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Recent experiments to fossilise microorganisms using silica have shown that the fossilisation process is far more complex than originally thought; microorganisms not only play an active role in silica precipitation but may also remain alive while silica is precipitating on their cell wall. In order to better understand the mechanisms that lead to the preservation of fossilised microbes in recent and ancient rocks, we experimentally silicified a Gram-positive bacterium, *Geobacillus* SP7A, over a period of five years. The microbial response to experimental fossilisation was monitored with the use of LIVE/DEAD staining to assess the structural integrity of the cells during fossilisation. It documented the crucial role of silicification on the preservation of the cells and of their structural integrity after several years. Electron microscopy observations showed that initial fossilisation of Gram-positive bacteria was extremely rapid, thus allowing very good preservation of *Geobacillus* SP7A cells. A thick layer of silica was deposited on the outer surface of cell walls in the earliest phase of silicification before invading the cytoplasmic space. Eventually, the cell wall was the only recognisable feature. Heavily mineralised cells thus showed morphological similarities with natural microfossils found in the rock record.

Short title: Long duration fossilisation of *Geobacillus* SP7A

Keywords: Fossilisation, *Geobacillus* SP7A, LIVE/DEAD staining, microfossils, electron microscopy

Introduction

Much progress has been made over the last decades towards the understanding of the mechanisms leading to the preservation of silicified microbial remains in rocks dating from the early Earth from the *in situ* study of fossilisation in contemporary environments as well as from the experimental fossilisation of microorganisms *in vitro* (see reviews in Konhauser et al. 2004; Westall and Southam, 2006; Westall 2011). The former described fossilisation in hot spring systems (e.g., cyanobacteria or anoxygenic photosynthetic bacteria) with studies following the early phases of fossilisation (Schultze-Lam et al. 1995; Phoenix et al. 2000) through to the final silicified remains (Walter et al. 1972; Schultze-Lam et al. 1995; Cady and Farmer 1996; Konhauser and Ferris 1996; Konhauser et al. 2001; Jones et al. 1998, 2001, 2003, 2004; Mountain et al. 2003; Handley et al. 2005, 2008; Tobler et al. 2008). Experimental fossilisation has been 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; Orange et al. 2013), diverse marine microbes (Westall et al. 1995), Gram-positive (Ferris et al. 1988; Westall 1997), Gram-negative bacteria (Birnbaum et al. 1989 ; Westall et al. 1995 ; Westall 1997; Toporski et al. 2002; Lalonde et al. 2005), archaea and their viruses (Orange et al. 2009, 2011a, 2011b). These different studies have shown that the silicification of microorganisms is the consequence of abiotic precipitation of silica. The organic microbial structures act as a template for the binding and nucleation of silica while their cell wall structure and binding site availability can also influence the rate of fossilisation (Westall 1997; Orange et al. 2009). In particular, Gram-positive bacteria have always shown high binding rates during fossilisation and metal binding studies due the greater availability of functional groups (Beveridge and Murray 1976;

Francis et al. 1978; Beveridge and Koval 1981; Ferris et al. 1988; Beveridge 1989; Westall 1997; Hetzer et al. 2006). Interestingly, the number of Gram-positive bacteria used in fossilisation studies has been quite limited, despite their high binding abilities.

Recently, microbial response to silicification has been investigated and it has been shown that microorganisms can implement various strategies to deal with a silica saturated environment (Lalonde et al. 2005; Konhauser et al. 2008; Saw et al. 2008; Iwai et al. 2010). The main effect of these strategies is that mineralisation is limited to the outer part of the cell wall (Phoenix 2000), thus allowing the cell to continue to live. Furthermore, the encapsulation of living microorganisms in silica gels for various biomedical applications is a growing topic and has also provided details on the way microorganisms survive within a wet silica matrix (see review in Livage et al. 2006 and Meunier et al. 2009).

The previous experiments to silicify microorganisms have been undertaken over relatively short periods and never beyond one year. This has allowed the very first stages of microbial fossilisation (i.e. mineralisation of the cell wall) to be examined in detail. But later steps, where other cellular structures are replaced by silica, have only been observed when accelerated silicification procedures have been used (Francis et al. 1978; Westall et al. 1995; Westall 1997) and to a lesser extent with natural silica concentrations (Toporski et al. 2002). In the continuation of these studies, this article presents the results of the first experimental fossilisation of a thermophilic Gram-positive bacterium, monitored over a period of five years. A combination of electron microscopy and LIVE/DEAD staining was used to track changes in the morphology and structural integrity of bacterial cells in order to assess the response of the cells to silicification on a long time scale. Our objectives were to reproduce experimentally the more

advanced steps of the fossilisation process in order to obtain additional information on the behaviour of the fossilising microorganisms.

Materials and Methods

Source of the bacterial strain and maintenance of cultures

Geobacillus strain SP7A was chosen as a representative of a thermophilic Gram-positive bacterium living in a hydrothermal environment. Strain SP7A was isolated from a coastal hot spring on Saint Paul Island (38°43'S, 77°31'E) in the South Indian Ocean (French Southern and Antarctic Lands). Saint-Paul Island is a 6.5 km^2 island corresponding to the emerged part of an otherwise submarine volcano. The central crater forms a lagoon. Saint Paul hot spring activity is related to ocean rifting (the Southeast Indian Ridge) and basaltic volcanism with geothermal waters that derive from subterranean heated aquifers mixed with seawater and groundwater. Water mixed with sediments was collected from a small stream of hot water appearing at low tide in the lagoon intertidal zone. The *in situ* characteristics of the water were pH 6.2, temperature 60-63°C, conductivity 51 $mS.cm^{-1}$ and a silica concentration of ~195-200 ppm SiO_2, resulting in few silica precipitates. No mineral deposits were present around the stream. The samples were stored anaerobically in sterile glass bottles at 4 °C. Strain SP7A was isolated from an initial enrichment culture using serial dilutions and inoculation on agar medium 162 (Degryse et al. 1978). The agar plates were incubated at 60°C. The purity of the isolate was tested microscopically using phase-contrast microscopy (Olympus CX 60). Phylogenetic analysis based on 16S rRNA sequences suggests that strain SP7A belongs to the genus *Geobacillus*, the closest

match being *Geobacillus stearothermophilus* (Nazina et al., 2001). On the basis of genetic differentiation and substantial differences in phenotype (data not shown), strain SP7A might represent a novel species of *Geobacillus* for which the name *Geobacillus sanctuspaulis* sp. nov. will be proposed. Strain SP7A is a Gram-positive facultative anaerobe, heterotrophic, non-motile rod, with an optimal growth temperature of 65°C at neutral pH. Cells sometimes develop an oval endospore visible with light microscopy (Figure 1a). For the purpose of this study, strain SP7A was grown aerobically in liquid 162 medium in 50 mL sterile and sealed culture vials.

Experimental fossilisation

We used a silicification procedure that attempted to reproduce the natural process as closely as possible (cf. Orange *et al.* 2009). Silicification was performed directly in liquid growth medium with the culture vials remaining sealed throughout the experiment. A pure sodium silicate solution containing ~27% SiO_2 and ~10% NaOH ($Na_2Si_3O_7$, M = 242 g/mol; Riedel de Haën) was diluted and filtered before being injected into vials to obtain a final silica concentration of 350 ppm Si (750 ppm SiO_2). Injection was performed at the end of the exponential growth phase of the microbial culture and marked “time zero” for silicification. The vials were incubated at 60°C until sampling, with subsampling at 24 hours, 15 days, 1 month, 10 months, 3 and 5 years. After the injection of silica, the medium pH increased slightly to 8 and remained at this value for the first year of experimental fossilisation. However, after 3 years, the pH had fallen to ~6.5.

LIVE/DEAD staining

Cell viability and structural integrity was monitored during the experimental fossilisation using the dual staining LIVE/DEAD® Bacterial Viability Kit (BacLight™) (L-7012, Molecular Probes, Eugene, OR). The LIVE/DEAD stain contains the fluorescent nucleic acid stain SYTO 9 and propidium iodide (PI) which allows differentiation of microorganisms with intact and damaged cytoplasmic membranes. This characteristic is often assumed to differentiate between viable and dead cells (Decamp and Rajendran 1998; Leuko et al. 2004; Quéric et al. 2004). While SYTO9 is able to penetrate through all types of cell walls, PI enters only cells with damaged cytoplasmic membranes. The microscopic assessment of LIVE/DEAD stained bacterial cells is usually simplified to either “green” (alive) or “red” (dead) cells. LIVE/DEAD staining has not been used previously for monitoring experimental fossilisation. As several studies have expressed doubts about the relationship between the staining and the actual viability of the cells (Renye et al. 2004; Teal et al. 2006; Berney et al. 2007), the results are here considered as an indicator of structural integrity rather than viability.

For the LIVE/DEAD staining, 50 µL of the sample were mixed with 50 µL of the stain solution and incubated for 15 minutes in the dark before observation using epifluorescence microscopy with UV excitation and blue light emission (Olympus BX60 and using manufacturer protocols). To compare the integrity of SP7A strain cells in prolonged culture (without silica), we conducted a parallel series of subsampling from vials not inoculated with sodium silicate. We processed samples immediately without prior glutaraldehyde fixation. In fact, it has been reported that glutaraldehyde could bias the results of the LIVE/DEAD staining, increasing the count of viable cells (Boulos et al. 1999). During this study, glutaraldehyde fixation also tended

to increase the count of living cells: during the first month of fossilisation, samples with prior glutaraladehyde fixation showed almost exclusively “green” cells, while similar samples without prior fixation showed lower “viable” cell counts (up to 90%) (data not shown), which is probably more representative of the actual culture state.

Scanning and transmission electron microscopy analysis

Samples for scanning and transmission electron microscopy (SEM, TEM) were collected on a 0.2 µm Millipore filter and stored in 2.5% glutaraldehyde solution at 4°C until preparation for SEM and TEM analyses. Preparation of samples for SEM was made using the critical point drying method whereas samples for TEM were prepared using a standard method involving dehydration by ethanol followed by embedding in resin before ultrathin sectioning (see Orange et al. (2009) for the complete procedure). SEM observations and analyses were carried with a Hitachi S-3200N SEM and a Hitachi S-4500 Field Emission Gun SEM equipped with an EDX detector (Oxford Instruments). TEM observations and analyses were made with a Philips CM20 Transmission Electron Microscope, equipped with an EDX detector (Oxford Instruments).

Results and Discussion

Viability of cells during prolonged growth

Geobacillus SP7A cell densities at the end of the exponential growth phase were high and cells were thus easily located during electron microscopy observations. The cells are 0.5-0.8 µm wide

and usually have lengths ranging between 1.5-3.5 µm (Figures 1b, 1c) reaching sometimes up to ~6 µm. The cells were usually turgid (Figure 1b) but signs of degradation and lysis were observed (Figure 1c). This was attributed to natural cell death which was observed in the case of growth prolonged beyond the exponential growth phase (Figures 2b, 2c). This was confirmed by the LIVE/DEAD staining which showed at first an important number of *Geobacillus* SP7A cells visible through fluorescence at the end of exponential growth phase, which appeared all green and thus supposedly alive (Figure 2a). However, during prolonged culturing in the absence of sodium silicate, cell viability rapidly decreased: the number of stained cells decreased, and these cells appeared mostly red after 24 hours (Figure 2b). All *Geobacillus* SP7A cells were apparently dead after 15 days (Figure 2c).

Monitoring of the fossilisation by electron microscopy

A few hours after silica injection, a loose colloidal silica deposit formed at the bottom of the vials. This early-formed silica precipitate was made of particles ranging from a few nm to ~40 nm in size (Figures 3a, 3d), which trapped numerous cells (Figures 3a, 3d, 3e). EDX analyses showed that this precipitate consisted solely of silica and did not include compounds from the growth medium (Figure 3g). The carbon signal comes from both organic compounds (i.e. cells) trapped in the precipitate and the underlying polycarbonate filter. Silica particles formed as a result of the spontaneous polymerisation of oversaturated dissolved silica, which started immediately after the injection of the concentrated silica solution in the culture vials. The silica precipitate consisted of particles formed in suspension, while particles seen on cell surfaces were

the result of the nucleation of either monomeric silica and subsequent particle growth or binding of pre-formed particles (Konhauser et al. 2004).

As already noticed in cultures kept beyond the exponential growth phase (Figure 1c), approximately half the cells had a deflated appearance after 24 hours indicative of degradation and lysis (Figures 3a, 3c), while the remainder were turgid and intact (Figure 3b). Most cells showed no major signs of degradation despite being encased in the silica matrix (Figures 3d, 3e, 3f) with individual silica nanoparticles already attached to the cell walls (Figures 3b, 3c, 3d arrows). During the following months of fossilisation, the already lysed cells did not show signs of further degradation (Figures 3h, 3i, 4a, 4b, 4c, 4d, 4e). A fine granular precipitate of nucleated silica particles progressively covered the cells (compare Figures 3b, 3c, 3i, 4c). By 10 months, cells were entirely covered by silica particles, sometimes making their identification difficult (Figures 4d, 4e). The strong carbon signal on the EDX spectra suggests that the silica deposit still contained substantial amounts of organic compounds, most likely cellular material (Figure 4f), although part of this signal also comes from the support filter. SEM micrographs suggest few structural changes to the cells between 10 months and 3 years. Cells (or cell remains) were present in substantial numbers and were easily recognizable (Figures 5a, 5b). No significant degradation of cells occurred during this period of time. No changes in the composition of the silica precipitate were noticed (Figure 5c), apart from a weaker carbon signal. By comparison with the early steps of the fossilisation, silica particle size was significantly more homogeneous after 3 years (compare Figures 3e and 5f). However, no continuous particle size growth nor additional silica deposition on the cells were observed during the several years of the fossilisation (maximum particle size remained around 40 nm) probably because of the lack of

further availability of the silica: the silica was added only once at the start of the experimental fossilisation in contrast to a continuous supply of silica, which would occur in natural settings (e.g. hot springs systems).

In parallel with precipitation on cellular surfaces, the cytoplasmic space was also progressively filled with silica particles, albeit at an apparent slower rate. As previously mentioned, a few cells already showed silica particles within their cytoplasm after 24 hours (Figure 3f). After 10 months, TEM micrographs showed heterogeneous mineralisation of the inner cellular structures: some cells had retained their cytoplasm (Figure 4g) while others were empty (Figure 4h) or completely filled with silica precipitate (Figure 4i). In some cases the bilayered plasma membrane was recognizable (Figure 4h), suggesting good preservation of the membrane. The endospore, when visible, was also preserved (Figure 4h). TEM observations made after three years of fossilisation confirmed the heterogeneities in cell preservation and cytoplasm mineralisation. Most of the cells observed within the silica precipitate appeared to be relatively well preserved, with the outline of the wall clearly visible and the cytoplasm still present and only slightly filled with silica particles (Figures 5d, 5e, 5f). Structures identified as the endospore were sometimes visible (Figure 5d, arrow) and showed similar mineralisation features. While some cells were completely mineralised and only identifiable by the faint outline of their cell wall (Figure 5g), others showed very limited filling by silica and no visible cytoplasm (Figure 5h). Numerous deformed remains of cells (cell wall fragments) also occurred in the silica precipitate (Figure 5i).

The very good preservation of *Geobacillus* SP7A cells was due to the rapid precipitation of a significant amount of silica on the cell wall and subsequently within the cytoplasm. Rapid

fossilisation is a paramount factor in the preservation of microorganisms in the geological record. Previous fossilisation experiments have shown that good preservation is only possible when the fossilisation process begins when microorganisms are alive, or very shortly after their death (Oehler and Schopf 1971; Oehler 1976; Francis et al. 1978; Bartley 1996; Schultze-Lam et al. 1995; Orange et al. 2009). The rapidity and the efficiency of the fossilisation process are highlighted by the fact that rapid lysis and death of *Geobacillus* SP7A occurred within two weeks of prolonged growth (Figures 1c, 2a, 2b, 2c). Furthermore, our results are consistent with previous studies that have shown that the fossilisation rate of Gram-positive bacteria is higher than that of Gram-negative bacteria or of archaea (Francis et al. 1978; Ferris et al. 1988; Westall 1997; Orange et al. 2009). For example, using an identical experimental protocol, Orange et al. (2009) found less precipitation of silica particles on the cell wall of the archaea *Pyrococcus abyssi* after one year than occurred in one month for *Geobacillus* SP7A. Furthermore, only limited intracellular silica precipitation occurred in *P. abyssi* after one year, while it was already noticed after 24 hours in *Geobacillus* SP7A (Figure 3f) and ubiquitous in this species after several months (Figures 4g, 4h, 4i, 5d, 5e, 5f, 5g, 5h, 5i). In addition, higher metal binding abilities of the Gram-positive *Bacillus subtilis* than the Gram-negative *Escherichia coli* have also been shown (Beveridge and Murray, 1976; Beveridge and Koval 1981; Beveridge 1989). Thus, the rapid fossilisation previously observed for mesophilic Gram-positive bacteria (Ferris et al. 1988; Westall 1997) also appears to apply to thermophilic species.

These differences are explained by the structure and composition of the cell wall of the different kinds of Prokaryotes. The anionic functional groups of the outer surface of the cell wall (carbonyl, hydroxyl, phosphoryl; Beveridge and Murray 1976; Beveridge and Koval 1981;

Schultze-Lam et al. 1996; Beveridge 2001; Yee et al. 2001; König 2002) have been identified as the main and primary binding sites (through hydrogen binding) for monomeric silica or colloidal silica particles. In the Gram-positive bacteria cell wall, these functional groups are mainly found in the thick, outer peptidoglycan layer (a hydroxyl and carboxyl-rich polysaccharide), which thus provides an excellent site for silica binding (Schultze-Lam et al. 1996; Westall 1997; Beveridge 2001; Orange et al. 2009). The peptidoglycan layer is thinner in Gram-negative bacteria walls and trapped between two membranes, the outer cell envelope and the inner cytoplasmic membrane, thus rendering its functional groups less available for binding. Silica or metal cations can however bind to the phospholipids of the anionic layer of lipopolysaccharides (LPS) that covers the outer membrane (Beveridge 2001; Yee et al. 2001). Cell wall structures among archaea are very diverse (König 2002) but have in common the lack of peptidoglycan. In most of these organisms only the plasmic membrane and the S-Layer are available as binding sites. Other mechanisms, specific to thermophilic species and members of the genus *Geobacillus*, may also be involved (Hetzer et al. 2006).

However, the fossilisation rate is not influenced only by the cell wall composition and structure. As will be detailed later in this section, the metabolic activity of the cell can also play a role on this rate by preventing silica from precipitating on the cellular structures. This is exemplified by the few cells showing initiation of mineralisation of the cytoplasm after 24 hours of silicification (Figure 3f). These cells showed signs of deformation and were thus likely already lysed, while intact cells showed no signs of cytoplasm mineralisation at that point.

Monitoring of the fossilisation by fluorescence microscopy with LIVE/DEAD staining

The rapid death of all *Geobacillus* SP7A cells within 2 weeks during prolonged growth had been verified by fluorescence microscopy (Figures 2a, 2b, 2c). On the other hand, during the experimental fossilisation, SEM and TEM observations showed how *Geobacillus* SP7A cells became progressively covered and filled by silica, and eventually preserved. Despite these observations, an important number of cells stained green with LIVE/DEAD staining after one month in cultures maintained in the presence of sodium silicate (Figures 2d, 2e, 2f). These cells were sometimes isolated (Figure 2e), but were found more often inside aggregates formed following silica precipitation (Figures 2d, 2f). Even after 3 and 5 years, numerous cells stained green in the silica aggregates (Figures 2g, 2h, 2i). In most cases up to 3 years the observed cells still appeared rod-shaped suggesting they were intact (Figure 2g). However, after several years of silicification, an increasing number of coccoid features (possibly the endospores) were also stained mostly green but also red (Figures 2h, 2i). Following these observations, we attempted to reanimate the fossilised cells. A portion of the 3 year-old sample was washed three times with fresh growth medium to remove the silica deposit and incubated in fresh medium at 60°C for a week. However, no cell growth was detected after repeated attempts.

We assume that the cells stained green after several months or years of fossilisation (Figures 2g, 2h, 2i) correspond to the best preserved cells seen under SEM (Figures 4d, 5a) and TEM (Figures 5d, 5i). In all cases, these cells comprised the majority of the cells (or cell remains) observed. No intact cell free of inner mineralisation was seen by TEM after three years (Figures 5d, 5e, 5f, 5g, 5h, 5i). Green staining indicates that these cells had kept an intact membrane and some nucleic acids, despite significant mineralisation of their wall and cytoplasm.

The few cells that stained red (e.g. Figures 2h, 2i) may have had their membrane damaged by silica binding while being generally well preserved by this same process. Finally, cells that had lost all their cytoplasm (Figures 3h, 4b, 4h) and those entirely filled with silica (Figure 4i) must have been invisible after the LIVE/DEAD staining.

Preservation of the cells and of their structural integrity

Geobacillus SP7A cells remained remarkably well preserved throughout the five years of experimental fossilisation (e.g., Figure 5a). SEM and TEM observations showed that the rod-like shape and major cellular structures of many cells remained recognizable for up to several years in the fossilising medium, while LIVE/DEAD staining indicated that cells retained intact membranes and nucleic acids (Figures 2g, 2h, 2i) for several years. The comparison with the prolonged growth experiment showed that, in a general sense, the influence of fossilisation acted on the preservation of the structural integrity of cells over much longer periods of time than it did for unfossilised cells, and thus provide them with a greater chance to be preserved in the rock record.

However, as even the best preserved cells were significantly mineralised after several years of silicification, we need to explain how these cells were able to keep intact membranes and nucleic acids in these conditions, and thus retain the capability of being positively stained by the LIVE/DEAD stain. This presupposes the question as to whether the *Geobacillus* SP7A cells remained alive or not during the experimental fossilisation

Despite evidence of rapid death during prolonged growth, it would have been possible for *Geobacillus* SP7A cells to remain alive during the first steps of the fossilisation. Several studies

have shown that microorganisms can be kept alive when trapped within a silica matrix. This is the aim of encapsulation methods (Finnie et al. 2000; Coiffier et al. 2001; Nassif et al. 2002, 2003; Chen et al. 2004; Ferrer et al. 2006; Muller et al. 2008; Rooke et al. 2008; Saw et al. 2008; Leonard et al. 2010), where microorganisms are immobilised in silica gel matrixes for biomedical applications and have been kept alive up to several months (Finnie et al. 2000; Leonard et al. 2010). In addition, *Geobacillus* SP7A was isolated from a hydrothermal environment rich in dissolved minerals where it is likely that the cells are able to deal with high concentrations of silica and that initial precipitation of silica on the outer surface of the wall is not detrimental to the cells. In a silicifying environment, cells can change their metabolisms (through protein expression, modification of the cell wall properties, production of EPS), thus implementing a survival strategy that limits the detrimental effects of the fossilisation (i.e. blocking of exchange with the surrounding environment, damage to cell structure and eventually death) (Lalonde et al. 2005, Konhauser et al. 2008; Saw et al. 2008; Iwai et al. 2010). In particular, while encapsulating a close relative to *Geobacillus* SP7A (the thermophilic *Anoxybacillus flavithermus*), Saw et al. (2008) noticed the ability of this species to survive silicification and to adapt its metabolism accordingly. Microorganisms can also actively repulse silica and thus avoid mineralisation (Westall et al. 1995; Lalonde et al. 2005; Rooke et al. 2008; Orange et al. 2009). More importantly, Phoenix et al. (2000, 2008) showed that cyanobacteria could remain intact and viable while mineralisation was limited to the outer surface of its continuously renewed sheath. A similar scenario could have applied for *Geobacillus* SP7A cells with the thick outer peptidoglycan layer forming a physical barrier to silica, which could be renewed as necessary (Figures 3d, 3e). However, on the basis of electron microscopy

observations, there is no evidence of a sustained specific behaviour of *Geobacillus* SP7A cells, such as silica repulsion, induced by the presence of silica. It can be stated that, in a general way, *Geobacillus* SP7A cells let themselves become rapidly fossilised. It is unknown when cell death occurred during the experimental silification, and if was a consequence of prolonged growth or of the thick silica precipitate formed on the cells limiting exchange between the cell and its environment. However, this cell death then allowed the more advanced steps of the fossilisation, *i.e.* mineralisation of the cytoplasmic space, to take place (Figures 4i, 5d).

Another reaction of bacteria to environmental stress is the production of endospores (Marquis et al. 2001). Certainly the physical protection offered by an endospore would be beneficial during fossilisation. Most *Geobacillus* species are known to produce endospores (e.g. Ahmad et al. 2000; Fortina et al. 2001; Nazina et al. 2001). We therefore cannot rule out endospore formation as a potential survival mechanism for *Geobacillus* SP7A in the face of mineralisation. Indeed a few endospores were observed (Figures 4h, 5d), but there is no evidence that they were produced as the result of the fossilisation. As spores require extended culture times and specific conditions to produce new cell growth, this would also be consistent with our failed attempts to revive rapidly fossilised samples. Endospores can be stained by the LIVE/DEAD stain (Laflamme et al. 2004). The observation of increasing numbers of coccoidal features by fluorescence microscopy after 5 years (Figure 2i) suggests that the endospores were at that point the only cellular component that could be stained, and therefore the best preserved.

Consequently, both electron and fluorescence microscopy observations indicate that, despite their apparent good morphological preservation, progressive decay of the *Geobacillus* SP7A occurred during the several years of experimental fossilisation. No intact cell free of

mineralisation could be observed at the advanced steps of the fossilisation (Figures 5d, 5e, 5f, 5g, 5h, 5i). Also, observations by fluorescence microscopy showing more coccoid than rod-shaped features after 5 years suggest the loss of the structural integrity of the cellular bodies, with only the endospores remaining quite intact. Finally, the pH of the medium was found being more acidic after 3 years after having remained constant at 8 for the whole beginning of the fossilisation. This might the result of the decaying of the organic matter, which could have acidified the medium.

As previously mentioned, the cells that stained green are probably those that were observed to be best preserved in the TEM preparations (Figure 5d). The presence of silica particles in the cytoplasm does not necessarily imply damage to the membrane: dissolved silica could simply have diffused through the membrane and nucleated to form these particles in the cytoplasm. It is possible that the stain could represent a false positive result: Nassif et al. (2003) have previously warned that enzymatic activities cannot be used as a reliable viability indicator, as lysed cells can still host active enzymes. However, as the LIVE/DEAD staining methods rely primarily on the state of the membrane, it is unlikely that lysed cells or cell remains would have stained green. Intuitively, mineralisation of the cytoplasm seems incompatible with the presence of the preserved DNA within it. On the other hand, the pattern of mineralisation of the cytoplasm is particular, with distinct areas left almost totally unmineralised alongside areas with dense silica deposits (Figure 5d). A similar pattern had already been observed by Orange et al (2011b) during the silicification of the archaea *Methanocaldococcus jannaschii*. Remains of nucleic acids could still be present in these unmineralised areas and available for the stain. Limited data are available on the behaviour of DNA during experimental fossilisation and the role of silica on the

preservation of DNA is unclear (Lorenz and Wackernagel 1987; Demanèche et al. 2001; Inagaki et al. 2005). For example, Schelble et al. (2008) documented negative effects on the preservation of microbial DNA during exposure to silica. On the other hand, Orange et al. (2011a) showed that naked DNA of hyperthermophilic viruses may be preserved over long periods during an *in vitro* experimental fossilisation experiment.

Although LIVE/DEAD staining in this experiment is not a reliable method of assessing the viability of the cells, it nonetheless provides information on the state of preservation of the cells during prolonged growth and during the experimental fossilisation. Combined with electron microscopy observations, it showed that silicification was a critical factor in the long term morphological preservation of the cells. LIVE/DEAD staining also indicated the slow but continuous advance of the fossilisation processes, which took place over a period of several years. Most of the cells had their structural integrity preserved during the initial phase of the fossilisation, but eventually became entirely encrusted. Endospores may have retained their integrity for a longer time than the actual cells, but were eventually also affected by the fossilisation.

Comparison with natural fossilisation

The chronology of silicification of *Geobacillus* SP7A cells is similar to that described for the early stages of microbial fossilisation in natural hot spring environments, namely the spontaneous precipitation of silica and its binding to the external surface of the cells, followed by the filling of the cytoplasmic space (Schultze-Lam et al. 1995; Tobler et al. 2008; Handley et al. 2005). In addition, our long term monitoring provides new insights into the later phases of

microbial fossilisation, where cellular structures are almost entirely replaced by silica, with the exception of the cell wall (Figures 5d, 5e).

Early experimental fossilisation studies previously reported similar observations (Francis et al. 1978; Westall et al. 1995; Westall 1997) but as they used tetraethylorthosilane (TEOS, an organosilicon), a silicifying agent with high silica concentrations, these experiments resulted in an accelerated, non-natural fossilisation process. Sodium silicate solutions has since been preferred since they allow a more faithful recreation of the natural fossilisation processes, although at slower pace (Birnbaum et al. 1989 ; Toporski et al. 2002 ; Phoenix et al. 2000; Benning et al. 2004a, 2004b ; Lalonde et al. 2005 ; Orange et al. 2009). Our results complement those of Toporski et al. (2002) who, using a sodium silicate solution, had obtained extensive mineralisation of the cytoplasm of the bacteria *Pseudomonas fluorescens* and *Desulphovibrio indonensis*. Similarly, TEOS had also been used for early encapsulation experiments (see review in Livage et al. 2006) until it was proven that the use of aqueous sodium silicate solutions was less detrimental for the viability of the encapsulated cells (Coiffier et al. 2001; Chen et al. 2004). Experimental fossilisation using sodium silicate solutions thus allow study of the complex interplay between microbial mineralisation and microbial reaction to this mineralisation. However, one important difference between our experimental design and natural silicification is the supply of silica, which was made with a single addition at the beginning of the experiment, instead of a more or less continuous supply. This probably resulted in the lack of significant evolution in the mineralisation of the cells over long time periods of fossilisation.

Nevertheless, after several years of experimental fossilisation, some of the *Geobacillus* SP7A microfossils produced in our experiment showed morphological similarities to

microfossils found in ancient rocks in terms of encrustation and infilling by silica, with the outline of the cell wall being the only preserved and recognizable feature in the silica matrix (Figures 4i, 5e, 5g), (see, for example, those of the Gunflint and Draken formations, 2 Ga and 700-800 Ma, respectively; Barghoorn and Tyler, 1965; Knoll 1982). Fossilisation of *Geobacillus* SP7A was accompanied by the progressive loss of cellular structures which could aid the distinction of microorganisms having different outer membrane architectures. The specific cell wall structure of Gram positive bacteria had completely disappeared in some cells after 10 months (Figure 4i) while it was still visible in others (Figure 4h). When present (Figure 4h, 5d), the fossilised spores may however be recognizable and aid interpretations about the nature of the original microorganism.

Taken together with earlier studies on the archaea *Methanocaldococcus jannaschii* and *Pyrococcus abyssi* (Orange et al. 2009, 2011b), this study adds new elements to our knowledge of the fossilisation of thermophilic microorganisms, thought to be among some of the earliest forms of life on Earth (Nisbet and Sleep 2001; Konhauser et al. 2003).

Conclusions

Here we described the first, long-term experimental fossilisation of microorganisms using the Gram positive species *Geobacillus* SP7A which provided additional and important information about the fossilisation of microorganisms. Our results confirm that the fossilisation of Gram-positive bacteria is faster than for other mesophilic or thermophilic Gram-negative bacteria and archaea, due to the composition and structure of their cell walls. Prolonged silicification allowed us to observe the later stages of the fossilisation, with the complete encrustation of the

microorganisms and the replacement of organic structures by silica, thus reproducing the chronology of the fossilisation as it may occur in natural settings. The good preservation of *Geobacillus* SP7A cells and the preservation of their structural integrity over five years of fossilisation appear to be the result of rapid silicification, which prevented early degradation. These new results contribute to enhance our knowledge of the mechanisms of preservation of microorganisms in the rock record.

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FIG. 1 Light and SEM micrographs showing examples of *Geobacillus* SP7A cells in a fresh culture. (a) Light micrographs of *Geobacillus* SP7A cells exhibiting a terminal endospore. (b,c) SEM micrographs showing (b) dividing, turgid and (c) lysed *Geobacillus* SP7A cells. SEM micrographs were taken at 10 kV (b) and 2 kV (c) voltages.

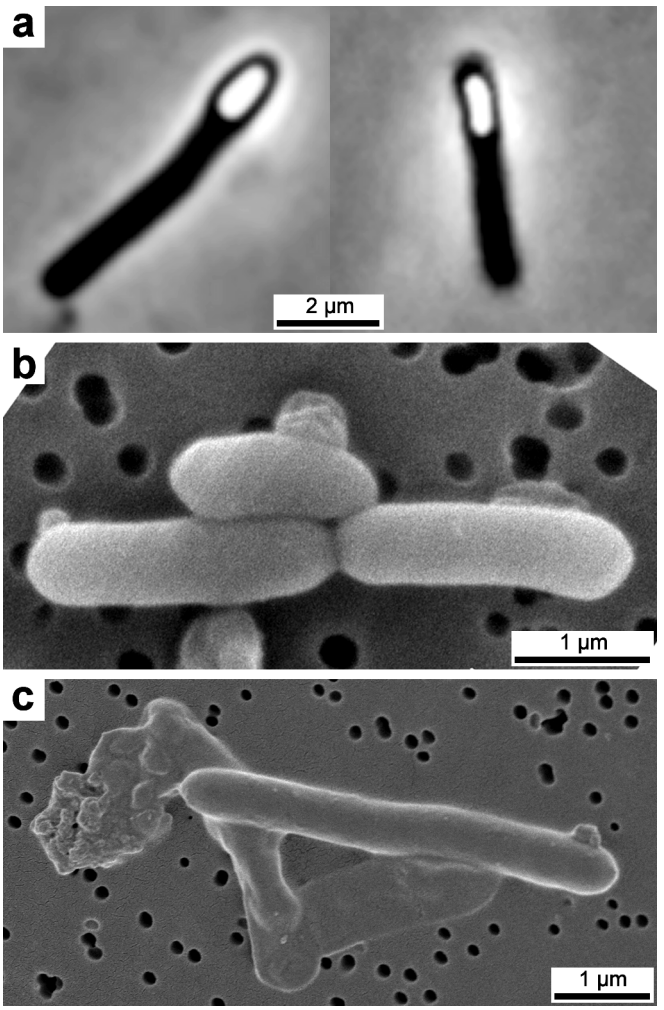

FIG. 2 Fluorescence micrographs of *Geobacillus* SP7A cells with LIVE/DEAD staining. (a-c) Prolonged growth of *Geobacillus* SP7A. (a) End of the exponential growth phase. (b) After 1 day. (c) After 15 days. (d-i) Experimental fossilisation of *Geobacillus* SP7A cells exposed to a ~350 ppm Si silica concentration. (d) 1 week. (e) 3 weeks. (f) 1 month. (g,h) 3 years. (i) 5 years.

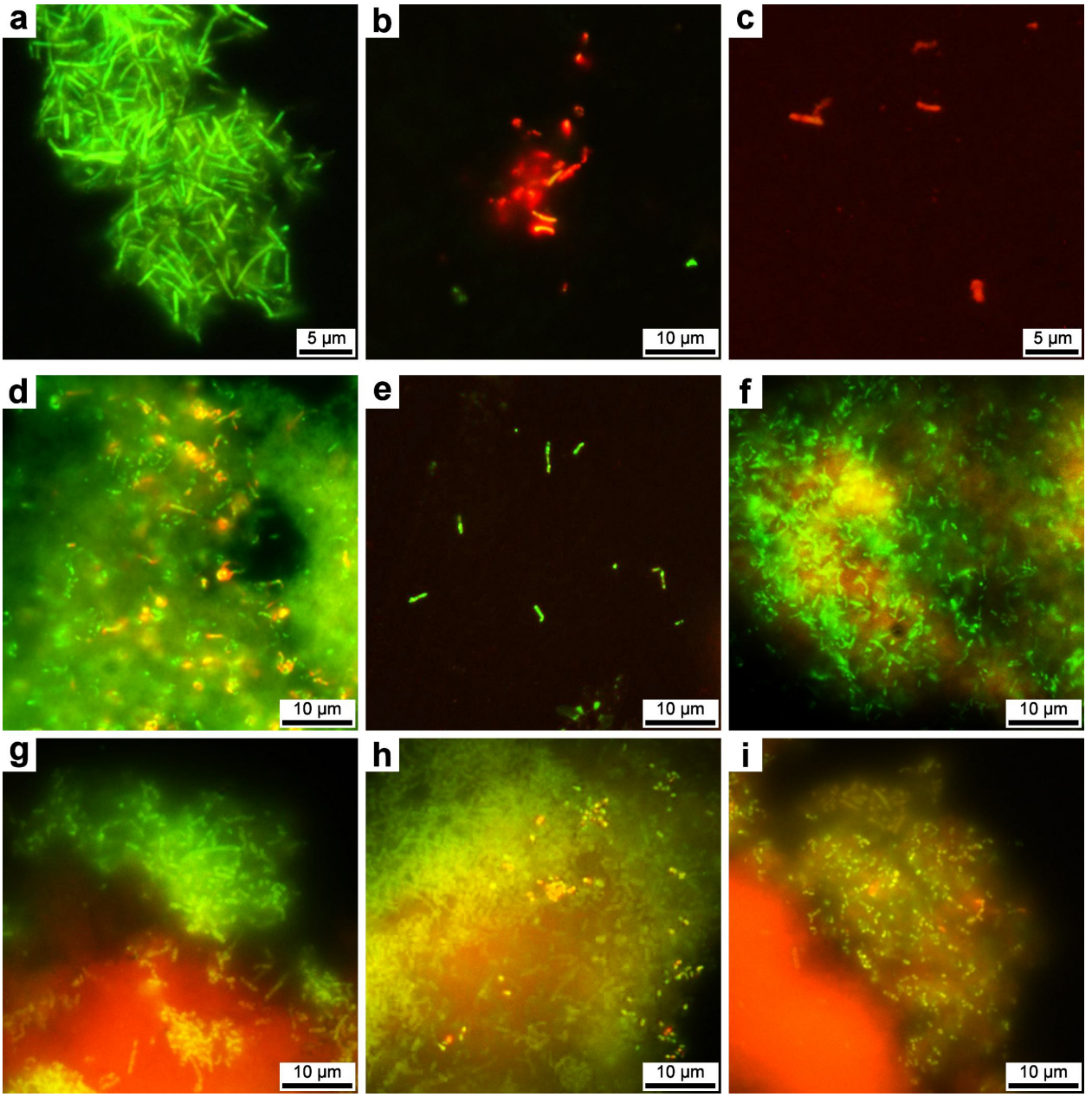

FIG. 3 Experimental fossilisation of *Geobacillus* SP7A. SEM and TEM micrographs showing *Geobacillus* SP7A cells exposed to a ~350 ppm Si silica solution for various lengths of time. (a-c) 24 hours, SEM. (a) lysed cells trapped in an amorphous silica precipitate with the corresponding EDX spectrum (g) made on the silica precipitate. (b,c) Extremities of *Geobacillus* SP7A cells; note the isolated silica particles on the cell wall (arrows). (d-f) 24 hours, TEM. (d) Unstained section, cells trapped in the silica precipitate, with the corresponding close-up view showing (e) an apparently intact cell in the precipitate, and (f) a lysed cell showing precipitation of silica in its cytoplasmic space. (h,i) 2 weeks, SEM. (h) Lysed cell trapped in the silica precipitate. (i) Extremities of a *Geobacillus* SP7A cell with an increasing number of silica particles on the cell wall. All SEM micrographs were made with a 2 kV voltage, the EDX spectra with a 5 kV voltage, and TEM micrographs at a 200 kV voltage.

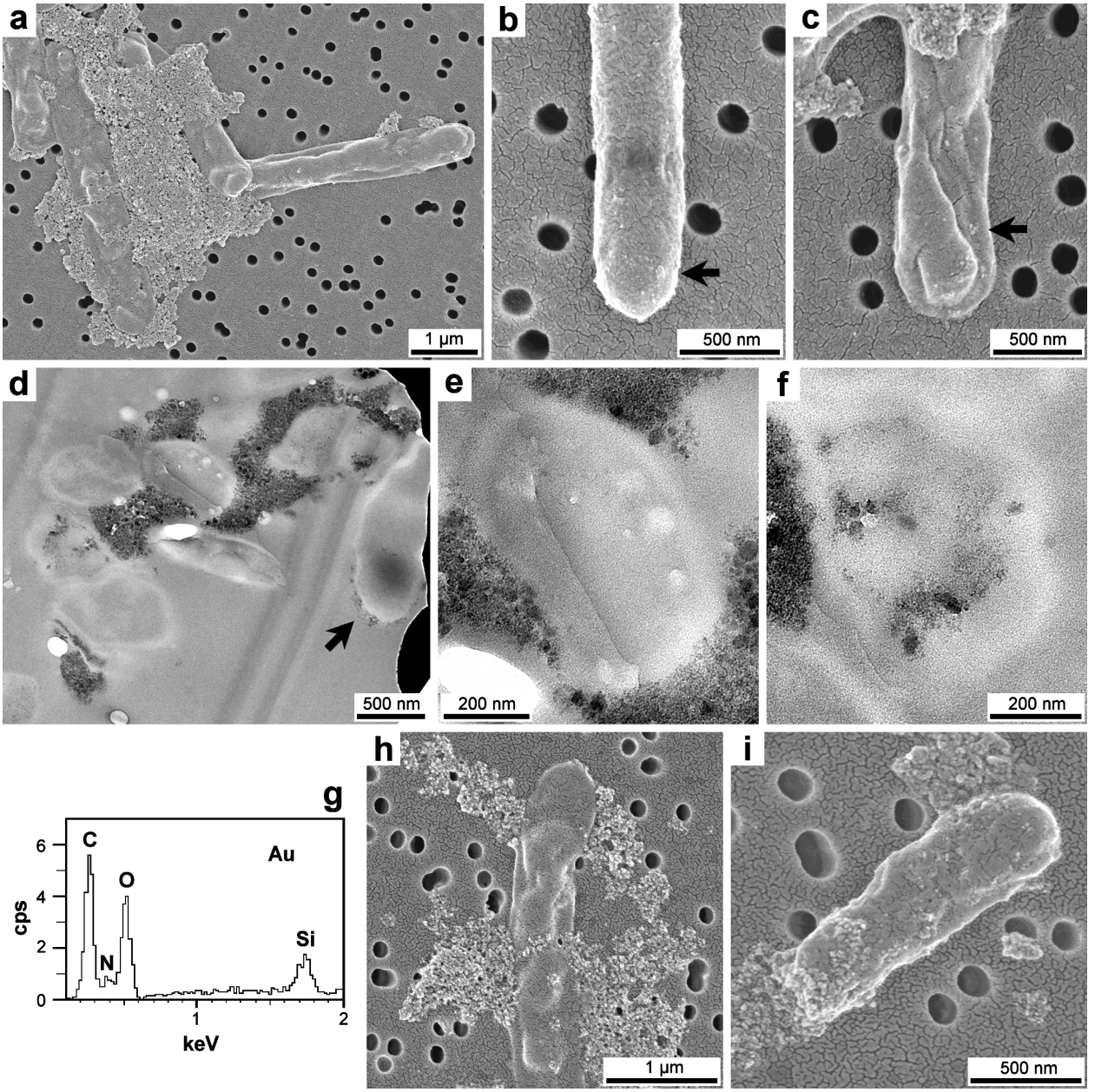
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FIG. 4 Experimental fossilisation of *Geobacillus* SP7A. SEM and TEM micrographs showing *Geobacillus* SP7A cells exposed to a ~350 ppm Si silica solution for various lengths of time. (a-c) 1 month, SEM. (a) 1 month, cell trapped in the silica precipitate. (b) Cell covered with fine silica particles (close-up in (c)). (d,e) 10 months, SEM, cells trapped in and covered by a silica precipitate with the corresponding EDX spectrum (f) made on the silica precipitate. (g-i) 10 months, TEM. (g) Stained section, cells trapped in the silica precipitate. (h) Stained section, empty cell showing visible wall and spore membranes. (i) Unstained section showing a *Geobacillus* SP7A cell whose structure is outlined by silica precipitation. All SEM micrographs were made with a 2 kV voltage, the EDX spectra with a 5 kV voltage, and TEM micrographs at a 200 kV voltage.

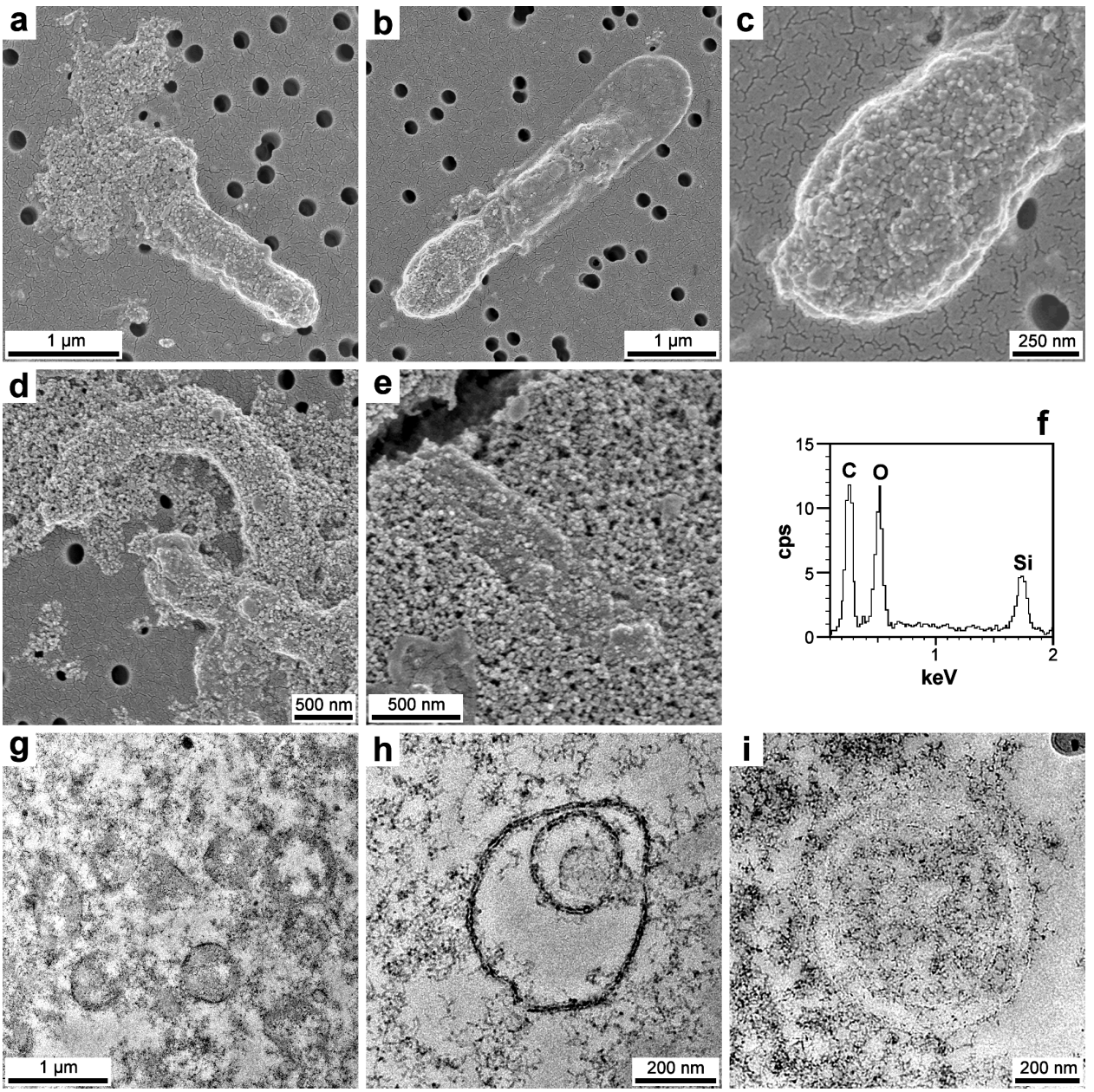
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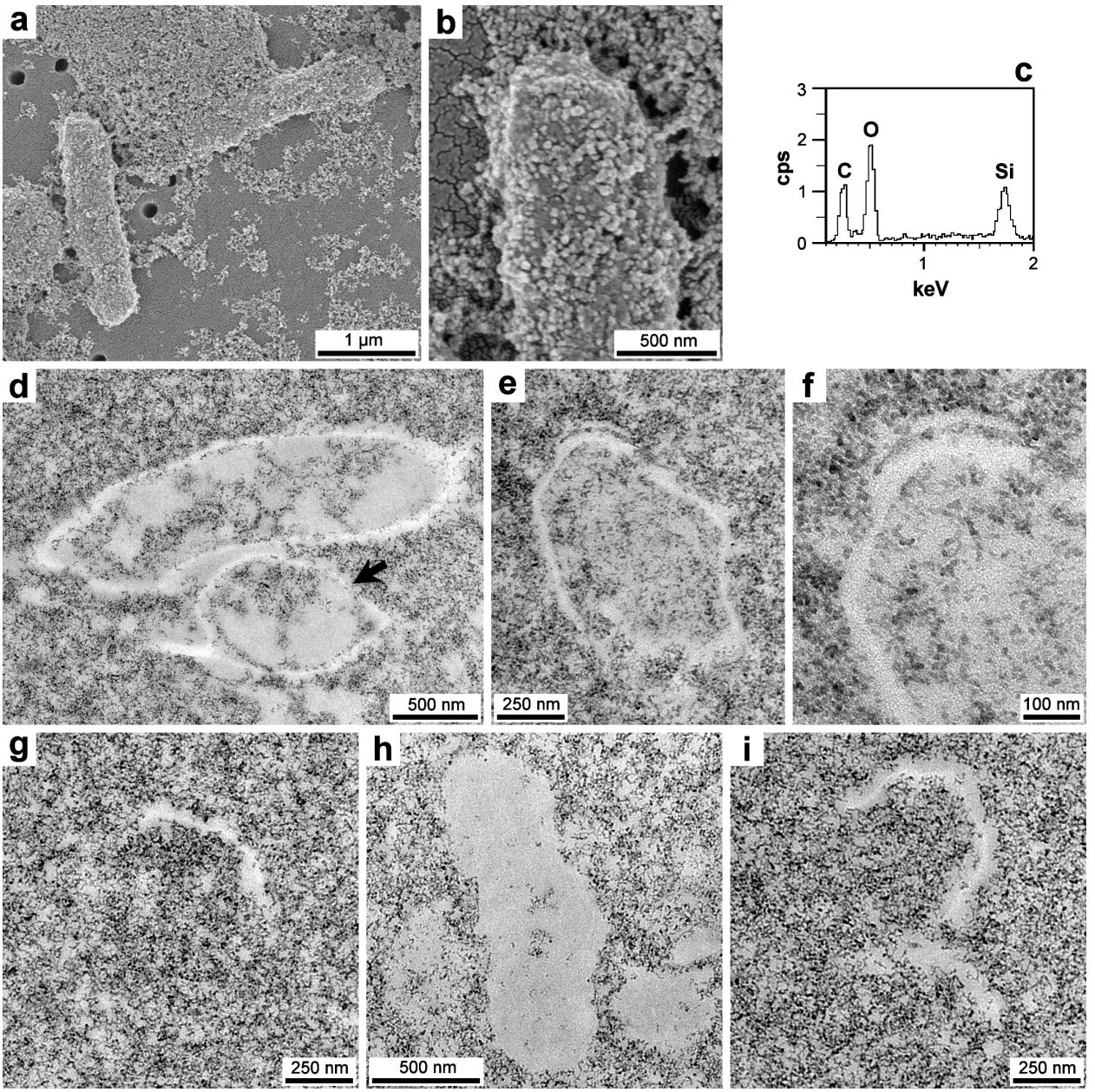
FIG. 5 Experimental fossilisation of *Geobacillus* SP7A. SEM and TEM micrographs showing *Geobacillus* SP7A cells exposed to a ~350 ppm Si silica solution for 3 years. (a,b) SEM. (a) Cells trapped in a silica precipitate and covered by silica nanoparticles (close-up in (b)), with the corresponding EDX spectra (c) made on the silica precipitate. (d-i) TEM, unstained sections. (d,e) Well-preserved cells, showing intact cell walls and limited silica precipitation in the cytoplasmic space (close-up in (f)). A possible endospore is visible and also mineralised (d, arrow). (g) Cell with cytoplasmic space entirely filled by silica. (h) Lysed cell, with cytoplasmic space free of mineralisation. (i) Heavily mineralised cell remains. All SEM micrographs were made with a 2 kV voltage, the EDX spectra with a 5 kV voltage, and TEM micrographs at a 200 kV voltage.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