl₂, 0.5 g of KCl, 0.3 g of KH₂PO₄ and 0.001 g of resazurin (as an anaerobic indicator). The pH was adjusted to 6.5 and the medium was sterilised by autoclaving. 500 mL of medium were dispensed into 1 L sterile bottles. Anaerobiosis was obtained by first applying vacuum to the bottle and then saturating it with N₂ (Balch and Wolfe 1976). The N₂ atmosphere was then replaced by a H₂/CO₂ atmosphere (80:20; 300 kPa). The medium was finally reduced by adding 5 mL of a sterile 10% (wt/vol) solution of Na₂S.9H₂O to each vial. The medium, inoculated to a final concentration of 1%, was incubated at 80 °C with shaking. Microbial growth was monitored with a phase-contrast microscope (Olympus CX 40).

Experimental fossilisation

The silicification procedure is derived from the methods used for previous silicification experiments (Birnbaum et al. 1989; Toporski et al. 2002), and is identical to that used by Orange et al. (2009).

Silicification was launched at the end of the exponential growth phase of the microorganisms. As a silicifying agent, we used a commercially available pure sodium silicate solution (Riedel de Haën) containing ~27% SiO₂ and ~10% NaOH. This pure solution was diluted 10 times to make a stock solution and filtered using a 0.2 µm polycarbonate Millipore filter for sterilisation and to remove particulate material. 9 mL of this stock solution were injected into each vial to obtain a final approximate silica concentration of 350 ppm Si. The injection of silica into the microbial cultures marked the start or 'zero time' of the silicification. Spontaneous polymerisation of silica occurred within a few hours after the injection of silica, leading to the formation of a white precipitate of colloidal amorphous silica at the bottom of the flasks. Separate bottles were prepared for silicification times of 24 hours to 1 year. After the silica injection, each vial was placed in an oven at 60 °C until sampled. The bottles remained sealed in anaerobic and contamination-free conditions until sampled.

Lyophilisation

At the end of each fossilisation period, the 500 mL cultures were centrifuged (Sorvall, SLA-1500 rotor, 7000 g, 20 min). For the purpose of lyophilisation, the silica/cell pellet was collected, along with ~50 mL of culture medium (the rest was discarded; thus only a small part of the EPS dissolved in the medium was kept). The silica/cell pellet was thereafter frozen at -80°C before being lyophilised. A *M. jannaschii* cell pellet, collected from a fresh culture with no silica added, was also lyophilised this way. After one year of fossilisation, the silica/cell pellet at the bottom of the culture bottles formed a very dense precipitate, which was lyophilised as described above. In addition, for this sample, a part of the medium was also kept and lyophilised for separate analysis. Although the microbial cultures had a volume of 500 mL, the amount of dried samples recovered after lyophilisation was always low, which prevented us from making

systematic replicates of the OM analyses. Instead, punctual replicates were made for each categories of organic compounds analysed.

EPS extraction

In addition to monitoring the experimental fossilisation made on lyophilised samples, the EPS and cells in a fresh *M. jannaschii* culture were separated in order to identify specific compounds and to follow their evolution during the experimental fossilisation. The EPS were recovered and purified using the cold ethanol extraction method (Antón et al. 1988; Underwood et al. 1995; Decho et al. 2005; Klock et al. 2007). A 500 mL *M. jannaschii* culture was centrifuged (2 hours, 20000 g). The pellet, hereafter called “cell” fraction and containing cells and some cell-bound EPS, was recovered. The dissolved EPS contained in the supernatant (hereafter called “EPS” fraction) were precipitated and recovered using the following procedure. NaCl was added to the supernatant to a final concentration of 20 g/L. The samples were kept at 4°C to allow precipitation and decantation of the EPS. The latter were finally recovered by centrifugation (5000 g) and rinsed in successive ethanol baths (from 70% to 100 % aqueous ethanol). The same rinsing procedure was applied to the “cell” fraction. The “EPS” and “cell” fractions were kept in absolute ethanol at 4°C until used.

To evaluate the presence of organic materials (e.g. EPS) in the silica precipitate that formed after the silica injection, a control experiment was performed where the silica was injected in a medium of a fresh *M. jannaschii* culture after having removed the cells by centrifugation (2 hours, 20000g). The silica precipitate was collected after 24 hours and fixed with 2.5% glutaraldehyde. The sample was then prepared for scanning electron microscopy (SEM) observation with the critical point drying method using the procedure described in Orange et al. (2009). SEM observations and analyses were carried with a Hitachi S-4500 Field Emission Gun SEM equipped with a EDX detector (Oxford Instruments).

Amino acid analyses

Amino acid (AA) compositions of the samples were determined by Thermo-Spectra High Pressure Liquid Chromatography (HPLC) after acid hydrolysis and phenylisothiocyanate (PITC) derivatisation, with the method used by Gautret and Trichet (2005). For the analyses, ~30 mg of lyophilised samples were used. 400 μ L of the “cell” fraction and 1 mL of the “EPS” fraction in absolute ethanol were collected and air dried. Due to the very small amount of material, it was not possible to determine the precise dry weight of these fractions used for this analysis (~1 mg). Hydrolysis was performed in 6N HCl at 110°C for 24 hours in sealed tubes under a N₂ atmosphere to prevent oxidation. The samples were neutralised by adding 200 μ l of a ddH₂O/methanol/triethylamine mixture (proportions 1:1:1 v/v/v) and then dried in a SpeedVac. The samples were derivatised for 20 minutes by phenylisothiocyanate (PITC) by adding, in each sample, 20 μ L of a stock solution of methanol/triethylamine/ddH₂O/PITC (proportions: 140, 20, 20 and 20 μ L, respectively). The samples were analysed by reverse phase chromatography using a Thermo Finnigan HPLC equipped with a Hypersil ODS C18 5 μ m column with an acetonitrile gradient (Eluent A: 0.8 mL of orthophosphoric acid per liter of ultrapure water, pH adjusted to 7.6 by addition of 30% NaOH solution; Eluent B: 50% eluent A / 50% acetonitrile; 1 hour gradient from 2% of Eluent B as the chromatographic starting condition to 50%, followed by a short 80% cleaning phase and equilibration back to the starting condition; flow rate: 1mL/min; injection volume: 20 μ L). The molecular composition was determined by calculating peak areas from the analysed compounds compared with a standard (STP : “Amino-acid standard for hydrolyse analysis”, Beckman System 7300/6300). The total mass of the soluble proteinic compounds (in μ g per g of dried sample) was obtained by adding the individual masses of all the analysed AAs. Due to a permanent contamination peak, arginine could not be quantified in the lyophilised samples but was measured in samples kept in absolute ethanol (“cell” and “EPS” fraction) (Table 1). Error bars were determined for each individual amino acid on the basis of 3 analyses of standard solutions prepared at 3 different concentrations. Errors thus differ for each AA.

Monosaccharide analysis

The monosaccharide composition was measured using gas chromatography (GC) after moderate acid hydrolysis (1.2 M H₂SO₄) and silylation (Comont et al. 2006, Disnar et al. 2008). Approximately 150 mg of lyophilised sample were used for the analyses. Moderate acid hydrolysis was performed with 2.5 mL of 1.2 M H₂SO₄ at 100°C for 3 hours, under vacuum. 6-deoxy D-glucose was used as an internal standard (Wicks et al. 1991). The samples were subsequently neutralised with solid CaCO₃. The precipitate was removed by centrifugation and the supernatant was evaporated to dryness before being redissolved in 0.5 mL of a 0.2% (m/v) LiClO₄ / pyridine solution (Bethge et al. 1966; Ogier et al. 2001). Samples were once again centrifuged, and the supernatant was placed in an oven at 60°C overnight in sealed vials. Finally, 100µL of a mixture of Trisyl (N,O-Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS); Sigma) was added and the samples were heated at 60°C for 1 hour in sealed and pre-ashed vials, before GC analysis. Monosaccharide analysis was performed by gas chromatography with a Auto System XL (Perkin-Elmer) equipped with a 25 m × 0.32 mm i.d. CP-Sil 5CB capillary column (0.25 µm film thickness) and a flame ionisation detector (FID). Helium was used as a carrier gas. After splitless injection at 240°C, analyses were performed in the following conditions : oven temperature kept at 60°C for 1 minute, then raised to 120°C at 30°C.min⁻¹, and finally to 240°C at 3 °C.min⁻¹ at which it was maintained for 30 minutes. A mixture of eight monosaccharides (ribose, arabinose, xylose, rhamnose, fucose, glucose, mannose and galactose) was used as external standard (i) for compound identification (through peak retention times), (ii) for individual response coefficient determination and (iii) for quantification of the different analysed compounds, and by comparison with the internal standard (6-deoxy D-glucose). Replicates analyses gave an analytical precision better than 15% (Comont et al. 2006; Disnar et al., 2008).

Fatty acid analyses

Prior to GC-MS analysis, fatty acids (FA) were released following two distinct procedures: thermochemolysis with tetramethylammonium hydroxide (TMAH, Disnar et al. 2008), and acid hydrolysis (Stefanova and Disnar 2000).

The first of these two procedures convert the FAs into methyl esters and the second one into free acids. The latter are esterified prior to analysis (details in Stefanova and Disnar 2000). Approximately 150 mg of lyophilised sample were used. As for the AA analyses, 400 μ L and 1 mL of the “cell” and “EPS” fractions, respectively, were also collected and evaporated.

For thermochemolysis, a mixture of a TMAH solution (10 % in MeOH), internal standard (heptylbenzoic acid dissolved in MeOH), and methanol (proportions 2:1:2 (v/v/v); 1:1:2 for the “cell” and “EPS” fractions) was added to the samples in test tubes, which were subsequently sealed and heated at 70°C for 1 hour. Vacuum was made in the tubes to prevent oxidation, and their bottom extremity was placed for 20 minutes in a sand bath heated at 250°C. After cooling, the products were extracted with 1 mL of ether. The ether solutions were transferred into vials, and evaporated until dryness at room temperature under a fumehood. The samples were then dissolved in dichloromethane, before being analysed by GC-MS.

For the acid hydrolysis, a mixture of a 6N HCl solution and methanol (proportions 1:1 v/v) was added to the samples in test-tubes along with the internal standard (5 α -cholestane in MeOH). The tubes were heated overnight in a water bath at 100°C. Afterwards, the samples were placed in a separation funnel with a few mL of ether. The ether fraction was recovered and evaporated. The samples were then dissolved in anhydrous methanol with a few drops of acetyl chloride and evaporated to dryness at 80°C for 2 hours. Finally, the samples were dissolved in dichloromethane, before GC-MS analysis.

Lipid analyses were performed by GC-MS on a TRACE-Polaris GCQ. The gas chromatograph was fitted with a Rtx-5MS capillary column (30 m, 0.25mm i.d., 0.25 μ m film thickness). The GC operating conditions were: temperature held at 40°C for 1 min, then increased from 40 to 120°C at 30 °C.min⁻¹, 120 to 240°C at 5°C.min⁻¹, with final isothermal hold at 240°C over 30 min. The sample was injected splitless, with the injector temperature set at 280 °C. The carrier gas was helium. The mass spectrometer was operated in the electron ionisation (EI) mode at 70 eV ionisation energy and scanned from 50 to 600 Da. Compounds were identified by using their retention time and their mass spectra. Quantification was made using the internal standard. Error margins were calculated from replicates analyses.

Results

Table 1 shows the analysis results for AAs, monosaccharides and FAs.

Amino acid analyses

Fresh *M. jannaschii* cultures contain more dicarboxylic (Asp, Glu) than basic AAs (Lys, His) (Fig. 1a). The amounts of neutral AAs (Gly, Ala, Val, Leu, Ile) are also significant, with slightly more of the smallest ones (Gly, Ala, Val), as well as proline (Pro). AA compositions of the “cell” and “EPS” fractions showed some differences. The “cell” fraction contained a relatively large amount of charged (Asp, Glu, His, Lys, Arg; 48.3%) and non-polar AAs (Ala, Ile, Leu, Val, Phe, Pro; 43.7%) and a low content of polar AAs (Cys, Ser, Thr, Tyr ; 8.0%) (Fig. 1a, Table 1). In comparison, the “EPS” fraction was characterised by a majority of non-polar AAs (71.0 %) and low amounts of dicarboxylic AAs (Asp, Glu; 7.5 %, vs. 34.2% for the “cell” fraction). The AA composition of the whole lyophilised *M. jannaschii* culture appeared logically as a mean of the “cell” and “EPS” compositions (Fig. 1a).

24 hours after the injection of the silica solution in the culture, the total amount of analysed AAs was three times higher in the freshly formed precipitate than in the fresh culture (Fig. 1b), probably as a consequence of the formation of this precipitate, which had fixed EPS originally dissolved in the medium (see Discussion). Afterwards, this amount decreased progressively in the first month of fossilisation to reach the fresh culture value. After one year, the total amount analysed was slightly higher than the fresh culture amount. The two months value was also significantly higher (see Discussion). Figs 1c, 1d, 1e and 1f show the evolution of the percentage of individual AAs during the experimental fossilisation, sorted by types. Basic and dicarboxylic AAs show opposite trends (Figs. 1c, 1d). Apart from glycine and serine, neutral and other AA proportions did not change significantly during the experiment (Figs 1e, 1f). The analyses made after one year of fossilisation showed that there was still a large amount of

AAs remaining in the dense silica precipitate, as well as in the medium (Fig. 1g). While the total amount of AAs in the silica precipitate after one year was twice the amount measured in the fresh culture (Fig. 1b; Table 1), the initial and final AA compositions were similar (Fig. 1h).

Monosaccharide analyses

The analysis of a fresh *M. jannaschii* culture showed the presence of four aldohexoses: mannose, allose, galactose and glucose (Table 1). Glucose was the most abundant compound (~80 % of the total monosaccharides analysed). According to the chromatograms, no other peaks related to other compounds were present. Due to the small amounts of samples available, individual analyses of the “cell” and “EPS” fractions failed to give relevant results.

Only glucose and mannose could be followed in the silica precipitate during the whole fossilisation, the former being always much more abundant than the latter (Table 1; Fig. 2b). The other monosaccharides were not identified in the fossilised samples, apart from traces of allose after one month (Table 1). Similar to the AAs, the total amount of analysed monosaccharides in the silica precipitate was twice as high as in the fresh culture 24 hours after the injection of silica in *M. jannaschii* cultures (see Discussion). This amount decreased strongly after 1 month and remained constant afterwards (Fig. 2a). For the individual monosaccharide compositions, while the quantity of glucose remained stable after one month, mannose progressively disappeared and was not detected after one year (Fig. 2b). The final glucose quantity in the precipitate was only about one third of the fresh culture value (Table 1, Fig. 2b).

Fatty acid analyses

Thermochemolysis with TMAH allowed the identification of a wide range of FAs in the fresh *M. jannaschii* culture from *n*-C_{12:0} to *n*-C_{22:0} (Fig. 3a). We noticed the expected predominance of compounds with an even carbon number and, in particular, high amounts of *n*-C_{16:0} and *n*-C_{18:0} which together represented almost 60 % of the total analysed (Table 1). There were also significant amounts of *n*-C_{20:0} and *n*-C_{22:0}. Acid hydrolysis allowed detection of a wider range of FAs,

up to $n\text{-C}_{30:0}$, as well as compounds that were not seen after thermochemolysis (e.g. $n\text{-C}_{19:0}$) (Fig. 3a). As in the preceding analysis, the even over odd carbon number compound predominance was the rule. $n\text{-C}_{16:0}$ and $n\text{-C}_{18:0}$ represented 70 % of the total FAs analysed (Fig. 3a, Table 1). The other FAs were usually only found in small quantities (< 5%). Thermochemolysis performed on “cell” and “EPS” fractions showed only minor compositional differences (Fig. 3b) with a distribution of FAs similar to that analysed in the whole culture.

Generally, the total amounts of FAs released were similar for the two extraction methods (Table 1, Fig. 3c). As for the AAs and monosaccharides, both methods showed a significant increase of the quantities analysed in the silica precipitate after 24 hours of fossilisation, followed by a quick decrease. After one week, the total amounts analysed seemed to stabilise above the fresh culture value for the TMAH extraction. The results of the acid hydrolysis extraction also showed an important increase after 2 months.

Monitoring of the FA composition during the fossilisation was limited to thermochemolysis, which gave the most reproducible results. After 24 hours of fossilisation, the amounts of FAs extracted in this way were ten times greater than those found in the fresh culture (Fig. 3c). This was accompanied by an increase in the proportions of the two main FAs identified ($n\text{-C}_{16:0}$ and $n\text{-C}_{18:0}$) and a depletion in the larger compounds ($n\text{-C}_{20:0}$ and $n\text{-C}_{22:0}$) (Fig. 3d). Only the two most abundant compounds ($n\text{-C}_{16:0}$ and $n\text{-C}_{18:0}$) could be monitored satisfactorily during the fossilisation (Fig. 3e). Since the analysed quantities of the other compounds was low and the peaks formed were small, it was difficult to follow their evolution during the fossilisation. After 24 hours, $n\text{-C}_{18:0}$ quantities decreased quickly before stabilising until the end of the experiment, while $n\text{-C}_{16:0}$ decreased more slowly after one month (Fig. 3e). After one year, only a few light FAs ($\leq n\text{-C}_{16:0}$) remained in the aqueous medium (Table 1) and those in the silica precipitate had the same distribution (Fig. 3f). The FAs extracted by acid hydrolysis in the fresh culture and in the silica precipitate after one year of fossilisation were similar (Fig. 3h), with similar total analysed masses in the two samples (Fig. 3c). This was not the case for the FAs extracted by thermochemolysis (Fig. 3g), which showed a final enrichment in $n\text{-C}_{14:0}$ and $n\text{-C}_{16:0}$, and the absence of the largest compounds ($n\text{-C}_{20:0}$, $n\text{-C}_{22:0}$), with total masses three times higher than in the fresh culture (Fig. 3c).

Discussion

This study represents a first attempt to monitor the degradation and preservation of the microbial OM during a one-year fossilisation experiment. The similarity between the AA composition of the fresh culture and the sample fossilised for one year (Fig. 1h) demonstrates consistency in the measurements and indicates that these AA analyses were not biased by the presence of silica. For the FAs, the use of an internal standard introduced at the start of the analysis was a guarantee of the reliability of the results. The two methods used to analyse these compounds (thermochemolysis and acid hydrolysis) theoretically release FAs involved in different chemical combinations (saponifiable amides, and hydrolysable esters, respectively; Stefanova and Disnar 2000, and references therein), giving complementary results. Moreover, both methods used could also detect free FAs (initially, or liberated during fossilisation).

Although replicate analyses could not be made on a systematic basis, the reliability of the measurements is also supported by the common trends followed by the total analysed masses of each category of organic molecules (Figs. 1b, 2a, 3c). However, these trends remain partly unexplained. Whereas the trends observed during the first week of the fossilisation can be linked to lysis of the *M. jannaschii* cells and the formation of the silica precipitate (see below), the peak observed after two months (Figs. 1b, 2a, 3c) cannot be related to a process that occurred during the experimental fossilisation: after the precipitation of silica and the cell lysis, a steady-state was observed during the fossilisation beyond 1 month with the only process observed being the continuous compaction of the silica precipitate (Orange et al. 2009). It may be due to an experimental artifact due to sampling or lyophilisation.

In this study we focused on a global analysis of compound families. As a consequence, the results provide no information regarding the origin of the analysed compounds (cytoplasm, cell wall, EPS) nor can we differentiate between molecules involved in polymers, those present in the free state in the medium, or those originating from the degradation of larger compounds during fossilisation. However, similarities between the initial and final compositions in AAs and FAs (Figs. 1h, 3h) suggest that there was only limited loss of monomeric compounds

from the organic macromolecules preserved in the silica precipitate. However, it may have affected the monosaccharides, which are likely the most reactive among the analysed compounds because of their carbonyl functional group.

OM composition of a *M. jannaschii* culture

Apart from monitoring changes in the OM during the experimental fossilisation of *M. jannaschii*, this study also provides information regarding the AA, monosaccharide and FA composition of a fresh *M. jannaschii* culture.

Previous proteomic studies of the AA composition of hyperthermophilic Archaea and *Methano(caldo)coccales* show ubiquitous higher amounts of charged AAs (Asp, Glu, His, Lys, Arg) in comparison with their mesophilic counterparts (Cambilleau and Claverie 2000; Das and Gernstein 2000; Haney et al. 1999; McDonald et al. 1999; Chakravarty and Varadarajan 2000). The charged AAs allow better stability of the protein in a high temperature environment. In addition, archaeal S-Layer proteins, in particular, are characterised by large quantities of non polar AAs (Ala, Ile, Leu, Val, Phe, Pro) (Akça et al. 2002; Claus et al. 2002; Eichler 2003). High amounts of charged and non-polar AAs were observed in the composition of *M. jannaschii* “cell” fraction (Fig. 1a ; Table 1) along with a lower dicarboxylic AAs content in the “EPS” fraction. The S-Layer and EPS were also identified as the most likely to have been preserved at the end of the experimental fossilisation (Orange et al. 2009) and as primary silica binding sites during the fossilisation process. If low dicarboxylic AAs amounts can be considered as characteristic of EPS during the subsequent monitoring of the fossilisation, non-polar AAs cannot be used as specific markers of the S-Layer (i.e. markers of cell remains) as they are also found in important amounts in the “EPS” fraction (Fig. 1a).

Monosaccharide analysis of the *M. jannaschii* culture identified two major components, glucose and mannose, and also small quantities of allose and galactose that could not be monitored during fossilisation (Table 1). Previous reports of monosaccharide composition of archaeal EPS (Rinker and Kelly 1996; Sowers and Gunsalus 1988; Antón et al. 1988; LaPaglia and Hartzell 1997; Poli et al. 2010; Hall-Stoodley et al. 2005; Schopf et al. 2008) have mainly shown the great compositional diversity among them, including some methanogenic

Archaea: only mannose for *Thermococcus littoralis* (Rinker et al. 1996), mainly mannose for *Haloferax mediterranei* (Antón et al. 1988), a mixture of rhamnose, mannose, galactose and glucose for *Methanobacterium formicium* (LaPaglia and Hartzell 1997), galactose and glucosamine for *Methanosarcina mazei* (LaPaglia and Hartzell 1997). Our results confirm this diversity.

Most of the literature concerning archaeal lipids deals with their specific membrane lipids (which were not analysed in this study; see review in Patel and Sprott 2006). Archaea and Bacteria are distinguished by the composition of their cell envelopes. While the bacterial membrane is made of diacyl-D-glycerol diesters, the archaeal membrane consists of isoprenoid L-glycerol diethers or di-L-glycerol tetraethers with long isoprenoid chains containing 20–40 carbon atoms (Kandler and König, 1998). Consequently, polar lipid FAs (PLFA) have thus been used as a marker of the presence of Bacterial and Eukaryotic biomass, while the presence of isoprenoid diether/tetraether is considered as indicative of the presence of Archaea (Jeanthon 2000). However, FAs were also identified in Archaea as a minor part of the total lipid fraction (Tornabene et al. 1978; Tornabene and Langworthy 1979; Nishihira et al. 2000; Carballeira et al. 1997). The FA composition of *M. jannaschii* shows similarities with the composition of the Archaea *Pyrococcus furiosus* (Carballeira et al. 1997), which has a similar range of FAs (*M. jannaschii* : $n\text{-C}_{12:0}$ to $n\text{-C}_{30:0}$; *P. furiosus* : $n\text{-C}_{12:0}$ to $n\text{-C}_{26:0}$), an even over odd carbon number compound predominance, and with $n\text{-C}_{16:0}$ and $n\text{-C}_{18:0}$ as the major compounds. However, in our case, all the FAs analysed were saturated, while the amount of monounsaturated FAs in *P. furiosus* is large.

General effects of the fossilisation on the OM

Electron microscopy observation of *M. jannaschii* fossilisation (Orange et al. 2009) showed that, while the EPS were preserved, most *M. jannaschii* cells lysed quickly and only a few badly damaged cells and cell remains could be observed after one year of fossilisation. In contrast to these morphological changes, the identity of the AA composition of the silica precipitate after one year of fossilisation with that of the fresh *M. jannaschii* culture (Fig. 1h) provides strong evidence that all the AAs were preserved, without any selectivity either for those more abundant in the EPS (Val, Leu, Ile, Pro, Phe) or for those in the cells

(dicarboxylic AAs: Asp, Glu) (Fig. 1a). Thus, the OM preserved by silica after one year of fossilisation appears not to be restricted to EPS, but also most probably includes the remains of degraded *M. jannaschii* cells. This thus differs from conclusions drawn from electron microscopy monitoring which had suggested that the preservation of cell remains in general was very limited (Orange et al., 2009). It is more difficult to establish the preservation of the FAs due to the two extraction methods used. However, similarities between the fresh culture and one year FA compositions (acid hydrolysis; Fig. 3h) suggest that these constituents were also very well preserved. On the other hand, the monosaccharides were significantly degraded during fossilisation (Fig. 2a; Fig. 4).

Consequently, as AAs were the most abundant and also the best preserved compounds over the duration of this experimental fossilisation (Fig. 4), we mainly base the following discussion on them, with the results from the others compounds being used as a complement.

OM behaviour during experimental fossilisation

The similarity between initial and final AA compositions are all the more surprising since important variations in the total analysed masses and in individual organic components were observed during fossilisation.

The first major changes in organic molecule composition were noticed during the first 24 hours of the experimental fossilisation, with a ubiquitous increase of the total analysed masses (Figs. 1b, 2a, 3c). Shortly after being injected in the *M. jannaschii* media, the polymerisation of silica led to the formation of a silica precipitate within a few hours. The formation of this precipitate is slightly faster in the cell containing samples than in control samples (without cells) (as observed in Orange et al. 2009), as dissolved EPS provide a good passive support for silica nucleation (Westall et al. 2000; Handley et al. 2008). These EPS could thus have been dragged to the bottom of the flasks with the silica precipitate, thus increasing the total analysed masses of OM. This hypothesis was verified by SEM observation of smooth EPS within the silica precipitate formed after injection of silica in a *M. jannaschii* culture growth medium whose cells had been removed (Figs. 5a, 5b). The increases in the AA amounts analysed after 24 hours could also be attributed to an EPS production by

M. jannaschii cells as a stress reaction to the injection of the silica solution. EPS production is one of the protective mechanisms used by microorganisms facing environmental challenges and has been demonstrated as a stress reaction of *M. jannaschii* (LaPaglia and Hartzell 1997; Johnson et al. 2005) and also during the experimental fossilisation of *S. azorensis* (Lalonde et al. 2005). EPS precipitation and production hypotheses are both consistent with previous electron microscopy observations (Orange et al. 2009). Consequently, the AA composition after 24 hours would be expected to be richer in AAs preferentially present in the “EPS” fraction (Val, Leu, Ile, Pro, Phe, Met) and poorer in AAs specific to the “cell” fraction (dicarboxylic AAs: Asp, Glu) (Fig. 1a). A depletion of dicarboxylic AAs (Asp, Glu) was indeed noticed, but no significant enrichment in EPS-specific AAs (Val, Leu, Ile, Pro, Phe) could be seen (Figs. 1c, 1e). It was not possible to verify this hypothesis from the FA analysis, as “cell” and “EPS” fractions showed no major differences (Fig. 3b).

Individual compound proportions between 24 hours and 1 month often evolved in the opposite way than during the first 24 hours. Molecules which became more abundant in the first 24 hours usually became depleted afterwards, for example, lysine and histidine for the AAs (Fig. 1c), glucose for the monosaccharides (Fig. 2b), *n*-C_{18:0} for the FAs (Fig. 3e). The behavior of aspartic acid, glutamine, glycine (Figs. 1c, 1e) and mannose (Fig. 2b) was the opposite. This suggests that the EPS precipitated or secreted during the first 24 hours were eventually released out of the silica precipitate due to compaction soon afterwards. As for a squeezed sponge, the observed compaction of the precipitate could have released organic molecules in the medium.

As previously mentioned, between one month and one year, a steady-state was reached with no significant changes being noticed by electron microscopy observation (see Orange et al. 2009). As most *M. jannaschii* cells had already lysed, no OM could have been produced. Accordingly, except for a limited number of individual organic molecules such as lysine or proline, the proportions of the individual compounds showed no significant evolution (Figs. 1c, 1d, 1e, 1f, 2b, 3e).

After one year of experimental fossilisation, in addition to the silica precipitate, significant amounts of OM were still present in the medium, as shown

by separate analyses made in this medium (Table 1; Figs. 1g, 3f). Several facts suggest that this material mainly originates from EPS, either dissolved in the medium, or released from the compacting silica precipitate. The AA composition of the medium shows similarities with the “EPS” fraction composition, being richer in neutral AAs (Gly, Ala, Val), and poorer in dicarboxylic AAs (Asp, Glu) in comparison with the composition of the precipitate (Fig. 1g).

Role of silica in the preservation of the OM

All the above observations suggest that OM was preserved over the experimental period of a year at a macromolecular (e.g. peptide, protein) or cellular level (EPS, membrane, S-Layer remains) rather than as monomers (individual AAs, FAs). The preservation of the OM during the experimental fossilisation was thus not related to a particular affinity between individual organic compounds and silica. In addition, since the degradation of the OM in culture bottles kept sealed and under anaerobic conditions must have been limited, silica is probably also not directly responsible for the preservation of the OM after one year of fossilisation.

However, by acting as a passive support for silica nucleation and polymerisation, cells and EPS contained in the medium found themselves quickly bound to or trapped in the silica precipitate that formed within a few hours after silica injection, as exemplified by the trapping of cell remains and EPS in the silica precipitate (Orange et al. 2009). In natural conditions, this silica matrix could then be able to provide a physical protection to organic compounds throughout its continuous compaction, thus limiting microbial or chemical degradation (Mongenot et al. 2001). For example, proteins and carbohydrates are known to show a high sensitivity to diagenetic degradation and are not expected to be preserved over geological time scale (de Leeuw and Largeau 1993; Vandembroucke and Largeau 2007), as opposed to lipids. However, several studies have reported the preservation of proteinaceous material and saccharides in kerogens or sediments (Tertiary to Jurassic sediments, Moers et al. 1994; Holocene microbialites, Camoin et al. 1999; 140 Ma Kashpir oil shale, Mongenot et al. 2001; Cretaceous microbialites, Neuweiler et al. 2002; Late Jurassic kerogen, Riboulleau et al. 2002; 4 Ma Pula kerogen, Nguyen and Harvey 2003,

and references therein), as the result of resistance of some molecules to degradation (de Leeuw and Largeau 1993; Moers et al. 1994; Tanoue et al., 1996), the formation of resistant macromolecules through condensation (Mongenot et al. 2001), or, more importantly, by immobilisation by fixation to minerals (Mayer 1994; Hedges and Keil 1995; Salmon et al. 1998; Six et al. 2002) or the encapsulation of these molecules in refractory organic substances (Nguyen and Harvey 1998, 2001, 2003; Knicker and Hatcher, 1997, 2001; Zang et al. 2000; Mongenot et al. 2001). Thus, in a similar way, silica precipitation probably stabilised and immobilised the OM through silica binding. Preservation of OM has been reported in silica sinters forming around hot springs. These sinters are formed as the result of the precipitation of silica, forming deposits of increasing thickness, and fossilising and entombing the microbial communities that thrive there. Preserved lipids that could be linked to the original microorganisms (Bacteria and Archaea) have recently been identified within these sinters (Pancost et al. 2005, 2006; Kaur et al. 2008). Our results suggest that proteinaceous compounds may also be found in recently formed sinters. However, entombment in a silica precipitate does not always prevent modifications at a cellular structural level (e.g. cell lysis, as demonstrated by the species *M. jannaschii* used in this experiment), or the degradation of highly reactive compounds (such as monosaccharides), or a possible recombination between primary organic molecules (e.g. reaction between AAs and monosaccharides, Maillard 1916).

The objective of the characterisation of OM contained in sediments and rocks is to try to identify the type of original microorganism through specific biomarkers. Previous fossilisation studies have shown that the earliest steps in fossilisation are crucial for the long term preservation of morphological and molecular traces. Our results show that, although it is no longer possible to identify *M. jannaschii* remains as Archaea using morphological features (e.g. cell wall structure) after one year of experimental fossilisation, it was possible to identify molecular traces that still retained information related to the molecular characteristics of Archaea. AAs and FAs, and likely membrane lipids as well, were well preserved during this first step of the fossilisation and still present characteristics of (hyper)thermophilic Archaea after one year of fossilisation, such as specific AA composition (high content in charged AAs, specific of hyperthermophilic Archaea, or in non polar AAs, from archaeal S-Layer, Fig. 1h)

or specific archaeal membranes lipids (di- or tetraethers linked to long isoprenoid chains) (Cambilleau and Claverie 2000; Das and Gernstein 2000; Haney et al. 1999; McDonald et al. 1999; Chakravarty and Varadarajan 2000; Akça et al. 2002; Claus et al. 2002; Eichler 2003; Kandler and König, 1998; Jeanthon 2000).

The aforementioned authors who studied the long term preservation of supposedly labile components have also highlighted important differences in the preservation potential of the different organic compounds (see review in de Leeuw and Laugeau 1993) and the importance for preservation of favourable environmental conditions (Poinar and Stankiewicz, 1999). This has also been shown by *in situ* and experimental fossilisation studies (e.g. Westall et al. 1995; Westall 1997; Toporski et al. 2002; Orange et al. 2009). The latter studies also documented the importance of rapid fossilisation to ensure good preservation of the morphological and molecular traces (e.g. Knoll et al. 1988; Bartley 1996; Schultze-Lam et al. 1995 ; Toporski et al. 2002; Konhauser et al. 2004; Orange et al. 2009). The results of our present study do not allow forecasting of the long term preservation potential of the organic compounds still present after one year of fossilisation. We have not simulated the long and complex diagenetic processes which degrade and transform the OM. However, by providing knowledge on the behaviour of the OM during the first steps of the fossilisation, this study helps to decipher some of the complex processes that took place immediately after the death of the organisms and during the beginning of the fossilisation, which are crucial for allowing the eventual preservation of OM in kerogens or sediments.

Conclusions

Monitoring of the fate of organic matter during the fossilisation of *M. jannaschii* over the period of a year has provided new information that helps to better assess the mechanisms that take place during fossilisation and the fate of OM during its initial degradation.

With AAs and FAs being the best preserved compounds over the duration of the experimental fossilisation, analyses showed that silica precipitation led to a

uniform and global preservation of the OM, with no distinction between the different microbial products (EPS or cells). This observation is particularly noteworthy because the *M. jannaschii* cells themselves were not well preserved. Furthermore, our analyses also provided new information concerning the chronology of EPS fossilisation, showing that their preservation was likely due to a very rapid association (within 24 hours) between polymerising silica and EPS dissolved in the medium, leading eventually to the precipitation of silica and EPS. Silica clearly played an important role in the preservation of the OM by stabilising it and providing physical protection against possible degrading agents.

This study thus provides new methods and ideas for the detection and the identification of OM during the fossilisation, which could be applied to fossilised remains of microorganisms in soil and in both ancient and recent geothermal formations.

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Figures captions

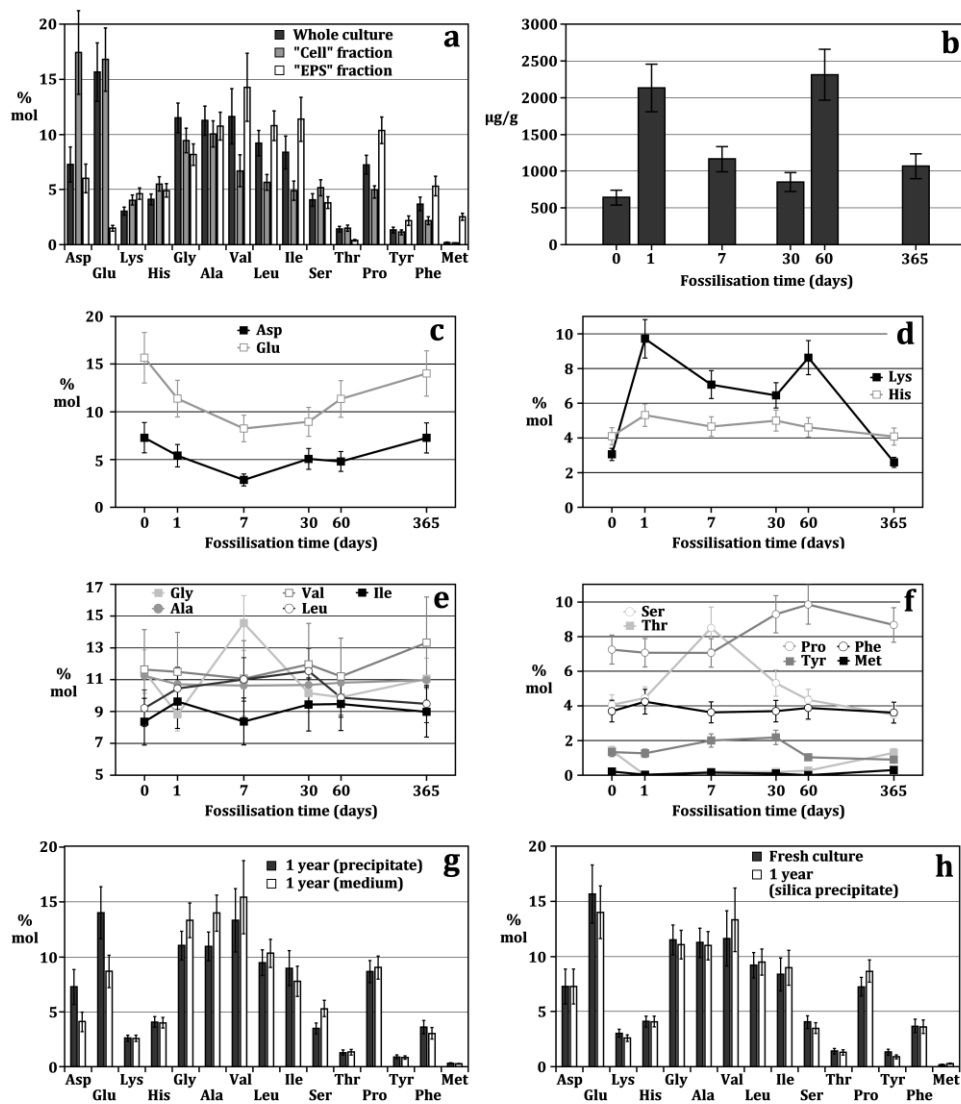


Fig. 1 Results of amino acid (AA) analyses made during the experimental fossilisation of *M. jannaschii* at a 350 ppm Si silica concentration. **a**, AA compositions of the fresh culture, and of the separated “cell” and “EPS” fractions; **b**, total analysed masses of AA in the fresh culture and in the silica precipitate during the experimental fossilisation; **c-f**, evolution of individual AAs percentages during experimental fossilisation. (**c**, dicarboxylic AAs; **d**, basic AAs; **e**, neutral AAs; **f**, aromatic and alcoholic AAs + proline); **g**, AA compositions of the silica precipitate and the medium after 1 year of fossilisation; **h**, comparison of AA compositions of the fresh culture and of the silica precipitate after one year of fossilisation

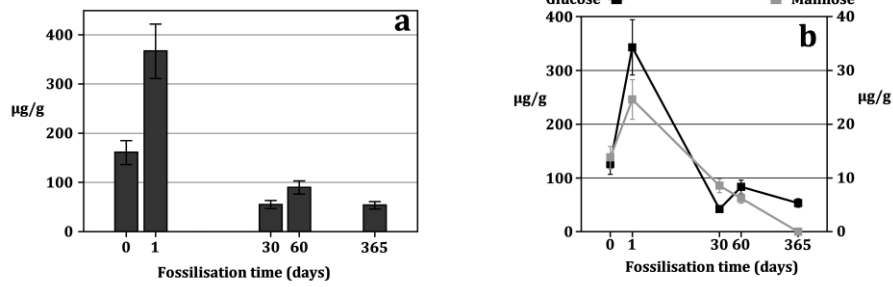


Fig. 2 Results of monosaccharide analyses made during the experimental fossilisation of *M. jannaschii* at a 350 ppm Si silica concentration. **a**, total analysed masses of monosaccharides in the fresh culture and in the silica precipitate during the experimental fossilisation; **b**, evolution of the analysed masses of glucose and mannose in the silica precipitate during the experimental fossilisation

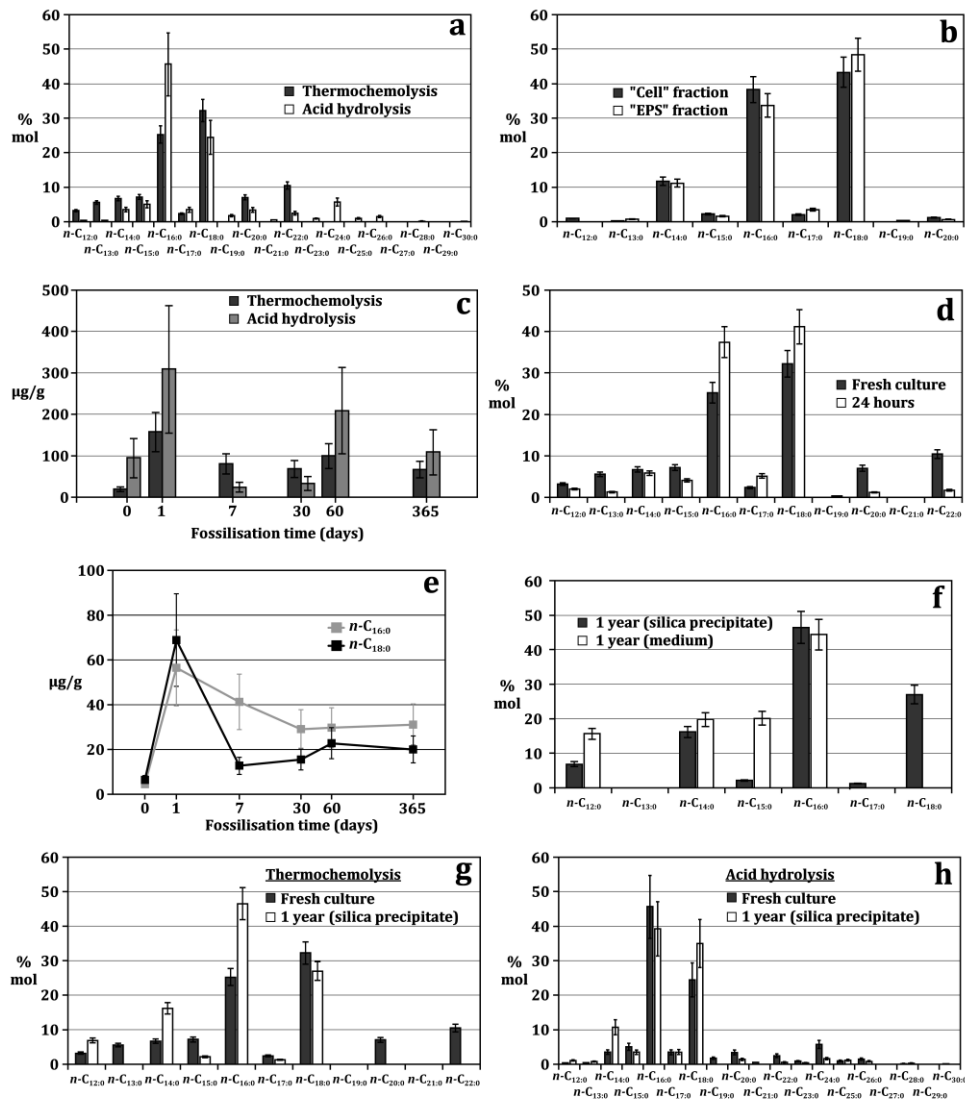


Fig. 3 Results of fatty acids (FA) analyses made during the experimental fossilisation of *M. jannaschii* at a 350 ppm Si silica concentration. **a**, FA compositions of the fresh culture, obtained

by thermochemolysis and acid hydrolysis, respectively; **b**, FA compositions of the separated “cell” and “EPS” fraction, obtained by thermochemolysis; **c**, total analysed masses of FAs in the fresh culture and in the silica precipitate during the experimental fossilisation, obtained by thermochemolysis and acid hydrolysis, respectively; **d**, FA composition of the fresh culture and of the silica precipitate after 24 hours of fossilisation, obtained by thermochemolysis; **e**, evolution of $n\text{-C}_{16:0}$ and $n\text{-C}_{18:0}$ masses in the silica precipitate during the experimental fossilisation, obtained by thermochemolysis; **f**, FA compositions of the silica precipitate and the medium after 1 year of fossilisation, obtained by thermochemolysis; **g**, **h**, comparisons of FA compositions of the fresh culture and of the silica precipitate after one year of fossilisation, obtained by thermochemolysis and acid hydrolysis, respectively

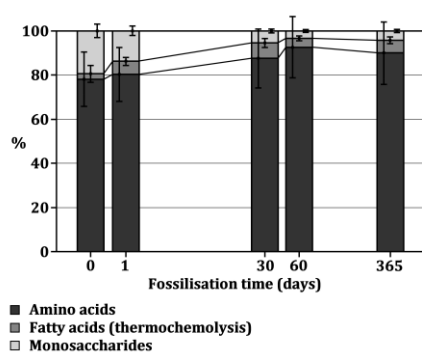


Fig. 4 Evolution of the proportions of the different kinds of organic compounds analysed during the experimental fossilisation of *M. jannaschii*; proportions were calculated from total analysed masses (Table 1).

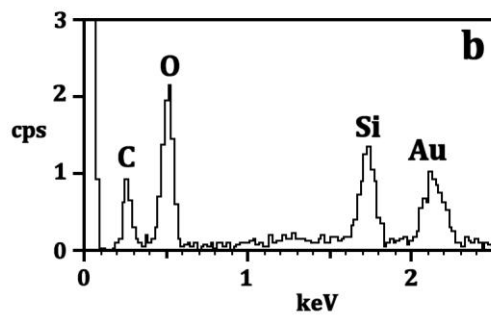
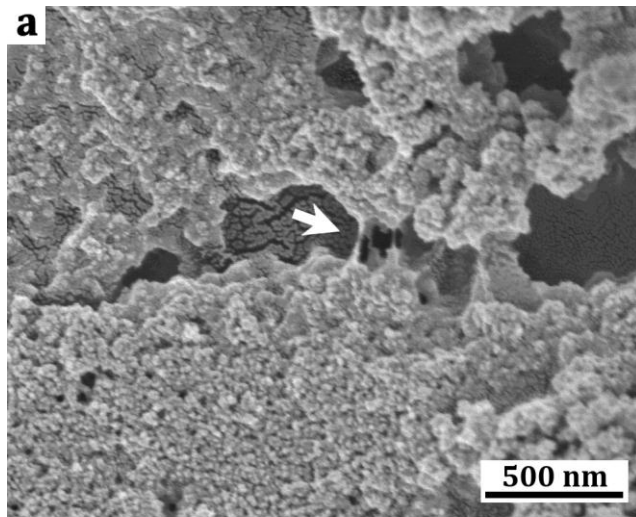


Fig. 5 Scanning electron microscopy (SEM) micrograph (**a**) of the silica precipitate formed after the injection of silica in a *M. jannaschii* growth medium after cells had been removed, with the corresponding EDX spectrum (**b**) made on the silica precipitate; note the smooth EPS inside the precipitate (arrow) and the carbon signal on the EDX spectrum, indicating important amounts of organic materials in the precipitate.