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The experimental silicification of Aquificales and their role in hot spring sinter formation

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ABSTRACT

Archean microfossils provide some of the earliest physical evidence for life on Earth, yet there remains a great deal of uncertainty regarding which micro-organisms were actually preserved. Because of the limited cellular detail remaining, interpretation of those microfossils has been based solely on size and morphology. This has led to significant controversy surrounding the presence or absence of cyanobacteria as early as 3.5 billion years. Accordingly, there has been an experimental bias towards studying their silicification. Here we report the very first findings on thermophilic bacteria–silica interactions, and investigate how *Sulfurihydrogenibium azorense*, a representative of the Aquificales often found as prominent members of modern hot spring vent communities, interacts with highly siliceous hydrothermal fluids. We show that adsorption of silica is limited to silica polymers and colloids, and that the magnitude of silica adsorption is dependent on its chemolithoautotrophic pathway. Intriguingly, when *S. azorense* is grown as a H_2-oxidizer, it responds to increasing silica concentrations by producing a protein-rich biofilm that may afford the cells protection against cell wall silicification. Although the biofilms of Aquificales could potentially contribute to or accelerate siliceous sinter formation under certain growth conditions, the cells themselves show a low preservation potential and are unlikely to have been preserved in the ancient rock record, despite phylogenetic analyses suggesting that they represent one of the most primordial life forms.

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INTRODUCTION

Much of what we understand about the early evolution of life comes from the examination of bacterial structures preserved in chert and cherty metasediments. The majority of the oldest Archean microfossils have filamentous or coccoid morphologies and appear to have grown as microbial mats in semirestricted basins where a combination of high productivity, limited water circulation and high salinity facilitated greater cellular preservation (Horodyski *et al.*, 1992). Additionally, rapid mineralization was necessary to limit postmortem degradation, a condition most commonly met by silicification in shallow, silica-supersaturated waters (Knauth & Lowe, 2003). The microfossils consist exclusively of the remains of cell sheath and cell wall material, whereas the cytoplasm and other cellular content have either completely decayed or formed remnant structures unrelated to the original structure (e.g. Knoll *et al.*, 1988). As a consequence of the poor preservation, comparisons between microfossils and modern microbes have largely been based on size and morphology. This has led to the suggestion that many of the Archean microfossils represent cyanobacteria (e.g. Schopf & Packer, 1987; Awramik, 1992) or alternatively, anoxygenic photosynthetic bacteria, e.g. *Chloroflexus* (Walter *et al.*, 1972).

A number of experimental studies have attempted to determine the physical changes associated with various bacteria during silicification (e.g. Oehler & Schopf, 1971; Oehler, 1976; Francis *et al.*, 1978; Westall *et al.*, 1995; Westall, 1997; Toporski *et al.*, 2002). These studies show that species-specific patterns of silicification exist, and that different microbes are capable of being silicified with different degrees of fidelity. Interestingly, all of the studies have focused on phototrophic or heterotrophic mesophilic bacteria, with an emphasis on cyanobacterial species.

Surprisingly, no studies that we are aware of have attempted to silicify thermophilic or chemolithoautotrophic bacteria.

There are a number of reasons why the earliest microfossils could represent micro-organisms that grew bathed in hot, silica-supersaturated fluids, perhaps sheltered from surface UV exposure (e.g. Nisbet & Sleep, 2001):

(1) It is believed that the early oceans were warm (Knauth & Lowe, 2003) and supersaturated with respect to amorphous silica (Siever, 1992; Tice & Lowe, 2004). At present, micro-organisms adapted to life at high temperatures and high silica concentrations are restricted to modern hot springs and hydrothermal vent systems, environments presumed to be much more prevalent in the early Archean (Farmer, 2000). Importantly, re-interpretation of the 3.46 billion years Apex Chert 'microfossils' of the Warrawoona Group, Western Australia, has led to the suggestion that the chert 'clasts' are not riverine, but instead hydrothermal deposits, and that if these structures are actually biogenic, they would likely have been thermophilic (Brasier *et al.*, 2002). Similarly, the recent discovery of pyritic, thread-like filaments in 3.2 billion years volcanogenic massive sulphides from the Pilbara Craton of Australia indicates that chemolithoautotrophic thermophiles lived in, or around, hydrothermal systems at that time (Rasmussen, 2000).

(2) In the high-temperature regimes around hot spring vents, photosynthesis is inhibited by elevated temperatures, and chemolithoautotrophic bacteria dominate microbial assemblages (Reysenbach & Shock, 2002). Many thermophilic bacteria and archaea survive by oxidizing reduced gases emitted from the vents (e.g. H_2, H_2S). Despite a general preference for O_2 as the terminal electron acceptor, many species of the Aquificales are tolerant of, or even dependent on, reducing conditions where the oxidation of H_2 is coupled to S°-reduction (Reysenbach & Shock, 2002). Such organisms likely thrived in the early Archean, and not surprisingly, phylogenetic evidence generally places such chemolithoautotrophic species as more deeply divergent than photosynthetic species, in accordance with a 'hot' early Earth and hydrothermal origin of life (Bocchetta *et al.*, 2000).

(3) Regardless of the potentially high sulphur gas concentrations in the Archean atmosphere, without the presence of oxygen absorbing radiation at wavelengths 220–300 nm, early photoautotrophs would have been subject to detrimental levels of solar radiation at ultraviolet wavelengths (Kasting, 1987). Conversely, an independence from sunlight meant that chemolithoautotrophs could have grown either as mats in deeper waters or under thick mineral crusts that would have sheltered the cells from the harmful UV rays (Pierson *et al.*, 1993).

(4) Experimental silicification studies have confirmed that silicification appears to be an inevitable consequence of microbes growing in polymerizing, silica-supersaturated solutions, and that conditions favouring the precipitation of amorphous silica are induced by rapid pH or temperature changes (Phoenix *et al.*, 2000; Yee *et al.*, 2003). Modern analogues exist at hot spring vents where hot, pressurized, silica-rich geothermal fluids are rapidly exposed to surface conditions (Fournier, 1985). At this interface, thermophilic chemolithoautotrophs thrive at the near-exclusion of other biota, where the geochemical conditions of their habitat render them more likely to be silicified.

Given the points above, it seems that interpretations of ancient microfossil assemblages based on experimental studies using only mesophilic species could be severely flawed. Additionally, little is known about the role of extracellular polymeric substances (EPS) secreted by many prokaryotes during the formation of biofilm, despite the fact that they may be more readily preserved than the bacteria that secreted them (Westall *et al.*, 2000). As a result, in this study, a preliminary attempt was made to address the above issues by experimentally silicifying *Sulfurihydrogenibium azorense,* a thermophilic, biofilm-forming, chemolithoautotrophic, anaerobic to microaerophilic member (Aguiar *et al.*, 2004) of what has been considered the most deeply branching order of life characterized to date, the Aquificales (Bochetta *et al.*, 2000). The role of two important chemolithoautotrophic metabolisms in silicification was assessed, and constraints are placed on potential interactions between silica species and the surfaces of *S. azorense*. The response of *S. azorense* to the initial stages of the mineralization process affects its cellular preservation potential as well as potential roles in hot spring sinter formation.

MATERIALS AND METHODS

Organisms and cultivation

Pure cultures of *S. azorense* Az-Fu1 were obtained from liquid nitrogen storage at the Oregon collection of methanogens (OCM 825) and are also available from Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany (DSMZ 15241). Recently recognized as a new terrestrial species of the Aquificales, the biofilm-forming *S. azorense* strain Az-Fu1 was isolated from a terrestrial hot spring (68.4 °C, pH 5.9) on São Miguel Island, Azores, in January 2001 (Aguiar *et al.*, 2004). Initial cultures were grown in modified MSH media previously described by Aguiar *et al.* (2004). While the controlled-atmosphere conditions necessitated the use of borosilicate glass Hungate tubes and serum bottles, the use of glassware was avoided wherever possible. Sodium hydroxide was used to adjust the pH of the media and was freshly prepared in order to prevent the introduction of leached silica during media preparation.

A final gas phase of CO_2 : O_2 : H_2 (41.6 : 1.8 : 56.6, v/v, 138 kPa) was established to contain O_2 (added after autoclaving) and H_2 (added upon inoculation) as the sole electron acceptor and donor, respectively. Constitutive metabolic limitation to H_2-oxidation was chosen for the starting cultures because *S. azorense* has been observed to grow in this combination of single electron donor and acceptor better than the other possible singular combinations (S. Lalonde, unpublished data). Small

cultures were repeatedly transferred in mid-exponential phase under these conditions to establish cell populations that have consistently metabolized via H_2-oxidation. To establish adequate biomass, cultivation was initially performed in 15 mL Hungate tubes containing 8 mL of media and then transferred to 120 mL glass serum bottles containing 60 mL of media.

Cultures consistently limited to metabolism by S°-oxidation were established by inoculation and successive transfer into modified MSH media to which approximately 4 g S° had been added per litre. Insoluble S° was added directly to serum bottles prior to dispensing and autoclaving to ensure equal distribution. Oxygen was added prior to inoculation for a final gas phase of CO_2 : O_2 (95 : 5, v/v, 106.4 kPa). The inoculum for S°-oxidizing cultures was initially obtained from the established H_2-oxidizing cultures. All cultivation and experiments were performed at 68 °C.

Experimental silicification

Silicification experiments were performed in 120 mL serum bottles with an initial volume of 60 mL. Actively growing cultures (10 mL derived from the cultures established in modified MSH media) were inoculated into 40 mL solutions consisting of a combination of sterile water and appropriate electron donors and acceptors, with 10 mL of a concentrated silica solution injected at initiation. All silicification experiments were performed at this 1/6 dilution of the modified MSH media to reduce interference with the molybdosilicate assay described in the next section. The initial concentrations of electron donors and acceptors remain fixed between cultivation and experiments, and growth during the experiments was uninhibited by the media dilution.

During preparation, glassware and other extraneous sources of silica were avoided wherever possible. Forty millilitres of anoxic 18 MΩ water was dispensed into the serum bottles under a CO_2 atmosphere. Experiments established for S°-oxidation received 0.2 mg S° directly into the serum bottle prior to the dispensing of the water, and 3 mL O_2 was added after autoclaving.

The solubility of amorphous silica (SiO_2) was calculated to be 96 PPM Si at 68 °C and pH 6.0 using the software package GEOCHEMIST'S WORKBENCH. A concentration of 90 PPM Si was chosen to evaluate the potential for bacterial silica adsorption slightly below the saturation state for amorphous silica at 68 °C (i.e. at undersaturated conditions). An initial Si concentration of 300 PPM was chosen to ensure supersaturation while investigating the microbial response to silica polymerization. Such high concentrations have been documented in silica-rich natural hot spring effluents (e.g. Jones *et al.*, 2003; Mountain *et al.*, 2003). To attain these concentrations, silica solutions of 540 PPM and 1800 PPM Si respectively, were prepared by the addition of $Na_2SiO_3{\cdot}5H_2O$ to anoxic 18 MΩ water (with an equilibrium pH > 11). The silica solutions were stored alkaline at 90 °C until the moment of initiation, to ensure that the silica remained entirely monomeric and molybdate-reactive.

Experiments were initiated by the injection of 10 mL of the appropriate concentrated silica solution, and were immediately adjusted to pH 6.0 by the addition of 1 M HCl (1 M NaOH for silica free controls) in an amount predetermined for each combination of silica solution and media. Biotic experiments received 10 mL of inoculum from the appropriate cell lines grown in modified MSH media. Conditions established for H_2-oxidation received H_2 gas to a pressure of 138 kPa as a final step. Experiments were performed in two ways: (1) with initiation occurring immediately upon inoculation, and (2) with initiation occurring approximately 20 h after inoculation, to characterize behaviour for both actively growing and stationary phase cultures, respectively. Heat-killed experiments were similarly prepared, but were autoclaved for 25 min at 121 °C approximately 20 h after inoculation, and immediately initiated upon cooling to 68 °C.

Abiotic controls received an injection of the appropriate sterile media preheated to 68 °C, and silica-free controls received injections of anoxic 18 MΩ water preheated to 90 °C. All media were preheated to 68 °C prior to inoculation, and all experiments were performed in triplicate. Samples (2.5 mL) were removed by syringe after gentle inversion at various intervals for (1) visualization by transmission electron microscopy (TEM), (2) analysis by modified Coomassie protein assay and (3) analysis using two molybdosilicate assays. Eight hundred microlitres of subsamples were preserved in 3% glutaraldehyde and stored at 4 °C until preparation for TEM. Additional experiments were terminated mid-experiment to evaluate pH change over time, and for all experiments the final pH was recorded. For all graphs, error bars representing 95% confidence intervals (CIs) were calculated as Z = 1.96 multiplied by the standard error of the mean about each triplicate data point.

Colorimetric techniques

Colorimetry was performed using a Beckman DU520 UV/VIS spectrophotometer and plastic cuvettes with a 1-cm path length. Molybdate-reactive silica determinations were performed at the time of sampling.

The heteropoly blue molybdosilicate method (Fanning & Pilson, 1973) was used to measure molybdate-reactive silica concentrations. This technique was chosen because it provides increased sensitivity and resistance to interfering substances over the traditional molybdosilicate yellow technique (Fanning & Pilson, 1973). Immediately upon sampling, 100 µL of sample was serially diluted to a factor of 200, and 2 mL of diluted sample was acidified with 40 µL of 2 M HCl. Eighty microlitres of 0.3 M ammonium molybdate solution (pH~8) was added simultaneously, followed 6 min later by the addition of 80 µL 0.6 M oxalic acid solution to eliminate interference from phosphate. Twelve minutes after the addition of the ammonium molybdate reagent, 80 µL of reducing agent solution (containing 0.01 M 1-amino-2-naphthol-4-sulphonic acid, 0.04 M Na_2SO_3, and 1.44 M $NaHSO_3$) was added, and the absorbance was read at 815 nm after 18 min.

In order to assess total silica concentrations, molybdate-unreactive silica was rendered molybdate-reactive by heated digestion with $NaHCO_3$ and H_2SO_4 (using methodology of Fanning & Pilson, 1973), and analysed using the heteropoly blue technique as above. The two methods were standardized against a 1000 PPM Si atomic absorption reference standard (Fisher Chemicals, Fairlawn, NJ), and the analytical uncertainties (2 σ) for the two silica determinations were ± 2.6% and ± 2.9%, respectively.

Protein concentrations were determined using the Coomassie Plus protein assay reagent kit (Pierce Biotechnology, Rockford, IL) and standardized to known concentrations of bovine serum albumin diluted into the medium of interest. A 0.5-mL aliquot of room temperature reagent was added to 1.0 mL of undiluted sample, incubated for 10 min, and the absorbance was read at 595 nm. This protocol produced linear absorbance values over a 1–25 μg mL^{-1} range for bovine serum albumin, and proved more sensitive for the experimental conditions than the suggested reagent to a sample ratio of 1 : 1.

Sample preparation for TEM

Samples stored in 3% glutaraldehyde were washed three times in PBS buffer, remaining in the buffer for 10 min between each wash. Samples were then stained with 2% osmic acid for 2 h, after which, the PBS washes were repeated. Serial dehydration was performed using ethanol concentrations increasing in 10% increments, from 20% to 100%, with an incubation of 15 min at each increment. Samples were further incubated in 1 : 1 propylene oxide/100% ethanol, followed by propylene oxide, for 15 min each, after which, they were left in 1 : 1 propylene oxide/spurr resin for 12 h. Samples were embedded in spurr resin and cured in a vacuum oven at 60 °C for 24 h, after which, they were sectioned using a Reichert-Jung ultracut microtome, collected on copper grids and examined with a Morgani 268 Philips electron microscope. Selected grids were subsequently stained with 1% uranyl acetate to enhance the contrast of cell material.

Acid-base titrations

A 50-mL aliquot of *S. azorense* culture, grown under silica-free conditions limited to H_2-oxidation, was centrifuged at 20 °C for 8 min at 10 400 g, and washed four times in 100 mL of 18 MΩ water with similar centrifugation. The pellet was resuspended in 0.01 M KNO_3 (pH ~ 5.5), transferred to a 100 mL titration flask containing 200 μL of 0.2 M HNO_3, and diluted with 0.01 M KNO_3 to a final volume of 50 mL. An airtight lid, prefitted with an N_2 gas line and a precalibrated pH electrode connected to an autotitrator (Metrohm Titrino GP 736, Metrohm Ltd., Herisau, Switzerland), was installed on the titration flask and the system was henceforth continuously purged with N_2 to prevent carbonate formation from CO_2 dissolution. The system was allowed to equilibrate until fluctuations in the pH reading were less than 0.5 mV min^{-1} for at least 25 min. Titrations were performed using the autotitrator's burette containing carbonate-free 0.01 M NaOH, delivered into the acidified suspension to increase the pH by increments of approximately 0.1 unit, over a range of 3–12 pH units. Ligand concentrations and disassociation constants (expressed as $pK_a = -\log_{10}K_a$) were genrated from the titration data using the Linear Programming Method (LPM) (Sokolov *et al.*, 2001), and control titrations were performed in the absence of *S. azorense*.

RESULTS

Growth under hydrogen-oxidizing conditions

Conditions undersaturated with respect to amorphous silica

In solutions with 90 PPM Si, the stability of monomeric silica and the absence of bacterial adsorption are illustrated by unchanging molybdate-reactive Si concentrations (Fig. 1). Although molybdosilicate colourimetry is often used to infer monomeric silica concentrations, silica polymers of low molecular weight may also remain molybdate-reactive upon polymerization, and therefore, such assays cannot yield true monomeric concentrations (Fannin *et al.*, 1973). Results obtained reflect the speciation between monomers and small polymers that are molybdate-reactive, and larger polymers, colloids and precipitates that are rendered molybdate-unreactive by increased polymer length. As a result, the proportion of molybdate-reactive to molybdate-unreactive Si species is not only dependent on the conditions governing polymerization, but to some degree on the assay protocol itself (i.e. reagent concentrations, sample dilutions). A pH change over the course of the experiment was observed in the biotic experiments, corresponding to an average increase of approximately 0.3 pH units after 48 h.

The abiotic controls mimic the biological experiments in that they show no removal of molybdate-reactive Si from undersaturated solutions. The slight covariations of Si

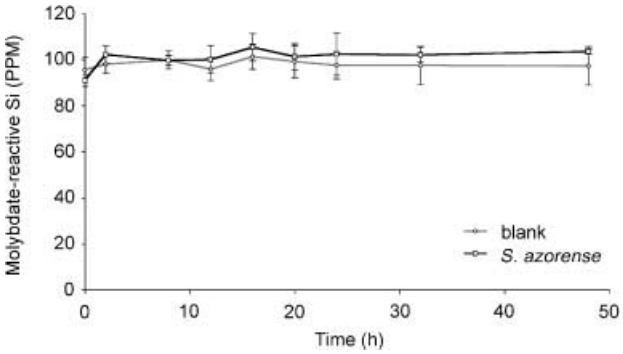

Fig. 1 Si concentrations in silica undersaturated solution. The blank controls contain no bacteria, and *Sulfurihydrogenibium azorense* was grown under hydrogen-oxidizing conditions and passed through exponential phase growth during the course of the experiment. Points are the average of triplicates, and error bars represent 95% confidence intervals.

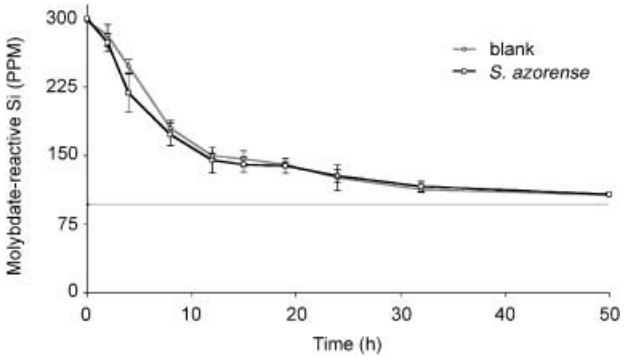

Fig. 2 Molybdate-reactive Si concentrations in silica supersaturated solution. The blank controls (without bacteria) illustrate spontaneous polymerization. *Sulfurihydrogenibium azorense* grew exponentially by hydrogen oxidation during the course of the experiment. Points are the average of triplicates, error bars represent 95% confidence intervals, and the grey line indicates the solubility of silica for the experimental conditions.

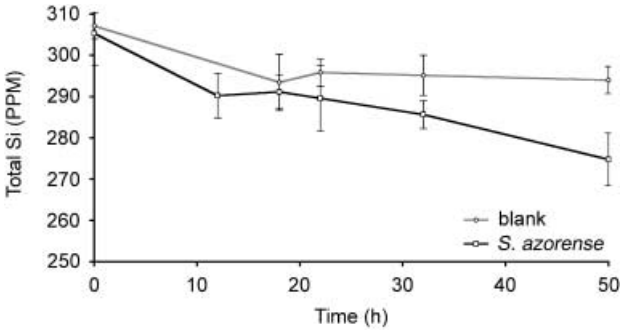

Fig. 3 Total silica concentrations over time in silica supersaturated experiments containing *Sulfurihydrogenibium azorense* grown by hydrogen oxidation. Points are the average of triplicates, and error bars reflect 95% confidence intervals.

concentration observed only in Fig. 1 were likely due to an inconsistency in the molybdosilicate technique specific to the time of sampling, as each culture and respective abiotic control were initiated, sampled and analysed in parallel.

Conditions supersaturated with respect to amorphous silica

In the 300 PPM Si experiments, spontaneous polymerization occurred in both abiotic and biotic experiments after the addition of silica and pH neutralization. This is indicated by the decrease in the molybdate-reactive Si concentrations over time in both sets of experiments (Fig. 2). In the abiotic experiments, approximately 50% of the monomeric silica was rendered unreactive to the molydosilicate technique within 15 h, after which, changes in the molybdate-reactive Si concentrations occurred slowly. These results highlight the strong chemical driving force for silica polymerization at high silica concentrations.

H_2-oxidizing cells of *S. azorense*, either growing exponentially (Fig. 2) or in stationary phase (results not shown) had a negligible effect on both the rate and extent of silica polymerization. Nonetheless, exponentially growing cells did immobilize up to 29 PPM Si (~12%) of the molybdate-unreactive silica, corresponding to approximately 7% of the total silica, after 50 h of exposure (Fig. 3). By comparison, any silica retained on the serum bottles in the abiotic experiments was within the experimental error of the molybdosilicate technique.

Protein concentrations

While difficult to quantify, EPS production was visually observed to be most extensive under the conditions of H_2-oxidation, with biofilm completely covering the area of the liquid–gas interface (230 cm^2) after 24 h. With experimental conditions of S°-oxidation, however, the production of EPS appeared less important and was largely limited to association with the insoluble elemental sulphur. Under these conditions, the protein concentrations often fell outside the linear range of the Coomassie assay, hence, there was no accurate means of protein quantification in these experiments. In all cases, growth was confirmed by light microscopy, although the biofilm-forming nature of growth made the monitoring of growth by cell counting unreliable.

Protein concentrations were monitored in the H_2-oxidizing experiments after the addition of silica solution. Experiments initiated during stationary phase growth were also monitored for protein concentrations after the injection of silica solution, with no increase in protein concentration or significant difference relative to silica-free controls observed (results not shown). For all exponentially growing cultures, a rapid increase in protein concentration was measured (Fig. 4), corresponding temporally to the onset of visible biofilm accumulation.

For experiments containing 0 and 90 PPM Si, exponential growth began approximately 12 h after inoculation, resulting in similar maximum protein concentrations (Fig. 4). The highest protein concentrations attained in the 0 PPM Si cultures occurred slightly later compared to those containing 90 PPM Si. This corresponded to a more rapid increase in protein

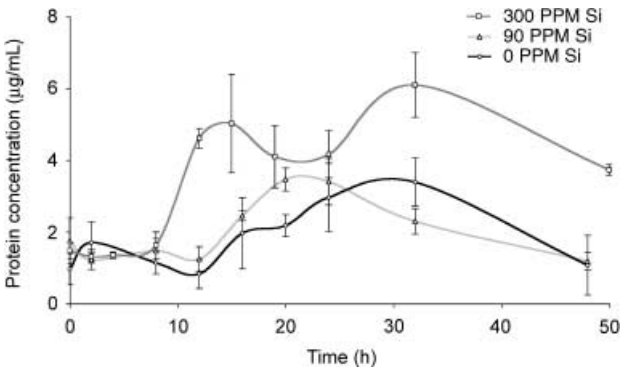

Fig. 4 Protein concentrations in hydrogen-oxidizing batch cultures of *Sulfurihydrogenibium azorense*, plotted as a function of time and at various Si concentrations. Graph begins with the simultaneous inoculation and silica solution injection. Points are the average of triplicates, and error bars indicate the 95% confidence intervals.

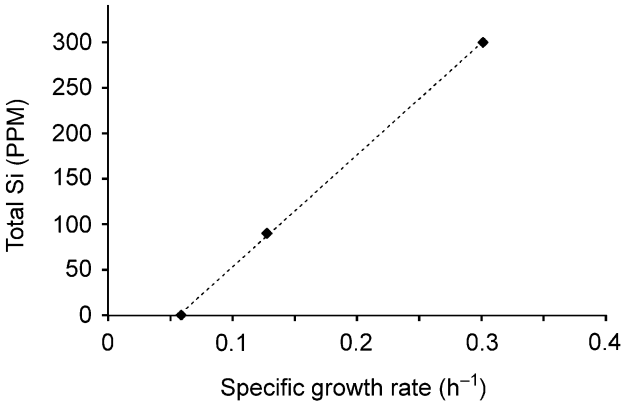

Fig. 5 The relationship of maximum growth rate achieved to Si concentration. The highest growth rate between two data points was chosen for any Si concentration, and fit is $R^2 = 0.9997$.

concentration in the 90 PPM Si cultures. For cultures containing 300 PPM Si, an even more rapid increase in protein concentration was observed, occurring approximately 4 h earlier than for cultures containing either 90 or 0 PPM Si. The unusual decrease and subsequent increase in protein concentration beginning at 14 h was observed for all of the triplicates containing 300 PPM Si. It was determined that such high silica concentrations had a negligible effect on the linear response of the Coomassie assay as performed.

Specific growth rates based on protein concentrations were calculated as follows:

$$\text{Specific growth rate } (\text{h}^{-1}) = \frac{\ln c_1 - \ln c_2}{t_1 - t_2}$$

where c represents the concentration (in this case, μg/mL BSA protein equivalent) at time t.

A plot of the maximum growth rate achieved vs. Si concentration (Fig. 5) revealed a linear relationship between growth rate and Si concentration of good fit ($R^2 = 0.9997$), although on the basis of three silica concentrations confidence in the relationship should be reserved.

Growth under sulphur-oxidizing conditions

Conditions undersaturated with respect to amorphous silica

Neither exponentially growing cells of *S. azorense* metabolizing by S°-oxidation nor their abiotic controls promoted the adsorption or polymerization of monomeric silica from solutions undersaturated with respect to amorphous silica (Fig. 6). The pH decreased a maximum of 0.3 pH units in concordance with the equation for metabolism by S°-oxidation:

$$\text{S}^\circ + 3/2\text{O}_2 + \text{H}_2\text{O} \Leftrightarrow \text{SO}_4^{2-} + 2\text{H}^+$$

Conditions supersaturated with respect to amorphous silica

The rate and extent of polymerization, as evaluated by the decrease in molybdate-reactive Si, was not affected by the presence of

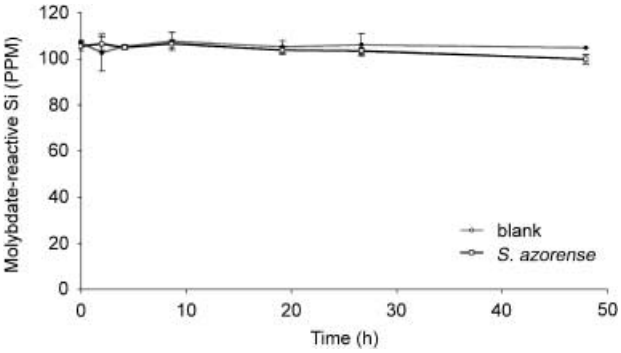

Fig. 6 Si concentrations in silica undersaturated solution. The blank experiments contain no bacteria, and *Sulfurihydrogenibium azorense* grew by sulphur-oxidation through exponential phase during the course of the experiment. Points are the average of triplicates, and error bars represent 95% confidence intervals.

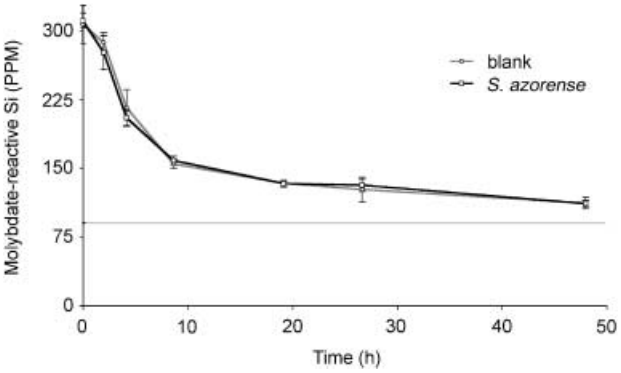

Fig. 7 Molybdate-reactive Si concentrations in silica supersaturated solution. *Sulfurihydrogenibium azorense* grew exponentially by sulphur-oxidation during the course of the experiment, and yielded silica polymerization comparable to the bacteria-free blanks. Points are the average of triplicates, error bars represent 95% confidence intervals, and the grey line indicates the solubility of silica for the experimental conditions.

S. azorense growing in either stationary phase (unpublished) or exponential phase (Fig. 7). In both the biotic and abiotic experiments, silica polymerization occurred spontaneously with a rate and magnitude comparable to those observed under conditions of H_2-oxidation. Again, a maximum pH decrease of 0.3 pH units was observed in experiments containing *S. azorense* passing through exponential phase growth, with apparently little affect on the depletion of molybdate-reactive Si. Growth of *S. azorense* occurs between pH 5.5 and 7 (Aguiar *et al.*, 2004).

Unlike the case of H_2-oxidation, no biological removal of silica outside of the experimental uncertainty was observed 50 h after the injection of concentrated silica solution into cultures containing exponential phase or stationary phase cultures of *S. azorense* grown via S°-oxidation.

Acid-base titration

The acid-base titrations were performed on whole cells, without discrimination between EPS and cell surface functional groups,

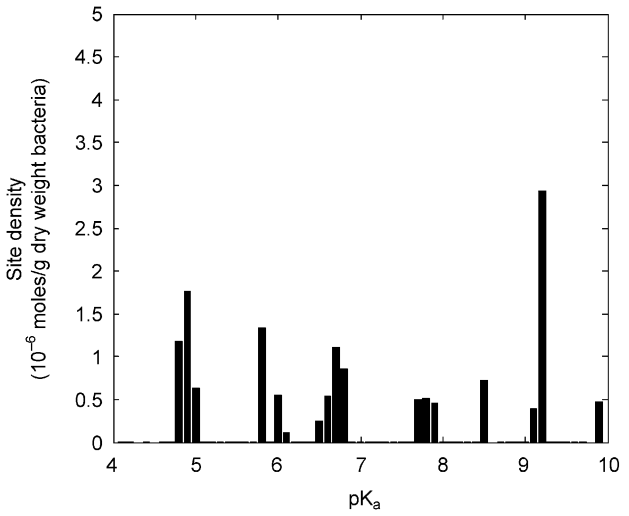

Fig. 8 Acid-base titration of *Sulfurihydrogenibium azorense* whole culture (cells + biofilm) grown in silica-free media under hydrogen-oxidizing conditions. Cluster A is suggested to represent carboxyl groups, clusters B and C, carboxyl or phosphoryl groups, cluster D, phosphoryl groups, and clusters E and F, amine groups.

through a pH range of 3–12. The discrete pK$_a$ plot generated by LPM has a number of distinct pK$_a$ clusters (Fig. 8) to which functional groups may be assigned following the criteria outlined by Phoenix *et al.* (2002). Cluster A falls within the pH range over which carboxylic functional groups deprotonate. Clusters B and C may represent either carboxylic or phosphoryl groups, although cluster B is closer to the former's range, and cluster C, the latter. Cluster D likely represents phosphoryl groups, and cluster E, amine groups, as in Phoenix *et al.* (2002). Although hydroxyl groups are an abundant component of the hydrated polysaccharides that comprise EPS (Whitfield & Keenleyside, 1995), they would not have contributed to the titration profiles because they do not deprotonate at pH values <11, with the exception of phenolic sites (Perdue *et al.*, 1985).

The most striking pK$_a$ peak (cluster F) can be assigned to amine functional groups. It appears that this functional group is present in greater abundance relative to any other group, as evident from the height of the site density peaks. This observation is interesting in that the amine peak is somewhat more pronounced than that observed with either *Bacillus subtilis* or *Calothrix* sp. Overall, the titration data indicate that on a dry weight basis, the surfaces of *S. azorense* are less reactive than those of *Calothrix* sp. whole filaments. This is not surprising considering that the hydroxyl-rich EPS would contribute significant weight while not contributing exchangeable protons over the pH range of the titrations.

DISCUSSION

The role of *Sulfurihydrogenibium azorense* during silicification

In recent years, a number of experimental studies have attempted to elucidate the bacterial role in silicification (see Konhauser *et al.*, 2004 for references). In this case, by considering both molybdate-reactive and total silica concentrations in the absence of silica flocculation, the extent of microbial silicification under the experimental conditions is be constrained to the adsorption of homogenously nucleated silica polymers and colloids, as discussed below.

Adsorption from silica-undersaturated conditions

Urrutia & Beveridge (1993) proposed that microbially mediated silica precipitation might occur by silicate anion adsorption onto specific organic surface sites, such as positively charged amine functional groups. This has been proven unlikely to occur from silica-undersaturated solution, as monomeric silica exists primarily as neutrally charged H_4SiO_4 at circum-neutral pH conditions (Iler, 1979). Under alkaline (approximately pH > 9.5) conditions, where anionic silica species may dominate the aqueous Si phase (Iler, 1979), bacterial cell walls are highly electronegative due to the deprotonation of carboxyl and phosphoryl groups, which invariably would inhibit electrostatic attraction between aqueous $H_4SiO_3^-$ and any positively charged amine groups that might exist under these conditions. The absence of monomeric silica adsorption to bacterial cell surfaces has recently been confirmed by several papers. Using *B. subtilis* (Fein *et al.*, 2002; Phoenix *et al.*, 2003) and *Calothrix* sp. (Yee *et al.*, 2003; Benning *et al.*, 2004), it was shown that adsorption at circum-neutral pH is unlikely to remove significant quantities of soluble silica, as the affinity of monomeric silica for these bacterial surfaces was found to be negligible under these conditions.

In a near-neutral hot spring environment, higher temperatures allow for elevated concentrations of silica to be achieved, while still maintaining silica-undersaturated conditions; the solubility of amorphous silica at pH 6 is 96 PPM Si at 68 °C vs. ~60 PPM Si at 30 °C. By investigating the adsorption of silica from only slightly undersaturated solutions, reactions of even weaker affinity (at a very slight cost of colourimetric resolution) between monomeric silica species and bacterial cell surfaces could potentially be revealed. The use of the heteropoly blue molybdosilicate method in this study provided increased resolution over the molybdate yellow technique used in similar studies (e.g. Yee *et al.*, 2003). With the increased sensitivity, the results confirm earlier observations; the adsorption of monomeric silica from undersaturated solutions does not significantly contribute to the bacterial silicification process.

Polymerization and precipitation in silica-supersaturated solutions

At hot springs, the sudden cooling of ascending hydrothermal fluids, decompressional boiling and degassing, fluid mixing, pH changes, and evaporation all contribute to a sudden increase in saturation state with respect to amorphous silica, leading to homogeneous nucleation (Fournier, 1985). In addition, it has been suggested that the high reactivity of microbial surfaces can lower activation energy barriers and promote the heterogeneous

nucleation of amorphous silica (Schultze-Lam *et al.*, 1995; Konhauser & Ferris, 1996; Konhauser *et al.*, 2001). Indeed, the separation of microbial and inorganic influences on basic geochemical processes remains a primary goal of geomicrobiology. From our results, it might appear that species such *S. azorense* would have a limited role in this regard because they do not affect the rate or magnitude of silica polymerization outside of experimental uncertainty, regardless of metabolism or growth phase. This is not surprising; in many hot springs, the high silica concentrations, continuous effluent flow, and rapid changes in silica solubility will result in the predominance of homogenous nucleation. Silica species heterogeneously nucleated on bacterial surfaces would be quantitatively insignificant, and such was the case during this study, where silica polymerization rates were unaffected by the presence of bacteria.

Without affecting the kinetics of polymerization, the biological immobilization of silica under the experimental conditions must have been limited to silica phases that are molybdate-unreactive (i.e. the large polymer, colloidal, and/or precipitate phases). Westall *et al.* (1995) have suggested that it is the polymeric/colloidal fraction that is bound to bacterial surfaces, and Heaney & Yates (1998) have discussed relations to hydrogen-bonding. Colloidal interaction is supported by frequent observations by electron microscopy that *in situ* bacterial silicification occurs by the immobilization of preformed nanosize siliceous spherules (Schultze-Lam *et al.*, 1995; Konhauser & Ferris, 1996; Phoenix *et al.*, 2000). The factors driving changes in saturation state will ultimately control the distribution of silica between monomeric, polymeric/colloidal, and precipitate phases, and therefore determine the proportion available for biomineralization. For example, visual precipitate accumulation was not noted in the experiments containing 300 PPM Si after 50 h, allowing for a greater polymeric/colloidal silica fraction. Correspondingly, TEM images (Fig. 9, discussed below) indicate that silicification occurred by the adsorption of nanosize silica spheres. Colorimetric and visual observations indicate that the biologically removed silica was neither molybdate-reactive nor precipitate at the time of adsorption, and that the silica must have been in the polymeric/colloidal form.

In the absence of significant heterogeneous nucleation, it follows that the immobilization of negatively charged, molybdate-unreactive silica species by bacterial surfaces is decoupled from the initial polymerization process. Although Yee *et al.* (2003) did not consider silica immobilization independent of polymerization, it has been subsequently demonstrated that in silica-supersaturated solutions, *Calothrix* sp. removed only approximately 3% more silica than bacteria-free systems, at the same time, becoming encrusted in amorphous silica precipitate (Benning *et al.*, 2004). However, Phoenix *et al.* (2003) found that *B. subtilis* failed to remove significant quantities of Si from silica-supersaturated solutions without the aid of cation bridging. With an apparent silica removal of approximately 7% for cells metabolizing via H_2-oxidation, our results indicate that *S. azorense* immobilized slightly more silica than *Calothrix* sp.

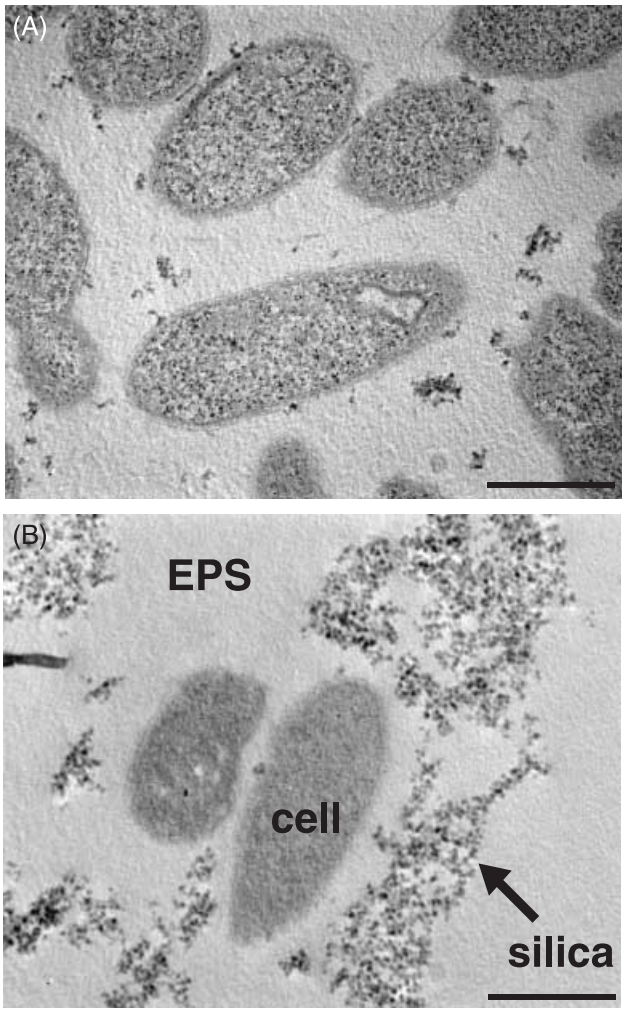

Fig. 9 TEM images of *Sulfurihydrogenibium azorense* grown metabolizing by hydrogen oxidation in silica supersaturated solution. Scale bars represent 500 nm. (A) Cells are observed at high density encased in the biofilm matrix. Siliceous spheres have no significant cell association. (B) After 50 h in silica-supersaturated solution, nanoscale silica spheres had been restricted from cell surfaces. Colloidal silica, EPS, and cells are labelled. Staining with uranyl acetate was not performed for the preparation imaged in (B).

from a 300 PPM Si solution, albeit at lower pH, lower biomass and higher temperature. The ability of a single bacterial cell to immobilize numerous large, preformed colloids, especially with the aid of the increased surface area offered by various exopolymers secreted by a wide range of thermophiles and mesophiles, might serve to explain potential discrepancies between *in situ* microbial silicification rates and laboratory simulations.

While the differing factors of pH and temperature between the Benning *et al.* (2004) study and our own experimental conditions undoubtedly yielded a colloidal fraction of different size and charge that is difficult to compare, at higher temperatures, more silica will remain in the solution. At concentrations supersaturated with respect to amorphous silica, the partitioning

of the silica phases will favour the polymeric/colloidal rather than precipitate fraction, relative to lower temperature systems. The obvious implication is that, with interactions between microbes and silica initially limited to the colloidal phase, the silicification of micro-organisms at higher temperatures would be accelerated relative to their counterparts inhabiting mesophilic niches, and even appear spontaneous at conditions where silica flocculation or precipitation is insignificant. Importantly, if the forces driving bacterial silicification are more important at higher temperatures, then micro-organisms inhabiting such niches (i.e. thermophiles) would need to be tolerant of the mineralization process relative to mesophilic counterparts.

The effects of chemolithoautotrophic metabolism

The different combinations of electron donors and acceptors used by *S. azorense* affected the amount of silica retained in the biomass. As discussed above, cells grown as H_2-oxidizers removed 7% of silica from solution, whereas the S°-oxidizers showed no significant quantity of silica removed from solution. This pattern may reflect a number of factors.

(1) *Sulfurihydrogenibium azorense* was observed to produce negligible EPS while metabolizing via S°-oxidation, and grew largely attached to the insoluble elemental sulphur particles. It appeared as though growth was less favourable under S°-oxidizing conditions, as indicated by unpublished cell counts, and lower to undetectable protein concentrations.

(2) Cell surface functional groups, and hence surface charge, may be characteristically different for cells grown under different metabolisms. The cyanobacterium *Calothrix* sp. varies sheath production under different growth conditions (Benning *et al.*, 2004), and the sheath and cell wall ligands contribute differently to overall surface charge (Phoenix *et al.*, 2002). It is also known that surface charge can respond to changes in growth phase (Daughney *et al.*, 2001), and it would be surprising if changes in metabolic conditions did not exert a similar effect.

(3) Proton extrusion (see equation 1) altered the pH of the batch culture, thereby potentially inhibiting silica adsorption to *S. azorense*. The pH differences between experiments containing *S. azorense* metabolizing via H_2-oxidation and S°-oxidation are indeed significant, with the H^+ concentration increased by a factor of 4 in the S°-oxidizing cultures, relative to H_2-oxidizing cultures. In highly acidic solutions, protons inhibit the silica polymerization process (Rothbaum *et al.*, 1979), but our experiments were run at pH 6.0 and thus it is unlikely that protons inhibited the bulk polymerization. No effect on silica polymerization could be attributed to the pH difference between abiotic and bacterial experiments. However, we cannot be certain that despite minimal changes to the bulk pH, there may have been immeasurable but drastic effects in the local microenvironment around the cell as the result of proton extrusion.

Irrespective of whether surface area, surface chemistry, or pH were the most significant factors in the metabolic effect, TEM micrographs of *S. azorense* grown by S°-oxidation in solutions supersaturated with respect to amorphous silica did not reveal silica aggregates on the cell surfaces. This result suggests that polymerization in those experiments did not lead to mineral formation. It is interesting to note that a phylogenetic survey of seven silica-depositing hot springs in Yellowstone National Park indicated that H_2-oxidation was the primary means of productivity (Hugenholtz *et al.*, 1998). It is possible that the retention of silica associated with *S. azorense* during growth by H_2-oxidation may represent acclimatization to the increased silica deposition rates associated with conditions favouring that metabolism. However, further surveys of the primary means of productivity in silica-depositing hot springs is required for any correlation to be stated with confidence. Regardless, it appears that H_2-oxidation in silica-supersaturated springs will allow for a microbial contribution towards siliceous sinter formation. Our results suggest that specific study of microenvironmental regulations by chemolithoautotrophs, which we have demonstrated here to be viable in heavily mineralizing fluids, would undoubtedly yield important insights regarding ancient silicification.

The response of *Sulfurihydrogenibium azorense* to silicification

It has previously been demonstrated that *Calothrix* sp. can perform photosynthesis and remain viable when encrusted in a matrix of amorphous silica (Phoenix *et al.*, 2000). It can do so because it produces a thick, electroneutral sheath that restricts silicification to the cell's outer periphery, and prevents cytoplasmic mineralization (Phoenix *et al.*, 2002). Sheath thickening during silicification has been implicated using synchrotron radiation Fourier-transform infra-red (SR-FTIR) studies on individual silicifying *Calothrix* filaments (Benning *et al.*, 2004). We present here preliminary findings that the EPS extruded by *S. azorense* could potentially serve to restrict silicification in an analogous fashion.

The restriction of silicification to EPS

Transmission electron micrographs of *S. azorense*, grown under conditions limited to H_2-oxidation and supersaturated with respect to amorphous silica, reveal the accumulation of spheroidal silica aggregates of ~20 nm in diameter after 50 h. The close proximity of the cells (Fig. 9A) illustrates their aggregation in the biofilm matrix, yet the silica spheres were generally not observed in direct association with the cell wall. Instead, silicification is limited to the EPS surrounding the cells (Fig. 9B). In some cases, the accumulation of silica spheres could be observed to follow the shape of cell contours, whereas restricted to a distance of ~70 nm from the cell wall. The influence of preparatory procedures on TEM representation of the initial stages of laboratory silicification is unknown, although it is unlikely that such preparation resulted in the removal of silica from bacterial cell walls as various studies have demonstrated colloidal silica in association with cell walls using similar

preparatory techniques (Konhauser & Ferris, 1996; Phoenix *et al.*, 2000). Despite being permeable to soluble ions, the lack of silica accumulation on the cell wall suggests that the cell proper has the means of protecting itself from silicification, possibly via the continual secretion of a protective EPS.

Fluxes in protein concentration during batch growth

Protein concentrations were observed to increase earlier, more rapidly, and achieve higher maximum values in cultures containing 300 PPM Si. Visually and in light micrographs, more extensive biofilm was also produced in those cultures. How the two observations are related cannot be demonstrated with our methods and results, but involves either (1) the cell doubling times being accelerated in the presence of silica or (2) individual cells producing more protein-containing product(s) without altering cell division rates. The answer likely lies in a combination of the two possibilities. While we are not aware of any nutritional role for Si during chemoautotrophic growth, the stress-induced production of biofilm has been documented for hyperthermophilic archaea and bacteria. For example, *Thermococcus litoralis* and *Thermotoga maritima* increase their production of exopolysaccharides with increasing dilution rates in continuous culture (Rinker & Kelly, 2000). *Archaeoglobus fulgidus* extrudes additional polysaccharides during pH, temperature and metal/solute stress (LaPaglia & Hartzell, 1997). Furthermore, the relationship between growth rate and Si concentration does not appear to follow Monod kinetics at high Si concentrations (i.e. the plot remains first order at high Si), suggesting that the increased protein production at high Si concentration is not due to any nutritional role.

As biofilms are chemically heterogeneous assemblages containing and, in some cases, dominated by acidic polypeptides (Beveridge, 1981), we propose that the increased protein concentrations observed may be attributed in some part to increased EPS production. Importantly, a protein-rich EPS is supported by the potentiometric titrations; amine functional groups are overly abundant on a dry weight basis, and the EPS contributes a significant portion of that weight. Differential protein expression (Oosthuizen *et al.*, 2002) as well as differential gene expression (Prigent-Combaret *et al.*, 1999; Whiteley *et al.*, 2001) has been previously indicated during biofilm formation. If EPS extrusion is a coping mechanism against cell silicification, biofilm-forming micro-organisms would be able to tolerate environments where silica is precipitated more rapidly, relative to species with exposed cell walls.

A potential mechanism of polymeric/colloidal silica immobilization

To date, two mechanisms for microbial silicification have been invoked. In the first instance, *B. subtilis* has been used to illustrate the case for cation-bridging, where the carboxyl-dominated cell wall of *B. subtilis* makes it unable to bind silica in the absence of a cation bridge due to electrostatic repulsion (Fein *et al.*, 2002; Phoenix *et al.*, 2003). Second, immobilization by hydrogen-bonding has been implicated for cyanobacterial silicification. For example, acid-base titrations of *Calothrix* reveal that the sheath is largely electroneutral, and the polysaccharides it contains provide abundant hydroxylgroups sites to hydrogen-bond with the hydroxyl groups of polymeric/colloidal silica (Phoenix *et al.*, 2002).

The immobilization of silica as colloids, as well the increase in protein concentration observed during growth at states of silica supersaturation, points toward a mechanism for silica–microbe interaction previously considered unimportant. Urrutia & Beveridge (1993) originally proposed that micro-organisms remove anionic silica by adsorption onto positively charged amine groups. For monomeric silica species, this has proven unlikely due to of the lack of silicate anions at pH values below 9, and no experiments to date have revealed a bacterial removal of monomeric silica in the absence of a cation bridge. In the case of *S. azorense,* hydrogen-bonding between a polysaccharidic EPS and the electronegative and neutral silica species cannot be discounted in explaining the silica immobilization observed. However, binding is electrostatically favoured by the abundant, positively charged amine groups inferred for surfaces of *S. azorense*.

As thermophilic organisms have evolved in niches with solute concentrations typically higher than those of their mesophilic counterparts, and because they have been observed growing in heavily mineralizing hot spring systems, it would be surprising if thermophilic organisms did not employ a more active system for coping with silicification, relative to their mesophilic counterparts. Such a system is implied by the titration and protein concentration data herein, whereby proteins inherently amine-rich, and potentially comprising a significant component of the biofilm matrix, serve to ensure the prevention of cell wall mineralization by providing highly reactive sites where silica can be immobilized and removed in a manner under biological control. While such interaction may ultimately lead to increased silica immobilization, and by deduction, increased rates of sinter formation, it also ultimately prevents the interactions between reactive silica species and the cell surface proper. While experiments ultimately leading to complete permineralization are desirable, our results imply that micro-organisms preventing the silicification of their cell wall, such as *S. azorense*, will less readily become encrusted in a fossilizing silica matrix and therefore will not be preserved in the ancient rock record.

CONCLUSION

In this study, we have found that *S. azorense* remains viable, and can grow exponentially, in the presence of high concentrations of silica. We also demonstrated that *S. azorense* does not adsorb monomeric silica from undersaturated solution, nor does it accelerate the rate or magnitude of polymerization of silica from supersaturated solution during growth by any of its metabolic pathways. There is, however, a metabolic role in silicification,

that being exponentially growing, H_2-oxidizing cultures of *S. azorense* remove small amounts of colloidal silica, whereas those cultures growing by S°-oxidation do not. Moreover, *S. azorense* remained viable in solutions supersaturated with respect to amorphous silica and yielded higher and more rapidly increasing protein concentrations in batch cultures with increasing Si concentration, possibly as a stress response. Although not confirmed quantitatively, biofilm production was visually noted to be more important in such cultures. Acid-base titrations indicate that amine functional groups are highly prominent on the surfaces of *S. azorense*, and likely serve as the ligands involved in silica colloid adsorption within the protein-rich biofilm matrix. Because silicification was observed to be restricted to the EPS, we propose that *S. azorense* may prevent cellular silicification to some degree by producing abundant reactive sites in the biofilm matrix and regulating EPS production appropriately, with potential contributions from metabolic effects. Finally, while we indicate that microbial silicification may lead to increased rates of sinter formation, we speculate that this does not necessarily equate to morphological fossil preservation, and propose that organisms such as *S. azorense* would not be preserved in the fossil record as a consequence of protection from cellular silicification.

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