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A novel hydrogen oxidizer amidst the sulfur-oxidizing *Thiomicrospira* lineage

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***Thiomicrospira* species are ubiquitously found in various marine environments and appear particularly common in hydrothermal vent systems. Members of this lineage are commonly classified as sulfur-oxidizing chemolithoautotrophs. Although sequencing of *Thiomicrospira crunogena*'s genome has revealed genes that encode enzymes for hydrogen uptake activity and for hydrogenase maturation and assembly, hydrogen uptake ability has so far not been reported for any *Thiomicrospira* species. We isolated a *Thiomicrospira* species (SP-41) from a deep sea hydrothermal vent and demonstrated that it can oxidize hydrogen. We show *in vivo* hydrogen consumption, hydrogen uptake activity in partially purified protein extracts and transcript abundance of hydrogenases during different growth stages. The ability of this strain to oxidize hydrogen opens up new perspectives with respect to the physiology of *Thiomicrospira* species that have been detected in hydrothermal vents and that have so far been exclusively associated with sulfur oxidation.**

Introduction

Thiomicrospira species are widespread in marine environments and have been isolated from hydrothermal vents, intertidal mud flats, marine Arctic sediments and continental shelf sediments (Kuenen and Veldkamp, 1972; Ruby and Jannasch, 1982; Jannasch *et al.*, 1985; Brinkhoff and Muyzer, 1997; Brinkhoff *et al.*, 1999a; 1999b; Knittel *et al.*, 2005; Takai *et al.*, 2004). All so far isolated *Thiomicrospira* species are capable of using reduced sulfur compounds as electron donors and are described as chemolithoautotrophs. However, according to Takai *et al.* (2004) *T. thermophila* and two *T. crunogena* strains, namely L-12 and TH-55, should rather be described as chemolithomixotrophs. Although *Thiomicrospira*'s close relative *Hydrogenovibrio* can use hydrogen as an electron donor for growth (Nishihara *et al.*, 1998, 2001), attempts to cultivate *Thiomicrospira* with hydrogen as sole electron donor have remained unsuccessful (Nishihara *et al.*, 1991). Sequencing of *T. crunogena* XCL-2's genome has revealed that genes encoding the large and small subunit of the NiFe hydrogenase as well as those of enzymes necessary for the assembly and maturation of the structural hydrogenase are available

suggesting that under certain conditions XCL-2 may indeed be able to use hydrogen (Scott *et al.*, 2006).

Thiomicrospira species have been repeatedly recognized in hydrothermal environments (for example, Brazelton and Baross, 2010, Perner *et al.*, 2011) where reduced hydrothermal fluids transport energy-rich inorganic electron donors like hydrogen or reduced sulfur compounds to the surface (Jannasch and Mottl, 1985). Phylogenetically diverse microorganisms can oxidize these reduced substrates whereby they gain energy that some can use for autotrophic CO_2 fixation (Campbell *et al.*, 2006, Jannasch and Mottl, 1985, Miroshnichenko and Bonch-Osmolovskaya, 2006). If *Thiomicrospira* indeed cannot utilize hydrogen in habitats like Lost City, where sulfide is particularly scarce, but hydrogen is highly abundant, the widespread predominance of *Thiomicrospira* is somewhat puzzling (Brazelton *et al.*, 2006, 2010; Brazelton and Baross, 2010).

We collected low-temperature hydrothermal fluids ($\leqslant 16\,^\circ$C) emanating near the Sisters Peak chimney on the Mid-Atlantic Ridge where mixed fluids had a pH of 6.6, 1.2 μM hydrogen and 82 μM sulfide (Perner *et al.*, 2013). From these emissions we isolated a *Thiomicrospira* species (SP-41) that can oxidize hydrogen. In the following we present *in vivo* hydrogen consumption rates, hydrogen uptake activity of partially purified protein extracts and hydrogenase transcript abundance at different growth stages of SP-41.

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Materials and Methods

Sample collection
Low-temperature hydrothermal fluids emanating near the Sisters Peak chimney structure were collected (Perner *et al.*, 2013). The Sisters Peak area is hosted in basalt and is located at 4°48′S Mid-Atlantic Ridge at a water depth of roughly 3000 m (Koschinsky *et al.*, 2008). Hydrothermal fluids were retrieved during dives with the remotely operated vehicle Kiel ROV 6000 (GEOMAR, Kiel, Germany) during the cruise MAR-SUED V (April/May 2009) with the RV Meteor. Hydrothermal fluids were collected using the pumped flow-through system KIPS (Kiel Pumping System) (Garbe-Schönberg *et al.*, 2006). To ensure that the retrieved fluids were indeed hydrothermally influenced liquids, we monitored the *in situ* temperature of the fluids online during sampling— temperature is a tracer for mixing of hydrothermal fluids with ambient seawater. Prior to fluid sampling, the fluid collection system was flushed for several minutes with the vent fluids, which exhibited a stable above ambient seawater temperature ($\leqslant 16\,°C$), before the valves were closed to collect the actual sample. Fluid chemistry conducted on these fluid samples evidenced that these collected fluids were hydrothermal fluids and not ambient seawater (cf. Perner *et al.*, 2013). For further details on sampling with the KIPS, see Perner *et al.* (2009).

Enrichment and isolation of Thiomicrospira sp. SP-41
Initially an enrichment culture containing SP-41 was grown by inoculating hydrogen-spiked artificial seawater medium (MJ medium, 10 ml) with 1 ml of low-temperature hydrothermal fluids. MJ medium was prepared as described before (Sako *et al.*, 1996), without yeast extract and without trypticase peptone, but 10 ml of vitamin solution was added (Balch *et al.*, 1979). A volume of 50 ml of medium was filled into 120 ml serum bottles closed with rubber stoppers and purged with a H$_2$:CO$_2$:O$_2$ (79:20:1) gas mixture (Westfalen AG, Münster, Germany). These flasks were inoculated with 2 ml of the 10 ml pre-culture. The reduction state of the medium was monitored with resazurin (0.5 mg l^{-1}). According to the color change in the medium (from blue to colorless), oxygen was being rapidly used up within 24 h. After 24 h incubation at 28 °C, the culture was transferred to fresh MJ medium as described above. In the home lab *Thiomicrospira* sp. SP-41 was isolated by plating the culture on T-ASW plates (containing 40 mM thiosulfate) (Dobrinski *et al.*, 2005) and then streaked on fresh T-ASW plates two more times to obtain isolated colonies. The purity of SP-41 was verified with fluorescence *in situ* hybridization and cloning and sequencing of 16S rRNA gene fragments (details see below). SP-41 was then routinely cultivated on MJ medium as described above, but with the addition of 0.6 mM thiosulfate (MJ-T medium).

Cultivation of other Thiomicrospira species
TH-55 and *T. thermophila* were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). When the experiments were performed *T. crunogena* XCL-2 was not available from public culture collections (for example, DSMZ and ATCC) and was therefore not included in our growth experiments. TH-55 was grown on T-ASW medium at 28 °C (Dobrinski *et al.*, 2005) and *T. thermophila* on DSMZ medium 1011 at 37 °C, using a H$_2$:CO$_2$ (80:20) gas mix (Westfalen AG) instead of N$_2$:CO$_2$.

In vivo hydrogen consumption experiments
For hydrogen consumption experiments all bacteria were grown in serum bottles on the respective media and a gas mix of H$_2$:CO$_2$:O$_2$:He (2:20:1:77) (Westfalen AG). TH-55 was grown on T-ASW medium and *T. thermophila* was grown on DSMZ 1011 medium as described above. For *T. thermophila* grown in DSMZ medium 1011 subsequently 20% of the headspace was replaced by sterile air. For comparison with SP-41 both strains were additionally grown in MJ-T medium (MJ medium with 0.6 mM thiosulfate) as described above. SP-41 cells were initially grown in MJ-T medium to gain sufficient inoculation material and incubated for 1–2 days at 28 °C. Cultures were then transferred 1:100 into MJ medium: (i) with 0.6 mM thiosulfate (MJ-T medium), (ii) with 0.5 mM cysteine but no thiosulfate (MJ-C medium) and (iii) without thiosulfate or cysteine (MJ medium). To monitor pH changes in the medium, 2 ml of filter-sterilized 0.5% phenol red solution was added. Hydrogen consumption was measured *in vivo* using a Trace GC Ultra gas chromatograph (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a ShinCarbon ST 100/120 column (Restek Corporation, Bellefonte, PA, USA) and a Pulse Discharge Detector (Vici Valco Instruments, Houston, TX, USA) with helium 6.0 as carrier gas (Linde Group, München, Germany). For every measurement 1 ml of the headspace was taken, diluted 1:87 and from this dilution 2 ml injected into the GC. Hydrogen consumption rates were calculated as described before (Perner *et al.*, 2010). Cell numbers were determined by direct cell counting using a counting chamber (Hawksley, Sussex, UK). The cultures were checked for purity at the end of the experiments with fluorescence *in situ* hybridization and/or PCR and subsequent sequencing of 16S rRNA or hydrogenase genes. All experiments were performed at least in triplicate, controls were performed in duplicate. Mean values and standard deviations are from three independent measurements.

Hydrogen uptake activity for Thiomicrospira protein extracts
For enzyme assays SP-41 and TH-55 were grown in MJ-T medium (0.6 mM thiosulfate) as described above and regassed with H$_2$:CO$_2$:O$_2$ (79:20:1)

(Westfalen AG). Filter-sterilized 0.5% phenol red solution (2 ml) was added to monitor pH changes in the medium. The purity of the strains was controlled before performing the enzyme assays. All further steps were performed in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) to prevent direct contact of hydrogenases with oxygen. After ~2.5 days of incubation, cells were harvested by centrifugation at 17 000 g (Sorvall RC 6 Plus Centrifuge, Thermo Fisher Scientific Inc.) and washed once in anoxic 50 mM Tris buffer pH 8. Hydrogenases were partially purified as described before (Adams and Hall, 1979) with a few variations: throughout the assay anoxic 50 mM Tris buffer pH 8 with 5 mM 1,4-dithiothreitol (Tris-DTT) were used in all steps. The cells were broken by sonication (UP50H, Hielscher Ultrasonics GmbH, Teltow, Germany) and cell debris and membranes harvested by ultracentrifugation at 40 000 g (LM-8 Ultracentrifuge, Beckman Coulter Inc., Brea, CA, USA) for 1 h. Membrane proteins were solubilized by using 3% sodium deoxycholate. Ammonium sulfate precipitation was performed by saturating the buffer consecutively at different concentrations (15%, 30%, 50% and 70%) and pelleting of proteins by ultracentrifugation with subsequent resuspension in Tris-DTT buffer.

Protein concentrations of the fractions were determined using the method from Bradford (1976). Hydrogen uptake activity was measured following previous descriptions (Muth *et al.*, 1987; Payne *et al.*, 1993) with oxidized methyl viologen as artificial electron acceptor. The change in absorption was monitored spectrophotometrically (V-630 UV-Vis Spectrophotometer equipped with an EHCS-760 Peltier Cell Holder, Jasco, Gross-Umstadt, Germany) in reaction buffer (50 mM Tris pH 8, 5 mM DTT and 10 mM methyl viologen dichloride) at 602 nm and 25 °C. The extinction coefficient was 12.04 mM^{-1} cm^{-1} at assay conditions. The anoxic reaction buffer was filled into cuvettes (Hellma GmbH & Co. KG, Müllheim, Germany) sealed with rubber stoppers during gassing with N$_2$ 5.0 ($\geqslant$99.999%) (Westfalen AG) and was reduced by adding 5 mM sodium dithionite solution. Protein fractions were added from an anoxic solution. Controls consisted of reaction buffer with protein solution where the enzymatic reaction was monitored under a N$_2$ atmosphere. For determining the hydrogen uptake activity of the protein extracts, subsequently nitrogen was replaced by H$_2$ 5.0 ($\geqslant$99.999%) (Westfalen AG). Hydrogen uptake activity calculations were performed from three independent measurements.

Hydrogenase transcripts at different growth stages

To quantify the hydrogenase large subunit transcripts of SP-41 during growth, cells were grown on MJ-T medium with H$_2$:CO$_2$:O$_2$:He (2:20:1:77) (Westfalen AG) in the headspace for up to 172 h.

Cells were harvested at different time points (8, 22.5, 46.5 and 172 h) of growth. For reverse transcription quantitative PCR (RT-qPCR) the following kits were used according to the manufacturer's protocols: Presto Mini RNA Bacteria Kit (Genaid Biotech Ltd., New Taipei City, Taiwan) for total RNA isolation, RTS DNase Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA), RNA Clean & Concentrator-5 (Zymo Research Corporation, Irvine, CA, USA), SuperScript VILO Master Mix (Life Technologies, Carlsbad, CA, USA) for synthesizing cDNA and SYBR Select Master Mix for CFX (Life Technologies). The primers 40 F (5′-CCA GTC ACC CGA ATT GAA GG-3′) and 189 R (5′-AGC AAT CAA ACC GGT ATC CC-3′) targeting the SP-41 hydrogenase large subunit gene were used (initial denaturation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 52 °C for 20 s, elongation at 72 °C for 15 s). Controls of the template lacking reverse transcriptase were performed by PCR. RT-qPCR results were evaluated with the Bio-Rad CFX Manager software (Bio-Rad Laboratories, Hercules, CA, USA). Transcription experiments were performed in triplicate.

Fluorescence in situ hybridization

Approximately 2×10^6 cells of each culture were fixed with formaldehyde (4% v/v) for 4 h, concentrated on polycarbonate filters (type: GTTP, pore size 0.2 μm, Millipore, Eschborn, Germany), washed twice with 1× PBS (phosphate-buffered saline) and stored at 4 °C until further treatment. In addition, 250 ml of low-temperature hydrothermal fluids were treated as described above and fixed on a polycarbonate filter. Fluorescence *in situ* hybridization analyses with cy-3 labeled oligonucleotide probes (final concentration 50 ng μl^{-1}) and 4′,6-diamidino-2-phenylindole (5 μg ml^{-1}) coloring were performed as stated in Glöckner *et al.* (1999). A new probe, Tcr69 (5′-CTT GTC GTT TCC GTC CG-3′) was designed based on the 16S rRNA gene sequences of several *T. crunogena* species, namely SP-41, TH-55, XCL-2, L-12 and JB-B2 as well as *T. halophila* and required a concentration of 35% formamide for successfully targeting *T. crunogena* species. The probe has one mismatch to *T. thermophila* for which it does not give any signals. In addition, the nonsense probe NON338 (Wallner *et al.*, 1993) was applied to all filters, which did not give any signals. Filter sections were inspected using an Axio Imager.M2 microscope (Carl Zeiss AG, Oberkochen, Germany).

DNA extraction and processing of 16S rRNA and hynL genes

DNA from SP-41 and TH-55 was extracted with the ultra clean microbial DNA isolation kit (Mo Bio Laboratories Inc.) according to the manufacturer's instructions. 16S rRNA genes were PCR amplified

using the primers 27 F and 1492 R (Lane, 1991) and the conditions described previously (Perner *et al.*, 2009). Details on the cloning for genes have been described before (Perner *et al.*, 2009). For sequencing of both DNA strands of the 16S rRNA genes from SP-41 and TH-55, the primers 27 F, 1492 R, 562 (5′-TAA TCT TGC GAC CGT ACT CC-3′), 719 (5′-CTG ACG CTG AGG TGC GAA AG-3′) and 1149 (5′-CCC AGT GTG GCT GAT CAT CC-3′) were used. The *hynL* (large subunit of the structural membrane-bound uptake hydrogenase) gene from TH-55 was partially amplified using the primers hynl110F and hynl410R (Takai *et al.*, 2005). PCR conditions were: initial denaturation 95 °C for 5 min followed by 32 cycles of denaturation at 95 °C for 45 s, annealing at 45 °C for 45 s and elongation for 70 s. The PCR product was cloned as described above. For double-stranded sequencing, the primers hynl110F, hynl410R, 208 (5′-TTA ACG CAG GGC GTT TAG GG-3′), 338 (5′-GCT CAC TTC CCG CTG AAT CC-3′), 614 (5′-TGC CAG CGG CAT TAT TTG GG-3′) and 714 (5′-CTT CGT CGG CAT ACC AGG AG-3′) were used. To sequence SP-41's full *hynL* gene, a fosmid clone containing the whole gene was used as template. The fosmid library was constructed with the Copy-Control Fosmid Library Production Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. For double-stranded sequencing, the primers hynl110F, hynl410R, 90 (5′-TCC TGC ACG GCT GCC AAA TC-3′), 502 (5′-TGA ACC GTT GTT TGC CAG TG-3′), 571 (5′-AGT GTC GTC AAA CGG TTC TG-3′) and 656 (5′-ACC GAT ATG AAT GCG GAT GG-3′) were used. Sequencing was performed with a 3730xl DNA Analyzer (Applied Biosystems, Life Technologies). All sequences were edited with Lasergene Software SeqMan (DNASTAR, Madison, WI, USA). 16S rRNA and *hynL* gene sequences were edited and analyzed as previously described (Perner *et al.*, 2009; 2010). Phylogenetic trees were calculated using PhyML (Guindon and Gascuel, 2003) and settings as described before (Perner *et al.*, 2009, 2010).

Data deposition
The 16S rRNA and *hynL* gene sequences from SP-41 and TH-55 were deposited in the National Center for Biotechnology Information under the accession numbers KJ573628, KJ573629, KJ573630 and KJ573631, respectively. The new strain SP-41 has been submitted to the DSMZ and is designated DSM 28671.

Results and Discussion

Phylogeny of Thiomicrospira sp. SP-41
Our isolate SP-41 was phylogenetically classified as a *Thiomicrospira* species (Figure 1a). On the basis of 16S rRNA genes, SP-41's closest relatives were three *T. crunogena* strains, namely, TH-55, MA-3 and XCL-2 (99% DNA identity), with 1, 2 and 3 nt

differences, respectively. The large subunit of SP-41's NiFe uptake hydrogenase (*hynL*) resembled *hynL* of XCL-2 (90% DNA and 98% AA identity) and the partial *hynL* sequence of TH-55 (89% DNA identity and 97% AA identity) (Figure 1b). Interestingly, the cluster containing the *hynL* genes of SP-41, XCL-2 and TH-55 exhibited the highest relatedness to *hynL* genes from *Epsilonproteobacteria* (⩽71% AA identity). The best hit to non-*Epsilonproteobacteria* was to *Shewanella* (*Gammaproteobacteria*, 70% DNA and 49% AA identity). Moreover, *Hydrogenovibrio*, which belongs to the same family as *Thiomicrospira*, showed only 40% AA similarity (Figure 1b). As SP-41, TH-55 and XCL-2 were all isolated from hydrothermal vent environments (Jannasch *et al.*, 1985; Wirsen *et al.*, 1998) and *Epsilonproteobacteria* are one of the most dominant groups found in these habitats (Campbell *et al.*, 2006), these *Thiomicrospira hynL* genes may have been acquired by a common ancestor through lateral gene transfer from *Epsilonproteobacteria*. Likewise, other genes in XCL-2 have also been posited to have been obtained through lateral gene transfer from different organisms (Scott *et al.*, 2006). This may indicate rapid evolution in this type of environments, but more information need to be gathered to investigate this hypothesis in detail.

Growth, H₂ consumption, hydrogenase activity and hynL expression of SP-41 with thiosulfate
SP-41 grown on artificial seawater medium supplemented with 0.6 mM thiosulfate (MJ-T medium) and hydrogen, consumed most of the hydrogen (∼86%) within the first 40 h of incubation (Figure 2). After ∼24 h of incubation 65% of the hydrogen was used up and cell density reached a maximum (2×10^7 cells ml^{-1}) (Figure 2). Despite no further increase in cell density, hydrogen was still being consumed until approximately only 5% of the originally present hydrogen was available. The hydrogen uptake activity of SP-41's partially purified membrane fraction was 1.26 ± 0.13 μmol H₂ per min per mg protein (Supplementary Figure S1). This is in the range of what has been measured for other hydrogenases from deep sea hydrothermal microorganisms (Takai *et al.*, 2005) and is ∼10-fold higher than the activity of *T. crunogena*'s close relative *H. marinus* (Nishihara *et al.*, 2001). Hydrogen uptake activity of SP-41's soluble proteins was negligible (Supplementary Figure S1). We also tested the transcription level of the *hynL* gene at different time points during growth on MJ-T medium with hydrogen, which demonstrated that the relative expression of the *hynL* gene varied in SP-41 with incubation time (Figure 3). The relative expression of the *hynL* gene transcript decreased to ∼45% after 46.5 h and ∼25% after 172 h of incubation. The reduction in *hynL* transcript products correlated with decreasing hydrogen concentration (23% and 5% of hydrogen were left after 46.5 and 172 h of
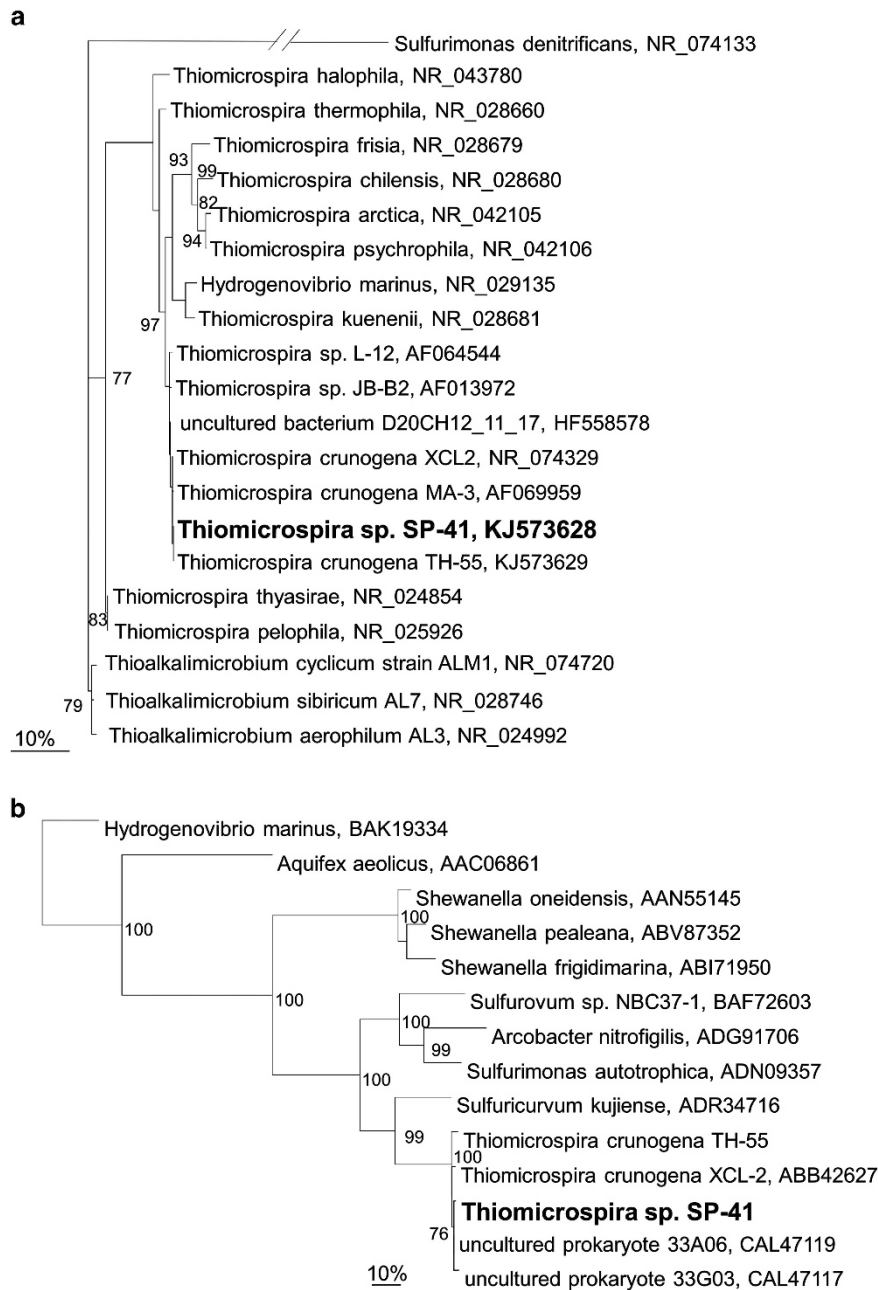
Figure 1 Phylogenetic relationships of *Thiomicrospira* sp. SP-41 (**a**) 16S rRNA genes and (**b**) the large subunits of the uptake hydrogenase (*hynL*) based on maximum likelihood analysis. The percentage of bootstrap resamplings is indicated at the nodes and only bootstrap values >75 are shown. The scale bar represents the expected number of changes per nucleotide (**a**) or amino-acid (**b**) position.

incubation, respectively) and the cells entering the stationary phase (Figure 3). In summary, SP-41 expresses an active hydrogen-uptake hydrogenase enzyme and can consume hydrogen when thiosulfate is present.

Growth and H₂ consumption of SP-41 without thiosulfate
As the genome of XCL-2 lacks genes encoding enzymes of assimilatory sulfate reduction (APS reductase and ATP sulfurylase), which are essential

for cysteine biosynthesis in the absence of thiosulfate or sulfide, it has been posited that XCL-2 cannot grow without adding respective sulfur sources (Scott *et al.*, 2006). To test how dependent SP-41 is for its growth with hydrogen on thiosulfate and cysteine, hydrogen growth experiments were also performed in MJ medium (no thiosulfate) with and without supplemented cysteine (0.5 mM). In both experimental setups most of the hydrogen was consumed within 118 h of incubations (Figure 4). While offering cysteine (0.5 mM), SP-41 consumed 95% of the available hydrogen (Figure 4a), which
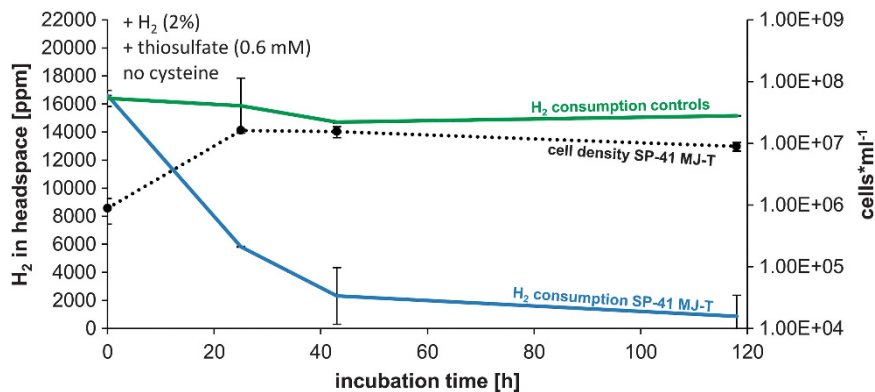
Figure 2 *In vivo* hydrogen consumption of SP-41 and cell numbers in MJ-T medium (0.6 mM thiosulfate) and $H_2:CO_2:O_2:He$ (2:20:1:77) in the headspace. SP-41's hydrogen consumption is shown in blue, respective cell numbers as broken black line and controls (only MJ-T medium) in green.
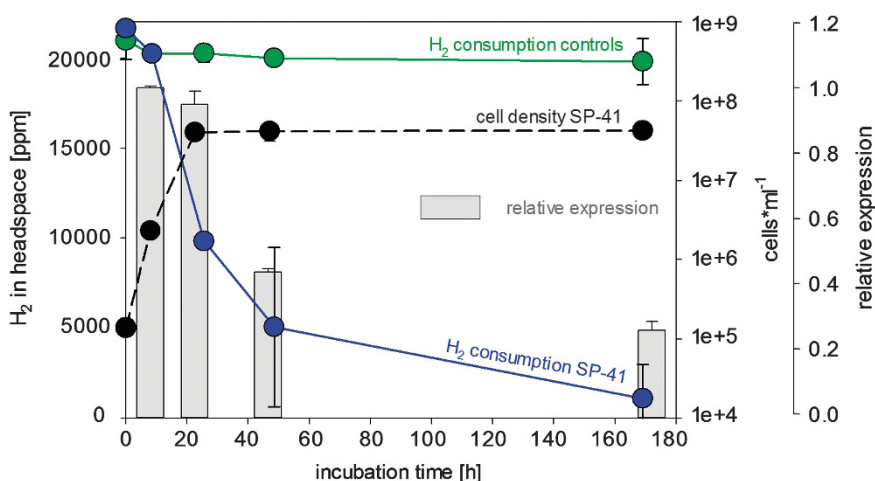

Figure 3 Transcription experiments showing hydrogen consumption, cell numbers and *hynL* gene expression level for SP-41 grown in MJ-T medium (0.6 mM thiosulfate). SP-41's hydrogen consumption is shown in blue, respective cell numbers as broken black line and controls (only MJ-T medium) in green. Relative expressions of the *hynL* gene from SP-41 at different time points during growth are indicated as gray bars.

compares with what we observed for SP-41 when thiosulfate (0.6 mM) was added (Figure 2). In contrast, when neither cysteine nor thiosulfate were supplemented, only 84% of the total hydrogen was used up within 118 h of cultivation (Figure 4b). The results indicate that SP-41 can consume hydrogen even in the absence of thiosulfate and cysteine.

Comparing different growth conditions of SP-41
When hydrogen is oxidized aerobically to water ($H_2 + \frac{1}{2}O_2 \gg H_2O$) the reaction yields 237 kJ mol^{-1} (Fuchs *et al.*, 2007). Theoretically, a relation should exist between the oxidation of electron donors (catabolism) and autotrophic CO_2 fixation (anabolism), governed by an empirical Gibbs energy dissipation coefficient (Heijnen and Van Dijken, 1992). For hydrogenotrophy $\sim$1060 kJ catabolic energy is required to fix 1 mol of carbon in biomass (Heijnen and Van Dijken, 1992). Given that we have 237 kJ mol^{-1} hydrogen oxidized aerobically to

water, we would expect a molar ratio of hydrogen consumption to CO_2 fixation on the order of five. In our experiments, during the exponential growth phase on MJ-T medium 1194 nmol H_2 per h or 29 860 nmol H_2 were oxidized—generating an estimated amount of 0.007 kJ energy—and 7.7×10^8 new cells were formed (Table 1). Assuming that all cells in the incubation consumed hydrogen, SP-41's hydrogen consumption rate in the exponential growth phase would be 1.47 ± 0.03 fmol H_2 per h per cell (Table 1), which is in the range of the consumptions of other bacterial strains (Häring and Conrad, 1991; Klüber and Conrad, 1993; Olson and Maier, 2002). If all these cells also fixed CO_2 autotrophically then an estimated amount of 0.29 fmol CO_2 per h per cell or 103 fg carbon per cell could be fixed in these incubations. This corresponds well with carbon contents measured for bacterial cells in the exponential growth phase in batch cultures (depending on the culture conditions between 39 ± 3 fg carbon per cell and 149 ± 8 fg of
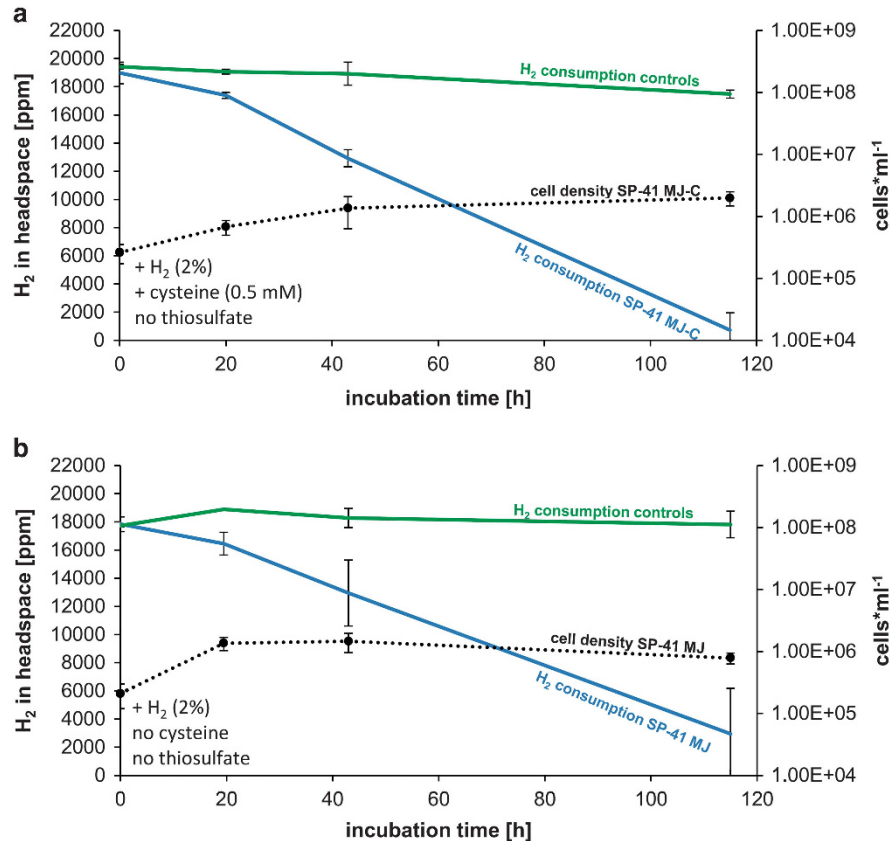
Figure 4 *In vivo* hydrogen consumption of SP-41 grown under different conditions: (**a**) SP-41 grown in MJ-C medium (without thiosulfate but with 0.5 mM cysteine), and in (**b**) MJ medium (without thiosulfate and without cysteine). Hydrogen consumption of SP-41 is shown in blue, respective cell numbers as broken black lines and controls (only MJ-C medium or MJ medium) in green.

Table 1 Hydrogen consumption and cell numbers in different media during exponential growth of SP-41 and TH-55

	SP-41			TH-55
	Type of medium			*Medium*
	MJ-T	*MJ-C*	*MJ*	*MJ-T*
H₂ consumption				
Total (nmol)	29 860	3655	7421	21 102
Per hour (nmol h⁻¹)	1194	183	381	898
Per cell and time (fmol H₂ per h per cell)	1.47	6.10	5.90	0.73
Energy (kJ)	0.0071	0.0009	0.0018	0.005
Total cell numbers	8.2×10^8	3.4×10^7	6.8×10^7	1.2×10^9

carbon per cell) (Vrede *et al.*, 2002). Conclusively, the amount of hydrogen consumed in the MJ-T medium is theoretically sufficient to form the number of cells that we counted.

In cultures without thiosulfate but with cysteine less hydrogen was consumed, that is, 3655 nmol hydrogen (or 183 nmol h⁻¹), less energy generated (estimated 0.001 kJ) and fewer new cells (2.1×10^7) formed during exponential growth (Table 1). During exponential growth in cultures without thiosulfate and without cysteine only 7421 nmol H₂ was used (that is, 381 nmol h⁻¹), theoretically generating 0.002 kJ energy and producing 5.8×10^7 new cells

(Table 1). Owing to lower cell densities in these cultures (Table 1) the estimated hydrogen consumption per cell was fourfold higher than in the incubations where thiosulfate was present: hydrogen consumption in the incubations without thiosulfate and with and without cysteine was 6.1 and 5.9 fmol H₂ per h per cell, respectively (Table 1). Assuming that all these cells fix CO₂ autotrophically and assuming a molar ratio of hydrogen consumption to primary production on the order of 5, theoretically 1.22 and 1.18 fmol CO₂ per h per cell, respectively, could be fixed in these incubations by SP-41.

In summary, conclusions drawn from these three types of growth experiments, that is, artificial seawater and hydrogen (i) with thiosulfate (MJ-T medium) (Figure 2), (ii) with cysteine but no thiosulfate (MJ-C medium) (Figure 4a) and (iii) without cysteine or thiosulfate (MJ medium) (Figure 4b), are manifold: SP-41 can grow on hydrogen without supplementing thiosulfate and cysteine and hydrogen consumption per cell is comparable for both types of experiments (Table 1). However, cell growth was significantly higher when thiosulfate was added to the medium (P-value <0.001). As cell growth in MJ-T medium was higher than in the MJ-C and MJ media, but hydrogen consumption per cell and per hour was ~fourfold lower in the MJ-T medium than in the other experiments, some of the newly formed biomass in the MJ-T medium is likely related to thiosulfate oxidation.

In vivo hydrogen consumption of reference strains and uptake hydrogenase activity of TH-55

To investigate whether the ability to consume hydrogen is a unique feature of our new strain SP-41, we tested experimentally if *T. thermophila* and TH-55 can consume hydrogen in the medium they were originally grown in and in the medium (MJ-T medium) we used to grow SP-41. *T. thermophila* grew on both media tested, that is, DSMZ medium 1011 with hydrogen and MJ-T medium with hydrogen (Figures 5a and b). It grew significantly denser

in the DSMZ medium 1011 than in the MJ-T medium (P-value <0.001), which is likely a consequence of higher cell density in the inoculum (Figures 5a and b). However, in neither experiments *T. thermophila* consumed hydrogen under the provided conditions (Figures 5a and b). This coincides with previous studies where it was shown that *T. thermophila* could not grow on hydrogen but required thiosulfate, elemental sulfur or sulfide as sole energy source (Takai et al., 2004). In line with this observation were our unsuccessful efforts to amplify *hynL* genes from *T. thermophila* with degenerate primers.

TH-55 was also not capable of consuming hydrogen when grown on the medium it was originally isolated on, that is, T-ASW medium (Figure 5c), which compares with previous attempts to cultivate *T. crunogena* including TH-55 on hydrogen (cf. Nishihara et al., 1991; Takai et al., 2004). In contrast, while cultivating TH-55 on MJ-T medium, hydrogen consumption and growth were observed (Figure 5d). The maximum consumption of TH-55 on MJ-T medium in the exponential growth phase was 0.73 ± 0.13 fmol H$_2$ per h per cell, which was half of SP-41's hydrogen consumption rate. As in the thiosulfate-rich medium T-ASW (40 mM thiosulfate) no hydrogen was consumed but in the MJ-T medium with nearly 67-fold lower thiosulfate concentration (0.6 mM) hydrogen was used, TH-55 may only revert to hydrogen oxidation under thiosulfate limitation. In both types of experiments 0.9 mM hydrogen was available at the start of the experiment. Besides the
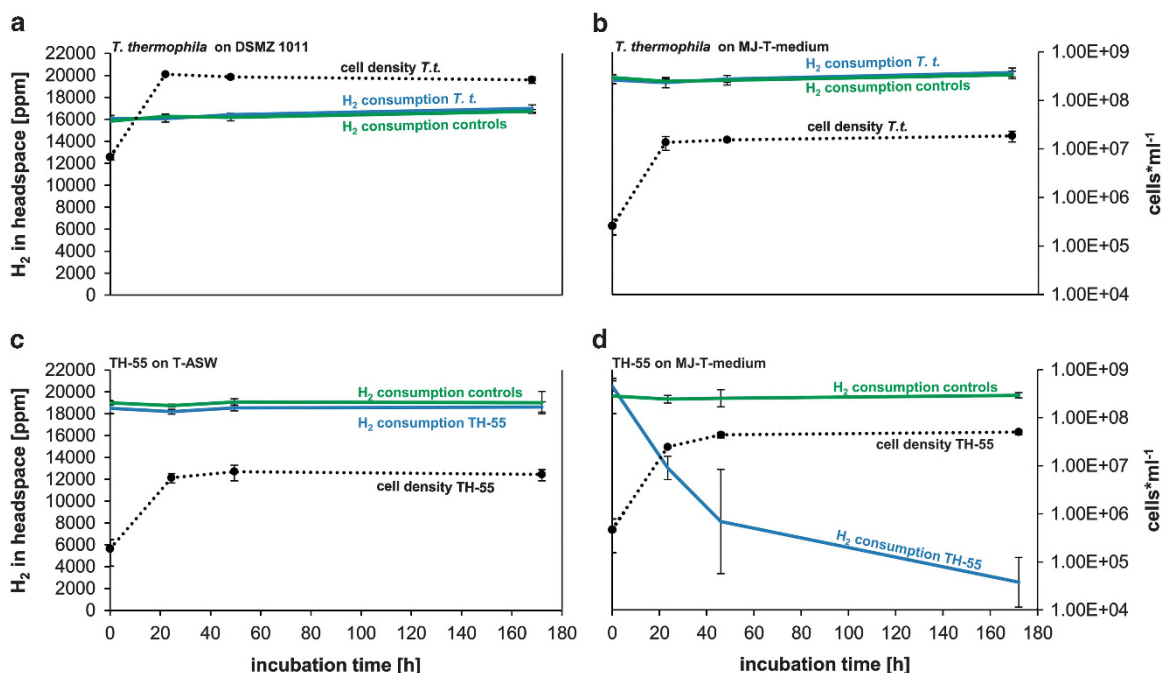

Figure 5 *In vivo* hydrogen consumption of TH-55 and *T. thermophila*. (**a**) *T. thermophila* in DSMZ medium 1011, (**b**) *T. thermophila* in MJ-T medium (0.6 mM thiosulfate), (**c**) TH-55 in T-ASW medium and (**d**) TH-55 in MJ-T medium (0.6 mM thiosulfate). Hydrogen consumptions are shown as blue lines, respective cell numbers as broken black lines and controls (only DSMZ medium 1011, T-ASW medium or MJ-T medium) as green lines.

large discrepancies between thiosulfate concentrations in the two tested media, other major differences between T-ASW and MJ-T medium include the pH (T-ASW pH 8 and MJ-T pH 6.9) and $NaHCO_3$ concentrations (T-ASW 2.4 mM and MJ-T 12 mM), which may have profound impact on TH-55's hydrogen oxidation ability. In other bacteria the hydrogen uptake ability has been shown to depend on a number of factors including growth and reaction conditions, such as availability of thiosulfate, hydrogen or CO_2 (Rákhely *et al.*, 2007). For *Thiomicrospira* species this would need to be tested in further experiments.

Interestingly, cell growth of TH-55 was significantly higher while offering hydrogen in MJ-T medium (with 0.6 mM thiosulfate) than while growing cells in T-ASW medium (with 40 mM thiosulfate) (*P*-value 0.001) (Figures 5c and d). We estimated whether the cultures would be oxygen-limited in the T-ASW medium faster than in the MJ-T medium. Cells in the T-ASW medium, where no hydrogen is consumed, reach lower cell densities than cells in the MJ-T medium, where hydrogen is consumed, because of the increased oxygen demand of thiosulfate oxidation; aerobic thiosulfate oxidation requires two oxygen molecules but aerobic hydrogen oxidation theoretically only needs half an oxygen molecule per reaction (Fuchs *et al.*, 2007). Our MJ-T and T-ASW incubations hold 1% oxygen in the headspace, which equals ∼30 000 nmol oxygen per incubation. Given that one oxygen molecule is theoretically required to oxidize two hydrogen molecules (Fuchs *et al.*, 2007) and a maximum of 60 000 nmol of hydrogen was provided in the MJ-T medium then 30 000 nmol of oxygen would be needed to aerobically fully oxidize all available hydrogen in these incubations, if hydrogen is the sole electron donor. Hence, the cells growing with hydrogen are theoretically not limited by oxygen during incubation. For T-ASW incubations supplemented with thiosulfate (2000 μmol in the incubations), oxygen would become a limiting factor with thiosulfate oxidation as the prevailing metabolism because aerobic thiosulfate oxidation requires two molecules of oxygen (Fuchs *et al.*, 2007) and thus 4000 μmol of oxygen would be needed to oxidize the available thiosulfate fully. On the basis of the stoichiometry, the oxygen availability (30 000 nmol) in these incubations would support oxidation of at most 15 000 nmol thiosulfate. In addition to growth stagnation related to oxygen limitation in the T-ASW medium, the higher cell densities in the MJ-T medium may be related to considerably higher carbonate concentrations in the MJ-T medium, which have been shown to stimulate autotrophic CO_2 fixation in *Thiomicrospira* (Dobrinski *et al.*, 2010). TH-55 also exhibited uptake hydrogenase activity (Supplementary Figure S1). The activity of partially purified membrane-associated proteins was 0.51 ± 0.05 μmol H₂ per min per mg protein, which is roughly half of the activity determined for SP-41 (Supplementary Figure S1). This is in line with the findings that TH-55's hydrogen consumption in the exponential phase is roughly half of that from SP-41. Soluble proteins from TH-55 did not show uptake hydrogenase activity and coincided with the findings for soluble proteins from SP-41.

Environmental implications

Our findings can explain the previously puzzling high abundance of *Thiomicrospira* species in sulfide-poor but hydrogen-rich hydrothermal habitats (Lost City) (Brazelton *et al.*, 2006, Brazelton and Baross, 2010; Brazelton *et al.*, 2010). They can also explain the hydrogen consumption measured in incubations with Lilliput hydrothermal fluids, which were enriched with *T. crunogena* (28%) at the end of the experiments (Perner *et al.*, 2011). In fact, in these incubations hydrogen consumption to CO_2 fixation ratios suggested that hydrogen would be fueling biomass production, but due to the absence of experiments demonstrating that *Thiomicrospira* can indeed oxidize hydrogen and due to relatively high sulfide concentrations in the hydrothermal fluids used for inoculating the incubations, oxidation of residual sulfide was postulated to be responsible for primary production (Perner *et al.*, 2011).

In the environmental hydrothermal fluid samples emanating at Sisters Peak, *Thiomicrospira* accounted for 0.7% of DAPI-stained cells and counts of cell numbers suggested 1.4×10^5 cells per ml hydrothermal fluids. This amounts to ∼990 *Thiomicrospira* cells in 1 ml Sisters Peak fluid sample. If all these cells can consume hydrogen (depending on the strain and conditions between 0.73 and 6.1 fmol H₂ per h per cell) then between 0.72 and 6.02 nmol H₂ per h could be consumed in 1 l of Sisters Peak low-temperature hydrothermal fluids. Given the molar ratio between hydrogen consumption to CO_2 fixation in autotrophic hydrogen oxidizers to be on the order of five, 0.14–1.2 nmol carbon could be fixed autotrophically if the gained energy would be used for biomass synthesis. It is very difficult to estimate fluxes from venting environments as diffused fluid emanations are highly variable. For the Sisters Peak hydrothermal fluid emission site no fluid flow rates are available, but flow rates for low-temperature venting sites have been estimated at 116–17 580 l h⁻¹ (Sarrazin *et al.*, 2009; Wankel *et al.*, 2011). Assuming that the Sisters Peak low-temperature fluids emanate at a rate as has been estimated for other venting sites and assuming that all the local *Thiomicrospira* species are active and consume hydrogen at the above mentioned rates then between 84 nmol (0.00002 kJ) and 105 μmol hydrogen (0.025 kJ) could be consumed and subsequently 17 nmol and 21 μmol CO_2 could be fixed at one venting site in 1 h by *Thiomicrospira*. For comparison, Petersen *et al.* (2011) calculated that 435 μmol hydrogen could be consumed per hour by

endosymbionts inhabiting a single vent mussel. The Sisters Peak endmember fluids—that is, 100% hydrothermal fluids without any mixed ambient seawater—holds an estimated 1.6 mM hydrogen (Perner *et al.*, 2014). The Sisters Peak low-temperature fluids, containing 6.9% hydrothermal endmember, have 1167 nM hydrogen (Perner *et al.*, 2013). However, if converting the hydrogen concentrations of the endmember fluids to mixed fluids with a 6.9% endmember portion, considerably more hydrogen should be available in the low-temperature emissions, namely 110 μM, suggesting that hydrogen is being consumed biologically. Likewise, in other low-temperature diffuse fluids hydrogen concentrations are 50–80% lower than predicted and the loss of hydrogen in such fluids has been attributed to biological activity (Wankel *et al.*, 2011). Conclusively, sufficient hydrogen is available in the Sisters Peak hydrothermal system to support our hydrogen consumption rates. *Thiomicrospira* species could account for considerable hydrogen consumption and respective primary production in this vent environment and possibly others as well.

Conclusion

This is the first report of a *Thiomicrospira* strain, namely SP-41, that expresses active hydrogenases and can consume hydrogen. In fact, hydrogen uptake ability does not appear to be a unique feature of our strain SP-41, but is also found for TH-55 and may even be valid for other *Thiomicrospira* species not tested here. Experimental data further suggest that under conditions of low thiosulfate concentrations hydrogen may become a promising substrate for some *Thiomicrospira* species. Future physiological experiments will have to address a set of key questions to better understand the role that environmental parameters have on *Thiomicrospira*'s presence and its hydrogen metabolism. This includes elucidating how widespread hydrogen consumption is among the culturable species of the ubiquitous *Thiomicrospira* lineage. Further, it will be vital to understand the role that the availability of thiosulfate and other reduced sulfur sources have for enhancing or reducing *Thiomicrospira*'s hydrogen oxidation ability.

Conflict of Interest

The authors declare no conflict of interest.

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