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ARTICLE

A novel bacterial sulfur oxidation pathway provides a new link between the cycles of organic and inorganic sulfur compounds

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Abstract

Dimethylsulfide (DMS) plays a globally significant role in carbon and sulfur cycling and impacts Earth's climate because its oxidation products serve as nuclei for cloud formation. While the initial steps of aerobic DMS degradation and the fate of its carbon atoms are reasonably well documented, oxidation of the contained sulfur is largely unexplored. Here, we identified a novel pathway of sulfur compound oxidation in the ubiquitously occurring DMS-degrader *Hyphomicrobium denitrificans* X^T that links the oxidation of the volatile organosulfur compound with that of the inorganic sulfur compound thiosulfate. DMS is first transformed to methanethiol from which sulfide is released and fully oxidized to sulfate. Comparative proteomics indicated thiosulfate as an intermediate of this pathway and pointed at a heterodisulfide reductase (Hdr)-like system acting as a sulfur-oxidizing entity. Indeed, marker exchange mutagenesis of *hdr*-like genes disrupted the ability of *H. denitrificans* to metabolize DMS and also prevented formation of sulfate from thiosulfate provided as an additional electron source during chemoorganoheterotrophic growth. Complementation with the *hdr*-like genes under a constitutive promoter rescued the phenotype on thiosulfate as well as on DMS. The production of sulfate from an organosulfur precursor via the Hdr-like system is previously undocumented and provides a new shunt in the biogeochemical sulfur cycle. Furthermore, our findings fill a long-standing knowledge gap in microbial dissimilatory sulfur metabolism because the Hdr-like pathway is abundant not only in chemoheterotrophs, but also in a wide range of chemo- and photolithoautotrophic sulfur oxidizers acting as key players in global sulfur cycling.

Introduction

In nature, the cycles of inorganic and organic sulfur compounds are intimately interwoven. The most oxidized inorganic form, sulfate (+6), is a ubiquitous electron acceptor used in the absence of energetically more favorable respiratory substrates like oxygen or nitrate. Sulfate respiration results in production of massive amounts of hydrogen sulfide (−2) reaching several hundred million tons annually, mainly in marine sediments and pelagic oxygen minimum zones [1]. Sulfide in turn serves as electron donor for sulfur-oxidizing microorganisms. Sulfur transformations driven by microorganisms also involve inorganic sulfur compounds of intermediate redox states, like thiosulfate ($^-$S–SO_3^-) or elemental sulfur. In addition, the natural sulfur cycle undergoes a considerable impact by organic sulfur compounds among which the malodorous, volatile dimethylsulfide (CH_3–S–CH_3, DMS) is especially important.

The main source for the ~300 million tons DMS produced per year is degradation of dimethylsulfoniopropionate, a stress protectant accumulated by some macroalgae and phytoplankton, in the upper mixed layers of the oceans [2–4]. Reduction of dimethylsulfoxide, breakdown of sulfur-containing amino acids and methoxylated aromatic compounds, methylation of sulfide or methanethiol (CH_3–SH, MT) as well as anthropogenic emissions from wastewater treatment, animal rendering, kraft pulping, and composting are other sources of DMS not limited to marine environments [4–6]. DMS is credited with a pivotal role in global climate control because its oxidation products initiate

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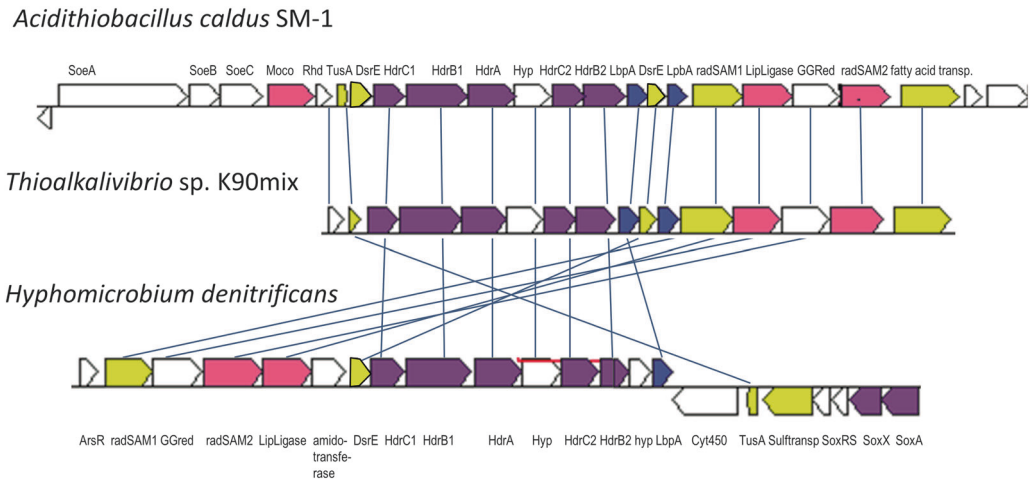

Fig. 1 Arrangement of *hdr*-like genes in selected bacteria capable of oxidizing inorganic or volatile organic sulfur compounds. Homologous genes are colored the same between organisms. Kegg/NCBI locus tag identifiers for the regions shown: *H. denitrificans*, Hden_0682-0703; *A. caldus*, Atc_2359-2337; *Thioalkalivibrio* sp. K90mix, TK90_0630-0645. SoeABC, sulfite-oxidizing enzyme [40]; LbpA, lipoate-binding protein; Rhd, rhodanese; GGred, geranylgeranyl reductase-like; radSAM, radical SAM domain-containing protein; LipLigase, lipoate:protein ligase

cloud formation over the oceans [7]. DMS-degrading microorganisms have vast importance in regulating Earth's climate, as they prevent the escape of about 90% of DMS to the atmosphere [8]. DMS can serve as a source of carbon and electrons and the sulfur contained can either be assimilated or is excreted as sulfate [9–12], thiosulfate [13], or tetrathionate [14], thereby establishing a tight link to the biogeochemical cycling of inorganic sulfur compounds.

Despite their obvious importance, neither the biochemistry of prokaryotic sulfur oxidation nor the microbial remineralization of DMS is fully understood [15–17]. A particular knowledge gap exists with regard to the intertwinement of both processes. Various DMS degradation pathways have been reported, some of these featuring MT and/or H_2S as intermediates [16]. Methanethiol oxidase catalyzes the reaction of MT with oxygen to formaldehyde, hydrogen peroxide, and sulfide [15]. Formaldehyde is either assimilated or oxidized to CO_2. The steps involved in sulfide oxidation have either not been elucidated or simply attributed as being the same as in inorganic sulfur compound oxidizers [9, 12, 14, 16, 18].

The major problem with the latter assumption is that the current picture of inorganic sulfur compound oxidation is far from being comprehensive. In a vast number of prokaryotes specialized on lithotrophic growth on reduced sulfur compounds, the periplasmic Sox pathway for thiosulfate oxidation to sulfate [19] is incomplete and lacks the SoxCD component responsible for the oxidation of SoxY-bound sulfane sulfur ($R–S^-$) to the sulfone state ($R–SO_3^-$). While a portion of these organisms employs the cytoplasmic Dsr pathway involving dissimilatory sulfite reductase [20], the central sulfur oxidation route is largely unknown for a huge array of other sulfur oxidizers including environmentally and biotechnologically highly relevant organisms like ore-leaching *Acidithiobacillus* species or thermoacidophilic archaea [17]. Metabolic reconstruction supported by microarray transcript profiling or comparative proteomics predicted the involvement of a heterodisulfide reductase-like HdrC1B1AHypHdrC2B2 complex in these organisms [17, 21–23] (Fig. 1) bearing resemblance to HdrABC from methanogens [24], although no coenzyme M-coenzyme B (CoM-S-S-CoB) heterodisulfide is present in the sulfur oxidizers. Direct experimental evidence for an involvement of an Hdr-like complex in oxidative microbial sulfur metabolism was so far not provided because the majority of the relevant organisms are obligate chemolithoautotrophs that strictly depend on reduced sulfur compounds as electron donors and are not accessible to manipulative genetics.

Here, we addressed the described major knowledge gaps in volatile organosulfur and inorganic sulfur compound degradation. We chose the Alphaproteobacterium *Hyphomicrobium denitrificans* X^T (ATCC 51888) as a model because it contains *hdr*-like genes (Fig. 1 [17, 25]) and is neither thermo-, acido-, alkali-, nor halophilic. As a typical member of the Hyphomicrobia, this appendaged, budding bacterium is ubiquitous in brackish and fresh waters as well as soils and also found in sewage treatment plants [26]. Most importantly, it is not obligately dependent on sulfur compound oxidation but grows as a restricted facultative methylotroph on substrates like methanol, methylamine (MA), or dimethylamine (DMA). This enabled us to compare the protein equipment of *H. denitrificans* cells on sulfur-containing and sulfur-free substrates to delineate a probable pathway for DMS oxidation involving thiosulfate as an intermediate and to genetically validate the sulfur-

oxidizing function of the Hdr-like proteins in *H. denitrificans*. The genetic potential for the Hdr pathway of sulfur oxidation is present in a wide spectrum of phylogenetic lineages and may co-exist with DMS/MT-degrading capability.

Material and methods

Bacterial strains, plasmids primers, and growth conditions

The bacterial strains, plasmids, and primers used in this study are listed in Supplementary Table S1. *Escherichia coli* BL21 (DE3) Δ*iscR* [27] was used for recombinant protein production and grown in LB medium. *E. coli* 10β was used for molecular cloning. *H. denitrificans* strains were cultivated in minimal media kept at pH 7.2 with 100 mM 3-(*N*-Morpholino)propanesulfonic acid (MOPS) buffer and containing per liter: 1 g NH_4Cl, 0.2 g $MgSO_4 \times 7\ H_2O$, 0.5 g $NaH_2PO_4 \times 2\ H_2O$, 1.55 g K_2HPO_4, and 0.2 ml l^{-1} trace element solution [28]. Methanol, dimethyl-amine (DMA), methylamine (MA), or dimethylsulfide (DMS) were added as carbon and electron source. Unless otherwise indicated, 200 ml cultures containing 24.4 mM methanol or 50 mM methylamine were shaken in 500-ml Erlenmeyer flasks at 200 rpm and incubated at 30 °C. For growth on DMS, 500-ml serum vials sealed with butyl rubber stoppers containing 100 ml medium were used. Varying concentrations of thiosulfate were supplied in growth experiments. Antibiotics for *E. coli* and *H. denitrificans* were used at the following final concentrations: kanamycin 50 μg ml^{-1}, tetracycline 15 μg ml^{-1}, ampicillin 100 μg ml^{-1}, rifampin 50 μg ml^{-1}, streptomycin 200 μg ml^{-1}, and chloramphenicol 25 μg ml^{-1}.

Recombinant DNA techniques

Standard methods for DNA manipulation and cloning were used unless otherwise indicated [29]. Restriction enzymes, T4 ligase, and Q5 DNA polymerase were obtained from New England Biolabs (Ipswich, UK) and used according to the manufacturer's instructions. Oligonucleotides for cloning were obtained from Eurofins MWG (Ebersberg, Germany). The genotypes of the *H. denitrificans* mutant strains generated in this study were confirmed by Southern hybridization and PCR. Southern hybridization was performed overnight at 68 °C. DNA probes for Southern hybridization were digoxigenin labeled by PCR as described in ref. [20]. Plasmid DNA from *E. coli* was purified using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, USA). Chromosomal DNA from *H. denitrificans* strains was prepared using the First-DNA all-tissue Kit (GEN-IAL GmbH, Troisdorf, Germany).

Transformation of *H. denitrificans* by electroporation

Electrocompetent cells of *H. denitrificans* were prepared using a modified protocol that had originally been developed for *Hyphomicrobium chloromethanicum* [30]. A *H. denitrificans* culture (400 ml) grown in minimal medium containing 24.4 mM methanol was harvested during early exponential phase at an optical density at 600 nm (OD_{600}) of 0.3 (4000 × *g*, 10 min, 4 °C). Cells were washed twice with ice-cold water (4000 × *g*, 10 min, 4 °C), once with ice-cold 10% (v/v) glycerol, and finally resuspended in 800 µl of 10% glycerol. Fifty-microliter aliquots of cells were mixed with 500 ng of plasmid DNA and incubated on ice for 10 min. Electroporation was carried out in 0.1 cm gap cuvettes (Bio-Budget Technologies GmbH, Krefeld, Germany) with a Bio-Rad gene pulser II (Bio-Rad Laboratories) with the following electrical settings: 2.4 kV and 200 Ω at a capacitance of 25 µF. After electroporation, 1 ml of minimal medium containing 24.4 mM methanol was added to the cuvette. Cells were transferred to an Eppendorf tube and incubated at 30 °C for 6 h. Transformants were selected by plating suitable dilutions of electroporated cells onto minimal medium agar containing 24.4 mM MeOH and the appropriate antibiotics. Plates were incubated at 30 °C for up to 14 days. The resulting antibiotic-resistant colonies were screened via PCR.

Construction of *H. denitrificans* mutant strains

For partial replacement of genes Hden_0691 (*hdrA*) and Hden_0692 (*hyp*) by a tetracycline Ω cassette and for markerless deletion of Hden_2748 (*tsdA*), respectively, splicing by overlap extension (SOE) [31] PCR fragments were constructed using primers Fwd_5′Δhdr, Rev_5′Δhdr, Fwd_3′Δhdr, Rev_3′Δhdr and Fwd_5′ΔtsdA, Rev_5′ΔtsdA, Fwd_3′ΔtsdA and Rev_3′ΔtsdA, respectively (see Table S1). The Δ*hdr* fragment was inserted into plasmid pET-22b (+) via XhoI and NcoI restriction sites resulting in plasmid pET-22bΔhdr. The tetracycline Ω cassette from plasmid pHP45Ω-Tc [32] was inserted into pET-22bΔhdr using HindIII restriction sites. The final construct pET-22bΔhdrTc was electroporated into *H. denitrificans* Sm200 (Table S1). Transformants were selected on minimal medium plates containing 24.4 mM methanol and the appropriate antibiotics. Double crossover recombinants were Tc^r and Sm^r but Ap^s because they had lost the vector-encoded ampicillin resistance. The genotype of double crossover recombinants was verified by Southern hybridization experiments. For complementation of the *H. denitrificans* Δ*hdr* mutant, plasmid pBBR1p264HdenHdrRif was electroporated in electrocompetent *H. denitrificans* Δ*hdr* cells. Transformants were selected on minimal medium plates

containing the appropriate antibiotics. The genotypes of recombinants were confirmed by PCR.

For markerless in-frame deletion, the Δ*tsdA* fragment was inserted into plasmid pk18*mobsacB* [33] using SphI restriction sites. The tetracycline cassette from pHP45Ω-Tc [32] was inserted into the resulting plasmid pk18*mobsacB*ΔtsdA using SmaI restriction sites. The final construct pk18*mobsacB*ΔtsdATc was electroporated into *H. denitrificans* Sm200. Transformants were selected on minimal medium plates containing 24.4 mM methanol and the appropriate antibiotics. Single crossover recombinants were Sm^r, Km^r, and Tc^r, verified by PCR screening and plated on minimal medium containing methanol and 10% sucrose. Double crossover recombinants survived in the presence of sucrose due to loss of the vector-encoded levansucrase (SacB).

Characterization of phenotypes and quantification of sulfur species

For growth experiments on 0.6 mM DMS, cultures were inoculated to a start OD_{600} of 0.01 with precultures in late-exponential growth phase cultured on 24.4 mM methanol. DMS was quantified using gas chromatography (GC). An aliquot of 50 µl samples was taken from the headspace and injected into a GC (PerkinElmer Clarus® 480, Rascon FFAP column 25 m × 0.25 micron) equipped with a flame ionization detector. Measurements were conducted at a column temperature of 200 °C, an injector temperature of 150 °C, and a detector temperature of 250 °C. N_2 was used as carrier gas. DMS concentrations were calculated by regression analysis based on a seven-point calibration with standard DMS solutions in minimal medium. For growth experiments on thiosulfate, media with 24.4 mM methanol and 2.5 mM thiosulfate were inoculated to a start OD_{600} of 0.005 with precultures in late-exponential growth phase cultured on the same medium. Thiosulfate, tetrathionate, sulfite, and sulfate were determined by colorimetric, turbidometric, and HPLC methods as described previously [34, 35].

Immunoblot analysis

H. denitrificans cells were resuspended in 1× PBS l (4 mM KH_2PO_4, 10 mM Na_2HPO_4, 115 mM NaCl) containing 0.2 mg ml^{-1} lysozyme and a few grains of DNase and disrupted by sonication (Bandelin Sonopuls UW 2070). After removal of insoluble cell material by centrifugation (16,100 × *g*, 15 min, 4 °C) protein content was determined with a BCA kit (Thermo Fisher Scientific) and cell extracts were separated by SDS-PAGE. Western analysis was performed using the Transblot SD semi-dry transfer apparatus (Bio-Rad Laboratories) and nitrocellulose membranes (Amersham Protran 0.45 NC, GE Healthcare). HdrA antigens were detected with antisera raised in rabbits (Eurogentec) against recombinant *H. denitrificans* HdrA purified from *E. coli* (see Supplementary information for recombinant protein production). Antisera were used at 1:2000 dilution. Binding of α-HdrA was detected with the SignalFire™ ECL reagent system (Cell Signaling Technology).

Proteomic profiling using tandem mass tags

For global proteomic profiling, *H. denitrificans* were grown in 300 ml minimal medium under two different regimes. In the first case, cultures contained 8 mM dimethylamine and were harvested during exponential growth phase at OD_{600} of 0.35. In the second case, cultures were pre-grown on 4 mM dimethylamine to an OD_{600} of 0.2 and then a total of 2.25 mM DMS was added in 0.25–0.5 mM portions. Cultures were harvested in the exponential growth phase before the last added DMS portion was completely used up. Harvesting proceeded at 10,000 × *g* for 12 min at 4 °C. Cells washed with 1× PBS were resuspended in lysis buffer (1× PBS containing 5% (wt/v) sodium deoxycholate (SDC) and protease inhibitor (cOmplete, Roche Diagnostics)) and disrupted by sonication. Cell debris was removed by centrifugation (16,100 × *g*, 30 min, 4 °C). The supernatant was adjusted to a protein concentration of 2 mg ml^{-1} with lysis buffer and stored at −20 °C. Cleared lysates were subjected to in-solution preparation of reduced, alkylated peptides as outlined in Supplementary Information. Peptides were labeled with amine-reactive Thermo Scientific Isobaric Mass Tagging (TMTsixplex) reagents. Experimental details concerning peptide separation, mass spectrometry, data processing, and statistical analysis are given in Supplementary Information. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [36] partner repository with the data set identifier PXD009030.

Identification of *hdr*-like gene clusters in bacterial genomes

Typical clusters of *hdr*-like genes were identified in microbial genomes based on BLASTP searches against assembled genomes at NCBI. HdrC1 and HdrB2 amino acid sequences from *Acidithiobacillus caldus* were used as queries. All hits used in further analysis had an *e* value of $1e^{-70}$ or lower. All positive hits were manually checked for co-occurrence of the other genes in the correct order of appearance (*hdrC1B1AhyphdrC2B2*). Furthermore, the occurrence of the set of *hdr*-like genes was checked in the Uncultivated Bacteria and Archaea (UBA) data set [37] using TBLASTN and the amino sequences of the *A. caldus*

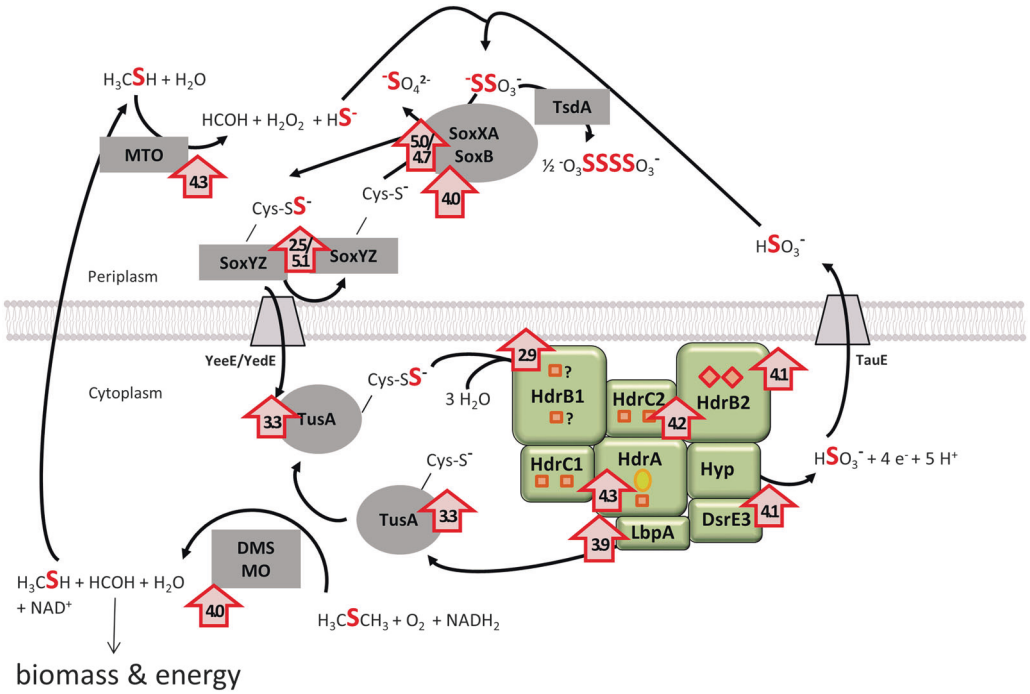

Fig. 2 Metabolic reconstruction of DMS and thiosulfate metabolism in *H. denitrificans* X^T based on genome sequence analysis, comparative proteomics, and reverse genetics. Fold changes (log2) in protein abundancies are given next to proteins detected as significant in the proteomic study. Prosthetic groups are indicated: orange boxes, [4Fe–4S]; red diamond, non-cubane [4Fe–4S], yellow oval, FAD. DMS, MO DMS monooxygenase; MTO, methanethiol oxidase; TsdA, thiosulfate dehydrogenase

Hdr-like proteins as queries with a cutoff in *e* value of $1e^{-50}$. Only those sequences were listed as positive that contained the complete *hdr*-like gene set on the same contig. The UBA data set, which is the largest currently available set of metagenome-assembled genomes, was also searched with the amino sequence of MtoX from *Hyphomicrobium* sp. VS [15] in order to identify metagenomes in which *mtoX* and *hdr*-like genes co-occur.

Results

Comparative proteomic analysis allows metabolic reconstruction of DMS oxidation in *H. denitrificans*

Some *Hyphomicrobium* isolates oxidize thiosulfate and sulfide as additional sources of electrons during chemoorganoheterotrophic growth [12] and several have been reported to grow on DMS as a sole carbon source [9, 11, 12, 15]. Contrary to earlier reports [12], we found that this also holds true for strain X^T. All sulfur contained in DMS is released as sulfate (Supplementary Fig. S1).

Detailed analysis of the genome sequence and proteomic comparison of *H. denitrificans* cells grown on DMS vs. DMA served as the basis for metabolic reconstruction of the DMS oxidation pathway (Fig. 2 and Supplementary Tables S2–S4). The proteome underwent substantial remodeling upon growth on the volatile organic sulfur compound. Thirty-one of 54 proteins that were statistically significantly more abundant during growth on the organosulfur compound are encoded within a range of 86 genes (operon structures Hden_0669–0759), localizing DMS degradation capacity to a defined genetic island. The initial step of DMS oxidation is catalyzed by DMS monooxygenase [9]. In the absence of genes encoding the characterized $FMNH_2$-dependent oxygenase DmoA from *Hyphomicrobium sulfonivorans* and its partner DmoB, a NADH-dependent FMN oxidoreductase [38], we tentatively assigned Hden_0730 annotated as FMN-dependent-azoreductase for this step. Methanethiol oxidase [15] is localized in the periplasm and releases hydrogen sulfide. Essential components of the thiosulfate-oxidizing Sox multienzyme complex, SoxYZ, SoxXA, and SoxB, were all much more abundant on DMS than on DMA, indicating thiosulfate as an intermediate en route to sulfate. A putative COG2392 membrane protein (also known as YeeE/YedE), Hden_0699, is encoded in the same gene cluster and downstream of *soxXA* (Fig. 1). *Serratia* proteins belonging to this group mediate transport of sulfur-containing compounds [39]. Hden_0699 could therefore be involved in transport of sulfur into the cytoplasm, where it is bound by the sulfane–sulfur carrier protein TusA, encoded by the neighboring gene. TusA is a central hub for sulfur in the cytoplasm, not only delivering to the sulfur oxidation

pathways in dissimilatory sulfur oxidizers [21], but also distributing to different biosynthetic pathways as has been shown for *E. coli* [41].

Prominent abundance changes were furthermore observed for the cytoplasmic proteins encoded in the cluster of *hdr*-like genes (Figs. 1 and 2), indicating that a Hdr-like complex indeed acts as a sulfur-oxidizing unit. Just as the proteins from lithotrophic sulfur oxidizers, the *H. denitrificans* HdrA-like protein appears to bind one flavin cofactor and only one instead of the six [4Fe–4S] centers in the protein from methanogens (Supplementary Fig. S2). HdrB2 is predicted to contain the two recently characterized non-cubane [4Fe–4S] centers of the heterodisulfide-reducing active site [24] while not all cluster-binding residues are conserved in HdrB1 (Supplementary Fig. S3). The HdrC-like proteins bind two [4Fe–4S] clusters (Supplementary Fig. S4) and may mediate electron transfer between HdrA and the two HdrB-like proteins. Proteins HdrAB1B2C1C2 have previously been co-purified from the hyperthermopilic bacterium *Aquifex aeolicus* but a functional role was not assigned [42]. The function of the hypothetical protein encoded between genes *hdrA* and *hdrC2* in all *hdr*-like gene loci from sulfur oxidizers is currently enigmatic. Related proteins are not encoded outside of *hdr*-like genes clusters, thus there is no relationship to any functionally characterized proteins.

Most likely, the Hdr-like proteins form sulfite which would then be exported to the periplasm, probably by Hden_0720, a TauE-like sulfite transporter [43]. Dedicated sulfite-oxidizing enzymes like the cytoplasmically oriented sulfite-oxidizing enzyme SoeABC [40] or periplasmic Sor-related sulfite dehydrogenases [44] are not encoded in *H. denitrificans* X^T, but once in the periplasm, hydrogensulfite could be removed by spontaneous chemical reaction with hydrogen sulfide [45] released in the MT oxidase reaction, thus regenerating thiosulfate as a substrate for the Sox system. Another possibility is oxidation of sulfite by the combined action of SoxXA, SoxYZ, and SoxB as has been described in vitro for the *Paracoccus pantotrophus* proteins [46].

Thiosulfate oxidation in *H. denitrificans* wild-type and mutant strains

To gain deeper insights into the suggested connection between metabolism of inorganic and organic sulfur compounds in *H. denitrificans*, we first assessed thiosulfate consumption by the wild-type (wt) strain. In contrast to older reports [12], it was well able to degrade thiosulfate. Besides the Sox proteins, *H. denitrificans* encodes a second thiosulfate-metabolizing enzyme, the tetrathionate-forming diheme cytochrome *c* thiosulfate dehydrogenase, TsdA [47]. *H. denitrificans* wt cultures growing

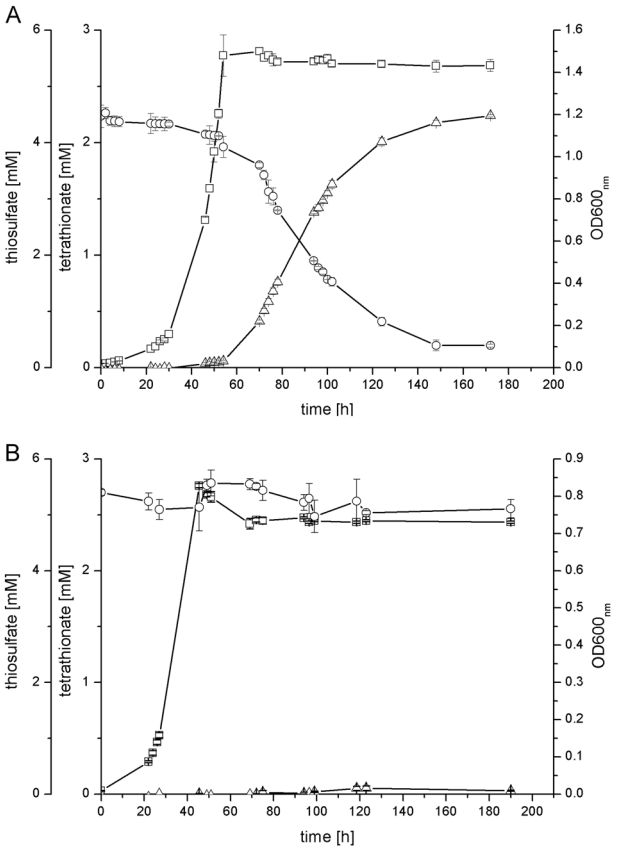

Fig. 3 **a** Stoichiometric formation of tetrathionate from thiosulfate by *H. denitrificans* X^T on 50 mM methylamine and 5 mM thiosulfate. **b** *H. denitrificans* Δ*tsdA* is unable to form tetrathionate from thiosulfate. Medium contained 24.4 mM methanol and 5 mM thiosulfate. Thiosulfate (○), tetrathionate (Δ), and OD_{600} (□) are given. Inocula had been grown on thiosulfate-free methanol-containing medium. All data are the average of two independent biological replicates. Errors bars indicate SD

chemoorganoheterotrophically in a medium buffered to pH 7.2 and containing 5 mM thiosulfate started thiosulfate consumption in stationary phase and produced stoichiometric amounts of tetrathionate (Fig. 3a). Basic published strategies for introduction of plasmids into *H. denitrificans* [48, 49] served as the starting point for reverse genetics enabling targeted gene knockouts and in-frame deletion of the *tsdA* gene. The Δ*tsdA* strain did not degrade thiosulfate under the described conditions and thiosulfate dehydrogenase was thus identified as the sole tetrathionate-forming enzyme in the organism (Fig. 3b). Tetrathionate is a dead-end product and not metabolized any further. When 0.5, 1, or 2.5 mM tetrathionate was added to methanol or methylamine-containing medium, concentrations stayed essentially the same even until late stationary phase (not shown).

The picture completely changed when cultures were grown in the presence of only 2.5 mM thiosulfate. Now the wild-type and the Δ*tsdA* strain did no longer form

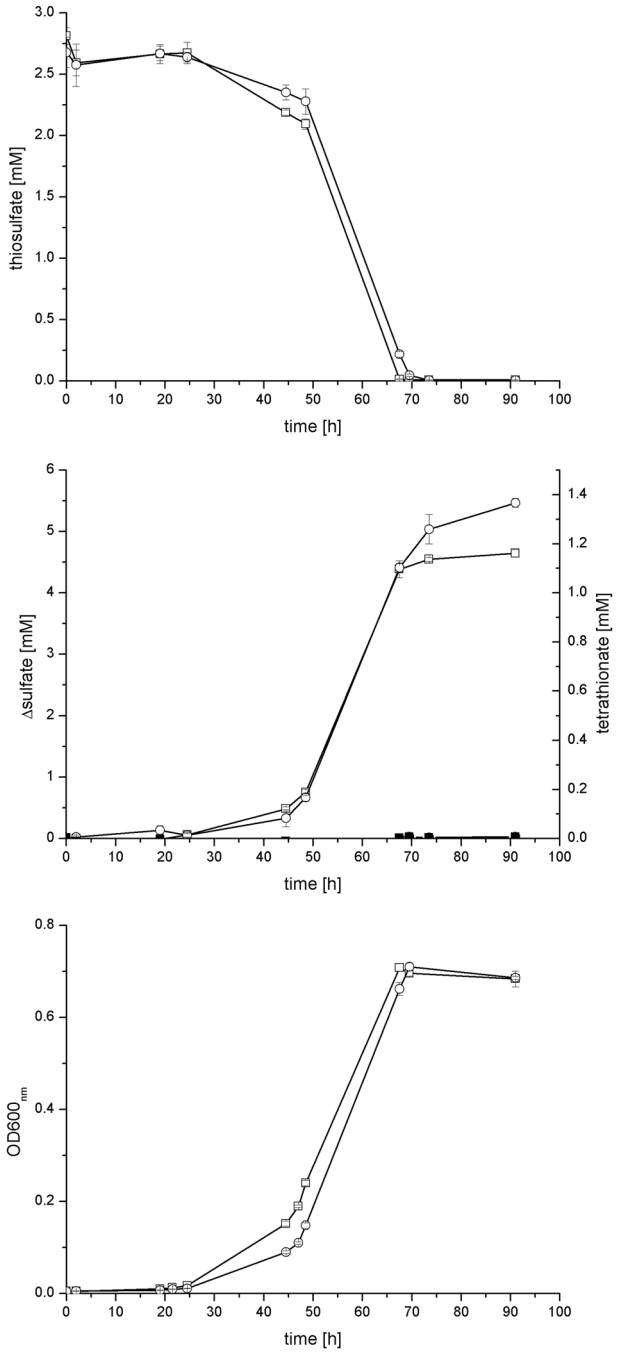

Fig. 4 Growth of *H. denitrificans* X^T (boxes, □, ■) and strain Δ*tsdA* (circles, ○, ●) on 24.4 mM methanol and 2.5 mM thiosulfate. Precultures were grown on methanol-containing medium with 2.5 mM thiosulfate. In the middle panel, open circles and boxes refer to sulfate, closed circles and boxes give tetrathionate concentrations. All data are the average of two independent biological replicates. Errors bars indicate SD

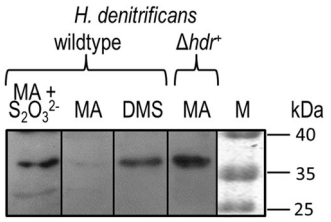

Fig. 5 Western blot analysis with antiserum against HdrA (37.6 kDa) performed with crude extracts of *H. denitrificans* wild type grown on methylamine plus thiosulfate ($MA + S_2O_3^{2-}$, 14.4 µg protein), methylamine (MA, 17.8 µg protein), and dimethylsulfide (DMS, 22.5 µg protein). Lane 4 was loaded with extract (17.5 µg protein) of *H. denitrificans* expressing plasmid-encoded genes *dsrEhdrClB1A-hyphdrC2B2hyplbpA* from a constitutive promoter. These cells were grown on methylamine. M, marker proteins. The antiserum was raised against HdrA produced recombinantly in *E. coli*

tetrathionate but both quantitatively oxidized thiosulfate to sulfate (Fig. 4). Metabolic reconstruction (Fig. 2) predicted the Hdr-like proteins to be involved in the latter process. This concept was supported by the presence of the HdrA-like protein in methylamine/thiosulfate, but not in MA-grown cells (Fig. 5). Proof was provided by our finding that sulfate formation absolutely required the presence of a functional Hdr-like complex and was no longer possible in *H. denitrificans* strain Δ*hdr* (Fig. 6a) in which part of the *hdrA*-like gene and the subsequent gene for a hypothetical protein were replaced by a tetracycline Ω cartridge preventing transcription and translation of the affected genes and all genes located downstream in the same transcriptional unit [32]. It should be noted that the strain lacking a functional Hdr-like system still degraded thiosulfate albeit at a much lower rate than the wild type. Tetrathionate was formed in stoichiometric amounts in this case (Fig. 6a). When the *hdr*-like gene cluster from *dsrE* to *lbpA* (Fig. 1) was reintroduced in trans on broad host range plasmid pBBR1p264 [50] such that it was expressed under a strong constitutive promoter from *Gluconobacter oxydans*, thiosulfate oxidation and sulfate formation capacities were completely restored (Fig. 6a).

DMS oxidation in *Hyphomicrobium denitrificans* X^T

Finally, *H. denitrificans* wild type, the Δ*tsdA* and Δ*hdr* deletion strains, and the Δ*hdr*$^+$-complemented strain were compared on DMS as the sole carbon and energy source. The Δ*tsdA* strain did not show any differences to the wild type excluding tetrathionate as an intermediate (Supplementary Fig. S4). The Δ*hdr* strain, however, proved completely unable to metabolize DMS and the complemented strain carrying the *hdr*-like genes in trans regained the ability to grow on DMS and to form sulfate (Fig. 6b), clearly identifying the proteins encoded by *H. dentirificans* *hdr*-like genes as essential for downstream processing of the sulfur contained in DMS.

As shown in Fig. 2, crucial steps of DMS/MT and thiosulfate oxidation occur in different cellular compartments and it is thus not immediately obvious why a block in downstream thiosulfate oxidation would completely prevent

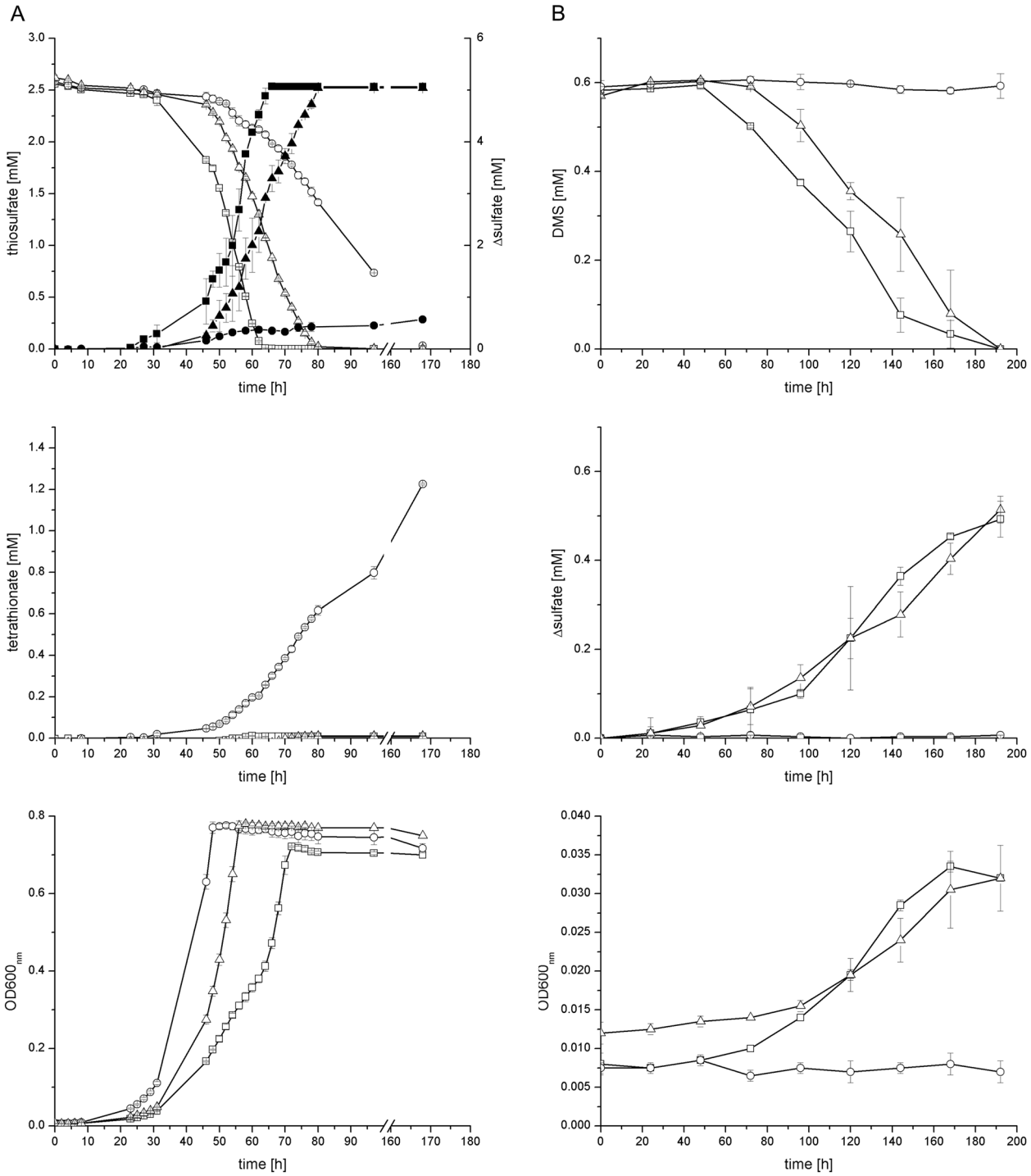

Fig. 6 Growth on and consumption of (**a**) thiosulfate in methanol-containing medium or (**b**) DMS by *H. denitrificans* wt (□), Δ*hdr* (○), and the complemented Δ*hdr*$^+$ strain (Δ). Consumption of thiosulfate and DMS, respectively, is shown in the upper panels. Stoichiometric production of sulfate from thiosulfate for the wt and the Δ*hdr*$^+$ strain is indicated by filled symbols in the upper left panel. Tetrathionate concentrations are shown in the middle left panel. Neither thiosulfate and tetrathionate nor sulfite were detected as intermediates during growth of the wild type on DMS, even with very sensitive HPLC methods. All data are the average of two independent biological replicates. Errors bars indicate SD

degradation of DMS. Here, we need to take into account that sulfide would accumulate in the course of the MT oxidase-catalyzed reaction in the absence of a functional cytoplasmic sulfur oxidation system. MT oxidase is subject to strong feedback inhibition by sulfide [15, 51], precluding any substantial MT and thus also DMS oxidation unless

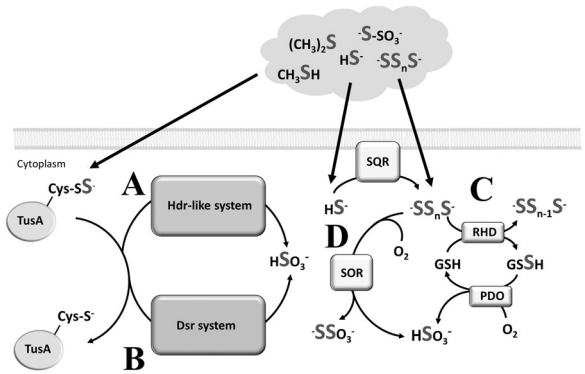

Fig. 7 Cytoplasmic sulfur oxidation pathways. For clarity, reactions are not given with exact stoichiometries. In cases *A* and *B*, the electrons released by formation of hydrogensulfite can be fed into respiratory or photosynthetic electron transport chains. In both cases, the TusA protein is proposed as a central sulfur carrier in the cytoplasm and collects sulfur stemming from the oxidation of organic and/or reduced inorganic sulfur compounds [21]. Initial steps take place outside of the cytoplasm. *A* Oxidation of (protein-bound) persulfide sulfur by the Hdr-like system discovered in this work. *B* Oxidation of protein-bound persulfide sulfur by the Dsr system involving dissimilatory sulfite reductase. The immediate sulfur substrate carrier for this enzyme is the protein DsrC [17]. *C* Sulfide detoxification in the cytoplasm via sulfide:quinone oxidoreductase (SQR), rhodanese (RHD), and sulfur/persulfide dioxygenase (PDO). Electrons resulting from the SQR-catalyzed reaction can be fed into the quinone pool. Sulfite is formed by direct reaction with molecular oxygen preventing energy conservation [52]. GSH (GSSH), reduced glutathione (persulfide). *D* Some archaeal and bacterial sulfur oxidizers contain sulfur oxygenase reductases (SOR). These enzymes usually catalyze a dioxygen-dependent disproportionation of elemental sulfur/and or poylsulfides to hydrogensulfite, thiosulfate, and hydrogen sulfide. Here, SOR is depicted without reductase activity as has been described for *Thioalkalivibrio paradoxus* [53]

sulfide is efficiently removed. In addition to the Hdr-like pathway, *H. denitrificans* contains the equipment for sulfide detoxification in the cytoplasm via sulfide:quinone oxidoreductase (SqrB, Hden_0718), a rhodanese-type sulfur transferase (Hden_0719, Rhd) and persulfide dioxygenase (Pdo, Hden_740) [52] (Fig. 7). Although SqrB and Pdo are significantly more abundant on DMS than in its absence (Table S2), their presence in the Δ*hdr* strain is obviously neither sufficient to sustain growth on this substrate nor are they involved in any significant production of sulfate.

Diversity of the Hdr-like system

To investigate the diversity of microorganisms in the environment that contain the novel sulfur-oxidizing heterodisulfide reductase-like system identified in *H. denitrificans* by reverse genetics, we checked all genome and metagenome sequences available through the National Center for Biotechnology Information. Stringent criteria were applied and only those organisms were listed that contain all six core *hdr*-like genes in the same order of appearance (Table S5). The gene set was identified in Archaea as well as in the domain Bacteria. The greatest number of organisms and metagenomes containing *hdr*-like genes is found among the Proteobacteria. With very few exceptions, all characterized organisms containing *hdr*-like genes are either established chemo- and photolithotrophs oxidizing reduced inorganic sulfur compounds or they have been reported as oxidizing mineral sulfides (FeS, FeS_2, and others) or organic sulfur compounds (DMS). In some instances, the ability of the respective organism to oxidize sulfur compounds has not been tested or reported. Notably, all of these organisms stem from sulfur-dominated habitats like solfataras or ocean sediments and are therefore likely sulfur compound oxidizers. Our database search revealed the occurrence of *hdr*-like genes in several assembled metagenomes assigned as unclassified Sulfolobales, Proteobacteria, Alpha-, Beta-, or Gammaproteobacteria. The respective metagenomics studies focused on subseafloor or subsurface aquifers, sediments and rocks/rock porewater as well as mine wastewater again emphasizing that the activity of organisms containing the Hdr-like system for sulfur oxidation may considerably impact the transformation of sulfur compounds at these sites.

A previous database survey focusing on the linkage between genes encoding sulfurtransferases and genes encoding putative cytoplasmic sulfur oxidation systems revealed an almost mutually exclusive occurrence of *hdr*-like genes and those for the Dsr system of sulfur oxidation [17]. Two organisms (*Thioalkalivibrio sulfidophilus* and *Thioalkalivibrio nitratireducens*) contained both genes sets. As evident from Table S5, the same holds true for two metagenomes assigned to the Betaproteobacteria and one gammaproteobacterial metagenome assigned to the Chromatiales. In total, only a 5.5% fraction of all analyzed sulfur oxidizer genomes contains *dsr* as well as *hdr*-like genes sets. This implies that using either one of the pathways is the rule rather than the exception.

The genetic capacity for sulfur oxidation in the cytoplasm via the Hdr-like and/or the Dsr system (Fig. 7a, b) can (but does not have to) be combined with the genetic equipment for sulfide detoxification involving Sqr, Rhd, and Pdo [52] (Fig. 7c) and/or cytoplasmically located sulfur oxygenase reductase (SOR), an enzyme that catalyzes oxygen-dependent disproportionation of elemental sulfur and/or polysulfides [53] (Fig. 7d). As already noted above, *H. denitrificans* encodes Hdr-like proteins as well as Sqr, Rhd, and Pdo. *Acidithiobacillus albertensis* DSM 14366^T may serve as an example containing *hdr*-like genes (BLW97_RS11375-RS11335), the pathway involving Pdo (BLW97_RS13940) and SOR (BLW97_RS13410). *T. nitratireducens* DSM 14787^T is equipped with all four different genes sets (*hdr*-like: TVNIR_3249-3244, *dsr*:

TVNIR-0860-0847, *pdo*: TVNIR_2193, *sor*: TVNIR_2721 and TVNIR_1174).

Linkage of aerobic DMS and MT oxidation and Hdr-like system

When DMS is used as a carbon and electron source under aerobic conditions, the first step is either catalyzed by a DMS monooxygenase or by an oxygen-independent methyltransferase [15, 16, 54]. Both reactions result in the formation of methanethiol. The presence of the recently identified methanethiol oxidase, MTO [15], can therefore be taken as an ideal indicator for all organisms pursuing the described pathways and also covers those microorganisms that might exclusively degrade methanethiol. We checked all organisms listed by Eyice and coworkers as containing MTO [15] for the co-occurrence of the *hdr*-like gene cluster and thereby detected an intertwinement of aerobic DMS degradation with Hdr-based sulfate formation not only for *Hyphomicrobium* species, but also for a number of *Thioalkalivibrio* species (Table 1). This finding expands the linked occurrence of aerobic DMS/MT degradation and sulfate formation by an Hdr-like system from ubiquitous environments colonized by mesophiles like *Hyphomicrobium* to habitats dominated by extreme conditions. Our database survey further revealed a set of other chemo- and phototrophs, including magnetotactic bacteria, *Thiobacillus*, and *Thiothrix* species as well as purple sulfur bacteria, that combine MT oxidase and the Dsr pathway of sulfur oxidation (Table 1) verifying that the final steps of sulfate formation in organosulfur compounds oxidizers are indeed catalyzed by the same modules as in bacteria restricted to oxidation of inorganic sulfur compounds.

Discussion

The findings presented here close several major knowledge gaps in prokaryotic oxidation of organic and inorganic sulfur compounds. Our study revealed a linkage of organosulfur compound and inorganic sulfur compound degradation present not only in *H. denitrificans*, but also in a range of haloalkaliphiles (Table 1). Our findings add a new aspect to the current understanding of organosulfur compound oxidation because an intertwinement of organic and inorganic sulfur compound oxidation via Hdr-like proteins is so far unprecedented and provides a novel shunt in the biogeochemical sulfur cycle. *H. denitrificans* occurs in a wide range of natural and industrial habitats and although its specific contribution to global DMS removal has not been determined, the possibility of sulfate formation from DMS via the combination of initial monooxygenase-catalyzed steps and the Hdr-like pathway by single organisms should no longer be underestimated.

Overall, DMS oxidation and concomitant assimilation of carbon into cell material in these organisms can be summarized as:

$$(CH_3)_2S + 4\,O_2 \rightarrow CO_2 + [CH_2O] + H_2SO_4 + H_2O$$

For *H. denitrificans*, thiosulfate produced in the periplasm was firmly established as an intermediate of this process. Just as externally provided thiosulfate, it is initially degraded in the periplasm by the well-established Sox system. This multienzyme complex releases sulfate and SoxY-bound sulfane sulfur (Fig. 2). The cytoplasmic Hdr-like proteins proved to be indispensable for further processing of sulfane sulfur, a finding that we consider a major breakthrough because our experiments provide first direct evidence for the function of Hdr-like proteins in any sulfur oxidizer.

The reaction mechanism of as well as the fate of the electrons released by the Hdr-like system is currently unresolved. The similarity with components of archaeal heterodisulfide reductase points at (protein-bound) persulfides (RSS^-) and/or disulfides (RSSR) as possible intermediates in the reaction cycle. The most probable end product is sulfite, the same product as formed by the much better studied Dsr system [20] (Fig. 7). Database analyses showed that genes encoding the Hdr-like and Dsr systems occur almost mutually exclusively in chemo- and phototrophic sulfur oxidizers (ref. [17] and Table 1 and S5)

In *H. denitrificans*, production of Hdr-like proteins appears dependent on the presence of oxidizable substrates as shown by comparative proteome and immunoblot analyses (Figs. 2 and 5). Induction of the hyphomicrobial *hdr*-like genes is probably mediated by the ArsR-type regulator encoded by the first gene in the operon (Fig. 1). ArsR/SmtB family of metal (toxic ion) responsive repressors are typically autoregulated and part of the operon that contains genes involved in metabolism of the ion itself [39].

Our results disclose proteins encoded in *hdr*-like gene loci as essential components of a novel cytoplasmic sulfur oxidation pathway and show that the pathway is present in a wide range of mesophilic and extremophilic bacteria and archaea. Homologous genes were not only found in cultivated prokaryotes previously recognized for their potential to oxidize reduced sulfur compounds, but also in a number of organisms for which this capacity had not been reported. In addition, metagenomes from several sulfur-dominated sites contained the typical arrangement of *hdr*-like genes. So far, identification of bacteria and archaea associated with oxidative dissimilatory sulfur metabolism and the ability to oxidize sulfane sulfur to sulfite in the environment has almost exclusively focused on testing for the presence of sulfite reductase (DsrAB) genes [55–59]. Our work strongly

Table 1 Occurrence of *mto*, *dsr*, and *hdr* genes in genome-sequenced *mto*-containing prokaryotes

Organism/group	MTO	Dsr complex[a]	Hdr complex[b]
α-Proteobacteria			
Hyphomicrobiaceae			
Hyphomicrobium denitrificans ATCC 51888^T	Hden_0743	No	Hden_0689-0694
Magnetospira sp. QH-2	MGMAQ_0496	MGMAQ_2110-2122	No
β-Proteobacteria			
Burkholderiaceae			
Burkholderiales bacterium JOSHI_001	BurJ1DRAFT_0065	BurJ1DRAFT_3336-3324	No
Thiobacillaceae			
Thiobacillus denitrificans ATCC 23644^T	B059DRAFT_02518	B059DRAFT_02130-02118	No
Thiobacillus thioparus DSM 505^T	B058_RS0108880	B058_RS0104165- 4100	No
γ-Proteobacteria			
Chromatiaceae			
Chromtiaceae bacterium 2141T.STBD.0c.01a	B1781_RS11525, B1781_RS11000	B1781_RS05315-375	No
Thiocapsa roseopersicina 1711 (DSM 217^T)	SAMN05421783_102223	SAMN05421783_10260-246	No
Thiorhodococcus drewsii AZ1 (DSM 15006^T)	ThidrDRAFT_0542 (wrongly stated in ref. [15]: ThidrDRAFT_3297	ThiDRAFT_2036-2022	No
Ectothiorhodospiraceae			
Thioalkalivibrio sp. AL5	F574_RS0114010	No	F574_RS0107460-7435
Thioalkalivibrio sp. ALJ2	F468_RS0112785	No	F468_RS0104125-4150
Thioalkalivibrio sp. ALJ4	C936_RS0113555	No	C936_RS0107740-7765
Thioalkalivibrio sp. ALJ5	C937_RS0112700	No	C937_RS0107450-7425
Thioalkalivibrio sp. ALJ9	G317_RS0100035	No	G317_RS0106450-6475
Thioalkalivibrio sp. ARh4	F465_RS0114060	No	F465_RS0107820-7795
Thioalkalivibrio versutus AL2 (DSM 13738^T)	C164DRAFT_2790	No	C164DRAFT_0453-0448 C164DRAFT_3541-3546
Thiotrichaceae			
Thiothrix disciformis DSM 14473^T	A3IEDRAFT_00868	A3IEDRAFT_03610--03597	No

[a]The *dsr* gene cluster comprises *dsrABCEFHMKLJOPN*

[b]The cluster consists of *hdrC1B1A-hyp-hdrC2B2*

indicates that general conclusions on the biogeochemical sulfur cycle based on sequence data should integrate the new Hdr-like pathway (Fig. 7). Given its occurrence in a wide range of extremely thermo-, alkali-, and/or acidophilic prokaryotes, this applies especially to habitats characterized by extreme conditions.

Prokaryotes harboring the equipment for inorganic sulfur compound oxidation as well as volatile organosulfur compound degradation via the Hdr-like pathway impact biogeochemical processes in terrestrial and marine sediments, subsurface ecosystems, aquifers, wastewater treatment plants as well as other industrial habitats. Our findings will certainly better inform future microbial trait-based ecosystem models relevant for the prediction of sulfur-based biogeochemical processes and the natural sulfur cycle.

Data deposition

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (http://www.ebi.ac.uk/pride) with the data set identifier PXD009030.

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Author contributions: TK and CD designed the research; TK performed the research, TK and CD analyzed data and wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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