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Influence of cell's growth phase on the sulfur isotopic fractionation during in vitro microbial sulfate reduction

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ABSTRACT

Culture experiments of sulfate reducing bacteria were conducted to produce large sulfur isotope fractionation in marine sediments. We determined the sulfur isotope fractionation factor for both the exponential growth phase and maintenance growth phase. The results show that the sulfur isotope fractionation during the maintenance phase is larger than it is in the exponential phase, irrespective of a temperature anywhere from 25 °C to 37 °C. In the natural environment, sulfate reducing bacteria may not dominantly grow exponentially. We suggest that sulfate reducers in the natural environment only metabolize at the minimum level to maintain their body. This may solve the apparent discrepancy between the large sulfur isotope fractionation observed in marine sediments and the smaller fractionation obtained from culture experiments.

1. Introduction

Microbial sulfate reduction is one of the common energy metabolisms within sediment. The sulfate reducers use organic substrates or molecular hydrogen as an electron donor, and sulfate as an electron acceptor. Sulfate reducing microbes extend across the domains Archea and Bacteria (Rabus et al., 2006). The sulfur isotope fractionation by sulfate reducing microbes is the key to understanding the ancient biogeochemical sulfur cycle from geological records (Johnston, 2011). Geochemical evidence of Precambrian sedimentary rocks suggests that sulfate reducers have emerged more than 3.4 billion years ago (Shen and Buick, 2004; Ueno et al., 2008). However, factor controlling the magnitude of the isotopic fractionation is still a matter of debate. It is known that isotope fractionation by sulfate reducing microbes changes with chemical conditions (e.g., sulfate concentration, electron donor type and concentration, temperature) based on a large number of culturing experiments (Harrison and Thode, 1958; Kemp and Thode, 1968; Chambers et al., 1975; Böttcher et al., 1999; Canfield, 2001; Habicht et al., 2002, 2005; Canfield et al., 2006; Hoek et al., 2006; Johnston et al., 2007; Mitchell et al., 2009; Sim et al., 2011a, 2011b; Leavitt et al., 2013).

The sulfate reduction rate has been believed to control the isotope fractionation between sulfate and the resulting sulfide. Previous experiments have shown a negative correlation between sulfur isotope fractionation and sulfate reduction rate (Harrison and Thode, 1958; Sim et al., 2011a; Leavitt et al., 2013). Large sulfur isotope fractionation over 47‰ can be attained when decreasing the sulfate reduction rate (Sim et al., 2011b; Leavitt et al., 2013). However, this negative correlation between sulfur isotope fractionation and the sulfate reduction rate is not always applicable to the culturing experiments (Canfield et al., 2006). The reason why some experimental results don't have a negative correlation is unclear. Other experiments have suggested that the negative correlation can only be obtained when the cell grows exponentially (Detmers et al., 2001; Johnston et al., 2007; Sim et al., 2011a). In the natural environment, however, the majority of sulfate reducers may maintain the cell without doubling rather than growing exponentially. Hence, the application of experimental results is limited when it comes to interpreting the geological records. In fact, averaged sulfur isotope fractionation between sulfate and sulfide in modern marine sediments is 51‰, while the averaged fractionation obtained from previous culturing experiments was only about 18‰ (Canfield and Teske, 1996).

In order to solve the discrepancy, it is important to understand sulfur isotope fractionation not only during the cell's exponential phase, but also during the cell's maintenance phase. Some recent studies have shown the shift of isotope fractionation according to the cell's growth phase (Canfield et al., 2006; Davidson et al., 2009). Here we present our new experiments, designed to investigate the difference in sulfur isotope fractionation between the exponential growth phase and the maintenance growth phase.

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2. Materials and methods

2.1. Culture experiments

We use the sulfate reducing bacterial isolate, *Desulfovibrio desulfuricans* (DSM 642) for the batch culture to determine variation of sulfur isotope fractionation during different phases of bacterial growth. This species is a typical sulfate reducing bacteria growing in brackish water and anaerobic conditions. Their optimum growth temperature is 37 °C (Thode et al., 1951; Harrison and Thode, 1958; Canfield et al., 2006). Complete genome sequences of the *D. desulfuricans* are available (Field et al., 2008).

We conducted 5 series of experiments by changing the temperature to 15 °C, 25 °C, 30 °C, 37 °C and 45 °C. First, we prepared 25 bottles of 100 ml glass vials for each series of experiments. Each glass vial was purged with N_2 gas and 40 ml of culture medium for the sulfate reducing bacteria (Modified DSMZ 63) is added with an excess amount of ferrous iron to precipitate sulfide ion as a form of FeS. Modified DSMZ 63 contains K_2HPO_4, 0.5 g; NH_4Cl, 1.0 g; Na_2SO_4, 1.0 g; $CaCl_2 \times 2H_2O$, 0.1 g; $MgSO_4 \times 7H_2O$, 2.0 g; $FeSO_4 \times 7H_2O$, 0.5 g; $FeCl_2 \times 4H_2O$, 3.0 g; lactic acid, 2.0 ml; yeast extract, 1.0 g; resazurin, 1.0 mg; ascorbic acid, 0.1 g; Na-thioglycolate, 0.1 g; distilled water, 1 L. pH was adjusted to 7.0. After autoclaving and storing the glass vials at an experimental temperature, about 20 μl of bacterial culture was inoculated into fresh mediums to start the experiment. As *D. desulfuricans* starts to generate H_2S, the H_2S is greeted by Fe^{2+} ions to precipitate black particles of iron sulfide. After the iron sulfide becomes visible in the culture medium, 2 or 3 culture bottles were retrieved at every 12-hour intervals to follow the degree of sulfate reduction progression and sulfur isotope fractionation. But the period between retrievals is set to be flexible in order to get sulfur isotope data at various points of the bacterial growth phase.

At 25 °C, 30 °C and 37 °C, large enough amounts of iron sulfide were precipitated to calibrate sulfide–sulfur isotope composition. However, the amount of precipitates obtained at 15 °C and 45 °C was insufficient. At 15 °C, we could not get the precipitates for more than two months. The experiment at 45 °C also produced only a very small amount of sulfides, which were extremely fine-grained feathery particulates. The total amount of sulfides is less than 2 mg, which is the minimum amount needed for sulfur isotope measurement. We could, therefore, calibrate sulfide–sulfur isotope composition at only 25 °C, 30 °C, and 37 °C. At 25 °C and 37 °C, we carried out a long run experiment about 1000 h. At 30 °C, we targeted the transition phase from exponential phase to maintenance phase, hence the collection time is shorter than it was at 25 °C and 37 °C.

2.2. Isotope analysis

Precipitated sulfide particles in the vial were recovered by centrifugation and dried using a freeze drier. These black precipitates underwent XRD analyses and were identified as Mackinawite (Fe_9S_8). Dried sulfide samples were transferred to a 100 ml beaker and oxidized with bromine. Dissolved sulfate was washed out by distilled water. After filtration, $BaCl_2$ is added to the solution to get a white precipitation of $BaSO_4$. Then, we filtrated the $BaSO_4$ precipitates transformed from FeS.

On the other hand, sulfate in the supernatant medium was precipitated and recovered as $BaSO_4$ by adding $BaCl_2$. At 25 °C and 37 °C, we rinsed this $BaSO_4$ with 1N HCl to remove barium salts other than barium sulfate.

Both of the $BaSO_4$ were weighed and basic concentrations of sulfate and sulfide are derived from these weights. The initial sulfate concentration calculated from the medium inclusion is 17.0 mM. We collected an average 16.5 ± 0.55 (2σ) mM of $BaSO_4$ from 5 uninoculated samples in the 37 °C experiment. These $BaSO_4$ are burned to produce SO_2 gas using the method of Bailey and Smith (1972). Then SO_2 gas was introduced to the Delta-plus mass spectrometer for sulfur isotopic measurements at the Department of Earth and Planetary Science, the University of Tokyo. Sulfur isotopic compositions are denoted as

$$\delta^{34}S = 1000\left(R_{sample}/R_{standard} - 1\right) \quad (1)$$

where R represents the isotope ratio ($^{34}S/^{32}S$) for the species of interest. All isotope analyses are reported as parts per thousand (‰) deviations from the Vienna Canyon Diablo Troilite (VCDT) standard. We calibrated with our house $BaSO_4$, whose sulfur isotope composition is already known as −0.71‰. The analytical reproducibility of measurements, determined by repeated analyses of our house $BaSO_4$, is ±0.66‰ (2σ). In our batch culture experiments, sulfur isotope fractionation factor, sulfur isotope enrichment factor, and the sulfur isotopic composition of initial sulfate are obtained from the relationship between the fraction of remaining sulfate and sulfur isotopic compositions of sulfate and sulfide (Nakai and Jensen, 1964).

$$\delta^{34}S_{total} = f\delta^{34}S_{SO4} + (1-f)\delta^{34}S_{H2\,S} = \delta^{34}S_{SO4,0}, \quad (2)$$

$$\delta^{34}S_{SO4} = \delta^{34}S_{SO4,0} + 1000\left({}^{34}\alpha_{SR} - 1\right)\ln f, \quad (3)$$

$$\delta^{34}S_{H2S} = \delta^{34}S_{SO4,0} - 1000\left({}^{34}\alpha_{SR} - 1\right) f \ln f/(1-f), \quad (4)$$

$${}^{34}\varepsilon_{SR} = 1000\left(1 - {}^{34}\alpha_{SR}\right), \quad (5)$$

$${}^{34}\alpha_{SR} = R_{H2S}/R_{SO4}. \quad (6)$$

where f represents the fraction of remaining sulfate; $\delta^{34}S_{SO4,0}$ represents the isotopic composition of initial sulfate; $\delta^{34}S_{SO4}$, $\delta^{34}S_{H2S}$ represents the isotopic composition of sulfate and sulfide respectively; ${}^{34}\alpha_{SR}$ represents the isotope fractionation factor of sulfate reduction and ${}^{34}\varepsilon_{SR}$ represents the isotope enrichment factor of sulfate reduction.

The fraction of remaining sulfate in the 25 °C and 37 °C experiments is calculated with the sulfate concentration of the medium in a collected sample, assuming the initial sulfate concentration is 17.0 mM. On the other hand, the fraction of remaining sulfate calculated with sulfate concentration possibly exceeds the actual value in the 30 °C experiment because of the lack of acid treatment. The fraction of remaining sulfate, therefore, is calculated by the sulfide concentration in the 30 °C experiment. If there is no sulfur species except for sulfate and sulfide, the sulfate concentration must be 17.0 − [sulfide] mM. However, the sulfate concentration calculated by the sulfide concentration in our 37 °C experiment always exceeds the actual sulfate concentration. This discrepancy is probably caused by the loss of sulfur during the oxidation process of sulfide because there seems to be no intermediate sulfur species in the 37 °C experiment, as discussed in Section 4.4. Since the sulfate concentration calculated by sulfide concentration exceeds actual sulfate concentration by an average 2.0 ± 0.5 mM (n = 18), the sulfate concentration is calculated as 17.0 − ([sulfide] + 2.0) mM by sulfide concentration.

3. Results

3.1. Temporal variations of sulfate and sulfide concentration

Concentration of remaining sulfate, product sulfide, and the sum of these are represented in Fig. 1. In the 25 °C experiment, the sulfate and sulfide concentrations collected at the same time have a large variation, especially before 550 h. This variation is consistent with the variety of FeS black precipitates observed in each vial and indicates the cell's growth phase in each vial is different. Sulfate and sulfide concentrations became nearly constant after 550 h, indicating that additional production of sulfide was minimal after 550 h, when the average concentration of remaining sulfate and sulfide was 6.3 mM and 7.8 mM, respectively.