A bacterial isolate from the Black Sea oxidizes sulfide with manganese(IV) oxide

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Mn is one of the most abundant redox-sensitive metals on earth. Some microorganisms are known to use Mn(IV) oxide (MnO₂) as electron acceptor for the oxidation of organic compounds or hydrogen (H₂), but so far the use of sulfide (H₂S) has been suggested but not proven. Here we report on a bacterial isolate which grows autotrophically and couples the reduction of MnO₂ to the oxidation of H₂S or thiosulfate (S₂O₇²⁻) for energy generation. The isolate, originating from the Black Sea, is a species within the genus *Sulfurimonas*, which typically occurs with high cell numbers in the vicinity of sulfidic environments [Y. Han, M. Perner, Front. Microbiol. 6, 989 (2015)]. H₂S and S₂O₇²⁻ are oxidized completely to sulfate (SO₄²⁻) without the accumulation of intermediates. In the culture, Mn(IV) reduction proceeds via Mn(III) and finally precipitation of Ca-rich carbonate [Mn(Ca)CO₃]. In contrast to Mn-reducing bacteria, which use organic electron donors or H₂, Fe oxides are not observed to support growth, which may either indicate an incomplete gene set or a different pathway for extracellular electron transfer.

manganese reduction | sulfide oxidation | *Sulfurimonas*

In stratified basins, for example the Black Sea, in between the oxygenated surface waters and sulfidic bottom waters a suboxic zone lacking oxygen (O₂), H₂S, and mostly also nitrate (NO₃⁻) has been frequently reported (1). Despite the absence of electron acceptors, high bacterial CO₂ fixation rates at the border with sulfidic waters were measured, without a known energy metabolism which could fuel growth under these environmental conditions (2, 3). Thermodynamically, a suitable electron acceptor for H₂S oxidation at this depth would be Mn. Even though Mn concentrations are low (Fig. L4), the oxidized form MnO₂ is particulate and is therefore transported much faster than dissolved electron acceptors to the sulfidic waters by gravitational sinking (4). Nevertheless, so far all attempts to cultivate microorganisms which oxidize H₂S with MnO₂ have failed.

During an expedition on the research vessel *Maria S. Merian* in November 2013 we sampled the water column of the Black Sea, focusing on the suboxic zone. We took a water sample at the depth of highest abundance of *Epistomobacteriaceae* [12 to 15% (2)] and transferred it into a gas-tight serum bottle containing MnO₂ (Fig. L4, red arrow). At this depth H₂S was detectable and neither O₂ nor NO₃⁻ was present. After a first enrichment with daily additions of H₂S resulting in ~10 to 20 µM concentrations, we transferred a small volume into an artificial medium.

As with H₂S, no detectable accumulation of S⁰ and SO₄²⁻ was observed with S₂O₇²⁻ as electron donor. Growth was observed concurrent with the reduction of Mn(IV) to Mn(III) from day 3 to 6 and continued with the reduction of Mn(III) to Mn(II), leading to the precipitation of Ca-rich particles (Fig. 2A and B), in the Latin notation of Pontus Euxinus, meaning *Sulfurimonas* from the Black Sea. The cells are slightly curved, with lengths of 1 to 4 µm and widths of 200 to 300 nm (Fig. 1B). ‘S. marisnigri’ grows autotrophically with doubling times of 9 to 13 h during the exponential growth phase and reaches a final cell density of 3 to 6 x 10⁷ cells per mL after 7 to 10 d (Fig. 2A). Toward the end of the growth phase, the medium turns from black to brownish-gray (Fig. 1C and D, Inserts), due to the reduction of MnO₂ and precipitation of Mn(Ca)CO₃ (Fig. 2B). Even though this may be an artifact due to the cultivation conditions, this particular mineral phase was reported in exceptional amounts from the anoxic basins of the Baltic Sea, but the mechanism of its formation is still under debate (6). Cultivation of ‘S. marisnigri’ with NO₃⁻ and successive additions of H₂S resulted in growth and undetectable H₂S levels in the culture, indicating a principal ability to use H₂S directly as electron donor. In contrast to *Shewanella oneidensis* and *Geobacter metallireducens*, attempts to cultivate ‘S. marisnigri’ with amorphous FeOOH, goethite (α-FeOOH), FeO₃-, and FeCl₃ were unsuccessful, leading to the conclusion that Fe(III) was not a viable electron acceptor under these conditions. This may be due to the absence of a critical protein component for the reduction of Fe oxides or could indicate that extracellular electron transfer onto MnO₂ might function in a different manner.

Growth of ‘S. marisnigri’ with MnO₂ and a constant supply of H₂S resulted in accumulation of SO₄²⁻ as cell numbers increased, and negligible concentrations of elemental sulfur (S⁰). In the sterile control, however, S⁰ accumulated and SO₄²⁻ increased just slightly (Fig. 2C). Likewise, in the central gyres of the Black Sea S₂O₇²⁻ and sulfite (SO₃²⁻) were undetectable (7) and S⁰ occurred in nanomolar concentrations (8). Growth with MnO₂ and S₂O₇²⁻ was accompanied by the complete oxidation of S₂O₇²⁻ to SO₄²⁻ with a stoichiometry of 1:2 (Fig. 2D) and following Eq. 1:

\[
4\text{MnO}_2 + S_2\text{O}_7^{2-} + 6H^+ \rightarrow 4\text{Mn}^{2+} + 2\text{SO}_4^{2-} + 3H_2O.
\]

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. MF563835-MF563475).

The authors declare no conflict of interest.

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A further indication that bacterial H$_2$S oxidation with MnO$_2$ may be of more general importance is the formation of intermediate Mn$^{3+}$ in our cultures, which we detected indirectly as dissolved reactive Mn (14) (Fig. 2B). Mn$^{3+}$ was reported to be a major constituent of the marine Mn cycle both in sediments (15) and in the water column of the Black Sea (16) in the absence of O$_2$ and H$_2$S (17). So far, known processes mediating Mn$^{3+}$ formation are the oxidation of organic matter with MnO$_2$ reduction, enzymatic oxidation of Mn$^{3+}$, and the abiotic reaction of MnO$_2$ with Fe(II) and H$_2$S (15, 18). Our study adds another biologically mediated process via lithotrophic MnO$_2$ reduction, which can promote the buildup of Mn$^{3+}$, as observed both in marine sediments and across pelagic redoxclines. In conclusion, we suggest that this bacterial metabolism, which we prove here in pure culture, may be widespread in pelagic redoxclines and to a minor extent in marine sediments where H$_2$S is produced and Mn is present in sufficient amounts with important consequences for Mn and S cycling.

In addition to its presence in pelagic environments, the genus *Sulfurimonas* is globally abundant in redox transition environments such as hydrothermal vents and marine sediments (12). In sediments, the addition of MnO$_2$ is thought to promote the production of SO$_4^{2-}$, apparently depending on microbial activity and leading to the precipitation of Mn(Ca)CO$_3$ (13). In those experiments, addition of FeOOH did not stimulate SO$_4^{2-}$ production. Those findings fit remarkably well to our observations in pure culture, suggesting that an organism with a physiology similar to that of *S. marisnigri* may have been responsible for the observed activity.

**Fig. 1.** (A) Water column profile of the Black Sea suboxic zone: O$_2$ (blue), NO$_3^-$ (green), H$_2$S (yellow), and particulate (part.) Mn (black triangles). Red arrow indicates sampling for cultivation. Data from ref. 19. (B) Scanning electron microscopy (SEM) picture of the isolate *S. marisnigri* (C and D) Bottle photo and SEM picture of the particulate Mn phase before (C, Insert) and after (D, Insert) growth. (Scale bars: B, 1 µM; C and D, 10 µm.)

Contrast to *S. oneidensis*, where growth is only supported by the reduction of Mn(III) to Mn(II) (9). Total inorganic carbon (TIC) analysis of the particulate fraction at the end of the growth phase confirmed the formation of carbonate. The Ca-to-Mn ratio of single Mn(Ca)CO$_3$ precipitates was determined using SEM and energy dispersive X-ray microanalysis (EDX). The mean ratio of 0.19 with an SD of 0.03 in cube-shaped precipitates was used to estimate the proportion of reduced Mn in the particulate phase (Fig. 2B, blue dashed line). With this approach, we can show that MnO$_2$ was almost completely reduced and transformed to Mn(Ca)CO$_3$.

We excluded disproportionation of S$_2$O$_3^{2-}$ as an alternate explanation for our findings. Cultivation of *S. marisnigri* solely on S$_2$O$_3^{2-}$ as an energy source did not result in growth and formation of H$_2$S or SO$_4^{2-}$. This is supported by cultivation of *S. marisnigri* with MnO$_2$ and a surplus of S$_2$O$_3^{2-}$, in which the concentration of S$_2$O$_3^{2-}$ remained constant after the depletion of MnO$_2$. Finally, disproportionation in the presence of Fe oxides would have led to the precipitation of FeS and FeS$_2$, which was not the case.

The isolate belongs to the group of *Epsilonbacteriaceae* which is reported to be highly abundant in the redox transition zones of, for example, the Black Sea (12% of the total bacterial community), the Baltic Sea (21%), and the Cariaco Basin (27%) (2, 10). In these systems, *Epsilonbacteriaceae* can be responsible for up to 100% of the dark CO$_2$ fixation activity in the absence of O$_2$ and often NO$_3^-$. Therefore, Jost et al. (3) and Taylor et al. (11) already suggested a potentially MnO$_2$-dependent H$_2$S oxidation by autotrophic bacteria.

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Materials and Methods

Gases and Nutrients. Gases in the water column were measured as reported in Schulz-Vogt et al. (19).

Cultivation. Water samples were taken at 44° – 16.7586 N and 36° – 18.9567 E at the depth indicated in Fig. 1. Culture purity was ensured by sequencing, lack of growth in organic-rich media, and by microscopy. Medium for cultivation and experiments was prepared anaerobically following the technique described by Widdel and Pfennig (20) based on a SO$_4^{2-}$-free artificial sea-water with a salinity of 21.1 and a pH of 7.5. The following sterile and anoxic components were added (milliliters per liter): 1 M NaHCO$_3$, 30; 1 mM NiCl$_2$, 20; Pfennig’s trace elements solution SLT, 1; 10 mM NaH$_2$PO$_4$, 1; and vitamin solution, 0.42. Vitamin solution contained (milligrams per milliliter) the following: B$_6$, 0.1; inositol, 0.1; biotin, 0.1; folic acid, 0.1; PABA, 1; nicotinic acid, 10; p-panthotenate, 10; and thiamine, 20. Chemical Mn$_2$O$_3$ provided as electron acceptor was purchased from Merck and additionally grinded with an agate ball mill.

Molecular Analysis and Bioinformatics. DNA was extracted using QiAamp DNA Mini Kit (51306) following the manual. For PCR we used Thermo Fisher Scientific Kit EP0072 according to the manufacturer’s protocol description and primers 27f -1492r. PCR products were purified with the Agentcort AMPure XP (Beckman Coulter GmbH) magnetic beads and cloned into the vector pSC-A-amp/kan (StrataClone SoloPack) following the manual. Clones were sequenced (LGC) forward and reverse using vector primers, trimmed, manually corrected, assembled and deposited at NCBI GenBank (accession nos. MF563385-MF563475).

Cell Counting. A gluteraldehyde-fixed sample (2.5% final concentration) was treated with at least five times the volume of a hydroxylamine solution (1.5 M NH$_4$OH·HCl dissolved in 0.2 M HCl) to dissolve Mn particles. The mixture was treated with ultrasonic for 5 min and subsequently filtered onto a 0.2-µm polycarbonate filter and embedded in DAPI-containing oil. Cells were enumerated with an epifluorescence microscope at 1,000 magnification.

$S_2O_3^{2-}$ and $SO_3^{2-}$. We added 0.5 µL 0.2 M·l$^{-1}$-filtered subsample to 25 µL of Hepes/EDTA buffer (200 mM/50 mM in MilliQ) and 25 µL of the monobromobimane solution (48 mM in acetonitrile) and incubated at room temperature for 30 min in the dark. Derivatization was stopped by adding 25 µL of methanethiosulfonic acid (324 mM in MilliQ water). Samples were diluted with MilliQ water and measured daily. Samples and calibration series were analyzed using a BioTek HPLC System with pump 525 (1 mL min$^{-1}$), oven 582 (25 °C), column LiChropher 100 RP-18 B (5 µm) 125 × 4, and a Lascar FP 1520 detector (excitation at 380 nm, emission at 480 nm). Data were analyzed with the program GenMius III Version 1.10.3.7. Eluent A contained 0.25% acetic acid and eluent B 100% methanol. The gradient protocol was as follows: 0 min 100% A, 2 min 100% A, 5.5 min 92% A, 8 min 68% A, 12 min 68% A, 13 min 0% A, 18 min 0% A, 19 min 100% A, and 23 min 100% A. With these adjustments, $S_2O_3^{2-}$ peak appeared after 10 min and $SO_3^{2-}$ peak after 8 min.

$S^2$. A 900-µL sample was added to 100 µL of zinc acetate solution (5% wt/vol). Then, 500 µL chloroform were added and intensively mixed for 2 min. One hundred microliters of the chloroform phase were diluted with 400 µL methanol and measured with HPLC, using BioTek HPLC System with pump 525 (1 mL min$^{-1}$), oven 582 (25 °C), column LiChropher 100 RP-18 B (5 µm) 125 × 4, and DAD 545V detector (wavelength 265 nm and 6-nm bandwidth). Data analysis was described above. An isocratic gradient with 100% methanol was applied. With these adjustments, the peak appeared after 4.4 min.

$Mn$ and $Ca$. Part. Mn and Ca dissolved in hydroxylamine solution as well as total diss. (≤0.2 µm) Mn and diss. reactive Mn (dMn$_{react}$, mainly comprising Mn$^{4+}$ (14)) were measured by ICP-OES (ICAP 7400 Duo; Thermo Fisher Scientific) using an external calibration and Sc as internal standard. Precision and accuracy were checked by international reference materials (SGR-1b for the part. and SLEW-3 for the diss. fraction) and were below 2%.

SEM-EDX. A Zeiss Merlin Compact SEM (variable pressure, in-lens SE and BSE detector) equipped with EDX (Oxford Instruments) was used to identify Mn(Ca)CO$_3$ precipitates and to directly quantify the Ca-to-Mn ratios. The sample preparation was done as described elsewhere (19). Reduced Mn in the part. phase was calculated with Eq. 2:

$$\text{part. } \text{red. } Mn = \frac{\text{part. } Mn - \text{total part. } Ca/Mn}{\text{by ICP - OES}}.$$  

TIC. Dried material was treated with 40% H$_2$PO$_4$ and the released CO$_2$ was analyzed by an IR detector (multi-EA 4000, Analytic Jena). Pure standard CaCO$_3$ (12.0% TIC) was used for calibration.

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