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Brief Report

Pressure effects on sulfur-oxidizing activity of
Thiobacillus thioparus

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Abstract

Carbon capture and storage technologies are crucial for reducing carbon emission from power plants as a response to global climate change. The CarbFix project (Iceland) aims at examining the geochemical response of injected CO₂ into subsurface reservoirs. The potential role of the subsurface biosphere has been little investigated up to now. Here, we used *Thiobacillus thioparus* that became abundant at the CarbFix1 pilot site after injection of CO₂ and purified geothermal gases in basaltic aquifer at 400–800 m depth (4–8 MPa). The capacity of *T. thioparus* to produce sulfate, through oxidation of thiosulfate, was measured by Raman spectroscopy as a function of pressure up to 10 MPa. The results show that the growth and metabolic activity of *T. thioparus* are influenced by the initial concentration of the electron donor thiosulfate. It grows best at low initial concentration of thiosulfate (here 5 g l⁻¹ or 31.6 mM) and best oxidizes thiosulfate into sulfate at 0.1 MPa with a yield of 14.7 ± 0.5%. Sulfur oxidation stops at 4.3 ± 0.1 MPa (43 bar). This autotrophic specie can thereby react to CO₂ and H₂S injection down to 430 m depth and may contribute to induced biogeochemical cycles during subsurface energy operations.

Introduction

Carbon capture and storage (CCS) technologies hold an important promise for long-term sequestration of anthropogenic CO₂ in the subsurface (Masson-Delmotte *et al.*, 2018). The storage of CO₂ in the form of carbonate minerals in natural basaltic or peridotitic geological formations has a high potential to store CO₂ in terms of volumes, safety and duration (Gíslason *et al.*, 2014; Masson-Delmotte *et al.*, 2018). Once mineralized as carbonate, the CO₂ is quickly immobilized for geological time scales, with negligible risk of return to the atmosphere. This operates successfully in Iceland. The original CarbFix1 industrial pilot experiment injected ca. 230 tons of CO₂ and purified geothermal gases composed of 75% CO₂ + 24.2% H₂S + 0.8% H₂ mixed with water into the subsurface. It was followed by a rapid removal of carbon from the fluid, including mineralization within 2 months after the injection stopped (Gíslason *et al.*, 2014; Sigfusson *et al.*, 2015; Snæbjörnsdóttir *et al.*, 2018). Approximately 165 tons of CO₂ were stored in biomass or precipitated into calcite, indicative of a sequestration efficiency of 72 ± 5% (Pogge von Strandmann *et al.*, 2019). Injection at the CarbFix1 pilot site induced partial dissolution of the basalt along the flow path of the acidic CO₂-rich water and liberated divalent cations into the fluid hence promoting precipitation of carbonate minerals. In 2012, the injection well at the pilot site clogged as a result of microbial activity stimulated by the injection of CO₂-charged water into the basaltic rocks at 350 m depth and temperature of 20–50°C (Gíslason *et al.*, 2018; Snæbjörnsdóttir *et al.*, 2020). As of summer 2014, the project has moved to industrial scale in Iceland and CO₂ and H₂S are mixed with water and directly injected at 700 m depth at a temperature over 250°C, therefore avoiding the stimulation of the subsurface biosphere at the hotter alternative injection site CarbFix2.

Trias *et al.* (2017) showed that subsurface groundwater microbial communities indeed reacted quickly to the anthropogenic injection of acidic CO₂-rich water. Prior to injection in February 2012, basalt mainly hosted heterotrophic bacteria living under aerophilic to microaerophilic conditions. The injection of a fast-flowing CO₂-rich fluid in

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March 2012 provoked the dissolution of the host-basalt, released polyaromatic hydrocarbons (PAHs), Fe^{2+} and other divalent cations, and reduced bacterial diversity. It also induced the development of lithoautotrophic iron-oxidizing *Betaproteobacteria* among which *Gallionellaceae* related species bloomed, and aromatic compound degraders. After 2 months under more anaerobic conditions (May 2012), *Firmicutes* bloomed along with *Thiobacillus* species, suggesting an important role of the latter sulfur-oxidizing bacteria after CO_2 injection into basalt. Among the markers detected by metagenomic analysis, those for sulfur metabolism were well expressed and included markers for sulfur oxidation (Trias *et al.*, 2017).

Unfortunately, we know very little about the potential activity of these sulfur-oxidizing microorganisms under subsurface pressures and whether they are strictly piezosensitive or potentially piezotolerant or piezophilic. Their metabolic activity may be limited to the surface or extend at depth. Sulfur-oxidizing microorganisms are primarily Gram-negative bacteria currently classified as species of the *Thiobacillus*, *Thiomicrospira* (recently divided into *Thiomicrospira*, *Hydrogenovibrio*, and *Thiomicrospira* – see Boden *et al.* 2017a) and *Thiosphaerae* genera, among others. The *Thiobacillus* genus includes obligate autotrophic organisms, which require inorganic carbon as carbon source. *Thiobacilli* may produce sulfuric acid as an oxidation product of thiosulfates, or polythionate including tetrathionate or sulfate to generate metabolic energy. These obligate chemolithoautotrophic betaproteobacteria include *Thiobacillus thioparus* that is the type species of the *Thiobacillus* genus and that we chose as a model to investigate growth and metabolic activity as a function of pressure, in order to evaluate the potential role of *Thiobacillus* species during or after CCS operations (Taylor and Hoare, 1969). *Thiobacillus thioparus* is a *priori* mesophilic and grows optimally at 25–30°C in the presence of oxygen and reduced sulfur compounds such as thiosulfate or tetrathionate and produces sulfur or sulfate under aerobic conditions, which is controlled by the bacterial oxidizing capacity and depends whether oxidation occurs by a complete or incomplete pathway (Houghton *et al.*, 2016). All *T. thioparus* strains are facultative anaerobes, also capable of using nitrate as an electron acceptor instead of oxygen producing nitrite (Orlygsson *et al.*, 2014).

The present study was therefore focused on the aerobic thiosulfate oxidation by a model strain of *T. thioparus* as a function of pressure at 30°C. To avoid bias due to compression and decompression cycles, experiments were performed in a controlled high-pressure device and sulfur metabolites measured *in situ* by Raman spectroscopy.

Results and discussion

In this study, we illustrated for the first time the effects of pressure on sulfate production by the sulfur-oxidizing bacterium *T. thioparus*, which has been identified as an autotrophic bacterium capable of oxidizing both organic and inorganic sulfur compounds (Gu *et al.*, 2018).

Thiosulfate oxidation by T. thioparus at ambient pressure

Growth of *T. thioparus* strain DSM 505 was monitored in glass tubes as a function of the initial concentration of thiosulfate at 30°C, with 5, 10, 15 and 20 g.l^{-1} thiosulfate. Bacterial growth that was quantified by measuring of the optical density at OD₆₀₀ nm of triplicates shows that the higher the thiosulfate concentration, the lower the growth of *T. thioparus* (Supporting Information Fig. S1). We obtained the highest growth at 5 g.l^{-1} of thiosulfate (31.6 mM). This agrees well with the observations of Perez and Matin (1980) who studied the growth of the closely related species *Thiobacillus novellus* on mixotrophic media, using thiosulfate and/or glucose at different concentrations. They observed that the use of thiosulfate as an electron donor decreases the growth rate of *T. novellus* at any concentration tested and concluded to a negative correlation between initial thiosulfate concentration and growth rate although *T. novellus* utilized thiosulfate. Different interpretations were proposed, including the inhibition of growth by small amount of sulfite or the alteration of the membrane respiratory chain (Perez and Matin, 1980).

As for bacterial growth, the highest metabolic activity of *T. thioparus* was measured using the lowest concentration of thiosulfate electron donor at ambient pressure (Table 1). Using an initial concentration of 5 g.l^{-1} (31.6 mM) of thiosulfate, the bacteria produced 9.3 ± 0.1 mM of sulfate after 244 h (10 days) of incubation, achieving an oxidation yield of almost $14.7 \pm 0.5\%$. Based on the calibration performed, the relative change in intensity of the sulfate and thiosulfate Raman peaks shows that the amount of sulfate

Table 1. Sulfate formed and thiosulfate oxidation yield at ambient pressure experiments by *T. thioparus* after 244 h at 30°C.

Initial thiosulfate concentration [$\text{S}_2\text{O}_3^{2-}$] ₀	5 g.l^{-1} 31.6 mM	10 g.l^{-1} 63.2 mM	15 g.l^{-1} 94.8 mM	20 g.l^{-1} 126.4 mM
SO_4^{2-} formed (mM)	9.3 ± 0.1	8.8 ± 0.1	7.7 ± 0.1	6.2 ± 0.1
Oxidation yield (%)	14.7 ± 0.5	7.0 ± 1.0	4.0 ± 0.3	2.4 ± 0.1

that is produced is twice the amount of thiosulfate that is oxidized. The pH, initially adjusted at 6.8, did not significantly change through the experiment. This experiment shows that sulfate in the medium results from the metabolic activity of *T. thioparus* at ambient pressure, since controls performed in the absence of the bacteria do not show any sulfate even after 320 h of incubation. We did not measure intermediate sulfur products, such as tetrathionate, or elemental sulfur, during the experiments, in agreement with Starkey (1935) although emphasized as mandatory by some authors (Boden *et al.*, 2017b).

In situ monitoring of thiosulfate oxidation by *T. thioparus* as a function of pressure

Thiobacillus thioparus was subjected to pressure between 0.5 and 10 MPa in the high-pressure cell and its metabolic activity measured by *in situ* Raman spectroscopy over 316 h (ca. 2 weeks). We used initial concentrations of 5 g l⁻¹ (31.6 mM) of thiosulfate in experiments at 0.5, 1, 4 and 10 MPa and 15 g l⁻¹ (94.9 mM) for pressures of 1.5, 2 and 3 MPa in order to evaluate independently the effect of pressure and metabolite concentration. Supporting Information Figure S2 illustrates the evolution of Raman spectra as *T. thioparus* oxidized thiosulfate into sulfate as a function of time during the experiment carried out at 1 MPa. Spectra are normalized to the intensity of the P(OH)₂ symmetric stretching band of H₂PO₄⁻ at 877 cm⁻¹, which serves as an internal standard in the present experiments (Fig. 1). Spectra exhibit a progressive transformation of thiosulfate in sulfate as a function of time, as shown by the simultaneous increase in intensity of the sharp S—O symmetric

stretching band of sulfate at 980 cm⁻¹ and the decrease in intensity of the broader S—O symmetric stretching band of thiosulfate at 995 cm⁻¹. While exponential growth of *T. thioparus* at ambient pressure starts after a lag time of 100–150 h (Supporting Information Fig. S1), metabolic activity starts quickly without any latency.

Figure 2 displays kinetics of sulfate production by *T. thioparus* in the high-pressure cell from ambient pressure to 10 MPa, at two initial thiosulfate concentrations. This shows that *T. thioparus* is able to produce sulfate up to 4 MPa, and that sulfur oxidation does not occur anymore at 10 MPa. Kinetic data could be adjusted to a first order kinetic reaction:

$$[\text{SO}_4^{2-}] = 2 [\text{S}_2\text{O}_3^{2-}] \times (1 - e^{-kt})$$

with [SO₄²⁻] as the sulfate concentration expressed in mM, [S₂O₃²⁻] as the concentration of thiosulfate oxidized to produce sulfate, expressed in mM (the factor 2 comes from the observed stoichiometry), *k* as the reaction constant in h⁻¹ and *t* the time in hours. The kinetic parameters give the final concentration of sulfate produced after a virtual infinite reaction time and the reaction constant *k*. They are reported in Table 2 as a function of pressure and initial thiosulfate concentration. They show that *T. thioparus* is definitely a pressure-sensitive bacterium as far growth and metabolic activity are concerned. At 0.5 MPa, the rate and yield of sulfur oxidation are already lower than at ambient pressure and the rate of the reaction is limited to one-fourth of the value at ambient pressure in the most favourable conditions with low initial concentration in thiosulfate.

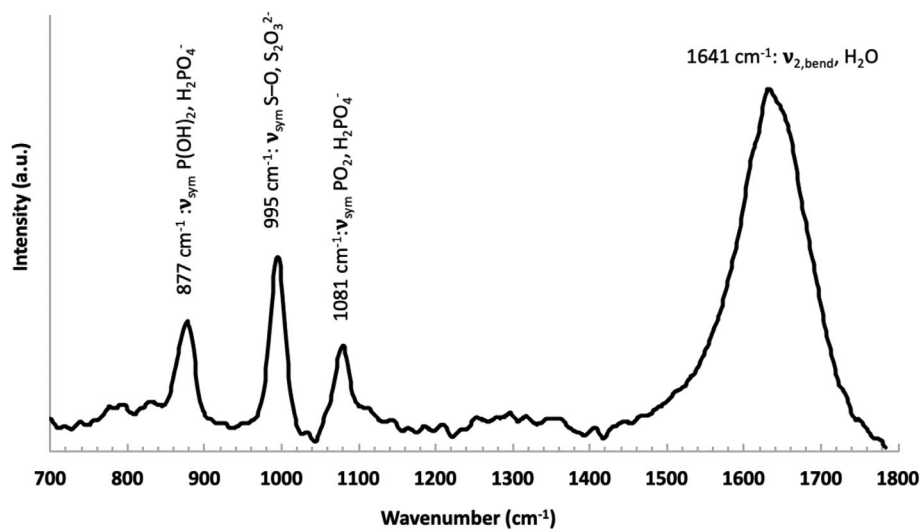


Fig. 1. Raman spectrum of the supernatant of the *T. thioparus* culture medium in 10 ml tubes at ambient conditions over a spectral range that allows the monitoring of the oxidation of thiosulfate into sulfate. The band at 995 cm⁻¹ corresponds to ν_{sym} S—O of thiosulfate. The bands at 877 cm⁻¹ and 1081 cm⁻¹ correspond to ν_{sym} P(OH)₂ of H₂PO₄⁻ and ν_{sym} PO₂ of H₂PO₄⁻, respectively, both serving as internal standard. At 1641 cm⁻¹, one sees the $\nu_{2\text{bend}}$ of H₂O.

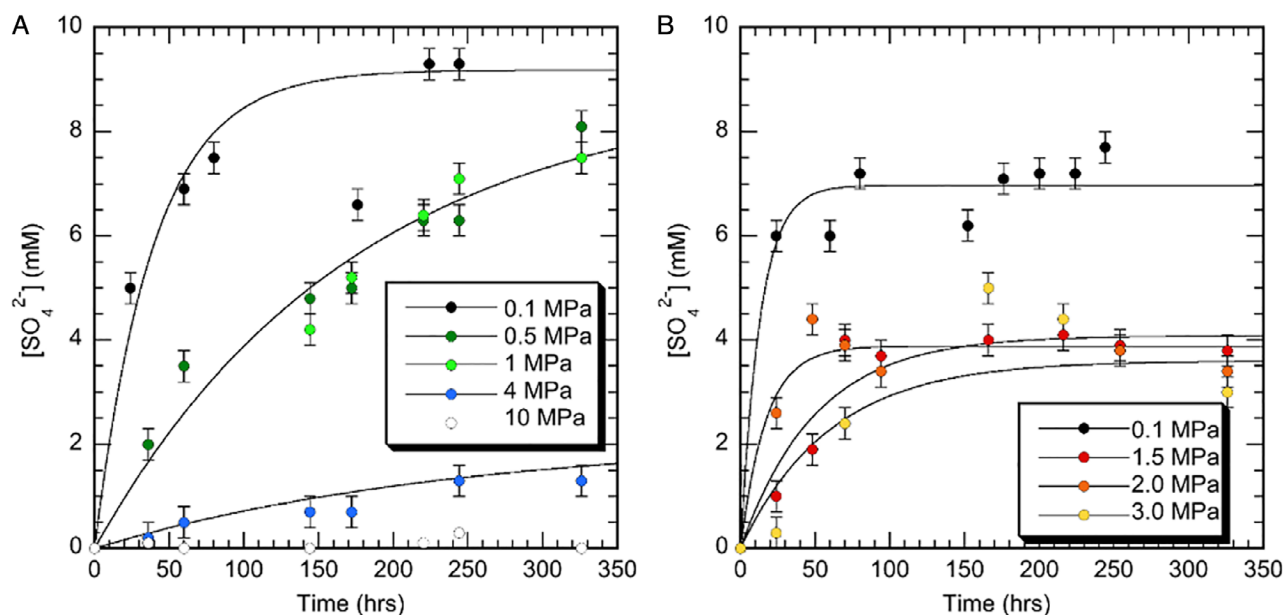


Fig. 2. Kinetics of sulfate production by *T. thioparus* as a function of pressure to 10 MPa with different initial concentrations of thiosulfate $[\text{S}_2\text{O}_3^{2-}]_0$ ranging from 5 g.l^{-1} (A) to 15 g.l^{-1} (B).

Table 2. Sulfate formed as a result of thiosulfate $\text{S}_2\text{O}_3^{2-}$ oxidation by *T. thioparus* as a function of pressure. Sulfate concentration $[\text{SO}_4^{2-}]_{316\text{ h}}$ and oxidation yield were evaluated after 316 h of experiment.

Pressure (MPa)	$[\text{S}_2\text{O}_3^{2-}]_0$ (mM)	$[\text{SO}_4^{2-}]_{316\text{ h}}$ (mM)	Oxidation yield (%)	$[\text{SO}_4^{2-}]_\infty$ (mM)	k (h^{-1})
0.1	31.6	9.3 ± 0.1	14.7 ± 0.5	9.2 ± 0.6	0.025 ± 0.009
0.5	31.6	8.1 ± 0.1	12.8 ± 0.7	8.8 ± 1.2	0.006 ± 0.001
1.0	31.6	7.5 ± 0.1	11.9 ± 0.4	ND	0.004 ± 0.001
4.0	31.6	1.3 ± 0.1	2.0 ± 0.2	2.2 ± 1.9	0.004 ± 0.005
10	31.6	0	0	0	0
0.1	94.9	7.7 ± 0.1	4.0 ± 0.3	6.9 ± 0.2	0.08 ± 0.02
1.5	94.9	3.8 ± 0.1	2.0 ± 0.1	4.1 ± 0.3	0.02 ± 0.005
2.0	94.9	3.4 ± 0.1	3.5 ± 0.1	3.9 ± 0.2	0.06 ± 0.02
3.0	94.9	3.0 ± 0.1	3.1 ± 0.1	3.6 ± 0.6	0.02 ± 0.01

When the experiment was performed with a higher initial concentration of thiosulfate, the initial speed of the reaction was already very low and pressure induced only a slight decrease of the reaction rate. For both initial thiosulfate concentrations, the yield of the oxidation reaction linearly decreased as a function of pressure. This is well illustrated in Fig. 3 that represents the decrease in the oxidation yield as compared to results at ambient pressure. It includes the results of experiments performed at low and high initial concentration of thiosulfate. They are consistent with each other, hence showing that the pressure effect is the same for both initial concentration in thiosulfate. The speed of the reaction decreases at a rate of $0.23(1) \text{ MPa}^{-1}$. At 4 MPa (400 m depth), only 1.3 mM sulfate was formed after 316 h and sulfur oxidation becomes fully ineffective at 4.3(1) MPa (430 m depth) and above as confirmed by the experiments at 10 MPa.

These results are in good agreement with those obtained by Tuttle and Jannasch (1976) on three different *Thiobacillus* sp. marine isolates from deep sea environments (3000–4000 m depth). On the one hand, results slightly differ since Tuttle and Jannasch (1976) performed their experiments over longer periods of time ranging between 22 and 208 days at very low temperature $0\text{--}2^\circ\text{C}$ and their expected oxidized product was polythionate and chiefly tetrathionate, definitely lacking in the present experiments (Gerding and Eriks, 1950). On the other hand, at 25°C and ambient pressure *Thiobacillus* sp. oxidized ca. 10% of the initial 40 mM thiosulfate under oxic conditions (Tuttle and Jannasch, 1976), which is very comparable to 4.7 mM oxidized under closed conditions in the present study (31.6 mM $[\text{S}_2\text{O}_3^{2-}]_0$). At 0.3 MPa, the *Thiobacillus* sp. selected by Tuttle and Jannasch (1976) used ca. 5 mM of thiosulfate over

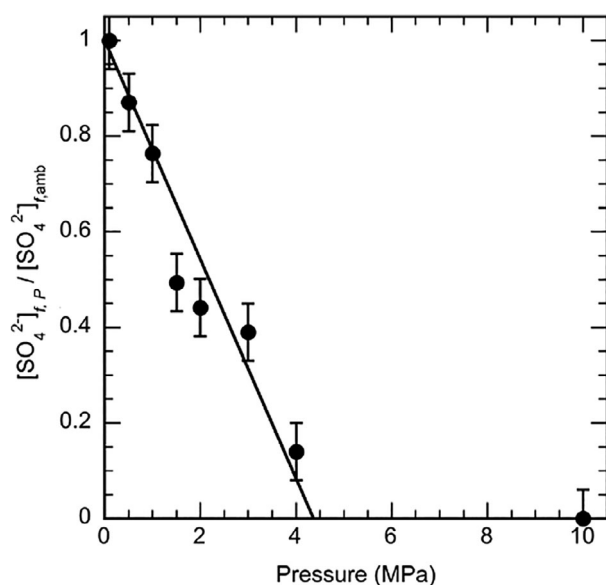


Fig. 3. Decay of sulfate production by *T. thioparus* as a function of pressure, normalized to ambient conditions.

22 days at 0°C and virtually none over 208 days at 53 MPa, 2°C despite their original *in situ* pressure in the ocean was 30–40 MPa (3000–4000 m depth). Again, this is very similar to the results in the present contribution.

Our results also generally agree also with those obtained by Teske *et al.* (2000) who investigated the diversity of thiosulfate-oxidizing bacteria from marine sediments and hydrothermal vents. Depending on the strain, their frequent population of heterotrophic acid-producing thiosulfate oxidizing bacterial strains isolated from slope sediments off the coast of New England produced between 2 and 4.6 mM sulfate over 20 days of aerobic incubation at 15°C and ambient pressure, corresponding to oxidation of approximately 10%–23% of the original 10 mM thiosulfate in the medium, as compared to the 9.3 mM sulfate produced (14.7% oxidation of 31.6 mM thiosulfate) in the medium by autotrophic *T. thioparus* at 30°C. Strains isolated from hydrothermal vents and members of the same cluster as the sediments isolates vents produced 3.45 to 4.98 mM of sulfate (oxidized approximately 17% to 25% of the 10 mM thiosulfate available in the medium), at slightly higher temperatures of 37 and 42°C (Teske *et al.*, 2000). This shows that many different bacterial taxa can significantly oxidize thiosulfate into sulfate under various environmental conditions and play a ubiquitous role in the subsurface sulfur cycle, with some depth limitations.

The kinetics of thiosulfate oxidation by *T. thioparus* do not show any positive response upon moderate pressure as observed for many metabolic or enzymatic processes. The Le Chatelier's principle indeed predicts that the application of pressure shifts an equilibrium toward the state

that occupies a smaller volume, and accelerates processes that involve a transition state with a smaller volume than the ground state (Eisenmenger and Reyes-De-Corcuera, 2009). The present results unfortunately do not bring any further insight on the characteristic of the sulfur oxidizing pathway of *T. thioparus* as in many studies since it is hard to explain the pressure effects on complex metabolic pathways based on a simple volume law (Abe, 2007). As many sulfur-oxidizing Betaproteobacteria, the metabolic pathway for thiosulfate oxidation by *T. thioparus* includes the S₄I pathway that does not necessarily involve tetrathionate (Kelly *et al.*, 1997; Alam *et al.*, 2013) and still holds gaps in its mechanistic understanding and complexity despite recent progress in deciphering the typical regulation elements for periplasmic thiosulfate metabolism in such autotrophic sulfur-oxidizing bacteria involving enzymes located in different compartments of the cell (Wang *et al.*, 2019).

Impacts of the deep biosphere on subsurface energy operations

We report here the first evaluation of the effects of pressure on the sulfur-oxidizing activity of *T. thioparus*, which appears to be pressure sensitive. Bacterial species belonging to this group have been scarcely reported as piezotolerant. In the deep ocean or oceanic subsurface, sulfur-oxidizing bacteria are often present together with ubiquitous sulfate-reducing bacteria (Zobell and Oppenheimer, 1950; Kallmeyer and Boetius, 2004; Bowles *et al.*, 2011; Vossmeier *et al.*, 2012) at oxic-anoxic interfaces. Interestingly, piezotolerance or piezophily is common among sulfate-reducing bacteria that are ubiquitous not only in deep natural environments but also in oil reservoirs where H₂S is highly problematic (Gieg *et al.*, 2011). Among sulfate reducers, some are piezophile like *Desulfovibrio profundus*, *D. piezophilus*, and *D. hydrothermalis* (Bale *et al.*, 1997; Alazard *et al.*, 2003; Khelaifia *et al.*, 2011) with high optimal growth pressures in the range of 10–40 MPa (1000–3000 m depth), while others are piezotolerant only like *D. vulgaris* or tolerate limited pressure like *D. salexigens* and *D. alaskensis* (Bale *et al.*, 1997; Williamson *et al.*, 2018). Beyond our understanding of their growth capability, evaluating their metabolic activity as a function of pressure would be of the highest relevance since the toxic, explosive, and corrosive nature of metabolic products H₂S poses significant health, facility, and environmental damage risks in subsurface energy operations. There are only a limited number of contributions that have investigated their metabolic activity as a function of pressure and potentially other stresses. For instance, Wilkins *et al.* (2014) showed that of CO₂ had less toxic effect on the metabolism of mesophilic *D. vulgaris* at 8 MPa than at low pressures and suppressed sulfate

reduction at pressures as low as 1 MPa. Similarly, the model strain *Thiobacillus thioparus* used here related to some extent to those identified by Trias *et al.* (2017) after CO₂ injection or by Menez *et al.* (in prep) after the injection of sour gas mixture into the subsurface of the pilot CarbFix1 pilot site in Iceland in 2012 may not be fully representative of the strains that bloomed after the injections and one might want to repeat the present investigation on the actual *Thiobacillus sp.* isolated from this specific environment when available. Such isolates could potentially be active to higher pressure. Nevertheless, *T. thioparus* already actively oxidized thiosulfate up to 4 MPa (400 m depth), which corresponds to the depth of CO₂ injection at the CarbFix1 site. This indicates that the lessons learned from the analysis of the biota sampled after the injection well was clogged as of March 2012 (Trias *et al.*, 2017) reflected intensive microbial metabolic activity potentially down to 400 m depth.

Until cultivable isolates from this unique engineered environment are available, it is important to continue evaluating the metabolic activity of model strains as closely phylogenetically related as possible to those identified during the events in 2012 after 230 tons of pure CO₂ and purified geothermal gases mixed with locally sourced groundwater were injected in basalts at 400–800 m depth. In particular, sequencing of the 16S rRNA encoding genes of the biota well developed in May 2012 showed that 16S rRNA encoding gene sequences shared also 99% of identity with the autotrophic facultative anaerobic strain *T. denitrificans* that also is closely related to *T. thioparus* (Trias *et al.*, 2017). The latter should definitely be investigated as a function of pressure, should it have a higher tolerance to pressure.

In short, sulfur oxidation by the mesophilic bacteria *T. thioparus* was reported for the first time as a function of pressure to 10 MPa (1000 m water or 385 m basaltic rock depth). At ambient pressure, the highest growth and metabolic rates were obtained at the lowest concentration in thiosulfate as electron donor. *T. thioparus* transformed directly thiosulfate into sulfate without any intermediate under all conditions investigated up to 4 MPa. The rate and the yield of sulfur oxidation decreased linearly as a function of pressure, independently of the initial sulfur concentration and reached 0 at 4.3(1) MPa. This suggests that the *Thiobacillus sp.* related to the operational taxonomic unit retrieved by 454 pyrosequencing of the 16S rRNA encoding gene in the groundwater of monitoring wells enriched in dissolved inorganic carbon after injection of CO₂ and purified geothermal gases at the pilot CarbFix1 site could potentially oxidize thiosulfate to a depth of 400 m below ground, which corresponds to the injection depth during the tests performed in 2012 (Trias *et al.*, 2017). Getting a complete picture of the metabolic activities of key microorganisms in the basaltic

subsurface of the CarbFix sites would require further experiments conducted under pressure and including different electron donors and representative cultivable microbes identified at depth at the CarbFix sites.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supplementary text: Experimental Procedures

Fig. S1. Growth curves of *T. thioparus* monitored by the optical density (OD) at 600 nm as a function of initial thiosulfate [S₂O₃²⁻]₀ concentration ranging from 5 to 20 g.l⁻¹ in the standard medium by Starkey (1935).

Fig. S2. Time-series of Raman spectra of the supernatant of *Thiobacillus thioparus* culture at 1 MPa and 30°C in the high-pressure cell. The band at 877 cm⁻¹ corresponds to $\nu_{\text{sym}} \text{P}(\text{OH})_2$ of H₂PO₄⁻ and serves as an internal standard, while the vibration at 980 cm⁻¹ is due to the $\nu_{\text{sym}} \text{S}-\text{O}$ of the metabolic product sulfate and the band at 995 cm⁻¹ to $\nu_{\text{sym}} \text{S}-\text{O}$ of thiosulfate.