A regulatory duet: sHdrR and SoxR team up for transcriptional repression of sulfur oxidation in *Hyphomicrobium denitrificans*

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ABSTRACT

The Alphaproteobacterium *Hyphomicrobium denitrificans* X^T, an obligately chemoorganoheterotrophic methylotroph, uses C_1 compounds like methanol and also C_2 compounds for two purposes: oxidizing them to CO_2 for energy conservation and assimilating them for biomass production. When present, thiosulfate is used as an auxiliary electron donor. Its oxidation is initiated outside of the cytoplasm, i.e. in the periplasm. Further oxidative steps occur in the cytoplasm.

The presence of thiosulfate causes growth retardation on methanol but not on formate. I set out to explain this puzzling observation and found that methanol must be oxidized to formate and re-reduced for assimilation via the serine cycle. Thiosulfate oxidation produces sulfite, which forms adducts with pyrroloquinoline quinone (PQQ), thereby inhibiting periplasmic methanol dehydrogenase and thus methanol degradation. Formate metabolism remains unaffected as it occurs in the cytoplasm.

In the next step, the transcriptional regulation of genes encoding enzymes involved in sulfur oxidation in *H. denitrificans* was analyzed. Understanding the transcriptional regulation of sulfur oxidation to adapt metabolic flux to environmental conditions is necessary. In *H. denitrificans*, there are two homologous sulfane-sulfur-responsive ArsR-type transcriptional repressors, sHdrR and SoxR, that are responsible for the transcriptional regulation of genes encoding Sox, sHdr and associated proteins. Phenotypic analysis of knockout strains demonstrated the importance of these regulators *in vivo*. Site-directed mutagenesis, mass spectrometry, and gel shift assays *in vitro* revealed that regulatory proteins undergo conformational changes prior to detaching from the target DNA. DNA binding sites and transcriptional regulatory activity were also analyzed. The combined regulatory role of both repressors was confirmed *in vitro* by EMSA experiments. EMSA was also used to map common binding regions. These overlap the putative -35 and -10 RNA polymerase binding sites upstream of the divergently transcribed *soxY* and *soxA*, and *soxT1A* and *shdrR* gene sets.

Genes for two potential sulfur compound transporters, SoxT1A and SoxT1B, which resemble YeeE/YedE-family thiosulfate transporters, are located in the same genetic island as those involved in sulfur oxidation (*sox* and *shdr*). SoxT1A was identified as being crucial for delivery of sulfur to the cytoplasm for oxidation, while SoxT1B plays a role in signal transduction for the transcriptional repressor SoxR. Mutants lacking these transporters exhibit disrupted sulfur oxidation, underscoring their distinct but essential roles.

Target genes regulated by the repressors sHdrR and SoxR were identified through RNA-Seq analysis of deletion mutants. SoxR regulates the *sox* genes for the enzymes of thiosulfate oxidation in the periplasm and the *lip-shdrR-lbpA* genes encoding proteins responsible for sulfite formation in the cytoplasm, while sHdrR affects only a subset of these genes, excluding the *sox* genes. Both repressors cooperate, potentially forming heterodimers, and interact with other transcriptional regulators. Their regulatory effect extends far beyond sulfur oxidation, significantly impacting anaerobic metabolism, particularly denitrification in *H. denitrificans*. Whether the interaction between the two repressors is direct or indirect *in vivo* is an important question for future research.

Keywords: *Hyphomicrobium denitrifican,* ArsR family regulators, sHdr system, sHdrR, SoxR, YeeE/YedE-family sulfur transporter, thiosulfate oxidation.

CHAPTER 1

GENERAL INTRODUCTION

1 Sulfur-oxidizing bacteria are diverse and ubiquitous

Thiosulfate ($S_2O_3^{2-}$), an inorganic sulfur compound at an intermediate redox state, serves as a substrate for microbial energy metabolism across diverse ecosystems. While aerobic thiosulfate oxidation dominates in marine environments, driving significant contributions to global sulfur cycles (Podgorsek and Imhoff, 1999; Marshall and Morris, 2013; Watsuji et al., 2016), anaerobic pathways, particularly nitrate/nitrite-dependent processes, are increasingly recognized as major drivers of thiosulfate turnover in oxygen-depleted zones such as hydrothermal vents (Teske et al., 2000), anoxic basins (Menezes et al., 2020), and oxygen minimum zones (Callbeck et al., 2021). Recent studies suggest that nitrate and nitrite reduction may account for the bulk of marine thiosulfate consumption (Ding et al., 2023). Thiosulfate-dependent denitrification to N_2 is best known for obligately autotrophic species such as *Thiobacillus denitrificans*, *Thiomicrospira denitrificans* or *Sulfurovum lithotrophicum* (Inagaki et al., 2004), has been found to be important in marine chemosynthetic symbioses (Paredes et al., 2021), and has also been reported for the facultatively autotrophic *Paracoccus pantotrophus* (Robertson and Kuenen, 1983).

Sulfur-oxidizing prokaryotes encompass a diverse array of organisms, ranging from autotrophic specialists to obligately organoheterotrophic bacteria that oxidize thiosulfate as an additional electron donor and are widely distributed in soil and natural waters (Trudinger, 1967; Tuttle and Jannasch, 1972; Sorokin et al., 1999; Ding et al., 2023). Among these, *Hyphomicrobium* species stand out for their ubiquity in freshwater, soil (Hirsch and Conti, 1964; Gliesche et al., 2015; Li et al., 2023b), and engineered systems like wastewater treatment plants (Holm 1996), where they contribute to nitrate removal under both aerobic and anaerobic conditions (Martineau et al., 2015). Despite their metabolic versatility and ecological prevalence (Deligeer et al., 2002; Yamaguchi et al., 2003; Yamaguchi et al., 2004), key gaps remain in understanding their sulfur metabolism, even as their denitrification enzymes (Martineau et al., 2015) and genetic regulation have been well characterized.

2 Bioenergetics of *Hyphomicrobium* species and their carbon and oxidative sulfur metabolism

Hyphomicrobium denitrificans is an Alphaproteobacterium, known for its distinctive shape. The cells appear as rods or ellipsoids with prosthecae, i.e. stalk-like extensions (Moore 1981, Vuilleumier et al. 2011). Bacteria of the genus *Hyphomicrobium* reproduce by budding, where a new cell forms at the end of the stalk (Urakami et al., 1995) (Fig. 1).

2.1 Habitats, respiratory energy conservation and C_1-metabolism

Hyphomicrobia are typically found in low-oxygen environments such as soil, freshwater and sewage treatment plants (Gliesche et al., 2015), where they perform aerobic respiration. *H. denitrificans* is a restricted facultative methylotroph that can neither grow autotrophically nor on compounds with three or more carbon atoms (Gliesche et al., 2015) and achieves its highest growth yields when utilizing methanol or methylamine(s) as carbon and energy sources (Chistoserdova, 2011). The substrate range of *Hyphomicrobium s*pp. further includes formate, acetate, ethanol, methylamine (MA), dimethylamine (DMA), dimethyl sulfoxide (DMSO) or dimethyl sulfide (DMS), though this range can vary even among strains of the same species (Gliesche et al., 2015; Koch and Dahl, 2018). When supplied with methanol as a carbon source *Hyphomicrobium* spp., particularly *H. denitrificans*, are commonly identified as key players in denitrification systems, where they reduce nitrate to nitrogen gas, thereby providing energy for growth (Martineau et al., 2015).

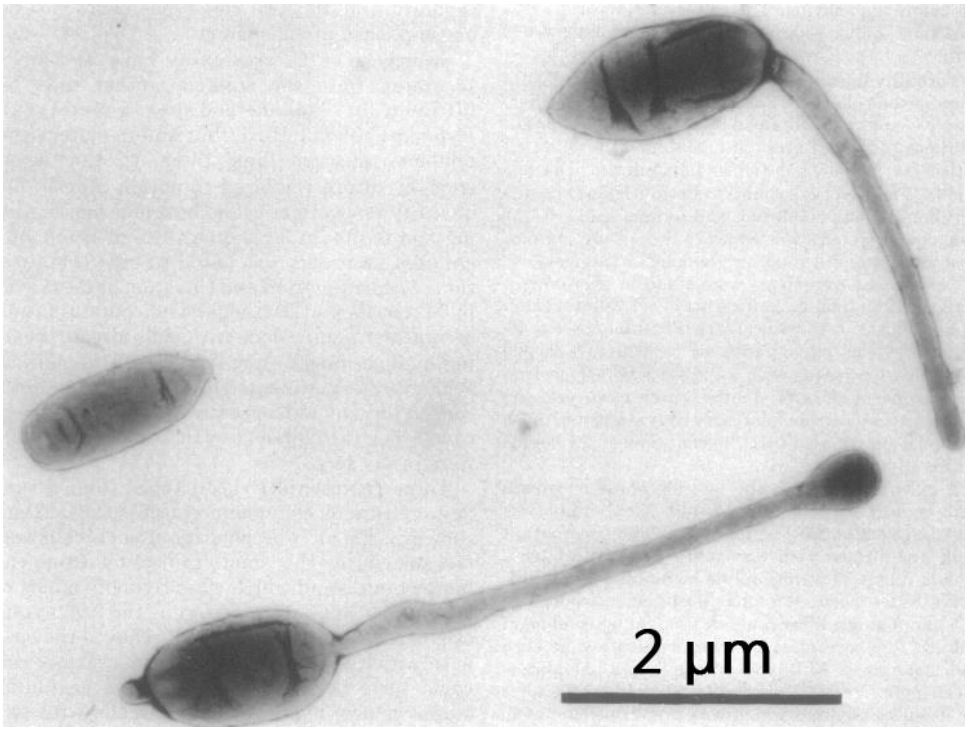

Fig. 1. Three cell types of *Hyphomicrobium* sp., illustrating morphological variation within the species. Image taken from (Moore and Hirsch, 1973).

In methylotrophic bacteria such as *Hyphomicrobium* species, methanol is used as a source of carbon and energy. In these organisms, methanol is first oxidized to formaldehyde by methanol dehydrogenase (MDH), a periplasmic enzyme that requires pyrroloquinoline quinone (PQQ) for its activity (Duine et al., 1978) (Fig. 2). In fact, very fundamental work on methanol dehydrogenase has been performed on the enzyme from the type species of *Hyphomicrobium denitrificans*, strain X^T (Duine et al., 1978; Duine and Frank, 1980b, a; Dijkstra et al., 1988; Frank et al., 1988; Dijkstra et al., 1989; Poels and Duine, 1989). Formaldehyde, a highly reactive and potentially toxic intermediate, is then further oxidized to formate, which is either assimilated into biomass via the serine cycle (Anthony, 2011) or oxidized to CO_2 by

formate dehydrogenase for energy production (Fig. 2). The oxidation of formaldehyde occurs in the cytoplasm through a pathway that employs tetrahydromethanopterin (THMPT) as a cofactor (Chistoserdova et al. 2011). However, the detailed mechanism of methanol metabolism in *H. denitrificans* and the specific enzymes involved in the methanol oxidation pathway need further clarification.

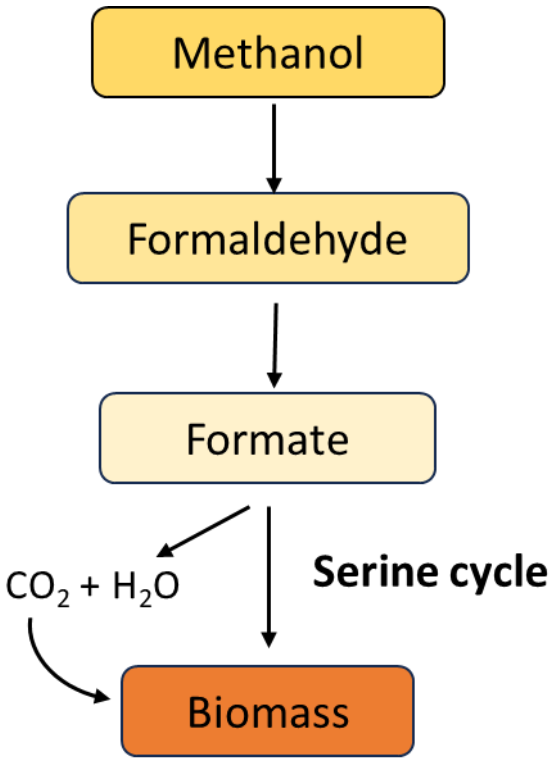

Fig. 2. Oxidation and assimilation of methanol in *Hyphomicrobium* species. Methanol is initially oxidized to formaldehyde by a periplasmic methanol dehydrogenase. Formaldehyde is then further oxidized to formate. Formate can also serve as a carbon source and be assimilated into biomass via the serine cycle. For detailed mechanistic insights and regulatory pathways, see (Anthony, 2011) and (Chistoserdova, 2011).

2.1 Oxidative sulfur metabolism in H. denitrificans

The capacity for thiosulfate oxidation in the obligate chemoorganoheterotroph *H. denitrificans* X^T has first been reported in 2018 (Cao et al., 2018; Koch and Dahl, 2018). Thiosulfate oxidation commences in the periplasm. Here, two thiosulfate molecules can be oxidatively linked to form the dead-end product tetrathionate, a reaction catalyzed by thiosulfate dehydrogenase (TsdA) (Koch and Dahl, 2018; Li et al., 2023b). Alternatively, thiosulfate can be completely oxidized to sulfate. This pathway is preferred at lower substrate concentrations (<2.5 mM) and involves the periplasmic SoxYZ carrier protein to which thiosulfate is oxidatively bound by the action of the *c*-type cytochrome SoxXA. Sulfate is then hydrolyzed off by SoxB and the sulfane sulfur remaining on SoxYZ is transferred to the cytoplasm. Once inside, the sulfur is delivered through a cascade of sulfur transfer reactions to the sulfur-oxidizing heterodisulfide-reductase-like enzyme complex, sHdr (Tanabe et al., 2024), in the cytoplasm (Fig. 2a). A type I system has been proposed, which is encoded by a *shdrC1B1AHC2B2* gene cluster (Fig. 2b). This system is typically accompanied by genes

encoding for diverse sulfur transferases and up to three genes for lipoate-binding protein (LbpA) (Cao et al. 2018). In the proposed model, LbpA plays a critical role in the energy conversion step: electrons can be transferred directly from the lipoamide cofactor to NAD^+, enabled by the low standard redox potential of the lipoamide/dihydrolipoamide couple. This makes LbpA a central component of the sHdr-dependent sulfur oxidation mechanism. How sulfur is transferred into the cytoplasm for further oxidation was still a mystery at the onset of this thesis and available knowledge about the import of inorganic sulfur compounds for assimilation provided starting points for experiments.

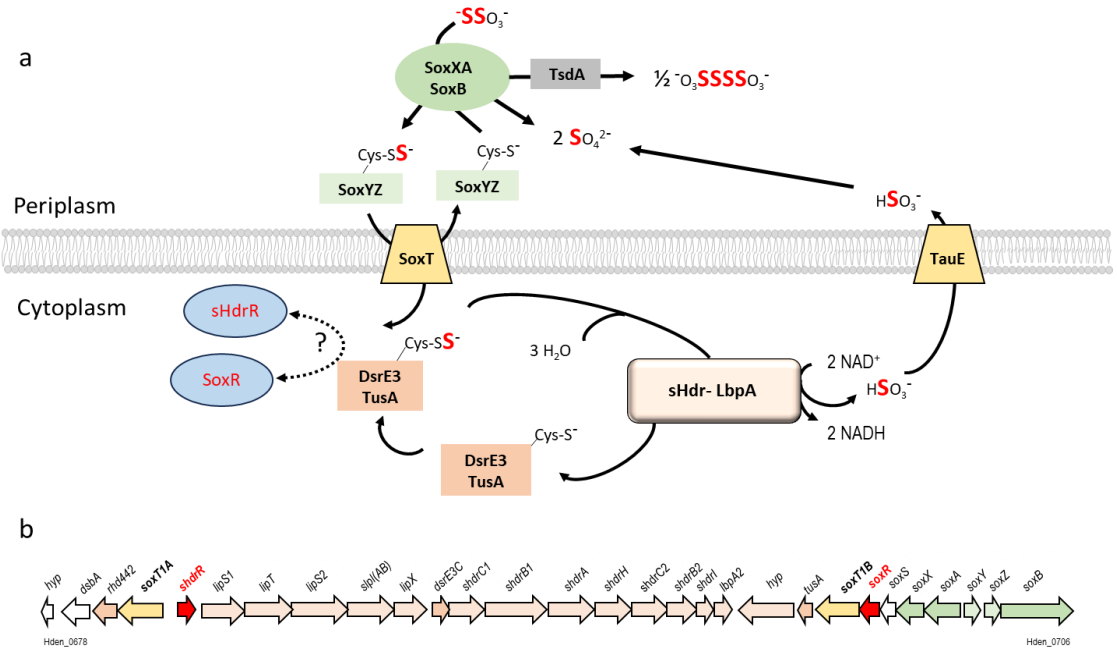

Fig. 3. (a) Model of thiosulfate oxidation in *H. denitrificans*. (b) The *sox* gene cluster, *shdr* gene cluster and its vicinity in *H. denitrificans.* Encoded proteins or functions as well as locus tags are given. Complete oxidation of thiosulfate to sulfate starts in the periplasm where enzymes SoxXA and SoxB act together in oxidative attachment of thiosulfate to the sulfur carrier protein SoxYZ and subsequent hydrolytic release of sulfate. The sulfane sulfur stemming from thiosulfate is then transferred to the cytoplasm and further oxidized by the proteins of the sulfur-oxidizing heterodisulfide reductase-like enzyme system, sHdr, in conjunction with the lipoate-binding protein LbpA. The resulting sulfite is transported back to the periplasm and oxidized to sulfate. The sHdr complex probably consists of several polypeptides, sHdrC1, sHdrB1, sHdrA, sHdrH, sHdrC2 and sHdrB2. TsdA, diheme cytochrome *c* thiosulfate dehydrogenase; LbpA, lipoate-binding proteins. Sulfur is an essential element in living organisms. Although sulfur transport across membranes is crucial for sensing and responding to sulfur compounds, it is less understood than the involved redox reactions.

Sulfur assimilation, which is essential for bacterial growth, involves both well-characterized and newly identified transport systems. In *Escherichia coli*, the CysUWA complex—an ATP-binding cassette (ABC) transporter encoded by *cysU*, *cysW*, and *cysA*—facilitates the uptake of sulfate and thiosulfate from the environment. This system functions in conjunction with periplasmic binding proteins: Sbp for sulfate and CysP for thiosulfate (Fig. 4). In addition to the CysPUWA system, newly identified transporters have broadened our understanding of thiosulfate uptake. *E. coli* and other bacteria, such as *Spirochaeta thermophila*, possess an alternative thiosulfate transport system involving the transmembrane protein YeeE, a member of the YeeE/YedE protein family (Tanaka et al., 2020). Efficient function of the YeeE-

mediated pathway also requires the cytoplasmic sulfur transferase YeeD. Members of the YeeE/YedE family are integral inner membrane proteins defined by a conserved sulfur transport motif and are proposed to mediate the translocation of sulfur-containing compounds across the membrane (Gristwood et al., 2011). This protein family is widely conserved across diverse bacterial phyla and is implicated in a variety of sulfur-related metabolic pathways (Tanaka et al., 2020). In sulfur-oxidizing prokaryotes, genes encoding YeeE/YedE-like transporters often co-occur with sulfur-metabolizing enzymes. For example, in *H. denitrificans,* the *sox* gene cluster, involved in thiosulfate oxidation, includes *soxT* genes encoding potential transporters (Figs. 3 and 4). Two *soxT* genes have been identified in *H. denitrificans*. One is part of a typical *soxSRT* arrangement and resides immediately upstream of the genes encoding a TusA-like sulfur carrier protein and a putative cytochrome P450. The second *soxT* gene is located downstream of the large set of genes that encode the enzymes for cytoplasmic sulfite formation and is transcribed divergently from them. However, the specific functions of these two putative SoxT transporters remained unknown.

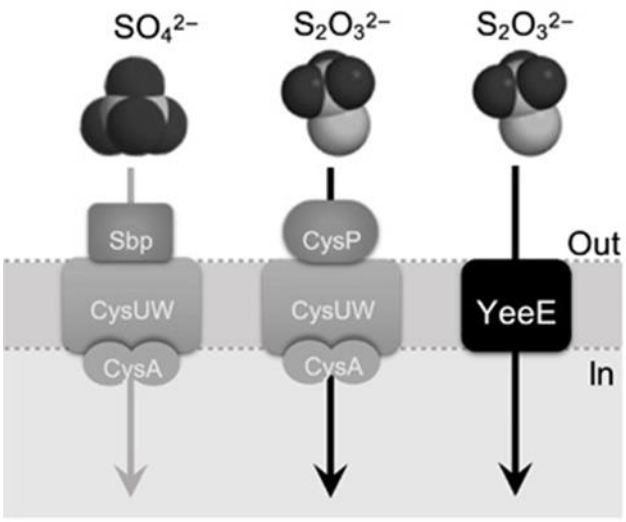

Fig. 4. Uptake of sulfate and thiosulfate in the inner membrane in *E. coli* (Tanaka et al., 2020). The *Escherichia coli* CysUWA (also called CysTWA) complex (the gene product of *cys*U, *cys*W, and *cys*A), an adenosine triphosphate–binding cassette transporter, takes up sulfate and thiosulfate ions from the environment as a sulfur source in combination with periplasmic binding proteins Sbp and CysP, respectively. *E. coli* strain YeeE also imports thiosulfate as a sulfur source.

3 Regulation of oxidative sulfur metabolism

In the environment, bacteria such as *H. denitrificans* must not only cope with constantly changing concentrations of respiratory electron acceptors (oxygen, nitrate), but may also encounter varying concentrations of reduced sulfur compounds such as thiosulfate. Regulatory mechanisms are necessary to adapt to fluctuating conditions. The regulation of bacterial sulfur metabolism often involves specific regulators that control the expression of genes within sulfur-related gene clusters. These regulators can respond to the presence of sulfur compounds or other environmental signals to activate or repress gene expression, thereby coordinating the bacterial response to sulfur availability (Giedroc et al., 2023). These regulators typically bind to promoter regions of the sulfur-related gene clusters and modulate

the transcriptional activity of the genes involved in sulfur metabolism, ensuring that the bacterial response is appropriately tuned to the environmental conditions

To date, very few transcriptional regulatory mechanisms have been established for lithotrophic sulfur oxidizers, where the corresponding genes are thought to be always highly expressed. One is the TspSR two-component system found in acidophilic, sulfur-oxidizing bacteria of the genus *Acidithiobacillus*, where the histidine kinase TspS senses sulfide and the response regulator TspR activates Sox expression (Li et al., 2017b). TspSR are encoded upstream of the *sox* genes in the *sox* I cluster on the chromosome of *Acidithiobacillus caldus* MTH-04 (Li et al., 2017b). Related genes are present in further lithotrophic sulfur oxidizers such as *Thermithiobacillus tepidarius* and *Thiohalorhabdus denitrificans* (Li et al., 2017b). In the same *A. caldus* an alternative thiosulfate oxidation pathway is present, the S_4I pathway, made up of thiosulfate:quinone oxidoreductase (Tqo or DoxDA) and a tetrathionate hydrolase (TetH). Transcription of the respective genes is regulated by another two-component system called RsrS-RsrR (Wang et al., 2016). A two-component system is also encoded in immediate vicinity of the *sox* genes in the purple sulfur bacterium *Allochromatium vinosum*, but here the regulator is a diguanylate cyclase response regulator (Grimm et al., 2011).

For facultative sulfur oxidizers, more complex regulatory patterns have been reported than for the metabolically restricted lithotrophs, with upregulation usually occurring only in the presence of metabolizable sulfur substrates. Alphaproteobacteria that are not restricted to sulfur oxidation, such as *Rhodovulum sulfidophilum*, *Paracoccus pantotrophus* or *Pseudaminobacter salicylatoxidans* may serve as examples. Here, the ability to oxidize thiosulfate and, depending on the organism, other reduced inorganic and organic sulfur compounds such as sulfide or dimethyl sulfide, is not constitutive but can be induced by the presence of oxidizable sulfur compounds (Rother et al., 2005; Mandal et al., 2007). In these organisms, transcriptional regulation is exerted through SoxR, which may also be in a two-component system with SoxS [not to be confused with the superoxide response regulator genes (SoxR/S) with the same nomenclature]. SoxR is a transcriptional repressor to the Sox pathway.

3.1 Response to reactive sulfur compounds

In many prokaryotes the oxidation of reduced sulfur compounds, particularly that of hydrogen sulfide, does not serve primarily for providing electrons for energy conservation through respiration of photosynthetic electron flow but for detoxification. Regulatory mechanisms observed in these organisms provide a valuable source of information.

Bacterial strategies for sensing and detoxifying toxic sulfur species are becoming increasingly well understood. These mechanisms often rely on DNA-binding transcriptional regulators that employ cysteine thiol-based redox sensing to detect and respond to specific reactive molecules. Under non-stress conditions, these regulators repress the transcription of downstream genes involved in cellular defense (Fig. 5a). Upon sensing oxidative or electrophilic stress, redox-sensitive cysteine residues undergo modification, leading to conformational changes in the regulators. This results in transcriptional de-repression or activation of genes encoding detoxification enzymes, which act by exporting the reactive species or converting them into less harmful compounds (Fig. 5b). Among these reactive molecules, reactive sulfur species (RSS), generated through the oxidation of hydrogen sulfide

(H_2S), have recently been recognized as key signaling entities, particularly in H_2S-mediated signaling via protein persulfidation (Giedroc et al., 2023).

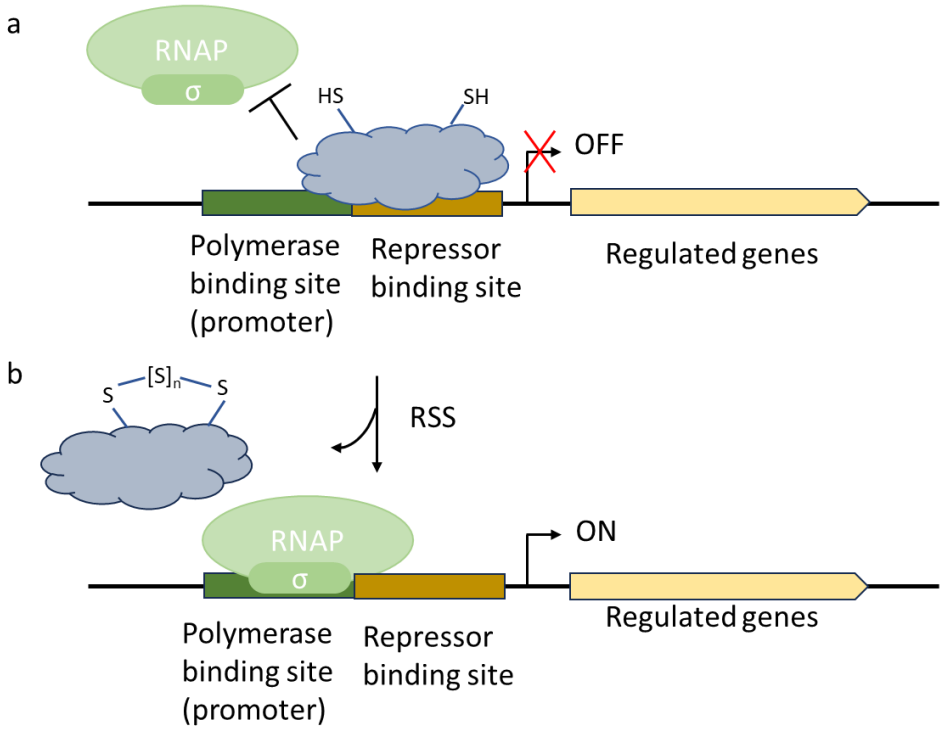

Fig. 5. Model of RSS-responsive transcriptional gene regulation. **(a)**The reduced form of the protein binds to the promoter region and represses the expression of downstream genes in the operon. **(b)** In the presence of reactive sulfur species (RSS), S-dependent oxidative modifications of protein displace from the promoter region. The RNA polymerase binds DNA and subsequently induces gene expression.

Hydrogen sulfide (H_2S) is a well-known toxic gas that can be produced endogenously in many organisms through dissimilatory sulfate reduction. An emerging consensus is that H_2S and sulfur species containing sulfur–sulfur bonds, known as reactive sulfur species (RSS), function as antioxidants and offer protection against oxidative stress and antibiotics (Giedroc et al., 2023). Maintaining H_2S and RSS homeostasis is crucial for bacterial survival. Bacteria regulate H_2S/RSS levels by expressing persulfide-sensing transcriptional regulators, which control the expression of genes involved in H_2S detoxification. Bacterial RSS-sensing transcriptional regulators detect RSS through persulfidation, triggering allosteric changes that modulate DNA binding or transcriptional activity. This leads to altered expression of genes involved in H_2S oxidation and restoration of H_2S/RSS homeostasis. These primary RSS sensors tightly control intracellular RSS levels by regulating genes encoding enzymes such as sulfide:quinone oxidoreductase (SQR), persulfide dioxygenase (PDO), flavin-dependent coenzyme A persulfide reductase, various sulfurtransferases (ST), and membrane transporters like TauE and YedE/YeeE (Giedroc et al. 2023).

Several primary RSS sensors have been identified in bacteria. One example is CstR (CsoR-like sulfurtransferase repressor), found in *Staphylococcus aureus* and some other bacteria. CstR represses the expression of genes involved in H_2S detoxification under low H_2S conditions (Luebke et al., 2014). When H_2S levels increase, CstR undergoes conformational changes that relieve repression, allowing the expression of detoxification enzymes like persulfide

dioxygenase (Luebke et al., 2014). Another example is BigR (Biofilm growth-associated Repressor), identified from the non-sulfur oxidizing plant pathogens *Xylella fastidiosa* and *Agrobacterium tumafaciens* where it regulates the expression of what is predicted to be a type II PDO (Blh) (Barbosa and Benedetti, 2007; Sattler et al., 2015).

Notably, unlike persulfide-sensing regulators such as CstR, SqrR, and BigR, FisR (Fis family transcriptional regulator), a member of the Fis family transcriptional regulators in *Cupriavidus pinatubonensis* (Proteobacteria), acts as a reactive sulfur species (RSS)-responsive transcriptional activator (Li et al., 2017a). FisR positively regulates σ54-dependent transcription by promoting open complex formation via ATP hydrolysis. It has three domains: an N-terminal regulatory domain that responds to signals, a middle AAA+ domain that hydrolyzes ATP, and a C-terminal DNA-binding domain. FisR contains conserved cysteine residues that form disulfide and tetrasulfide cross-links, altering its ATPase activity without affecting DNA binding or oligomerization (Li et al., 2017a).

In contrast to primary sensors that are directly involved in H_2S/RSS homeostasis, secondary RSS sensors play distinct roles, such as in reactive oxygen species (ROS) sensing, detoxification, or the regulation of virulence genes. For example, H_2O_2 sensors like OxyR and PerR may undergo cysteine persulfidation in response to mild or nonphysiological stress (Hou et al., 2019; Liu et al., 2023). Increasing evidence shows that ROS-regulated enzymes, such as peroxiredoxins and glutaredoxins, also help eliminate excess H_2S or RSS (Cuevasanta et al., 2019; Liu et al., 2022).

3.2 ArsR-type transcriptional repressors in the regulation of sulfur metabolism

The SoxR transcriptional regulator proteins of *Paracoccus pantotrophus* and *Pseudaminobacter salicylatoxidans* belong to the arsenic repressor (ArsR-SmtB) family of prokaryotic repressors (Cook et al., 1998; Ma et al., 2009). Members of this family are widely abundant in bacteria. ArsR/SmtB homologs are small (~15 kDa) proteins containing several alpha helices that adopt multimeric complexes to bind DNA (Busenlehner et al., 2003).

ArsR superfamily proteins are compact, homodimeric winged helical DNA-binding proteins characterized by a core secondary structure of α1-α2-α3-α4-β1-β2-α5 (Fig. 6). Some members extend at either or both N- and C-terminal ends, which, if α-helical, are referred to as α0 and α6 helices, respectively, aiding sequence comparisons. The DNA-binding function is mediated by the helix-turn-helix (HTH) motif, specifically the α3-α4 segment, which engages with successive DNA major grooves. The β1-β2 wing extends from the periphery of the dimer and may interact with adjacent DNA minor grooves.

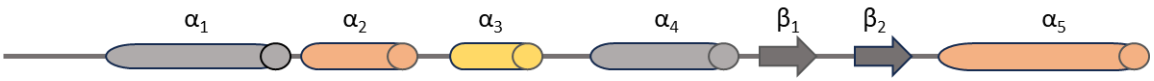

Fig. 6. Secondary structure of ArsR-SmtB family repressors. The conserved structural elements typical of the ArsR-SmtB family transcriptional repressors are shown, including five α-helices that form the DNA-binding helix-turn-helix (HTH) motif and a dimerization interface.These structural features enable the repressors to sense environmental stress signals and regulate gene expression accordingly.

Members of the ArsR-SmtB family were originally recognized as metal-responsive transcriptional regulators that repress metal related genes in the absence of a regulatory metal cofactor (Osman and Cavet, 2010). Coordination of a metal effector promotes a conformational change in the ArsR complex, allowing de-repression of the metal related genes. There are also members in the ArsR-SmtB family that have been shown to sense reactive oxygen or sulfur species (Capdevila et al., 2017). In the cyanobacterium *Nostoc* sp. PCC 7120, the ArsR-SmtB transcriptional regulator RexT responds to H_2O_2 by upregulating thioredoxin expression to maintain redox homeostasis (Li et al., 2022). RexT uses disulfide bond formation to modulate DNA binding. High-resolution crystal structures of RexT in reduced and H_2O_2-treated states reveal that it forms a vicinal disulfide bond in response to H_2O_2. BigR from *Xylella fastidiosa* belongs to the ArsR-type proteins sensing reactive sulfur species and controls the transcription of genes involved in sulfide-dependent photosynthesis and the detoxification of H_2S derived from associated host plants (Guimarães et al., 2011). SqrR (Sulfide Quinone Reductase Repressor), found in *Rhodobacter capsulatus,* serves as a master regulator of sulfide-dependent photosynthesis in this purple sulfur bacterium. SqrR directly senses persulfides, leading to de-repression of genes involved in persulfide detoxification and H_2S oxidation (Shimizu et al., 2017). Two ArsR proteins from *Vibrio cholerae*, HlyU (VC_0678) and BigR (VC_0642), also primarily respond to inorganic and organic persulfides rather than to hypoxia or H_2O_2 (Capdevila et al., 2021). HlyU-mediated activation of the *V. cholerae hlyA* gene in response to intracellular RSS is an adaptation that might help the pathogen evade the gut inflammatory response (Capdevila et al., 2021).

In contrast to BigR, SqrR and HlyU, knowledge about SoxR is comparatively sparse. While binding regions for the transcriptional repressor have been identified in promoter-operator segments within the *sox* gene clusters of *P. denitrificans* and *P. salicylatoxidans* (Rother et al., 2005; Mandal et al., 2007) no information is available on factors that control its DNA-binding capacity. In *H. denitrificans*, two distinct but closely related ArsR-type transcriptional repressors, SoxR and sHdrR, are encoded in the *sox-shdr-lbpA* gene region. They could well be responsible for the transcriptional regulation of genes encoding Sox, sHdr and associated proteins (Fig 2).

4 Aims of the thesis

This study sought to advance our understanding of sulfur compound metabolism in methylotrophic bacteria and the complex regulatory networks involved. Specifically, the regulatory processes were compounds, such as thiosulfate, as supplementary electron donors during methylotrophic growth.

This research focused on the following topics:

1) **Thiosulfate metabolism and C_1 compound utilization in *H. denitrificans***: To investigate how thiosulfate impacts C_1 metabolism, particularly why thiosulfate oxidation inhibits methanol consumption, by analyzing the interaction between sulfur oxidation pathways and carbon metabolism.

2) **Regulation of sulfur oxidation by SoxR**: To determine whether thiosulfate oxidation in *H. denitrificans* is regulated by SoxR, through the identification of target genes, mapping of

binding sites, and characterization of DNA-binding properties of the repressor protein. How are sulfur-related signals sensed?

3) **Regulation of sulfur oxidation by sHdrR**: To explore the regulation of the sHdr system by sHdrR, using similar methods as for SoxR, and to understand its role in sulfur metabolism.

4) **Co-regulation of sulfur oxidation by SoxR and sHdrR**: To investigate whether and how *sox* and *shdr* genes are co-regulated, and to elucidate the underlying regulatory mechanisms.

5) **Function of SoxT transporters:** To decipher the roles of potential SoxT transporters in *H. denitrificans* by analyzing their distribution, constructing mutant strains, and characterizing their phenotypes and transcription levels. Characterize the interplay between putative sulfur import systems and regulatory elements. How are signals transmitted to coordinate downstream sulfur oxidation reactions?

6) **Global role of SoxR and sHdrR:** Do the regulators have functions beyond sulfur oxidation? Is there a connection between sulfur oxidation and anaerobic metabolism, particularly denitrification?

Publications and manuscripts included in this thesis

Li, J., Koch, J., Flegler, W., Garcia Ruiz, L., Hager, N., Ballas, A., Tanabe, T. S., & Dahl, C. (2023). A metabolic puzzle: Consumption of C_1 compounds and thiosulfate in *Hyphomicrobium denitrificans* X^T. *Biochimica et Biophysica Acta - Bioenergetics* **1864**: 148932. DOI: 10.1016/j.bbabio.2022.148932

Li, J., Törkel, K., Koch, J., Tanabe, T. S., Hsu, H. Y. & Dahl, C. (2023) In the Alphaproteobacterium *Hyphomicrobium denitrificans* SoxR serves a sulfane sulfur-responsive repressor of sulfur oxidation. *Antioxidants* **12**: 1620. DOI: 10.3390/antiox12081620

Li, J., Göbel F., Hsu, H. Y., Koch, J., Hager N., Flegler, W., Tanabe, T. S., & Dahl, C. (2024) YeeE-like bacterial SoxT proteins mediate sulfur import for oxidation and signal transduction. *Communications Biology* **7**: 1548. DOI: 10.1038/s42003-024-07270-7

Li, J., Schmitte, N., Törkel, K., & Dahl, C. (2025) In *Hyphomicrobium denitrificans* two related sulfane-sulfur responsive transcriptional repressors regulate thiosulfate oxidation and have a deep impact on nitrate respiration and anaerobic biosynthesis. *Molecular Microbiology* (under review). DOI: 10.1101/2025.02.17.638619

Li, J., Schmitte, N., Törkel, K., & Dahl, C. (2025) The sulfane-sulfur responsive transcriptional repressor sHdrR: Properties and binding sites. *Manuscript in preparation.*

CHAPTER 2

A metabolic puzzle: Consumption of C_1 compounds and thiosulfate in *Hyphomicrobium denitrificans* X^T

Li, J., Koch, J., Flegler, W., Garcia Ruiz, L., Hager, N., Ballas, A., Tanabe, T. S. & Dahl, C.

Biochimica et Biophysica Acta - Bioenergetics **2023,** *1864*, 148932.
DOI: 10.1016/j.bbabio.2022.148932

Hyphomicrobium denitrificans X^T (ATCC 51888^T) is an Alphaproteobacterium widely distributed in various natural environments, including soils, brackish water, sewage, and freshwater ecosystems. As a chemoorganoheterotroph, it plays an essential role in environmental carbon cycling by metabolizing a variety of single-carbon compounds, such as methanol, formate, methylamine, dimethylamine, and dimethylsulfide. However, *H. denitrificans* has a restricted substrate range, as it cannot utilize carbon compounds containing more than two carbon atoms nor sustain autotrophic growth using carbon dioxide.

Many obligately heterotrophic methylotrophs are known to utilize thiosulfate as an auxiliary electron donor to enhance energy yield during growth on C_1 compounds such as methanol or formate. In *H. denitrificans* X^T, two distinct thiosulfate oxidation pathways are occurred. However, rather than improving growth, the simultaneous presence of methanol and thiosulfate significantly impairs cellular proliferation. This paradoxical effect underscores the complex regulatory and metabolic interactions between C_1 compound utilization and sulfur compound oxidation in this organism.

The first pathway involves the periplasmic thiosulfate dehydrogenase TsdA, which catalyzes the conversion of thiosulfate into tetrathionate, a metabolic dead-end production that cannot be further oxidized, thereby potentially limiting the energy gain from sulfur oxidation. This accumulation may also impose a burden on the periplasmic environment or interfere with other periplasmic redox reactions.

A second pathway also initiates in the periplasm, where the SoxXA complex oxidatively fuses thiosulfate to the carrier protein SoxYZ. The sulfane sulfur attached to SoxYZ is then processed by SoxB to release sulfate. Further oxidation of the remaining sulfur species occurs in the cytoplasm via the sulfur-oxidizing heterodisulfide reductase-like system (sHdr). Beyond its metabolic function, the sHdr pathway in *H. denitrificans* is subject to complex regulation. Transcriptional control of the *shdr* gene cluster is proposed to be mediated by an ArsR-type regulator, sHdrR (Koch and Dahl 2018). To explore this regulatory mechanism, we compared thiosulfate consumption between *H. denitrificans* Δ*tsdA* Δ*shdrR* and *H. denitrificans* Δ*tsdA*. When cultivated in a medium containing methanol and thiosulfate, *H. denitrificans* Δ*tsdA* Δ*shdrR* exhibited a significantly higher specific oxidation rate for thiosulfate but suffered a marked reduction in growth rate compared to the reference strain *H. denitrificans* Δ*tsdA.* Interestingly, exposure of *H. denitrificans* Δ*tsdA* Δ*shdrR* to thiosulfate during the growth experiment further enhanced its specific oxidation rate, suggesting that additional regulatory mechanisms beyond sHdrR may be involved in controlling thiosulfate metabolism.

One proposed explanation for the observed growth retardation is that thiosulfate oxidation may interfere with methanol assimilation. In *H. denitrificans*, methanol undergoes a five-step

oxidation pathway to formate, which is subsequently incorporated into biomass via the serine cycle. It was hypothesized that excessive reduction of the NAD^+/NADH and cytochrome *c* pools caused by thiosulfate oxidation might disrupt electron flow from methanol oxidation, thereby impairing methanol assimilation and biomass production. However, experimental findings challenged this hypothesis. When the carbon source was switched from methanol to formate, growth inhibition was no longer observed. Unlike methanol, formate can be directly assimilated via the serine pathway without requiring oxidation, indicating a distinct interaction between thiosulfate oxidation and methanol metabolism. Supporting this conclusion, we found that the NAD^+/NADH ratio was significantly higher in *H. denitrificans* Δ*tsdA* Δ*shdrR* grown on either formate or methanol without thiosulfate. However, the addition of thiosulfate reduced this ratio, contradicting the notion that over-reduction alone was responsible for inhibiting carbon assimilation.

To resolve this apparent contradiction, we investigated whether a toxic metabolic intermediate might be responsible for the inhibitory effect of thiosulfate oxidation on methanol assimilation. This led to the discovery of previously unreported sulfite accumulating in the medium. The presence of this toxic sulfur species is believed to inhibit periplasmic methanol dehydrogenase activity, thereby disrupting methanol oxidation and assimilation. In contrast, formate metabolism remains unaffected, as its assimilation occurs in the cytoplasm, where it is protected from sulfite toxicity by the cell membrane and specific sulfite export mechanisms. Sulfite is a reactive intermediate known to form covalent adducts with pyrroloquinoline quinone (PQQ), the essential redox cofactor in periplasmic methanol dehydrogenase. Thus, the growth retardation observed in the presence of thiosulfate can be attributed to sulfite accumulation, which interferes with periplasmic methanol metabolism.

This sulfite-PQQ interaction likely disrupts methanol oxidation by compromising methanol dehydrogenase function, thereby creating a specific bottleneck in methanol metabolism in the presence of thiosulfate. Meanwhile, formate oxidation, which occurs in the cytoplasm and relies on a distinct enzymatic machinery, remains unaffected. Taken together, these findings suggest a metabolite-level inhibition mechanism linking sulfur compound oxidation to impaired methanol utilization in *H. denitrificans*, and they highlight the intricate cross-talk between sulfur and C_1 compound metabolism. Studying *H. denitrificans* also advances understanding of microbial sulfur metabolism, with broader implications for sulfur cycling. Future research should explore the molecular mechanisms of sulfite transport and detoxification, as well as regulatory factors controlling the sHdr pathway, to deepen insights into sulfur-based energy metabolism and microbial community dynamics.

J.L. contributed to this study by conceptualization, investigation, validation, figure design and writing: the investigation and construction of the Δ*tsdA* Δ*shdrR* strain, overproduction of the sHdrR protein, Western blot and Electrophoretic mobility shift assay, phenotypic characterization, quantification of sulfur compounds and protein content were conceptualized, carried out, analyzed, and validated by J.L.

CHAPTER 3

In the Alphaproteobacterium *Hyphomicrobium denitrificans* SoxR serves a sulfane sulfur-responsive repressor of sulfur oxidation

Li, J., Törkel, K., Koch, J., Tanabe, T. S., Hsu, H. Y. & Dahl, C.

Antioxidants **2023**, *12*, 1620
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Sulfur-oxidizing bacteria play a crucial role in global sulfur cycling by utilizing various sulfur compounds as electron donors for energy production. Among these, thiosulfate serves as a key intermediate in sulfur metabolism and is commonly used by many sulfur-oxidizing bacteria as either a primary or supplementary electron source. Depending on the metabolic pathway employed, thiosulfate can undergo oxidation via different mechanisms during chemolithotrophic or photolithotrophic growth. One major pathway involves its conversion into tetrathionate, catalyzed by thiosulfate dehydrogenase. Alternatively, thiosulfate can be oxidized through the periplasmic Sox system, a well-characterized sulfur oxidation pathway found in a wide range of bacteria.

Complete oxidation of thiosulfate via the Sox pathway requires the coordinated action of multiple protein components, including SoxXA, SoxYZ, SoxB, and SoxCD (Friedrich et al. 2001). SoxXA initiates the process by catalyzing the formation of a disulfide bond between the active site cysteine of SoxYZ and thiosulfate-derived sulfane sulfur. SoxB hydrolytically cleaves the sulfone group from SoxYZ, releasing sulfate and leaving behind a sulfane sulfur-bound SoxYZ intermediate. The final oxidation step, in which SoxYZ-bound sulfane sulfur is fully converted to sulfate, is mediated by SoxCD, a key sulfur dehydrogenase.

However, several sulfur-oxidizing chemolithotrophic and photolithotrophic bacteria have evolved an incomplete version of the Sox system, known as a truncated Sox system, which lacks SoxCD. In these bacteria, the oxidation of SoxY-bound sulfane sulfur remains incomplete due to the absence of SoxCD. Consequently, these organisms rely on alternative pathways to further oxidize sulfane sulfur within the cytoplasm. In *H. denitrificans*, the truncated Sox system can be combined with cytoplasmic sulfur oxidation system, the sulfane sulfur-oxidizing heterodisulfide reductase-like (sHdr) system, for complete oxidation to sulfate. However, the regulatory mechanisms controlling the expression of these genes remain an area of active investigation.

Previous studies have proposed that sulfur oxidation genes in *H. denitrificans* are regulated by an ArsR-type repressor, sHdrR. This regulator likely modulates gene expression in response to intracellular sulfur levels. However, additional regulatory factors are suspected to be involved in fine-tuning this process. In this study, we identified a second transcriptional regulator SoxR. To gain genetic evidence of SoxR's role in *H. denitrificans*, we constructed the mutant strain *H. denitrificans* Δ*tsdA* Δ*soxR*. This strain exhibited a significantly higher specific oxidation rate of thiosulfate than the reference strain *H. denitrificans* Δ*tsdA*, but its growth rate was

markedly reduced. Pre-exposure to thiosulfate further enhanced the oxidation rate, a pattern also observed in *H. denitrificans* Δ*tsdA* Δ*shdrR*, suggesting that both regulators influence thiosulfate oxidation. It appears to interact, either directly or indirectly, with sHdrR, forming a complex regulatory network that governs thiosulfate oxidation in *H. denitrificans*.

To further characterize the regulatory function of SoxR, RT-qPCR was performed to analyze transcript levels of twelve selected genes in *H. denitrificans* Δ*tsdA* Δ*soxR* compared to the reference strain during thiosulfate oxidation and under sulfur-limiting conditions. The target genes included several *sox* and *shdr* genes. The results revealed that SoxR significantly influences the expression of *sox* genes, while exerting a weaker effect on *shdr*, *lbpA,* and *tusA*. Interestingly, *soxT1B* and *soxR* transcription remained unchanged in response to thiosulfate, suggesting that SoxT1B may play an additional role in sensing thiosulfate and modulating the sHdr and Sox pathways.

Recombinant SoxR was used to identify its DNA binding sites within the *shdr/sox* gene cluster in *H. denitrificans*, revealing four distinct binding regions. Non-reducing SDS-PAGE analysis and gel permeation chromatography showed the SoxR form an intramolecular disulfide bond between Cys^{50} and Cys^{116}. The oxidized SoxR Cys^{50}Ser and SoxR Cys^{116}Ser variants formed intermolecular dimers connected by the remaining cysteine on each of the monomers. Further biochemical analysis using EMSA and MALPEG assays showed that polysulfide led to the persulfidation of the single remaining cysteines of SoxR. Either polysulfide merely leads to the formation of a Cys^{50}–Cys^{116} bridge, or one or more sulfur atoms are enclosed by the two cysteines. Mass spectrometry confirmed SoxR forms an intramolecular tri-, tetra-, or penta-sulfide bond between Cys ^{50} and Cys ^{116} upon interaction with reactive sulfane sulfur species.

The structural change in SoxR activates the transcription of the *shdr/sox* gene cluster, coordinating the thiosulfate oxidation process. These discoveries significantly advance our understanding of sulfur oxidation regulation in *H. denitrificans*. By elucidating the roles of SoxR this research sheds light on how sulfur-oxidizing bacteria fine-tune their metabolism in response to environmental sulfur availability.

J.L. contributed to this study by conceptualization, investigation, data validation, visualization, and writing: J.L. contributed to the construction of the *H. denitrificans* Δ*tsdA* Δ*soxR* strain, site directed mutation of SoxR variants *in vitro*, overproduction and purification of SoxR proteins and its variants, EMSA assays, gel filtration, and mass spectrometry for all the mutants. RNA extraction and RT-qPCR for *H. denitrificans* Δ*tsdA* and Δ*tsdA* Δ*soxR* strains were carried out by J.L.

CHAPTER 4

YeeE-like bacterial SoxT proteins mediate sulfur import for oxidation and signal transduction

Li, J., Göbel, F., Hsu, H., Koch, J., Hager, N., Flegler, W., Tanabe, T. & Dahl, C.

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The biogeochemical sulfur cycle is largely driven by prokaryotes. Many sulfur-oxidizing prokaryotes use substrates like sulfide and thiosulfate as alterative or additional electron donors, initiating oxidation in the periplasm and completing it in the cytoplasm. Sulfur transport across the cytoplasmic membrane is likely involved in the sensing and response to externally available reduced sulfur compounds. Although the enzymatic pathways and transcriptional regulators involved in sulfur oxidation are well studied, two key aspects remain poorly understood: how sulfur compounds are imported into the cytoplasm, and how cells sense and respond to external sulfur sources to regulate gene expression.

Sulfur assimilation is essential for all living organisms, and prokaryotes acquire it from inorganic sulfate or organosulfur compounds such as sulfonates, sulfate esters, and sulfur-containing amino acids. This uptake is mediated by various ABC-type transporters, with solute-binding proteins determining substrate specificity. In *E. coli*, the CysUWA complex imports sulfate and thiosulfate in coordination with periplasmic proteins Sbp and CysP, respectively (Kredich et al. 2008, Barajas et al. 2011). Additionally, the YeeE/YedE-family transporter TsuA facilitates thiosulfate uptake, relying on the adjacent cytoplasmic partner TsuB, which is functionally distinct from the central sulfur hub TusA (Morigasaki et al. 2020, Ikei et al. 2024). YeeE/YedE homologs also appear in other organisms, including selenium transporters in *Methanococcus maripaludis* and sulfur-containing ion transporters like PmpA/B in *Serratia*, with some contributing to cellular sulfane sulfur uptake rather than assimilation (Funkner et al. 2015, Lin et al. 2015, Gristwood et al.2011).

In sulfur-oxidizing bacteria such as *Hyphomicrobium denitrificans*, *Paracoccus spp.,* and *Roseovarius*, YeeE-like transporters (SoxT) often co-occur with *sox* gene clusters. These clusters encode periplasmic and cytoplasmic enzymes for thiosulfate oxidation and are regulated by SoxR repressors. In *H. denitrificans,* two soxT genes are also found and play important roles in the sulfur oxidation process. Thiosulfate oxidation begins in the periplasm, where SoxXAB proteins oxidatively conjugate one sulfur atom of thiosulfate to a conserved cysteine on the substrate-binding protein SoxYZ, releasing a sulfate molecule. The second sulfur atom from the original thiosulfate is transferred into the cytoplasm by an unknown mechanism. There it is further oxidized to sulfite by the cytoplasmic sHdr-LbpA system.

Here, we set out to decipher the function of the two different potential SoxT transporters in *H. denitrificans.* Specifically, we investigated the role of *soxT1A* and *soxT1B*, which encode

membrane proteins resembling YeeE/YedE-family thiosulfate transporters. Both genes are located in the vicinity of genes involved in sulfur oxidation and transcriptional regulation.

To elucidate their functions, we analyzed the distribution and phylogeny of related transporters across sulfur-oxidizing prokaryotes and constructed targeted mutants lacking the transporter genes as well as the transcriptional regulators *soxR* and *shdrR*. Phenotypic characterization of these mutants, along with comparative transcriptional analysis of key sulfur oxidation genes, enabled us to assign functional roles to SoxT1A and SoxT1B.

Functional analysis revealed that SoxT1A is essential for transporting sulfur into the cytoplasm for further oxidation. Mutants lacking *soxT1A* are unable to oxidize sulfur, despite exhibiting high transcription levels of sulfur oxidation genes, indicating that SoxT1A is responsible for substrate delivery rather than transcriptional regulation. Its expression significantly upregulated in the presence of thiosulfate, suggesting its central role in importing sulfur into the cytoplasm for further processing by the cytoplasmic sHdr-LbpA system.

In contrast, SoxT1B appears to function in signal transduction rather than sulfur import. SoxT1B-deficient mutants also exhibit a sulfur oxidation-negative phenotype; however, this results from reduced transcription of sulfur oxidation genes rather than impaired sulfur uptake. This defect is rescued by deletion of the transcriptional repressor SoxR, suggesting that SoxT1B interacts with SoxR to modulate gene expression in response to external sulfur availability. Consistent with a regulatory function, SoxT1B expression is rarely induced in response to thiosulfate, supporting its role in signaling rather than transport. Furthermore, the genomic region surrounding *soxT1B* is associated with genes involved in the periplasmic thiol-disulfide oxidoreductase SoxS and TusA, suggesting that SoxT1B could participate in a regulatory or signaling-related sulfur transport pathway distinct from bulk import.

Together, SoxT1A and SoxT1B represent distinct but complementary components of sulfur metabolism in *H. denitrificans*. SoxT1A mediates the import of sulfur into the cytoplasm for oxidation, while SoxT1B regulates gene expression in response to external sulfur availability. These findings underscore the dual role of membrane-associated proteins not only in facilitating metabolic flux but also in environmental sensing and regulatory control of sulfur oxidation.

J.L. contributed to conceptualization, investigation, data validation, writing: J.L. contributed to the construction of the *H. denitrificans* Δ*tsdA* Δ*soxR,* Δ*tsdA* Δ*shdrR,* Δ*tsdA* Δ*soxT1A* Δ*soxR,* Δ*tsdA* Δ*soxT1A* Δ*shdrR,* Δ*tsdA* Δ*soxT1B* Δ*soxR* and Δ*tsdA* Δ*soxT1B* Δ*shdrR* strains. The investigation and validation of RNA extraction and RT-qPCR for all the mutants were carried out by J.L.

CHAPTER 5

In *Hyphomicrobium denitrificans* two related sulfane-sulfur responsive transcriptional repressors regulate thiosulfate oxidation and have a deep impact on nitrate respiration and anaerobic biosyntheses

Li, J., Schmitte, N., Törkel, K.& Dahl, C.

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In *H. denitrificans,* an obligately chemoorganoheterotrophic Alphaproteobacterium, thiosulfate serves as a supplemental electron donor. However, its utilization is tightly controlled by two homologous ArsR-type transcriptional repressors, sHdrR and SoxR, which are responsive to sulfane sulfur. These regulators not only govern sulfur oxidation pathways but also play a crucial role in modulating broader metabolic processes, particularly anaerobic respiration and denitrification.

To better understand the role of sHdrR, we investigated its phylogenetic distribution among Alphaproteobacteria and other bacterial taxa. Comparative genomic analysis revealed that sHdrR homologs are widely conserved among bacteria that utilize sulfur compounds, particularly within the Hyphomicrobium genus and related methylotrophic or facultatively sulfur-oxidizing bacteria. Phylogenetic reconstructions suggest that sHdrR evolved from ancestral ArsR-type regulators but has acquired specialized functions in sulfane-sulfur sensing. Notably, sequence alignments indicate the presence of two highly conserved cysteine residues, which are hypothesized to serve as key thiol-based redox switches, mediating the regulator's response to intracellular sulfane sulfur levels.

To experimentally validate the functional importance of these conserved cysteines, sHdrR variants were constructed in which the cysteine residues were replaced with serine. The resulting mutant strains exhibited significant defects in sulfur-responsive transcriptional regulation, confirming that these cysteines play a central role in sHdrR function. Their ability to sense and bind sulfane sulfur may enable *H. denitrificans* to fine-tune gene expression in response to fluctuating environmental sulfur levels.

To comprehensively identify genes regulated by sHdrR and SoxR, we conducted both targeted RT-qPCR assays and global RNA-seq analyses in wild-type and regulator-deficient mutant strains. RNA-seq data revealed that the deletion of sHdrR and SoxR affected 165 and 170 genes, respectively, with substantial overlap: 138 genes were co-regulated by both repressors. SoxR predominantly controls the expression of the *sox* operon, encoding key enzymes for periplasmic thiosulfate oxidation and sulfane sulfur transport into the cytoplasm. Additionally, SoxR regulates the *lip-shdr-lbpA* gene cluster, which encodes cytoplasmic enzymes essential for sulfite formation. These genes facilitate the oxidation of sulfane sulfur to sulfite, which can then be further processed or excreted depending on the metabolic state of the cell. In contrast, sHdrR affects a subset of these genes but does not regulate *sox* operon transcription. This suggests that sHdrR is not essential for periplasmic sulfur oxidation but plays a more

specialized role in modulating intracellular sulfur processing and related metabolic pathways. In summary, both regulators exhibit overlapping yet distinct regulatory influences, indicating potential cooperative interactions. The transcriptional profiles suggest that sHdrR and SoxR may act together, possibly forming heterodimers or interacting with additional transcription factors to fine-tune gene expression in response to environmental sulfur availability.

Beyond their roles in sulfur oxidation, sHdrR and SoxR exert significant influence over broader metabolic networks, particularly anaerobic respiration and denitrification. Transcriptomic data indicate that the absence of either regulator leads to altered expression of multiple genes involved in anaerobic metabolism, suggesting that sulfur oxidation is functionally linked to the regulation of electron transport chains and alternative respiratory pathways.

The integration of sulfur oxidation with denitrification in *H. denitrificans* highlights a key metabolic strategy: the bacterium efficiently balances electron flow between sulfur oxidation and nitrogen reduction, optimizing energy conservation under oxygen-limited conditions. This regulatory coupling may enable *H. denitrificans* to dynamically adjust its metabolism depending on the availability of sulfur and nitrogen compounds in its environment.

Further supporting this metabolic interplay, we observed that the absence of sHdrR and SoxR also had a significant impact on genes associated with fatty acid biosynthesis. This unexpected finding suggests a potential link between sulfur metabolism and lipid homeostasis, possibly mediated through redox-sensitive regulatory mechanisms. Given that fatty acid synthesis is an energy-intensive process requiring NAD(P)H and other reducing equivalents, sulfur oxidation may influence lipid metabolism by modulating intracellular redox balance. The precise molecular connections between these pathways remain to be elucidated, but our findings indicate that sulfur metabolism extends far beyond energy conversion, influencing diverse aspects of cellular physiology.

Our findings provide new insights into the intricate regulatory networks governing sulfur metabolism in *H. denitrificans*. The ArsR-type repressors sHdrR and SoxR serve as key transcriptional regulators that not only control sulfur oxidation pathways but also exert broader effects on anaerobic metabolism, redox homeostasis, and fatty acid biosynthesis. Their overlapping yet distinct regulatory targets suggest a cooperative mode of action, possibly involving heterodimer formation or interactions with additional transcription factors. Future studies should focus on characterizing the biochemical mechanisms underlying SoxR-sHdrR interactions, identifying potential co-regulatory factors, and exploring how sulfur oxidation is integrated with other metabolic processes at a systems level.

J.L. contributed to this study by conceptualization, investigation, validation and writing: J.L. contributed to perform the EMSA experiments. The construction of the *H. denitrificans* Δ*tsdA shdrR* Cysteine mutants *in vivo,* the growth experiment, and investigation of RNA extraction, RT-qPCR and RNA-seq experiments for *H. denitrificans* Δ*tsdA,* Δ*tsdA* Δ*shdrR*, Δ*tsdA* Δ*soxR* strains were performed by J.L.

CHAPTER 6

The sulfane-sulfur responsive transcriptional repressor sHdrR: Properties and binding sites

Li, J., Schmitte, N., Törkel, K.& Dahl, C.

Manuscript in preparation

Bacteria have developed diverse mechanisms to detect and respond to the availability of inorganic reduced sulfur compounds, such as thiosulfate, sulfide, and elemental sulfur. These regulatory systems are essential for maintaining redox homeostasis, optimizing energy metabolism, and integrating sulfur oxidation pathways with other cellular processes. Inorganic sulfur compounds, key players in the global sulfur cycle, are sensed through a variety of sophisticated strategies. Among these, thiosulfate serves as a central intermediate in sulfur oxidation pathways. In the Alphaproteobacterium *Hyphomicrobium denitrificans*, this environmental sensing is mediated in part by a regulatory protein belonging to the ArsR-SmtB family of transcriptional repressors. One such protein, sHdrR, serves as a central node in the regulation of sulfur metabolism. sHdrR functions as a sulfane sulfur-responsive repressor, modulating the transcription of genes required for oxidative thiosulfate metabolism based on the availability of oxidizable sulfur compounds in the environment.

Electrophoretic mobility shift assays (EMSAs) demonstrated that sHdrR specifically binds to promoter regions of the *soxT1A-shdrR* and *soxY-soxA* operons, which are divergently transcribed and critical for sulfur oxidation. These promoter regions contain both direct and inverted repeat sequences that overlap with the canonical -35 and -10 elements, indicating that sHdrR exerts its regulatory influence by directly obstructing RNA polymerase binding and transcription initiation. Biochemical and electrophoretic analyses revealed that a truncated, yet stable, version of sHdrR (sHdrR-trunc) retains the ability to bind specifically to the intergenic regions between *soxT1A* and *shdrR*, as well as between *soxA* and *soxY.* These regions are key regulatory hubs upstream of genes critical for sulfur oxidation. Sequence analysis of these intergenic regions identified multiple AT-rich palindromic consensus operator motifs (ATA-N_2-A-N_2-AT-N_4-ATA), which are characteristic of ArsR-family repressor binding sites. Notably, these motifs overlap with the -10 promoter elements essential for RNA polymerase binding, suggesting that sHdrR represses transcription through direct steric hindrance of transcription initiation.

Comparative analysis revealed that sHdrR shares structural and functional characteristics with SoxR, another member of the ArsR-SmtB regulator family. Notably, sHdrR contains three cysteine residues, two of which—Cys^{50} and Cys^{116}—are conserved across homologous proteins in related bacterial taxa, suggesting a conserved mechanism of redox sensing. Upon exposure to sulfane sulfur species, such as polysulfide, *in vitro* biochemical assays showed that sHdrR undergoes persulfidation, leading to the formation of an intramolecular sulfur bridge between Cys^{50} and Cys^{116}. This modification induces a conformational change that significantly reduces sHdrR's DNA-binding affinity, thereby lifting repression of its target genes.

Importantly, a mutant variant of sHdrR, in which Cys^{63} is replaced with serine ($Cys^{63}Ser$), retains responsiveness to polysulfide while remaining unreactive to other oxidants, pinpointing Cys^{63} as a residue critical for specificity in sulfur compound sensing. This finding underscores the finely tuned redox sensitivity of sHdrR and highlights the importance of specific cysteine residues in regulating its activity.

Further supporting its central role, sHdrR was shown to act in coordination with SoxR to regulate the *shdr-lbpA-sox* gene cluster, which participates in thiosulfate oxidation (Li et al. 2025). Evidence suggests that both regulators do not sense thiosulfate directly but instead respond to intracellular polysulfide, a reactive intermediate generated during thiosulfate metabolism.

Altogether, this study provides the first comprehensive view of the DNA-binding dynamics and regulation of sHdrR. It expands our understanding of transcriptional control in sulfur-oxidizing bacteria.

J.L. contributed to conceptualization, investigation, data curation, visulalization and writing of the original manuscript: J.L. contributed to the construction of the *H. denitrificans* shdrR-trunc and variants *in vitro*, and EMSA experiments for sHdrR-trunc and variants with the tested regions under different conditions. EMSA for proteins with subfragments of the *soxT1A-shdrR*, *soxA-soxY* intergenic regions were performed by J.L.

CHAPTER 7

SUMMARY AND DISCUSSION

This study investigates the regulatory mechanisms underlying sulfur oxidation in *Hyphomicrobium denitrificans,* a methylotrophic Alphaproteobacterium capable of using thiosulfate as an additional electron donor during growth on C_1 compounds. Sulfur oxidation is initiated by Sox enzymes in the periplasm and proceeds via import of sulfane-sulfur into the cytoplasm, where it is processed by the sulfur-oxidizing heterodisulfide-reductase-like enzyme complex (sHdr) system, producing sulfite. Two ArsR-type transcriptional repressors, SoxR and sHdrR, regulate the expression of the genes involved in this process. SoxR is regulated by formation of a (poly)sulfur bridge between conserved cysteine residues, influencing a broader regulon including periplasmic *sox* genes and cytoplasmic *lip-shdr-lbpA* genes, whereas sHdrR affects only a subset of these genes. Additionally, two YeeE/YedE-family transporters, SoxT1A and SoxT1B, are involved in sulfur import and regulatory signaling, respectively. This study also explored how sulfur oxidation intersects with anaerobic metabolism, notably denitrification.

1. Consumption of C_1 compounds and thiosulfate oxidation in *H. denitrificans*

The metabolic versatility of *H. denitrificans* X^T enables it to simultaneously oxidize reduced C_1 compounds, such as methanol, and inorganic sulfur compounds like thiosulfate, making it a model organism for studying both carbon and sulfur metabolism. In this study, we examined how *H. denitrificans* X^T coordinates these two pathways.

Methanol oxidation occurs in the periplasm and is initiated by a PQQ-dependent methanol dehydrogenase, producing formaldehyde (Duine et al., 1978). This intermediate is either assimilated via the serine cycle or fully oxidized to CO_2 through tetrahydromethanopterin- and tetrahydrofolate-linked pathways, generating reducing equivalents (NADH/NADPH) critical for energy metabolism and biosynthesis (Chistoserdova, 2011). Formate, as an intermediate, is further oxidized in the cytoplasm with additional NADH production (Marison and Attwood, 1980). This electron flow is tightly regulated by redox cofactors including cytochrome *c*, NADH, and ubiquinone (Smejkalova et al., 2010; Chistoserdova, 2011). Concurrently, thiosulfate oxidation begins in the periplasm via the SoxAXYZ complex, generating sulfane sulfur, which is transferred to SoxYZ and eventually imported into the cytoplasm via the SoxT1A transporter. In the cytoplasm, the sulfur is processed by rhodanese-like enzymes (Rhd442), DsrE3C, and ultimately the sHdr-LbpA system, resulting in the release of sulfite (SO_3^{2-}) (Cao et al., 2018; Koch and Dahl, 2018; Ernst et al., 2021; Li et al., 2023b; Li et al., 2024; Tanabe et al., 2024).

Initial hypotheses suggested that thiosulfate oxidation might lead to the over-reduction of redox carriers such as cytochrome *c* or NAD(P), limiting their availability for methanol oxidation. However, experimental data refuted this, instead identifying sulfite as a central metabolic disruptor (Li et al.2023a). Sulfite, a reactive electrophilic molecule, interferes with methanol metabolism by forming adducts with the PQQ cofactor of methanol dehydrogenase (McIntire, 1989; Dewanti and Duine, 1998). This modification impairs enzyme function and slows methanol oxidation and thus growth rate, while cytoplasmic formate dehyhdrogenase

remains essentially unaffected. This effect underscores the importance of tightly regulating sulfite concentrations, not only to avoid its general toxicity but also to preserve cofactor functionality essential for C_1 metabolism.

Overall, these results provide new insights into how *H. denitrificans* X^T integrates carbon and sulfur oxidation, revealing a complex balance between energy acquisition and metabolic compatibility. Understanding such interactions is crucial for optimizing the use of methylotrophs in biotechnological applications involving mixed-substrate environments, and for refining models of microbial metabolism in sulfur-rich ecosystems.

2. Two YeeE-like bacterial SoxT proteins play different roles in *H. denitrificans*

Many sulfur-oxidizing prokaryotes oxidize sulfur compounds through a combination of initial extra cytoplasmic and downstream cytoplasmic reactions. Facultative sulfur oxidizers adjust transcription to sulfur availability. Two SoxT proteins, SoxT1A and SoxT1B, which resemble YeeE/YedE-family thiosulfate transporters and are encoded alongside sulfur oxidation and transcriptional regulation genes, were studied to better understand the role of sulfur-oxidizing enzymes and transcriptional repressors in the Alphaproteobacterium *H. denitrificans*.

SoxT1A-deficient mutants retain high expression levels of sulfur oxidation genes but fail to oxidize sulfur compounds, indicating that cytoplasmic sulfur delivery is an essential step in the metabolism of *H. denitrificans.* This role is consistent with the broader function of YeeE family proteins in sulfur compound transport and supports the view that SoxT1A is closely integrated into the core sulfur oxidation machinery. Adjacent to *soxT1A*, Hden_0679 encodes a periplasmic DsbA-like thioredoxin with two Cys-X_2-Cys motifs, one at the C-terminus. Thioredoxins function as disulfide oxidoreductases, facilitating redox reactions via reversible cysteine oxidation. We propose that *H. denitrificans* DsbA aids in releasing sulfane sulfur from persulfidated SoxYZ, transferring it into the cytoplasm via SoxT1A (Li et al., 2024) (Fig. 1). There, sulfur is processed by Rhd442 (Hden_0680), a rhodanese-like sulfur transferase, before being delivered to DsrE3C and oxidized to sulfite via the sHdr-LbpA system, possibly involving TusA (Tanabe et al., 2024).

SoxT1B primarily modulates gene expression (Li et al., 2024). The loss of SoxT1B results in diminished transcription of sulfur oxidation genes, but this repression is lifted in SoxR-deficient backgrounds, confirming that SoxT1B acts upstream of SoxR in regulatory cascades. These insights underscore the importance of coordinated sulfur uptake and transcriptional control in facultative sulfur oxidizers. Additionally, our findings suggest that SoxT1B could function as a sensor that links extracellular sulfur availability to intracellular transcriptional responses (Fig. 2). The genes in the vicinity of *soxT1B* appear to encode a second module dedicated to the transport of sulfur, albeit for a different purpose. Similar to SoxT1A, it likely receives sulfur from SoxYZ, aided by SoxS, and passes it to TusA. In *E. coli*, YeeD and YeeE work together to provide a specialized thiosulfate uptake and processing pathway (Tanaka et al., 2020; Ikei et al., 2024). YeeD is a small protein that closely resembles TusA. YeeD and TusA share a conserved cysteine residue essential for their function as sulfur transferases. Once arrived on TusA, sulfur then be transferred to the repressors encoded in the *shr-lbpA-sox* region (Fig. 2).

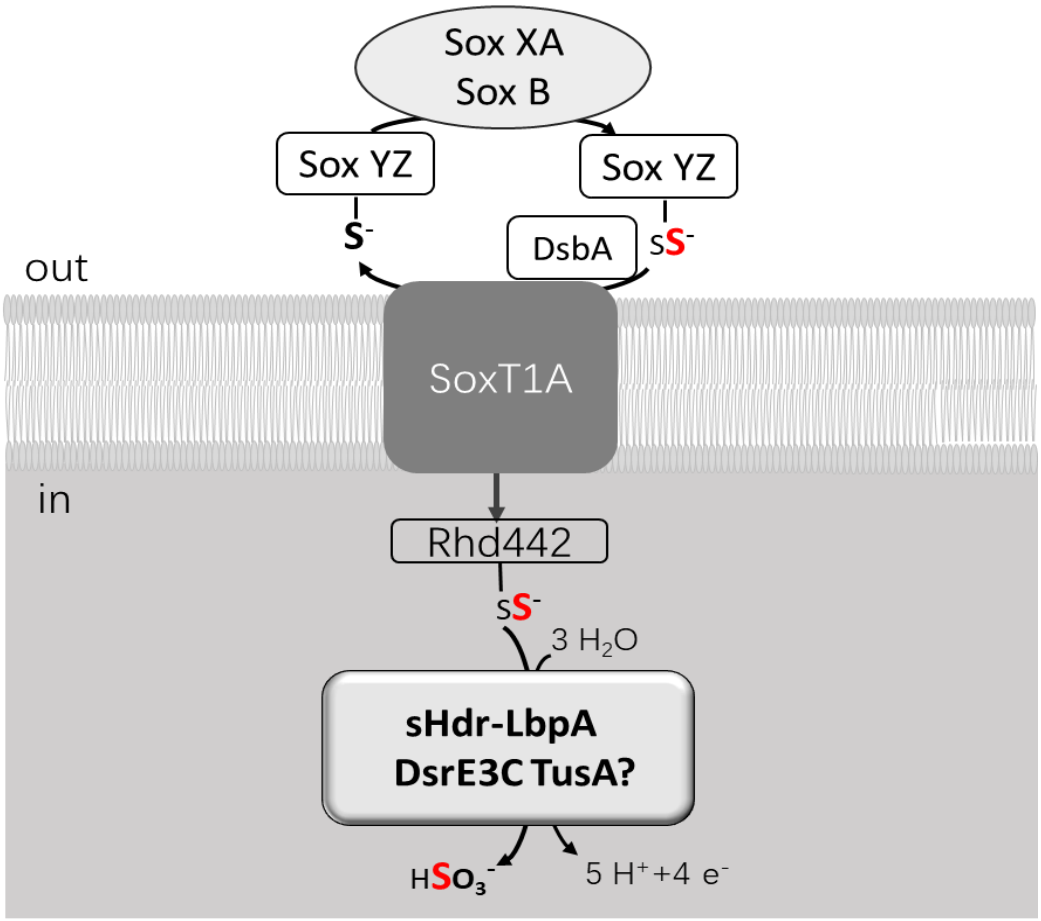

Fig. 1. Model of thiosulfate oxidation in *H. denitrificans* integrating the sulfur transport function of SoxT1A. Sulfur atoms printed in red stem from the sulfane sufur atom of thiosulfate, the oxidation of which is initiated in the periplasm.

These findings raise intriguing questions about the broader roles of SoxT-like proteins. SoxT1A and SoxT1B appear functionally distinct from typical YedE/YeeE family members, which are generally regarded as passive transporters for thiosulfate or other sulfur anions. While traditionally associated with transport, the observed impact of SoxT1A on *sox* gene expression suggests a possible regulatory function, either directly or indirectly through metabolite levels or signaling pathways. Elucidating these roles will be critical for establishing a comprehensive understanding of sulfur transport, signaling, and regulation in *H. denitrificans*. The precise mechanisms underlying SoxT1A and SoxT1B function remain to be elucidated. Future studies should focus on their biochemical properties, structural dynamics, and interactions with other sulfur oxidation components. Further research is also needed to identify the exact sulfur species transported by SoxT1A and SoxT1B.

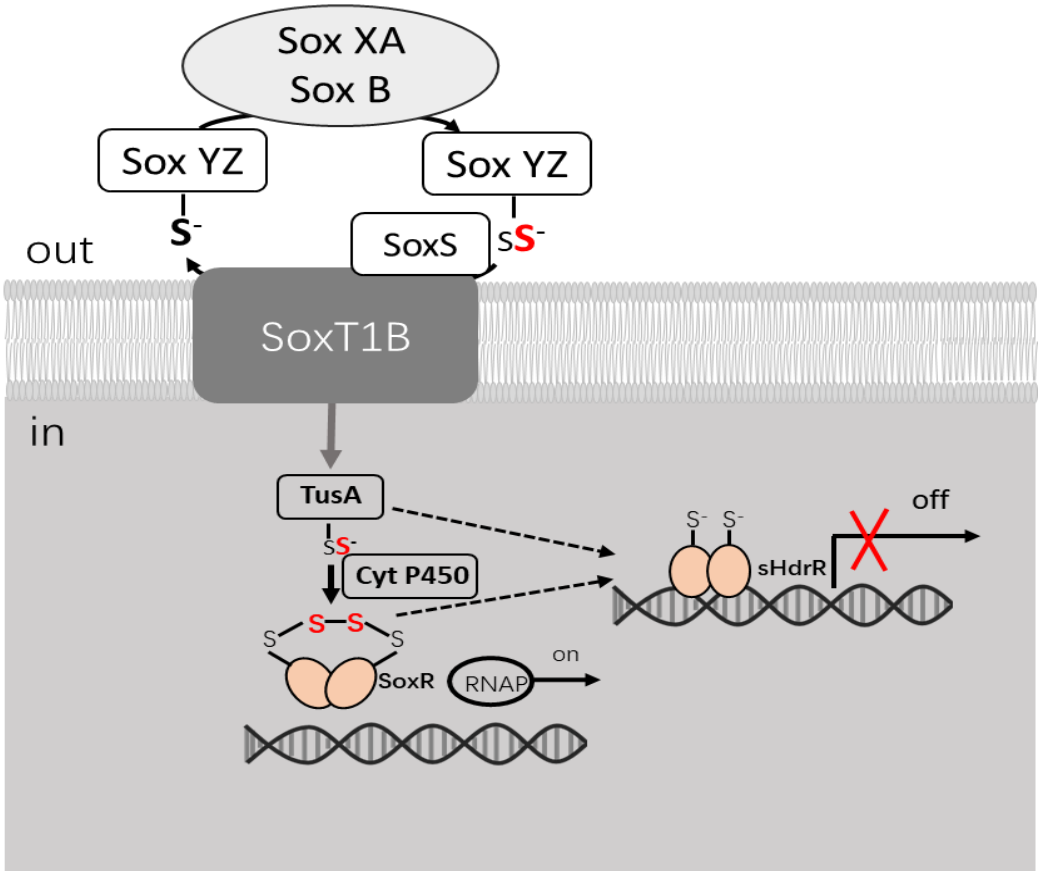

Fig. 2. Model of thiosulfate oxidation in *H. denitrificans* integrating the signal transduction function of SoxT1B. Sulfur atoms printed in red stem from the sulfane sulfur atom of thiosulfate, the oxidation of which is initiated in the periplasm. RNAP, RNA polymerase.

3. Two ArsR type regulators co-regulate the thiosulfate oxidation pathway in *H. denitrificans*

Two closely related ArsR-family transcriptional repressors, SoxR and sHdrR, are central to the regulation of sulfur oxidation genes in *H. denitrificans*. SoxR has emerged as the master regulator of the periplasmic and cytoplasmic components of the sulfur oxidation pathway. Functional assays and transcriptomic analyses show that SoxR governs the expression of *sox* genes involved in thiosulfate oxidation, as well as *lip, shdr,* and *lbpA* genes essential for cytoplasmic sulfite production. This breadth of regulatory influence supports the model that SoxR acts as a global integrator of sulfur availability signals.

Biochemical studies reveal that SoxR contains two highly conserved cysteines (Cys^{50} and Cys^{116}), which form disulfide or polysulfide bonds in response to oxidation or incubation with polysulfide, respectively (Fig. 3) (Li et al., 2023a). In contrast, the oxidized SoxR Cys^{50}Ser and Cys^{116}Ser variants formed intermolecular dimers, linked via the remaining cysteine residues on each monomer (Fig. 4). These results indicate that native SoxR exists as a homodimer, with the dimeric conformation bringing Cys^{50} and Cys^{116} of opposing monomers into close proximity, thereby enabling disulfide bridge formation under oxidizing conditions. *In vitro*, exposure to polysulfides induces the formation of tri-, tetra- and pentasulfide linkages (Fig. 3), reducing DNA binding activity and promoting transcription. This activity allows SoxR to act as

a dynamic switch that modulates gene expression in response to fluctuating environmental thiosulfate concentrations.

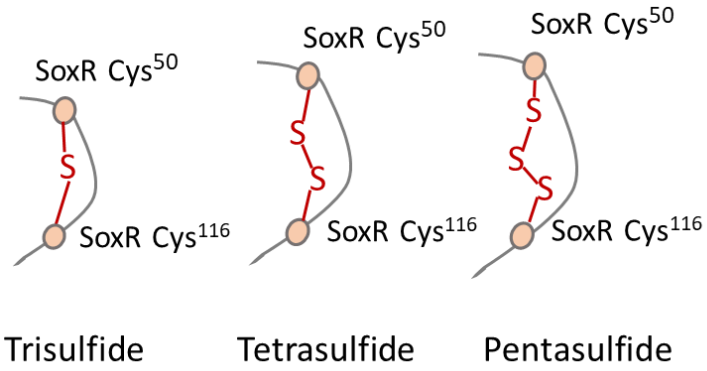

Fig. 3. Reaction of SoxR with polysulfide results in a mixture of tri-, tetra- and pentasulfide intramolecular linkages.

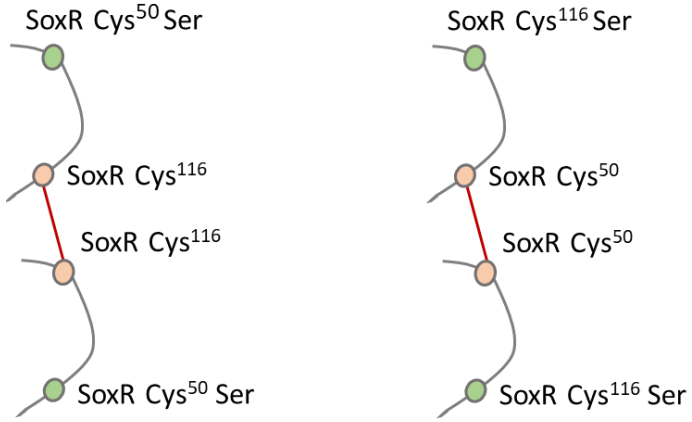

Fig. 4. Intermolecular disulfide linkages in SoxR variants lacking one of two conserved cysteines.

While SoxR controls both genes for periplasmic and cytoplasmic enzymes involved in the oxidation of thiosulfate to sulfite, sHdrR primarily targets the cytoplasmic *shdr-lbpA* cluster and related genes (Li et al. 2025). The sHdrR protein shares two conserved cysteine residues, Cys^{50} and Cys^{116}, located in the α2 and α5 helices, with other regulators such as SoxR and SqrR. These cysteines are critical for sensing sulfane sulfur species, as demonstrated by the functional analysis of cysteine mutants. Specifically, a *H. denitrificans* strain carrying sHdrR with Cys^{116}Ser mutation expressed the sulfur oxidation genes constitutively, whereas a strain with a Cys^{50}Ser exchange could not oxidize thiosulfate, i.e. the respective genes were constantly repressed (Li et al. 2025). Notably, sHdrR contains a third cysteine, Cys^{63}, in the α3 helix, similar to the dithiol protein RexT (Li et al. 2022), which reacts with H_2O_2 via two proximal cysteine residues in α3. Initial *in vitro* experiments indicate a role of Cys^{63} in sensing redox signals (see Appendix 5), but further studies are necessary. To clarify the function of cysteines in sHdrR, more experiments need to be performed, including analyses on non-reducing gels, and mass spectrometry analyses of wild-type sHdrR and its variants.

A major question concerns how SoxR and sHdrR interact—do they function redundantly, hierarchically, or cooperatively? The relationship between sHdrR and SoxR is particularly

intriguing, as multiple lines of evidence suggest that these two regulators may work together in a coordinated manner. Various experimental observations and genetic analyses indicate potential interactions between them, pointing to a possible cooperative or interdependent regulatory mechanism. Here, we present several key pieces of evidence supporting this hypothesis. Firstly, in Δ*soxR* or Δ*shdrR* strains, overlapping sets of sulfur oxidation genes are derepressed in the presence of oxidizable sulfur, suggesting that both regulators suppress transcription in the absence of thiosulfate (Li et al., 2023a; Li et al., 2025). Regulatory profiles obtained by RT-qPCR and RNA-seq analyses show that SoxR has broader control, and its deletion elicits stronger transcriptional responses than sHdrR (Li et al., 2025). While sHdrR functions as a repressor controlling expression of the genes for the sHdr complex, SoxR is additionally involved in the regulation of the *sox* genes which encode the enzymes of thiosulfate oxidation in the periplasm (Fig. 5).

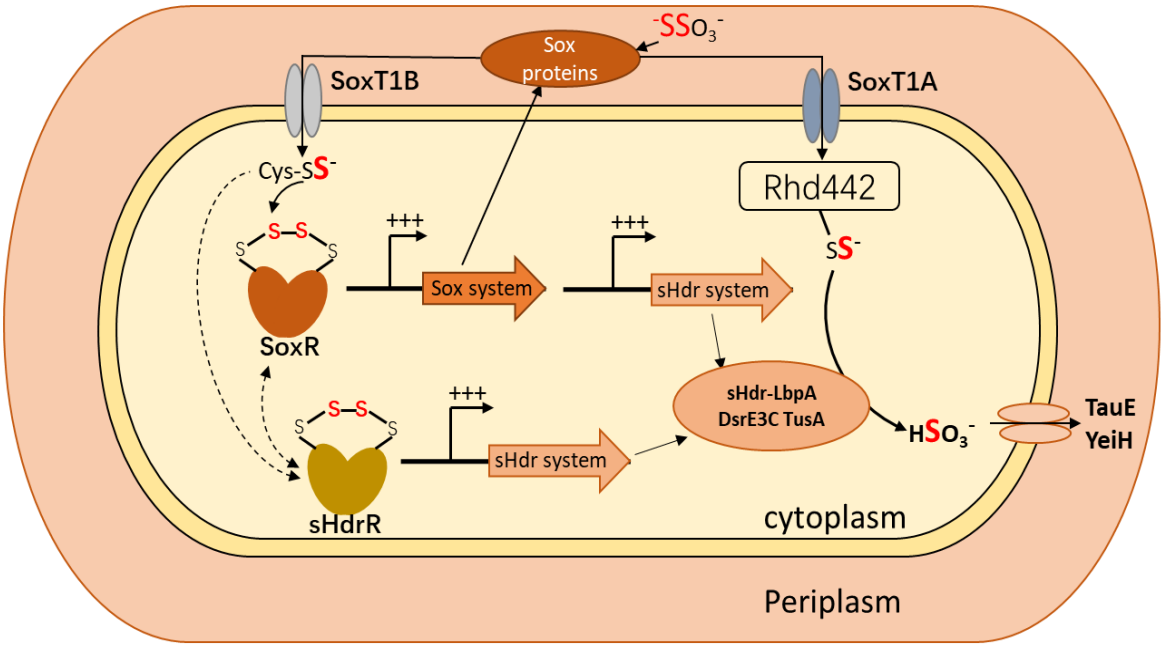

Fig. 5. Proposed function of SoxR and sHdrR in the regulation of sulfur metabolism in *H. denitrificans*. Sox proteins include SoxXA, SoxB, SoxYZ, SoxS. In *H. denitrificans*, thiosulfate oxidation is initiated in the periplasm, where the periplasmic carrier protein SoxYZ binds thiosulfate oxidatively through the action of the c-type cytochrome SoxXA (Li et al., 2023b). Sulfate is subsequently hydrolyzed off by SoxB, and the remaining sulfane sulfur on SoxYZ is transferred to the cytoplasm via the membrane transporter SoxT1A (Li et al., 2024). Once in the cytoplasm, the sulfur is delivered through a cascade of sulfur transfer reactions to the sulfur-oxidizing heterodisulfide-reductase-like enzyme complex, sHdr (Tanabe et al., 2024), which releases sulfite as a product that can be transported by TauE and YeiH. The transporter SoxT1B, which acts as a signal transduction unit for SoxR. SoxR affects the *sox* genes for periplasmic thiosulfate oxidation and sulfane sulfur import into the cytoplasm, as well as the *lip-shdr-lbpA* genes encoding the cytoplasmic enzymes essential for sulfite formation. sHdrR affects only a subset of these genes. TauE, a putative sulfite exporter is encoded by Hden_0720 (Weinitschke et al., 2007). YeiH (Hden_0834) is another candidate for sulfite export, with increased transcript abundance in the presence of oxidizable sulfur (Li et al., 2025). Sulfur atoms printed in red stem from the sulfane sulfur atom of thiosulfate.

This observation implies that SoxR may act as the dominant repressor, with sHdrR refining the response under specific cellular or environmental states. Given that both regulators respond to sulfane sulfur species, their co-regulation likely ensures a balanced response to sulfur

availability, optimizing sulfur metabolism. In addition, the two genes encoding a sulfite exporter (Hden_0834, YeiH) and a LysR family transcriptional regulator (Hden_0835) could also be involved in the regulation (Li et al., 2025).

To explore this further, several approaches are possible. For instance, a competitive binding assay could be designed to investigate sHdrR and SoxR interactions, as evidence suggests that they co-regulate certain genes. One strategy we can use is Western blot to differentiate these two proteins using specific antibodies. The sHdrR protein is purified with a His-tag, for which we already have an antibody to verify its presence. The SoxR protein was constructed with a Strep-tag, allowing us to distinguish these two proteins based on their respective tags. Another strategy is to label DNA with different fluorescent markers. Fluorescent labeling can be incorporated during primer design. For example, we can label the intergenic region between *soxT1A* and *shdrR* with green fluorescence and the intergenic region between *soxA* and *soxY* with red fluorescence. After labeling, we can incubate these DNA fragments with sHdrR or SoxR proteins. Following EMSA, the fluorescence intensity can be analyzed to determine the binding affinity of each protein to the corresponding DNA region. This approach would allow to identify the protein which binds more strongly to a specific region and provide insights into the regulatory mechanisms governing gene expression.

Overall, evidence from RT-qPCR, RNA-seq, and mutant growth studies supports a cooperative model. ArsR-family repressors are widespread in bacteria, often regulating metal detoxification, redox homeostasis, and stress responses (Saha et al. 2017). Comparative sequence and phylogenetic analysis indicate that SoxR and sHdrR diverged from a common ancestor, with conserved residues related to DNA binding retained, while redox-modulated regions evolved to accommodate sulfur-specific signals. The presence of similar systems in other Alphaproteobacteria supports the idea that sulfur-responsive ArsR-family regulators are an adaptive innovation for life in redox-dynamic habitats. To fully understand SoxR and sHdrR regulation, several questions remain: Do these proteins form heterodimers, and under what conditions? What are the precise binding motifs in target promoters? Further research is needed to address these questions. Moreover, extending this research to environmental isolates of *H. denitrificans* may reveal how regulatory plasticity contributes to niche adaptation.

5. Sulfur oxidation and anaerobic metabolism in *H. denitrificans*

One of the central insights emerging from this thesis is the link between sulfur oxidation and anaerobic metabolism, particularly denitrification, in *H. denitrificans* (Li et al., 2025). In microbial ecosystems, metabolic pathways often overlap or interconnect to optimize energy harvesting under dynamic environmental conditions. The findings presented in this thesis expand studies with obligate heterotrophs that can oxidize thiosulfate anaerobically, forming tetrathionate instead of sulfate (Sorokin et al., 1999). While *H. denitrificans* X^T can pursue both pathways, the Δ*tsdA* reference strain studied here exclusively produces sulfate. The energy yield from thiosulfate oxidation with oxygen or nitrate is comparable, though slightly less favorable under anaerobic conditions due to a more reduced respiratory chain (Li et al. 2025).

RNA-seq analyses of SoxR and sHdrR mutants revealed that the absence of either regulator protein not only disrupts sulfur oxidation gene expression but also leads to strong

downregulation of denitrification enzymes, ubiquinone biosynthesis, and O_2-independent PQQ synthesis, essential for methanol dehydrogenase activity. The dual role of SoxR and sHdrR in regulating not only sulfur metabolism but also genes involved in nitrate respiration reveals a complex regulatory network that allows *H. denitrificans* to thrive under both aerobic and anaerobic conditions. It is possible that regulatory crosstalk involving SoxR and sHdrR is bidirectional: sulfur oxidation influences the redox state and energy availability, while denitrification efficiency may impact sulfur intermediate processing. Certain metabolites, such as sulfite, formate, and ubiquinone, serve central roles in both pathways. For example, sulfite, produced during sulfur oxidation, can interfere not only with methanol oxidation but also with nitrate metabolism (Oblath et al., 1981; McIntire, 1989; Dewanti and Duine, 1998; Shi et al., 2019), while ubiquinone acts as a shared electron carrier whose redox state may be sensed to balance metabolic fluxes (Li et al., 2025).

Heterotrophic thiosulfate-oxidizing nitrate reducers like *H. denitrificans* play critical roles in the global sulfur and nitrogen cycles, particularly in hypoxic and anoxic ecosystems (Gliesche et al., 2015; Martineau et al., 2015). Regulatory flexibility likely offers an advantage in oxygen-limited environments like sediments, wastewater systems, and rhizospheres, where simultaneous availability of reduced sulfur compounds and nitrate may favor bacteria capable of integrating both metabolic strategies. The cooperative action of SoxR and sHdrR in regulating sulfur oxidation and denitrification highlights a previously unrecognized level of control over energy metabolism in these bacteria.

Future studies should further explore the sulfur-denitrification interface by investigating genetic interactions among SoxR, sHdrR, and denitrification genes, modeling redox fluxes under varying oxygen conditions, and analyzing electron transport proteins in cells under different electron donor/acceptor regimes. Such work will deepen our understanding of microbial contributions to sulfur-nitrogen cycling and their environmental impacts.

4. Perspective and outlook

This thesis provides insights into sulfur metabolism regulation in *H. denitrificans,* particularly the roles of SoxR and sHdrR. Due to the complexity of the regulatory networks, and the technical challenges associated with genetic manipulation of this organism several questions remain open for further investigation.

Firstly, SoxR contains two conserved cysteine residues. While their function was extensively studied *in vitro*, cysteine substitution variants still need to be investigated in the *in vivo* context. Similarly, sHdrR requires further study, particularly through the construction and phenotypic characterization of a sHdrR $Cys^{63}Ser$ mutant strain.

Secondly, we now know that SoxR and sHdrR function together in regulating sulfur metabolism, but the precise mechanism of their interaction remains unclear. It is uncertain whether they act independently, in a hierarchical manner, or even tightly together as a heterodimer. Further studies are needed to determine whether their DNA-binding sites overlap, whether they compete or cooperate in gene regulation, and how sulfane sulfur sensing influences their activity. Investigating their interaction through EMSA, DNase I footprinting, and Western blot analysis under varying sulfur conditions will help clarify their

regulatory dynamics. Developing a functional reporter system remains a priority, as attempts using *eGFP*, *mCherry*, and *uidA* were unsuccessful.

Thirdly, the transcriptional regulation of sulfur oxidation is further complicated by regulators such as YeiE, a LysR family member. High *yeiE* transcription suggests a role in sulfur metabolism, but its regulatory targets and activation signals remain unknown. Future studies should investigate YeiE and its possible broader role in sulfur metabolism.

Additionally, sulfur transport and signal transduction mechanisms require further exploration. SoxT1A is upregulated in response to thiosulfate and functions as a primary sulfur importer, while SoxT1B is likely involved in signal transduction. However, the specific sulfur species transported by these proteins remain unclear. Although single mutants for *soxT1A* and *soxT1B* showed a loss of thiosulfate oxidation, constructing a double mutant is necessary to fully elucidate their roles.

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APPENDIX 1

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A metabolic puzzle: Consumption of C_1 compounds and thiosulfate in *Hyphomicrobium denitrificans* X^T

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ABSTRACT

Many obligately heterotrophic methylotrophs oxidize thiosulfate as an additional electron source during growth on C_1 compounds. Although two different pathways of thiosulfate oxidation are implemented in *Hyphomicrobium denitrificans* X^T, a pronounced negative effect on growth rate is observed when it is cultured in the simultaneous presence of methanol and thiosulfate. In this model organism, periplasmic thiosulfate dehydrogenase TsdA catalyzes formation of the dead-end product tetrathionate. By reverse genetics we verified the second pathway that also starts in the periplasm where SoxXA catalyzes the oxidative fusion of thiosulfate to SoxYZ, from which sulfate is released by SoxB. Sulfane sulfur is then further oxidized in the cytoplasm by the sulfur-oxidizing heterodisulfide reductase-like system (sHdr) which is produced constitutively in a strain lacking the transcriptional repressor sHdrR. When exposed to thiosulfate, the Δ*shdrR* strain exhibited a strongly reduced growth rate even without thiosulfate in the pre-cultures. When grown on methanol, cells exhibit significantly increased NAD^+/NADH ratios in the presence of thiosulfate. In contrast, thiosulfate did not exert any negative effect on growth rate or increase NAD^+ levels during growth on formate. On both C_1 substrates, excretion of up to 0.5 mM sulfite as an intermediate of thiosulfate (2 mM) oxidation was recorded. Sulfite is known to form adducts with pyrroloquinoline quinone, the cofactor of periplasmic methanol dehydrogenase. We rationalize that this causes specific inhibition of methanol degradation in the presence of thiosulfate while formate metabolism in the cytoplasm remains unaffected.

1. Introduction

In respiratory organisms, energy can be conserved from electrons flowing from reduced inorganic or organic compounds (litho- vs. organotrophy) to more oxidized acceptor molecules. While electron donors of autotrophic organisms – be it organic or inorganic – are oxidized but not assimilated, electron donors of heterotrophic organisms (e.g. sugars) are assimilated into biomass and serve as carbon sources. In nature, where bacteria encounter multiple energy and/or carbon sources at the same time, separation between the different metabolic modes is not strict. Instead, many bacteria can draw on different sources of electrons in parallel and may not thrive as exclusive organohetero- or lithoautotrophs, especially when their substrates are present only in low concentrations [1,2]. A number of these organisms can use chemolithoheterotrophy, a mixed metabolic mode in which an organocarbon compound is used with simultaneous oxidation of an inorganic species such as thiosulfate, Mn^{2+} or molecular hydrogen as an auxiliary electron donor. True chemolithoheterotrophs can thereby generate additional proton-motive force (Δp) or sodium motive force (ΔNa^+) used in turn to generate ATP [3–7].

Based on observations of phenotypes, two physiological groups of obligately heterotrophic sulfur-oxidizing bacteria have classically been distinguished [8]: the long-known organisms mostly encountered in marine or saline environments which oxidize sulfur compounds incompletely to tetrathionate [7,9–13] and the increasing number of those organisms that oxidize sulfur compounds all the way to sulfate [14–18]. In 2018, we described that the Alphaproteobacterium *Hyphomicrobium denitrificans* X^T (DSM 1869^T, ATCC 51888^T) is capable of both, formation of tetrathionate and sulfate from thiosulfate (Fig. 1) and that the product formed is dependent on the initial substrate concentration in batch culture [19]. The organism is a representative of the Hyphomicrobiaceae (order Hyphomicrobiales), a family of

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phenotypically quite diverse, mostly aerobic bacteria [20]. Like other *Hyphomicrobium* species, the appendaged, budding *H. denitrificans* is ubiquitous in soils as well as fresh and brakish waters. As a restricted facultative methylotroph it can neither grow autotrophically nor on compounds with three or more carbon atoms [21]. Highest growth yields are reached with methanol or methylamine(s) [22,23]. The substrate range of *Hyphomicrobium* spp. further includes formate, acetate, ethanol, dimethyl sulfoxide or dimethyl sulfide and can vary even within strains of the same species [21].

It is well established that selected *Hyphomicrobium* strains and also representatives of other families of the Hyphomicrobiales use inorganic sulfur compounds like thiosulfate and sulfide as additional electron donors during methylotrophic growth, thereby increase their growth yield and thus appear as true chemolithoheterotrophs [15,26–28]. However, although even two different pathways of thiosulfate oxidation are implemented in *H. denitrificans* X^T (Fig. 1), no growth benefits from the inorganic sulfur compound could be detected in batch culture [19]. To the contrary, a significant reduction in growth rate was observed in the simultaneous presence of thiosulfate and methanol or methylamine [19,25]. Here, we aimed to explain this puzzling observation by combining several different experimental strategies. First, the involvement of Sox proteins in the initial degradation of thiosulfate in the periplasm was rigorously verified by reverse genetics. Second, information was collected on the regulation of the sHdr pathway. Third, growth experiments were performed with informative mutant strains on different C_1 compounds. In this context, the genetic basis of C_1 metabolism in *H. denitrificans* was analyzed in detail. To shed first light on the bioenergetics of the interplay between sulfur compound oxidation and C_1 carbon compound oxidation and assimilation, NAD^+/NADH ratios were determined in cells growing on C_1 compounds with or without thiosulfate. Increased levels were observed on thiosulfate/methanol but not on thiosulfate/formate and can be explained by excretion of sulfite as an intermediate of thiosulfate oxidation. Sulfite thus appears to inhibit methanol but not formate consumption.

2. Materials and methods

2.1. Bacterial strains, plasmids, primers, and growth conditions

Table S1 lists the bacterial strains, and plasmids that were used for this study. *Escherichia coli* strains were grown on lysogeny broth (LB) media under aerobic conditions [29] at 37 °C unless otherwise indicated. *Escherichia coli* BL21(DE3) was used for recombinant protein production. *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 buffer as described before [19]. Media contained either 24.4 mM methanol or 25 mM formate and were supplemented with thiosulfate in the indicated concentrations when needed. Antibiotics for *E. coli* and *H. denitrificans* were used at the following concentrations (in μg ml^{-1}): ampicillin, 100; kanamycin, 50; streptomycin, 200; chloramphenicol, 25.

2.2. Recombinant DNA techniques

Standard techniques for DNA manipulation and cloning were used unless otherwise indicated [30]. Restriction enzymes, T4 ligase and Q5

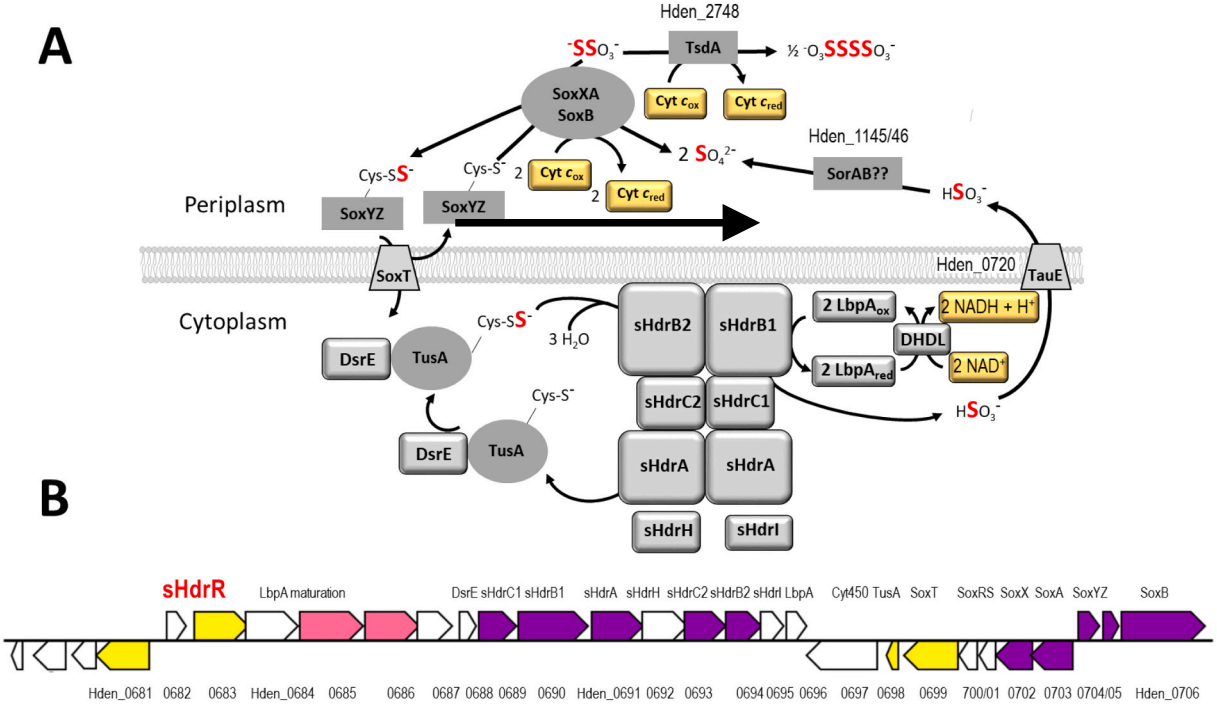

Fig. 1. (A) Current model of thiosulfate oxidation in *H. denitrificans*. The diheme cytochrome *c* thiosulfate dehydrogenase, TsdA (EC 1.8.2.2, thiosulfate:ferricytochrome *c* oxidoreductase), resides in the periplasm and catalyzes oxidative condensation of two thiosulfate molecules to tetrathionate, which is a dead-end product and not metabolized any further [19]. Complete oxidation of thiosulfate to sulfate also starts in the periplasm where enzymes SoxXA (EC 2.8.5.2, L-cysteine S-thiosulfotransferase) and SoxB (EC 3.1.6.20, S-sulfosulfanyl-L-cysteine sulfohydrolase) act together in oxidative attachment of thiosulfate to the sulfur carrier protein SoxYZ and subsequent hydrolytic release of sulfate from the thiosulfonate adduct. The sulfane sulfur stemming from thiosulfate is then transferred to the cytoplasm and further oxidized by the proteins of the sulfur-oxidizing heterodisulfide reductase-like enzyme system, sHdr, in conjunction with the lipoate-binding protein LbpA [19,24,25]. The resulting sulfite is transported back to the periplasm and oxidized to sulfate in a so far unresolved manner that may involve the products of genes Hden_1145/45. Locus tags are given for those proteins that are not encoded by any of the genes shown in (B). DHDL, dihydrolipoamide dehydrogenase (EC 1.8.1.4, dihydrolipoyl dehydrogenase): four different genes encoding this enzyme are present (Hden_0791, Hden_0896, Hden_1300, Hden_3225) (B) The *shdr* gene cluster and its vicinity in *H. denitrificans*. Encoded proteins or functions as well as locus tags are given. Color codes of genes are according to KEGG (https://www.kegg.jp/kegg/kegg1c.html).

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). Plasmid DNA from *E. coli* was purified using the GeneJET Plasmid Miniprep kit (Thermo Scientific, Waltham, USA). Chromosomal DNA from *H. denitrificans* strains was prepared using the First-DNA all-tissue Kit (GEN-IAL GmbH, Troisdorf, Germany).

2.3. Construction of H. denitrificans mutant strains

For markerless *in frame* deletion of the *H. denitrificans shdrR* (Hden_0682), *soxYZ* (Hden_0704/05) and *soxXA* (Hden_0702/03) genes by splicing overlap extension (SOE) [31], PCR fragments were constructed using primers listed in Table S1. The Δ*shdrR* fragment was inserted into pk18*mobsacB* [32] using BamHI and XbaI restriction sites. The SmaI-excised tetracycline cassette from pHP45Ω-Tc [33] was inserted into the SmaI site of the resulting plasmid pK18*mobsacB*ΔshdrR. The Δ*soxYZ* and Δ*soxXA* fragments were inserted into the XbaI site of pK18*mobsacB*-Tc which had been constructed by insertion of the SmaI-excised tetracycline cassette from pHP45Ω-Tc into the SmaI site of pK18*mobsacB*. The final constructs pK18*mobsacB*ΔshdrR-Tc, pK18*mobsacB*ΔsoxYZ-Tc and pK18*mobsacB*ΔsoxXA-Tc were electroporated into *H. denitrificans* Δ*tsdA* and transformants were selected using previously published procedures [19,25]. Single crossover recombinants were Cm^r and Tc^r. Double crossover recombinants were Tc^s and survived in the presence of sucrose due to loss of both, the vector-encoded levansucrase (SacB) and the tetracyclin resistance gene. The genotypes of the *H. denitrificans* mutant strains generated in this study were confirmed by PCR.

2.4. Characterization of phenotypes, quantification of sulfur compounds and protein content

Growth was followed by turbidity measurements at either 430 nm or 600 nm. A factor of 1.5947 ($R^2 = 0.9994$) was determined for conversion of OD_{600} into OD_{430} (Fig. S1). In general, a small wavelength is preferable for OD measurements especially when cell density is low, because the sensitivity of the measurement is then higher [34]. For growth experiments, media with 24.4 mM methanol and varying concentrations of thiosulfate were inoculated to a start OD_{430} of 0.008 with pre-cultures in late-exponential growth phase cultured on the same medium (50 ml culture in 100 ml-Erlenmeyer flasks). For phenotypic characterization, main cultures of 200 ml in 500 ml Erlenmeyer flasks were shaken at 200 rpm and 30 °C. Samples were taken in regular intervals and optical densities as well as thiosulfate, methanol, sulfite and sulfate were determined as necessary. Alternatively, growth experiments were run in 48-well microtiter plates. Plates were continuously shaken at 200 rpm and growth was followed by measuring optical density at 600 nm every 5 min using an Infinite 200Pro (Tecan, Crailsheim, Germany) plate reader. Each well contained 1 ml medium inoculated to an OD_{600} of 0.025. For each set of experiments, five wells were run in parallel, one of which remained untouched throughout and served exclusively for following growth. From the other 1-ml parallel cultures, samples were taken for thiosulfate measurements at regular time intervals. Sampling of the same well was restricted to twice 100 µl because sampling would otherwise have substantially reduced the culture volume and changed growth conditions, i.e. the ratio culture volume/surface area and thus aeration of the cultures.

Thiosulfate, sulfite and sulfate were determined by previously described colorimetric and turbidometric methods [35]. Experiments run in microtiter plates were restricted to quantification of thiosulfate which was performed with technical triplicates in a miniaturized format. Fifty µl H_2O and 40 µl 200 mM sodium acetate (pH 4.8) were added to 40 µl culture supernatants, mixed, followed by addition of 10 µl 200 mM NaCN and 10 µl 40 mM $CuCl_2$. After mixing thoroughly again, 10 µl iron nitrate solution (30 g l^{-1} $Fe(NO_3)_3 \times 9\ H_2O$ and 40 ml 55 % (v/v) HNO_3 made up to 100 ml with distilled water) were added, mixed again and absorption at 460 nm was read in 96-well microtiter plates against a reagent blank with a Sunrise Tecan microplate reader. The same instrument was used for miniaturized sulfite determinations that were performed as follows: 175 µl sample containing varying amounts of culture supernatant were mixed with 50 µl 2% zinc acetate and 25 µl 0.04% fuchsin (in 10% (v/v) H_2SO_4), incubated at room temperature for 10 min and measured against a reagent blank at 570 nm.

For the *H. denitrificans* type strain, the relationship between dry weight and turbidity measured at 430 nm has been reported to be linear to an optical density of 2.0, with OD_{430} 2.0 = 0.60 mg dry wt ml^{-1} [36–38]. Biomass values given in this work are all based on this conversion factor. We also calculated the protein content of cultures in Erlenmeyer flasks from OD_{430}: A linear correlation between OD_{430} and protein in cultures was found for cells growing on methanol up to an OD_{430} of 1.4 and for cells growing on formate up an OD_{430} of 0.9 (Fig. S2). Conversion factors of 0.1476 ($R^2 = 0.9883$) and 0.1322 ($R^2 = 0.9802$) were determined for growth on methanol and formate, respectively, i.e. an OD_{430} of 1.0 amounts to 0.146 mg protein ml^{-1} on methanol and to 0.132 mg protein ml^{-1} on formate. Protein thus constitutes 48.7 % and 44,1 %, respectively of the dry mass (ratios dry weight/protein 2.01 and 2.27), which is well within the range of values available for other species. For *Hyphomicrobium* strain EG, ratios of dry weight/protein between 2.3 and 2.8 were reported on different substrates [28]. The protein content was determined by the Biuret method as follows. One or two ml of cell suspension was centrifuged for 15 min at 16,100 ×*g* and room temperature. The cell pellet was washed once with 1 ml 1 % NaCl and resuspended in 0.5 ml dH_2O. After addition of 50 µl 5 M NaOH, the samples were incubated at 95 °C for 5 min and cooled down to room temperature. Then, 200 µl of copper sulfate reagent (6.25 g Na—K tartrate, 1.25 g $CuSO_4 \times 5\ H_2O$, 3.12 g KI and 5 g NaOH in a final volume of 500 ml H_2O) were added, followed by incubation for 30 min at room temperature and centrifugation at 16,100 ×*g* for 10 min. The absorbance at 546 nm was measured against a reagent blank.

Specific thiosulfate oxidation rates were calculated as follows: Thiosulfate concentrations determined in growth experiments were plotted graphically against time and fitted by a polynomial trend line between the second and fifth degree. The coefficient of determination helped to determine the correct degree of the polynomial. The first derivative of the polynomial function equation was then calculated and corresponded to the slope, i.e. thiosulfate oxidation rate in µM h^{-1} at each time point, from which the specific oxidation rate [µmol thiosulfate h^{-1} (mg protein)$^{-1}$] was derived.

All growth experiments were repeated three to five times. Representative experiments with two biological replicates for each strain are shown. All quantifications are based on at least three technical replicates.

2.5. Quantification of methanol

Methanol was determined with an analytical high performance liquid chromatography system (Knauer, Germany) equipped with a refractive index detector and an Eurokat H (Knauer, Germany) column system consisting of a pre-column (Eurokrat H, 10 µm, 30 × 8 mm) and a main column (Eurokat H, 10 µm, 300 × 8 mm). The system was run with 5 mM sulfuric acid in ultrapure water at a flow rate of 0.6 ml/min and a temperature of 65 °C. Results were evaluated using the ClarityChrom program.

2.6. Overproduction and purification of recombinant sHdrR

The 411-bp *shdrR* gene was amplified from *H. denitrificans* genomic DNA and cloned between the NdeI and NotI sites of pET22b (+), resulting in pET22b-*shdrR*. Recombinant sHdrR was overproduced in *E. coli* BL21(DE3). The cells were grown at 37 °C in 200 ml LB medium

containing ampicillin up to an OD_{600} of 0.6. Expression of *shdrR* was induced by adding 0.5 mM IPTG. IPTG-induced *E. coli* cells were grown over night at 20 °C. Cells were harvested at 14,000 ×*g* for 20 min and the pellet was washed with 50 mM Tris-HCl, pH 7.4. Three ml resuspending buffer (20 mM sodium phosphate buffer pH 7.4, 500 mM NaCl, 20 mM imidazole containing a spatula tip of deoxyribonuclease I and protease inhibitor) were added per g wet weight for homogenization. Cell lysis was achieved by sonification and followed by centrifugation (16,100 ×*g*, 30 min, and 4 °C) and ultracentrifugation (145,000 ×*g*, 1 h, *4 °C*. The supernatant was applied to a Ni^{2+}-NTA column equilibrated with lysis buffer. The column was washed with six volumes of lysis buffer and eluted with 50–500 mM imidazole in the same buffer. The protein was assessed for its purity by 12.5 % SDS-PAGE.

2.7. Electrophoretic mobility shift assay (EMSA)

Gel electrophoretic mobility shift assays are used to detect interactions between proteins and nucleic acids. In the assay, solutions of protein and nucleic acid are combined and the resulting mixtures are subjected to polyacrylamide under native conditions. After electrophoresis, the distribution of nucleic acid species is determined. In general, protein-nucleic acid complexes migrate more slowly than the corresponding free nucleic acid [39]. The binding reaction mixture (15 µl final volume), contained purified sHdrR protein (52 nM), 2 µl 50 % glycerol and 1.5 µl 10 × binding buffer (100 mM Tris-HCl, 500 mM KCl, 10 mM DTT, 5 % glycerol, pH 8.0). Reaction mixtures were pre-incubated for 20 min at room temperature followed by a further 30 min incubation at 30 °C after adding the DNA probe to a final concentration of 17 nM. The DNA probe consisted of a 362-bp fragment situated between the *shdrR* gene and the gene (Hden_0681) upstream and was generated by PCR using primers EMSA-Fr and EMSA-Rev. The reaction mixtures were loaded onto 6 % native polyacrylamide gels after these had been pre-run at 100 V for 1 h at 4 °C with 0.25 × TBE buffer (25 mM Tris/borate, 0.5 mM EDTA). The loaded gels were electrophoresed in 0.25 × TBE with 0.5 % glycerol at 180 V for 2 h at 4 °C. Gels were subsequently stained for 20 min with SYBR green I. The bands corresponding to sHdrR-bound and free DNAs were visualized with a ChemiDoc Imaging System (BioRad).

2.8. Immunoblot analysis

H. denitrificans cell extracts were prepared as described before [19]. Western analysis was performed using the Trans-Blot Turbo Transfer system (Bio-Rad Laboratories, Munich, Germany) and nitrocellulose membranes (Amersham Protran 0.2 µm NC, GE Healthcare). sHdrA antigens were detected with antisera raised in rabbits (Eurogentec) against recombinant *H. denitrificans* sHdrA [19]. Antisera were used at 1:500 dilution. Binding of α-HdrA was detected with the SignalFire™ ECL reagent system (Cell Signaling Technology) and visualized with a ChemiDoc Imaging System (BioRad).

2.9. Quantification of the intracellular NAD(H/$^+$) ratio

Four milliliter culture broth were first cooled in an ice bath for 10 min to retard cell metabolism, collected by centrifugation (16,100 ×*g* at 4 °C for 5 min) and resuspended in 400 µl of 0.4 M HCl (for NAD^+) or 0.4 M KOH (for NADH). The mixtures were then incubated at 30 °C (NADH) or 50 °C (NAD^+) for 10 min and centrifuged at 16,100 ×*g* for 10 min at 4 °C. Then, 300 µl supernatant was neutralized by adding 300 µl of 0.4 M HCl or 0.4 M KOH, respectively. The neutralized samples were immediately used in a microcycling assay for NADH and NAD^+ determination [40] performed in 96 well plates and followed using a Sunrise Tecan microplate reader. Samples of 30 µl were combined with 120 µl of 125 mM Tris-HCl, pH 8.0 containing 2.5 mM phenazine methosulfate, 0.65 mM 3-(4,5-dimethyl-thiazoyl-2)-2,5-diphenyltetrazolium bromide (MTT), 778 mM ethanol and 33 U/ml yeast alcohol dehydrogenase (A3263, Sigma-Aldrich, St. Louis, MO). The rate of MTT reduction was followed at 570 nm and 25 °C. Calibration was performed by measuring samples containing 30, 60, and 90 pmol NAD^+ or NADH. To ensure reliability of the assay, samples with 30, 60, and 90 pmol of the nicotinamide dinucleotides underwent the same procedure as described for the cells.

3. Results and discussion

3.1. Thiosulfate oxidation in H. denitrificans X^T

As already pointed out, *H. denitrificans* X^T has the genetic potential for two distinct pathways of thiosulfate oxidation, both of which occur or begin in the periplasm and use *c*-type cytochromes as electron acceptors [19,41,42] (Fig. 1A). We reported earlier that at 5 mM, all thiosulfate is stoichiometrically converted to tetrathionate. Thus, thiosulfate dehydrogenase (TsdA) causing oxidative condensation of two thiosulfate molecules to tetrathionate is the predominant catalyst under these conditions. A mutant strain lacking the *tsdA* gene is unable to form tetrathionate [19].

When exposed to thiosulfate concentrations of 2.5 mM or less, *H. denitrificans* X^T wildtype switches gears and the majority of the sulfur compound is no longer transformed to tetrathionate but completely oxidized to sulfate [19]. It is not surprising that the same holds true for the strain lacking *tsdA*. In Fig. 2A growth and methanol consumption in the absence and presence of thiosulfate are compared for the *H. denitrificans ΔtsdA* strain. Just as expected for an organism incapable of autotrophic growth, the increase in biomass was inversely proportional to the decrease of the carbon source methanol. Thiosulfate was completely oxidized to sulfate (data not shown). In full agreement with earlier results, growth of the *ΔtsdA* strain was significantly retarded in the presence of thiosulfate (compare growth curves with open and filled circles in Fig. 2A), fully in line with a much slower consumption of methanol in the presence of thiosulfate (compare open and filled boxes in Fig. 2A).

The genes encoding the enzymes acting together in the oxidation of thiosulfate to sulfate are all situated in the same genomic *shdr-lbpA-sox* island (Fig. 1B). Here, we show by individual markerless deletion of *soxXA* and *soxYZ* that the products of these genes are absolutely essential for thiosulfate oxidation in the *ΔtsdA* strain (Fig. S3). We can thus state with confidence that a typical incomplete Sox pathway without involvement of a sulfane sulfur dehydrogenase, SoxCD, [43,44] is operated in *H. denitrificans* X^T (Fig. 1A). SoxXA catalyzes the oxidative fusion of thiosulfate to a conserved cysteine of the sulfur carrier protein SoxYZ encoded by Hden_0704/05. The other four SoxYZ homologs encoded in *H. denitrificans* (Hden_1399/1400 and three *soxYZ* fusions, Hden_0338, Hden_1000 and Hden_1147) cannot functionally replace the protein encoded in immediate vicinity of *shdr-lbpA*. Just as in other organisms lacking bona fide SoxCD, sulfane sulfur still bound to SoxYZ cannot be oxidized in the periplasm but has to be transferred into the cytoplasm in a so far unresolved manner. Here, oxidation of sulfur to sulfite is strictly dependent on the proteins of the sulfur-oxidizing heterodisulfide reductase-like (sHdr) system (Fig. 1A) [19]. The four electrons released in this step are in all probability transferred to NAD^+ [24,45] (Fig. 1A). The lipoate-binding protein LpbA is essential for this step [25]. In *H. denitrificans* strains lacking functional sHdr or LbpA proteins the strong reduction of growth rate caused by thiosulfate in the wildtype was released [19,25]. It reappeared when the Δ*shdr* strain latter was re-equipped with the *shdr* genes *in trans* [19].

3.2. Regulation of shdr genes in Hyphomicrobium denitrificans

To even more clearly attribute the observed effects of thiosulfate on methanol assimilation and thus growth rate to the sHdr system, we first collected information on its regulation. A previous comparative proteomics approach had already shown that the proteins encoded in the

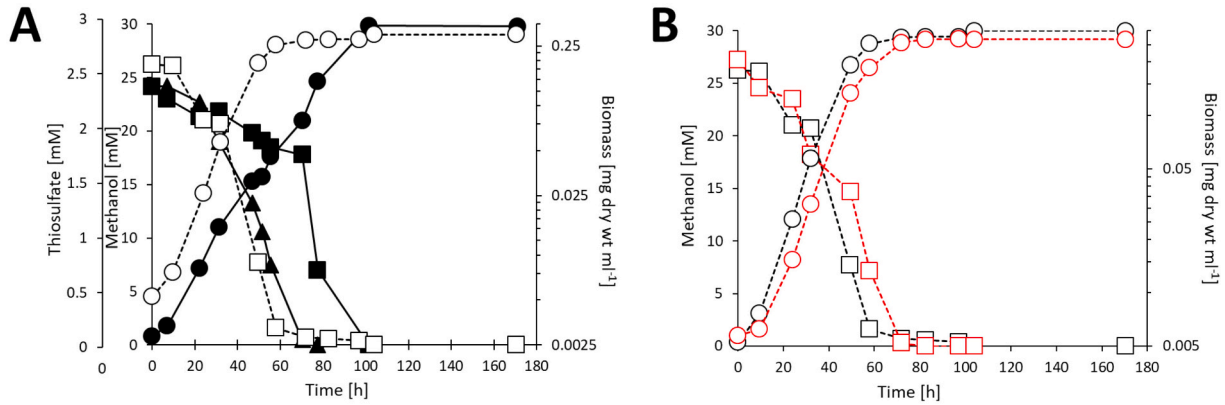

Fig. 2. Part A shows *H. denitrificans ΔtsdA* growing on 24.4 mM methanol (boxes). Precultures contained 2 mM thiosulfate. Cultures without (open symbols, dashed lines) and with thiosulfate (filled symbols, bold lines) are compared. Biomass is given as mg dry weight per ml (○ no thiosulfate, ● with thiosulfate). Methanol concentrations are indicated in the absence (□) and in the presence (■) of thiosulfate. Thiosulfate is given as black triangles (▲). In part B growth and methanol consumption in thiosulfate-free minimal medium are compared for *H. denitrificans ΔtsdA* (black symbols and lines) and *H. denitrificans ΔtsdA ΔshdrR* (red symbols and lines). Methanol concentrations (□, □) and biomass content (○,○) are displayed. Error bars indicating SD are too small to be visible. Sulfate was also quantified but is not shown for clarity. Sulfate concentrations increased by 5 mM in cultures initially containing 2.5 mM thiosulfate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hyphomicrobial *shdr* cluster are much more abundant in the presence of dimethylsulfide (DMS) than in the absence of an oxidizable sulfur compound [19]. Thiosulfate is an intermediate of DMS degradation. We postulated that the transcription of the *shdr* genes is regulated by the ArsR-type regulator encoded by the first gene in the operon, *shdrR* (Hden_0682) (Fig. 1B) [19]. ArsR-family regulators function as transcriptional repressors and include a wide range of metal-, metalloid- and non-metal-sensing proteins [46]. Here, we proved the function of sHdrR as a repressor by construction of *H. denitrificans ΔtsdA ΔshdrR*, a mutant strain with a markerless deletion of *shdrR* in a *ΔtsdA* background. In the absence of thiosulfate, this strain uses methanol just as the Δ*tsdA* reference strain (Fig. 2B) but produces the sHdr system constitutively as shown by immunoblot analysis with an antibody directed against sHdrA (Fig. 3A). *H. denitrificans* sHdrR was produced as a His-tagged recombinant protein in *E. coli*. Electrophoretic mobility shift assays verified that it specifically binds to the 362 bp DNA fragment between its own gene and the divergently transcribed gene Hden_0681 (Fig. 3B).

3.3. Growth and thiosulfate oxidation on a reduced C_1 carbon source: Methanol

In the next step, the effect of thiosulfate during growth on the reduced C_1-carbon compound methanol was assessed in detail for the *H. denitrificans ΔtsdA* reference strain. The growth rate-decreasing effect of thiosulfate became stronger the more thiosulfate was added to the cultures (Figs. 4 and S4). The effect of thiosulfate was particularly impressive when cultures were inoculated with thiosulfate-induced cells (Figs. 4 and S4). Specific thiosulfate oxidation rates rose with the thiosulfate concentration and were about four-fold higher when precultures had already been exposed to thiosulfate (Figs. 4 and S4).

The picture substantially changed for the *ΔtsdA ΔshdrR* strain. This strain, that constitutively produces the sHdr complex, exhibited a very strongly reduced growth rate and a high specific thiosulfate oxidation rate even without induction of pre-cultures. As soon as thiosulfate was consumed, the growth rate increased substantially (Fig. 4). The *H. denitrificans ΔtsdA ΔshdrR* strain exhibited somewhat higher specific thiosulfate consumption rates when pre-cultures had been exposed to thiosulfate than for the non-induced case (Figs. 4 and S4), possibly indicating an additional regulator involved in the overall process.

To further demonstrate the negative effect of thiosulfate on growth rate, thiosulfate was added in early exponential growth phase to cultures of *H. denitrificans ΔtsdA ΔshdrR* growing on 25 or 50 mM methanol. As soon as thiosulfate consumption set in, a drastic decrease in growth rate was observed (Fig. S5). To exclude with certainty that the growth rates observed for the *H. denitrificans ΔtsdA* and *ΔtsdA ΔshdrR* strains in the presence and absence of thiosulfate were negatively influenced by insufficient oxygen supply, growth was followed in 500 ml Erlenmeyer flasks filled with different culture volumes (100 and 200 ml). Consistent with earlier reports of reduced growth rates at oxygen tensions above 65 % air saturation [28], the better oxygenated 100 ml cultures grew significantly slower, irrespective of whether thiosulfate was present (Fig. S6). Slowest growth was observed for the *ΔtsdA ΔshdrR* strain in 100 ml medium containing thiosulfate.

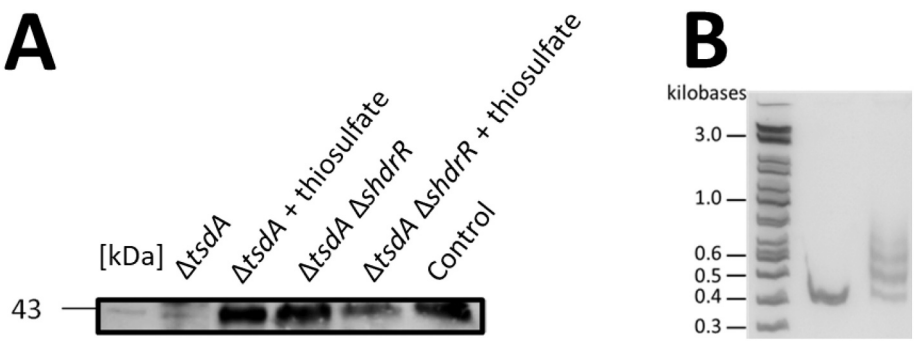

Fig. 3. (A) Western blot analysis with antiserum against sHdrA [19] performed with crude extracts of *H. denitrificans* strains *ΔtsdA* and *ΔtsdA ΔshdrR* grown either on 24.4. mM methanol alone or on 24.4. mM methanol plus 2 mM thiosulfate. Ten μg protein were loaded per lane. Recombinant sHdrA produced in *E. coli* [19,24] was used as the control. (B) Binding of sHdrR to the promoter region of the *shdr* gene cluster assessed by EMSA. Lane 1: DNA size marker; lane 2: 17 nM of DNA fragment; lane 3: Combination of 17 nM of DNA fragment and 52 nM recombinant sHdrR. Reaction mixtures were incubated at 30 °C for 30 min.

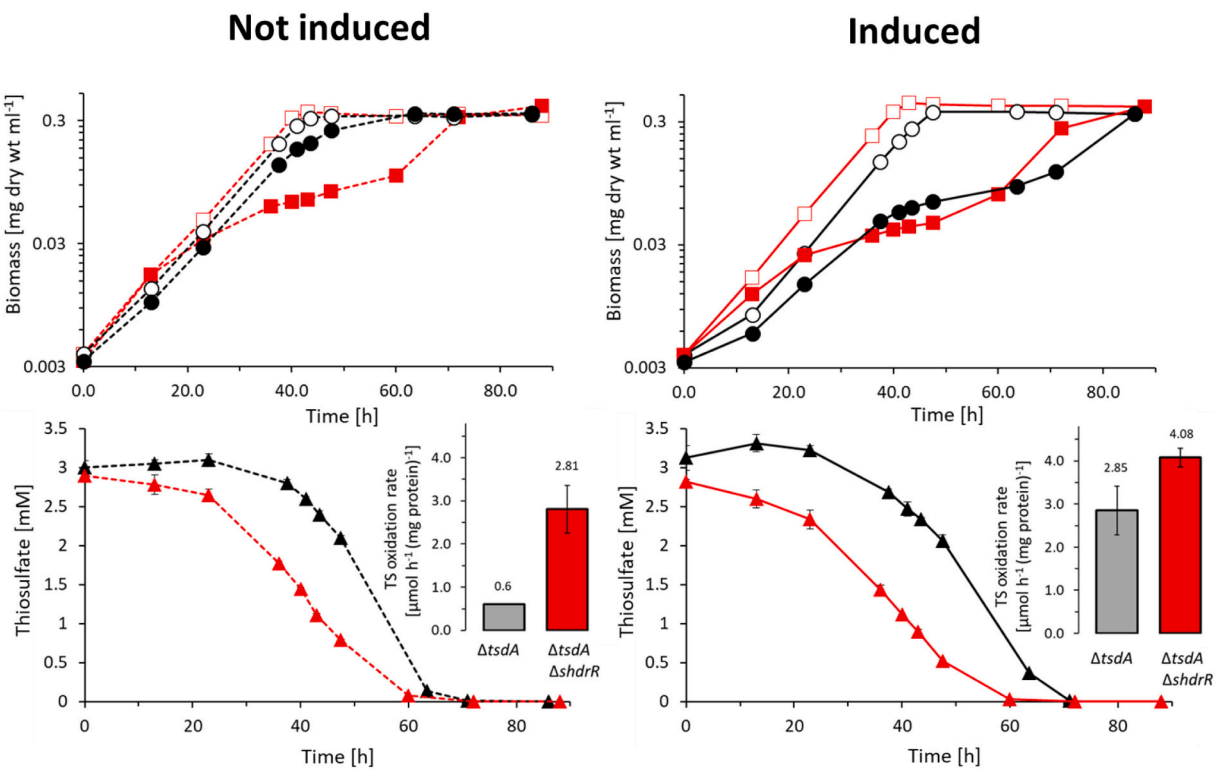

Fig. 4. Growth and thiosulfate consumption of *H. denitrificans ΔtsdA* (black circles and lines) and *ΔtsdA ΔshdrR* (red boxes and lines). Cultures were grown on methanol-containing medium (24.4 mM methanol) without (open symbols) or with 3 mM thiosulfate (filled symbols). Precultures contained either no thiosulfate (not induced, broken lines) or 2 mM thiosulfate (induced, solid lines). In the lower panels, triangles indicate thiosulfate concentrations for *H. denitrificans ΔtsdA* (black) and for *H. denitrificans ΔtsdA ΔshdrR* (red). Specific thiosulfate oxidation (TS) rates are depicted in the same color code. Error bars indicating SD are too small to be visible for determination of biomass. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Growth and thiosulfate oxidation on an oxidized C_1 carbon source: formate

We set out to collect more information on the interplay between sulfur oxidation and carbon metabolism in *H. denitrificans* by replacing methanol with a more oxidized C_1-carbon source. While externally added formaldehyde does not appear to be a suitable substrate for this species [21], we confirmed previous reports that the type strain studied here can grow on formate as the sole source of carbon and electrons [47].

The simultaneous presence of thiosulfate in formate cultures exerted a completely different effect than observed on methanol. Growth rates appeared essentially independent of the presence of thiosulfate in precultures and in main cultures and also independent of the constitutive presence of the sHdr system in the *H. denitrificans ΔtsdA ΔshdR* strain (Fig. 5). Whether the presence of thiosulfate as accessory electron donor during growth on formate causes significant increases in growth yield of the *Hyphomicrobium* strain studied here, has to await further studies, at best in continuous culture.

3.5. Respiratory electron transport and C_1-metabolism in Hyphomicrobium denitrificans X^T

From the reported growth experiments, it appeared that in the *H. denitrificans* type strain thiosulfate as an auxiliary electron donor prevents effective assimilation of a reduced C_1 carbon source, i.e. methanol, but does not negatively influence the rate of biomass production from an oxidized C_1 carbon compound. In order to understand this finding, we performed a detailed analysis of respiratory electron transport and C_1 metabolism and in *H. denitrificans* X^T on the basis of previously published results in combination with an analysis of the strain's genome sequence using the KEGG pathways database (www.genome.jp/kegg/pathway.html) [48]. BLASTP [49] was used to confirm the absence or presence of genes. The results are depicted in Figs. 6 and S7. Additional information is provided in Table S2.

Briefly, electrons from reduced carriers like NADH/$FADH_2$, ubiquinol or reduced cytochrome *c* can enter a respiratory chain composed of complex I, II and III and an array of terminal reductases that can mediate both oxygen respiration and anaerobic respiration on nitrate. In methylotrophs like *H. denitrificans*, substrates like methanol serves two purposes: first they are oxidized to CO_2 producing reducing equivalents for energy conservation via respiration and second they are assimilated for biomass production (Fig. 6A, B). The ability of *H. denitrificans* to oxidize and assimilate methanol is based on a combination of pathways and enzymes similar but not identical to those of the model methylotroph, *Methylorubrum extorquens* (formerly *Methylobacterium extorquens* [50]) (reviewed in [23,51]).

Methanol is first oxidized to formaldehyde by periplasmic pyrroloquinoline quinone (PQQ)-containing methanol dehydrogenase [52]. Formaldehyde is further processed in the cytoplasm by an elaborate pathway employing tetrahydromethanopterin (THMPT) as a cofactor [23]. Formate release from formyl-THMPT is catalyzed by the formyltransferase/hydrolase complex. The step comprises two consecutive reactions where methylofuran acts as an intermediate carrier of the one-carbon unit [53,54]. Formate is finally oxidized to CO_2 by formate dehydrogenase [55]. In many methylotrophs, an oxidative C_1 transfer pathway analogous to the THMPT pathway is proposed that is linked to tetrahydrofolate (THF) instead of THMPT. The first step in this pathway would be the reaction of formaldehyde with THF. An enzyme catalyzing this reaction has not been found in any organism so far and it has been proposed that a spontaneous chemical reaction may be a sufficient source of methylene-THF. However, recent result severely questions this concept [23,56].

Assimilation of C_1 carbon in *H. denitrificans* X^T occurs via the serine

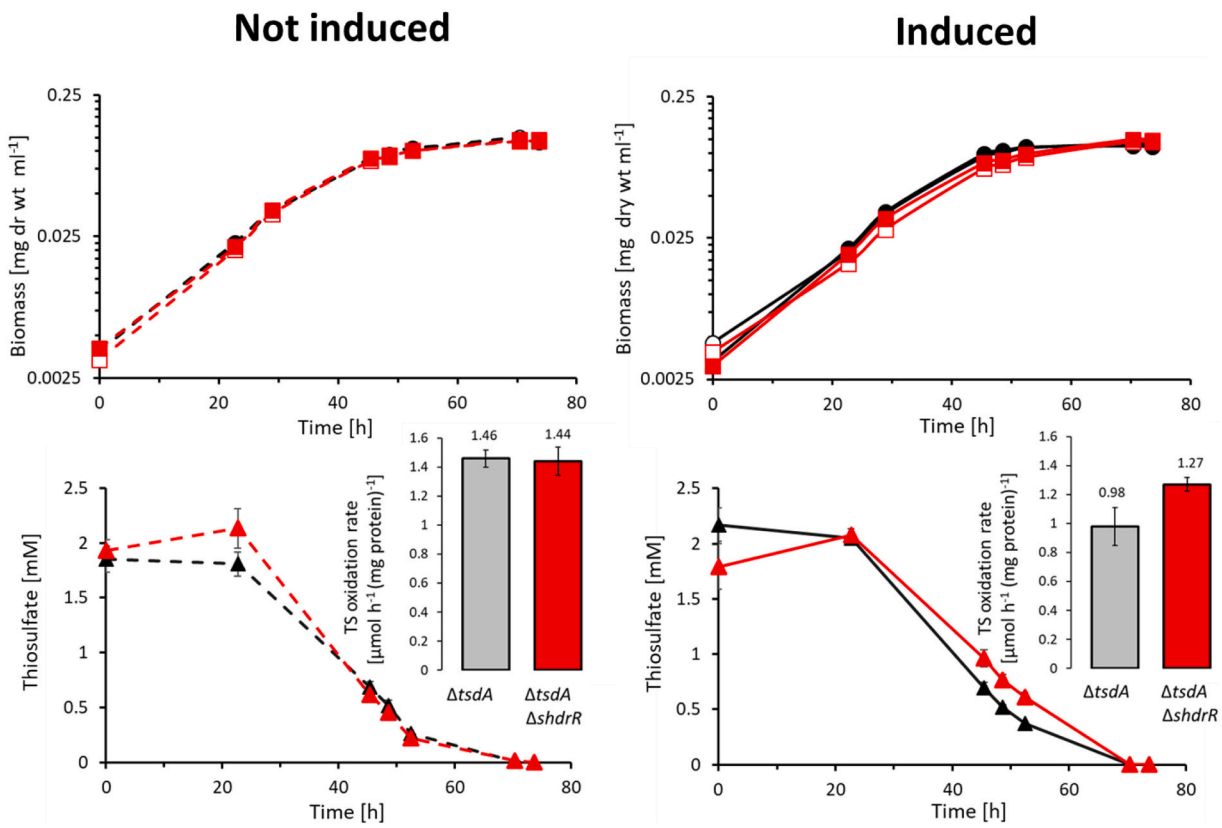

Fig. 5. Growth and thiosulfate consumption of *H. denitrificans ΔtsdA* (black circles and lines) and *ΔtsdA ΔshdrR* (red boxes and lines). Cultures were grown on formate-containing medium (25 mM formate) without (open symbols) or with 2 mM thiosulfate (filled symbols). Precultures also contained 25 mM formate and either no thiosulfate (not induced, broken lines) or 2 mM thiosulfate (induced, solid lines). In the lower panels, triangles indicate thiosulfate concentrations for *H. denitrificans ΔtsdA* (black) and for *H. denitrificans ΔtsdA ΔshdrR* (red). Specific thiosulfate (TS) oxidation rates are depicted in the same color code. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

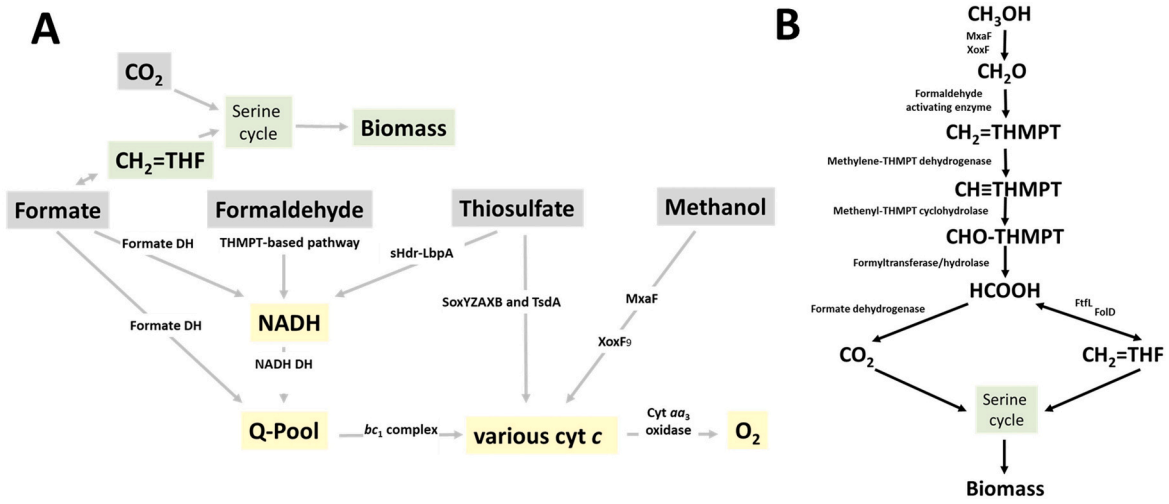

Fig. 6. (A) Overview of electron input and output modules in *Hyphomicrobium denitrificans* X^T during growth on methanol in the presence of thiosulfate at high ambient oxygen concentrations. Proteins are omitted here that were not detected in a proteomic study during growth on dimethyl sulfide (DMS) at high oxygen concentration [19]. Electron carriers/acceptors are highlighted in yellow and carbon assimilation is shown in green. Fig. S7 provides additional information on alternative electron pathways at low oxygen tension or during growth on nitrate as well as on further electron input modules. (B) Schematic representation of *H. denitrificans* methanol oxidation and assimilation pathways. THMPT, tetrahydromethanopterin; THF, tetrahydrofolate; MxaF and XoxF, methanol dehydrogenase; FtfL, ATP-dependent formate-tetrahydrofolate ligase; FolD, combined methylene-tetrahydrofolate dehydrogenase/methenyl-tetrahydrofolate cyclohydrolase. Table S2 provides additional information (EC numbers, locus tags in *H. denitrificans* X^T, published enzyme activity and/or purification). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cycle [57] as briefly depicted in Fig. 6. Formation of serine from methylene-THF and glycine is catalyzed by glycine hydroxymethyltransferase [58]. Serine is then converted to glycerate-2-phosphate and phosphoenolpyruvate (PEP). CO_2 is co-assimilated by carboxylation of PEP to oxaloacetate. In addition to the serine cycle, *H. denitrificans* X^T harbors the genetic equipment for the ethylmalonyl-CoA pathway and a functional glyoxylate shunt [23]. For *H. denitrificans* it is most likely that THF-based C_1 metabolism is run exclusively in the reductive direction and serves purely assimilatory purposes (Fig. 6B). Non-reversible formyl-THF hydrolase (PurU), that is usually present in organisms running an oxidative THF-linked pathway, is not encoded in *H. denitrificans* [23]. In summary, biomass formation from methanol in *H. denitrificans* appears to involve a long THMPT-dependent oxidative route yielding formate that is then hooked up to THF in an ATP-dependent manner and re-reduced up to the level of formaldehyde before delivery into the serine pathway (Fig. 6B).

3.6. Bioenergetic status of thiosulfate-grown H. denitrificans and formation of a sulfur intermediate

We rationalized that the observed negative effect of thiosulfate on biomass production from methanol but not from formate can in principle have two different reasons: First, thiosulfate oxidation may cause an over-reduction of the cellular nicotinamide dinucleotide and cytochrome *c* pools. Such over-reduction would then prevent effective assimilation of methanol into biomass, as methanol must first be oxidized all the way to formate before it can be hooked up to tetrahydrofolate, re-reduced up to the level of formaldehyde and finally delivered into the serine pathway for assimilation. Second, thiosulfate oxidation may lead to production of an intermediate inhibiting methanol but not formate metabolism.

To solve these questions, we first assessed the bioenergetic status of *H. denitrificans* during growth on methanol or formate in the absence versus the presence of thiosulfate by measuring the NAD^+/NADH ratio of the Δ*tsdA* Δ*shdrR* strain. As apparent by the comparison of Fig. 7A and B the NAD^+/NADH ratio drastically rose in methanol-grown cultures in exponential phase from 1.8 ± 1.3 to 4.6 ± 0.5 when cells were actively oxidizing thiosulfate, while it remained unchanged at a ratio of about 4 on formate. As expected, the ratio further increased in all cases when cells reached stationary phase (Figs. 7A, B and S8) and oxidizable substrates were no longer available. These results clearly discounted the idea that thiosulfate may cause an over-reduction of the nicotinamide dinucleotide pool.

In the next step, we assessed the possibility of the formation of potentially inhibitory intermediates during the oxidation of thiosulfate. Indeed, *H. denitrificans* Δ*tsdA* Δ*shdrR* as well as the Δ*tsdA* reference strain excreted up to 0.5 mM sulfite into the medium when exposed to 2 mM thiosulfate, irrespective of the C_1 compound present (Figs. 7C and D, S8). The highly reactive and potentially toxic sulfite is widely used as a food preservative [59]. Sulfite formed in the cytoplasm of *H. denitrificans* via the sHdr pathway is most probably shuttled across the cytoplasmic membrane by a TauE like-sulfite exporter (Fig. 1). An enzyme catalyzing efficient sulfite oxidation is obviously not present in the periplasm of *H. denitrificans*. The sulfite oxidation rates observed in cultures of about 0.1 mM h^{-1} are in the same range as those we determined for 0.5 mM and 1 mM sulfite dissolved in cell-free medium and incubated at the same temperature and shaking frequency (~0.12 mM h^{-1} and ~ 0.18 mM h^{-1}, respectively). While growth on formate is driven completely by cytoplasmic enzymes including formate dehydrogenase, growth on methanol is initiated in the periplasm by methanol dehydrogenase. We rationalize that formate utilization is shielded from effects exerted by sulfite through its efficient export, while periplasmic PQQ-containing methanol dehydrogenase is negatively affected. It should be noted that the formation of PQQ-sulfite adducts is well established [60,61] and

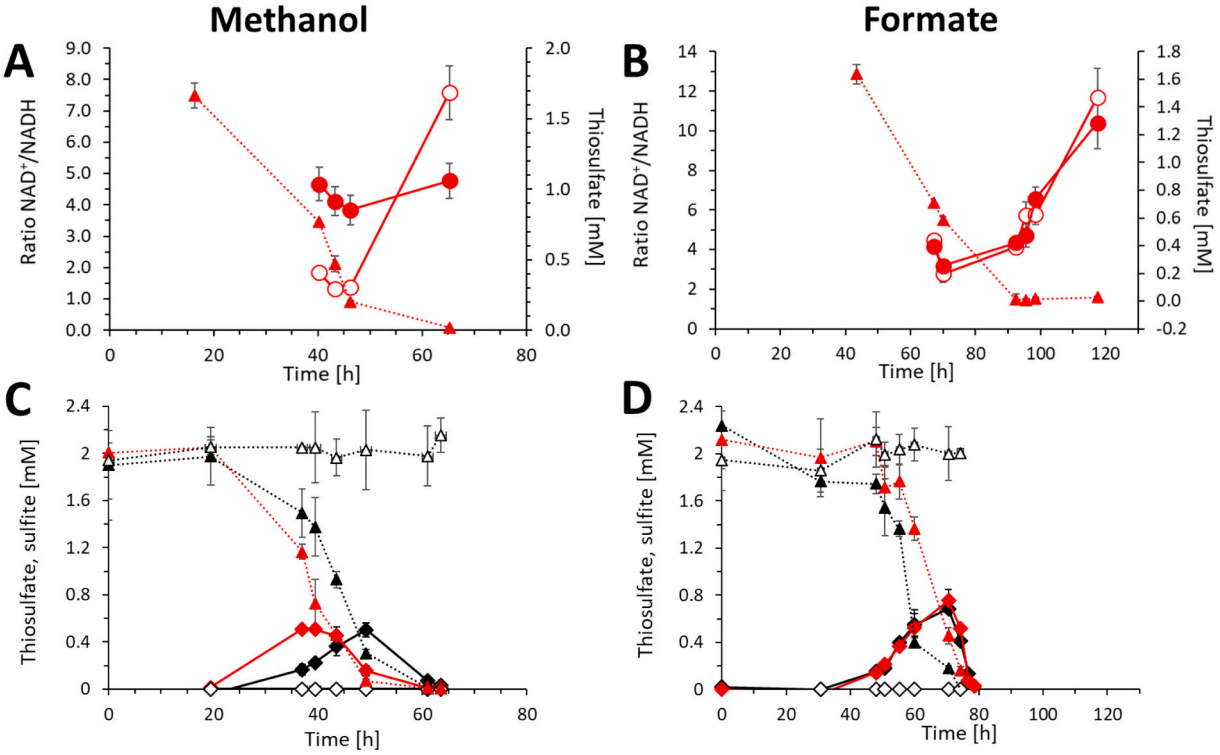

Fig. 7. NAD^+/NADH ratio in *H. denitrificans* Δ*tsdA* Δ*shdrR* on 50 mM methanol (A) and 50 mM formate (B) in the absence (open circles) and presence of 2 mM thiosulfate (filled circles). Thiosulfate is indicated by red filled triangles and was added at a biomass of 0.033 mg dry wt ml^{-1}. Precultures did not contain thiosulfate. In the lower panels, triangles indicate thiosulfate concentrations for *H. denitrificans* Δ*tsdA* (black), for *H. denitrificans* Δ*tsdA* Δ*shdrR* (red) and the thiosulfate-oxidation negative strain (open). Sulfite concentrations are given by diamonds following the same color code. Cultures shown in panels C and D were grown on methanol and formate, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

may be the basis for inhibition of methanol dehydrogenase.

3.7. Occurrence of tsdA, sox and shdr genes in Hyphomicrobium species

The negative influence of thiosulfate on the assimilation of reduced C_1-carbon compounds in a *Hyphomicrobium* strain that has two different thiosulfate oxidation pathways, one of which involves the sHdr complex and results in excretion of inhibitory concentrations of sulfite, led us to the question whether this is a general feature within the genus or an adaptation of a specific strain. We searched all genomes available for strains of the genus *Hyphomicrobium* for the presence of *tsdA* encoding periplasmic tetrathionate-forming thiosulfate dehydrogenase, the genes for the periplasmic Sox system and the genes for the cytoplasmic sHdr proteins (Tables 1 and S3). Even when only strains with complete genomes or assemblies on the scaffold level are considered, it is apparent that the equipment with sulfur oxidation pathways is strain specific (Table 1). While some strains do not contain any gene encoding proteins for thiosulfate oxidation, others contain solely *tsdA*, a combination of *sox* and *shdr* genes or all three of the latter. This observation is fully in line with reports for other methylotrophs of the genus *Methylobacterium/Methylorubrum* (Methylobacteriaceae, order Hyphomicrobiales) [26].

4. Conclusions

Here, we provide an experiment-based explanation for growth retardation of *H. denitrificans* X^T by thiosulfate during growth on methanol and shed first light on the bioenergetics underlying simultaneous use of an inorganic sulfur compound and C_1 compounds as electron donors. A first explanation model hypothesized that the oxidation of the auxiliary inorganic electron donor leads to an over-reduction of electron acceptors, i.e. cytochromes *c* and $NAD(P)^+$ that are thus not available in sufficient concentrations to allow efficient oxidation of methanol. This reduced substrate has to be oxidized to formate before it can serve as a substrate for assimilation into biomass. This idea was experimentally discounted and instead sulfite was detected as an exported intermediate of thiosulfate oxidation. While formate metabolism in the cytoplasm remained unaffected, the strong negative effect on biomass formation from methanol is probably attributed to inhibition of the periplasmic methanol dehydrogenase, which is likely due to formation of a sulfite-adduct of its PQQ cofactor.

Whether thiosulfate can be oxidized at all and, if so, by which pathways appears to be strain-specific among the genus *Hyphomicrobium* as well as among other methylotrophs. At elevated thiosulfate concentrations, TsdA catalyzing tetrathionate formation appears as the enzyme of choice, substantiated by the observation that *H. denitrificans* X^T exclusively forms tetrathionate at thiosulfate concentrations above 2.5 mM [19]. Possessing the sHdr pathway may be advantageous at low thiosulfate concentrations in a range not leading to accumulation of inhibitory amounts of excreted sulfite. This can probably occur in oxygenated environments, where the toxic compound is effectively chemically oxidized, or in habitats, where sulfite is removed rapidly by other members of the community.

Different equipment with thiosulfate oxidation pathways thus probably allows fine-tuned adaptation to environmental conditions. It is well established that in soils, a major habitat for *Hyphomicrobia* [21], methanol is present and its concentrations can be spatially and heterogeneously distributed. In proximity to plant material, hot spots of methanol might exist that are not detectable in mixtures with bulk soil due to a dilution effect [69]. While thiosulfate is typical for certain marine environments, particularly in aerobic/anaerobic interfaces, it

Table 1
Occurence of genes for enzymes involved in thiosulfate and cytoplasmic sulfur oxidation in the genus *Hyphomicrobium*. Only those strains are shown whose genome assembly is either complete or on the scaffold level. Information on genomes available on the level of contigs is provided in Table S2. The gene *soxX* is put in brackets, because it is not always present. In classical heterodimeric SoxAX proteins, SoxX serves as the site of electron storage and transfer, while SoxA harbors the catalytically active site. It is therefore well conceivable that SoxA alsone is active and transfers electrons directly to a separate cytochrome *c* acceptor encoded elsewhere in the genome.

Hyphomicrobium species/strain	*tsdA*	*soxA(X)BYZ*	*shdrC1B1AHC2B2*	Accession	Assembly level	Reference
H. denitrificans X^T SM1869	Hden_2748	Hden_0703-0706	Hden_0689-0694	GCA_000143145.1	Complete	[47,62]
H. denitrificans 1NES1	–	–	–	GCA_000230975.3	Complete	[63]
H. denitrificans SCN18_30_10_14_R2_B_61_9	J0H36_02460	J0H36_02935 06390, 07480-5	J0H36_02865-02890	GCA_017304115.1	Scaffold	[64]
H. denitrificans SCN18_30_10_14_R3_B_61_7	J0H04_09105	–	–	GCA_017307215.1	Scaffold	[64]
H. denitrificans SCN18_30_10_14_R1_P_61_7	–	J0H37_09715-30	J0H37_09135-40[a] J0H37_09200-05	GCA_017305615.1	Scaffold	[64]
H. nitrativorans NL23^T (ATCC BAA-2476)	–	–	–	GCA_000503895.1	Complete	[65,66]
H. sulfonivorans WDL6	–	–	–	GCA_001541235.1	Scaffold	Albers, P., unpublished
H. zavarzinii ATCC 27496^T	-	F812_RS23075, 0107895, 930-940	–	GCA_000383415.1	Scaffold	[21]
H. sp. AWTP1-2	EKK30_01635	–	–	GCA_003987855.1	Scaffold	[67]
H. sp. AWTP1-10	EKK38_19120			GCA_003987725.1	Scaffold	[67]
H. sp. MC1	HYPMC_2182	–	–	GCA_000253295.1	Complete	Ge-scope
H. sp. ghe19	HYPP_04503	–	–	LR743509	Complete	Cremers, G. unpublished
H. sp. 99	G359_RS04385	–	–	GCA_000384335.2	Scaffold	Chistoserdova, L. et al, unpublished
H. sp. 12-62-95	–	–	–	GCA_002279935.1	Scaffold	Kantor, R.S. et al., unpublished
H. sp. 32-62-53	–	–	–	GCA_002280885.1	Scaffold	Kantor, R.S. et al., unpublished
H. sp. SCN 65-11	–	–	ABS54_03385-03410	GCA_001724295.1	Scaffold	[68]
H. sp. SCN18_10_11_15_R1_B_65_8	–	J0J14_11055-70[a] J0J14_14585[a]	J0J14_15020-040[a]	GCA_017306765.1	Scaffold	[64]
H. sp. SCN18_10_11_15_R2_B_65_9	–	J0I57_14080-90[a]	J0I57_20835-50[a]	GCA_017306735.1	Scaffold	[64]
H. sp. SCN18_30_10_14_R3_B_64_9	–	J0I75_12920-30[a]	J0I75_04305-030	GCA_017306765.1	Scaffold	[64]
H. sp. SCN18_26_2_15_R2_B_61_8	J0I81_01825	–	–	GCA_017306805.1	Scaffold	[64]

[a] Partly present.

has also been reported to be present in most soils except in very humid regions [70]. Low-molecular-weight organic acids such as formate, are also ubiquitous in soils, as they are important root exudates [71] and are intermediates and by-products of anaerobic carbon metabolism [72]. For a facultative denitrifyer like *H. denitrificans* X^T, formate as a carbon source may be particularly important in the absence of oxygen and simultaneous oxidation of thiosulfate under anaerobic conditions may provide significant growth advantages. Clearly, future research has to address this issue more comprehensively and in more detail.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbabio.2022.148932.

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Supplementary Information

A metabolic puzzle: Consumption of C_1 compounds and thiosulfate in *Hyphomicrobium denitrificans* X^T

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This document contains supplementary information including additional detail concerning bacterial strains, primers and plasmids (Supplementary Table S1), methods used in growth experiments (Supplementary Figures S1 and S2), growth experiments (Supplementary Figures S3-6, S8), electron input and output modules in *H. denitrificans* (Fig. S7), enzyme commission numbers, locus tags and references for enzymes of electron transport, respiration and C_1 metabolism in *H. denitrificans* (Table S2) and occurrence of the *tsdA* gene and *sox* and *shdr* gene clusters in *Hyphomicrobium* species/strains (Table S3).

Supplementary Figures

Fig. S1. Graph illustrating determination of the factor for OD_{600} to OD_{430} conversion for *H. denitrificans.* Samples with turbidities exceeding the linear range (here optical densities above 0.3) were diluted with culture medium.

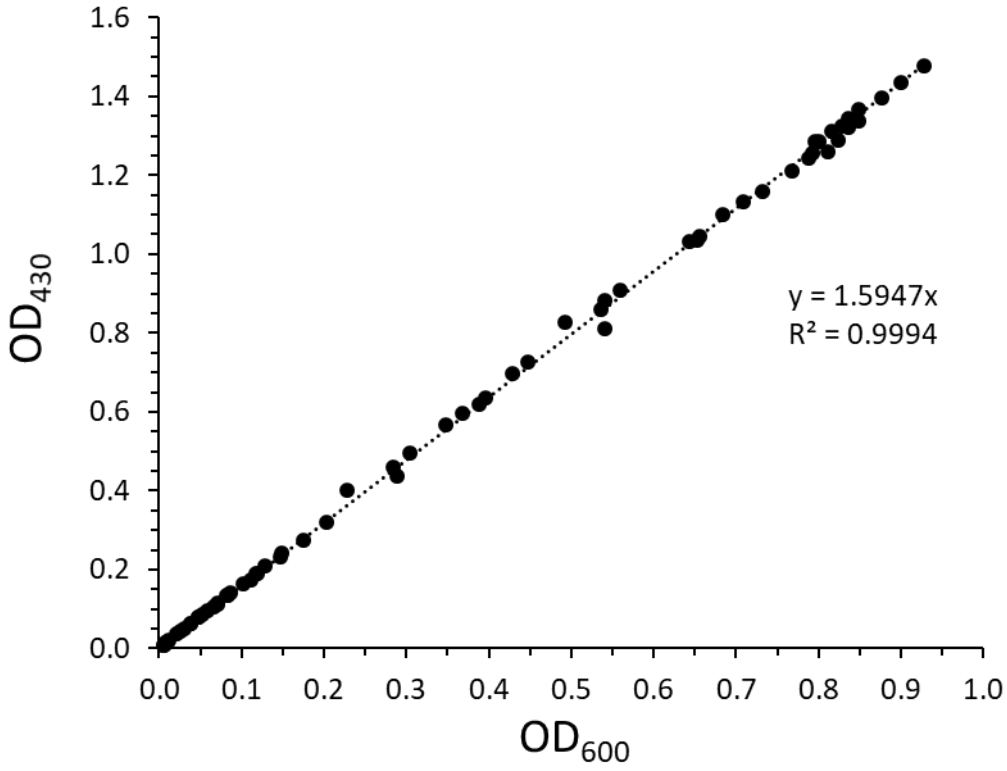


Fig. S2. Graph illustrating determination of the factors for OD_{430} to protein conversion for *H. denitrificans* growing on methanol (A) and formate (B).

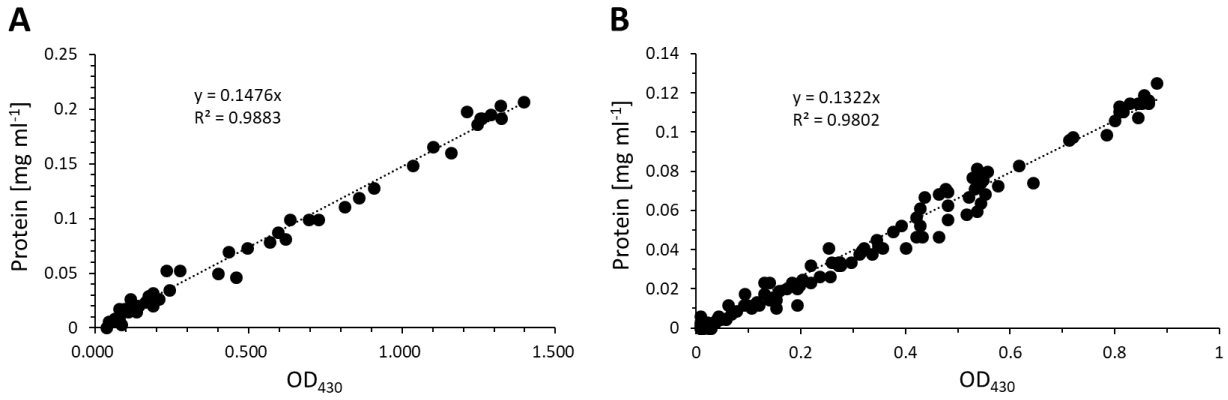


Fig. S3. Growth and thiosulfate oxidation by *Hyphomicrobium denitrificans ΔtsdA* strains lacking either *soxXA* (**A**) or *soxYZ* (**B**). The experiments were performed in 48 well plates in a plate reader with continuous shaking. Solid lines show optical density measurements at 600 nm for cultures on 24.4 mM methanol and 2 mM thiosulfate inoculated with precultures not containing thiosulfate (black, not induced) or containing 2 mM thiosulfate (red, induced). Black triangles represent thiosulfate concentrations for the non-induced cultures. Thiosulfate also stayed constant in the other cases but concentrations are omitted for clarity. Error bars indicate standard deviation for three technical replicates.

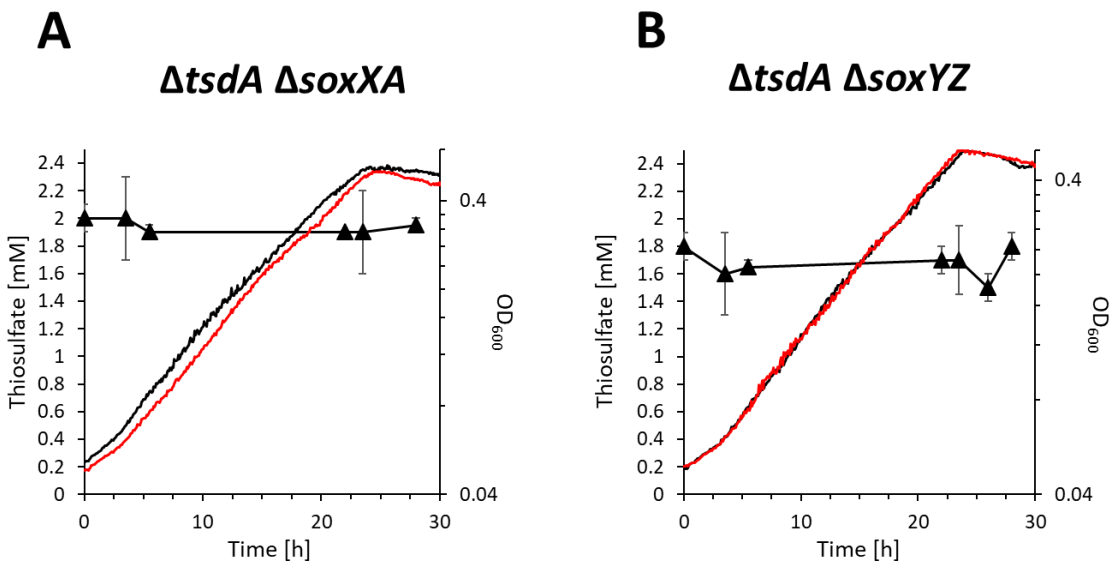


Fig. S4. Growth and thiosulfate consumption of *H. denitrificans ΔtsdA* (upper panels, circles) and *ΔtsdA ΔshdrR* (lower panels, boxes). Cultures were grown on methanol-containing medium without (open symbols) or with increasing thiosulfate concentrations (filled symbols: ○, 0.5 mM; ○, 1 mM; ○, 2 mM; ● 3 mM thiosulfate). Precultures contained either no thiosulfate (not induced, broken lines) or 2 mM thiosulfate (induced, solid lines). Specific thiosulfate oxidation (TS) rates are depicted as insets and follow the same gray shading code as stated earlier. Error bars indicating SD are too small to be visible.

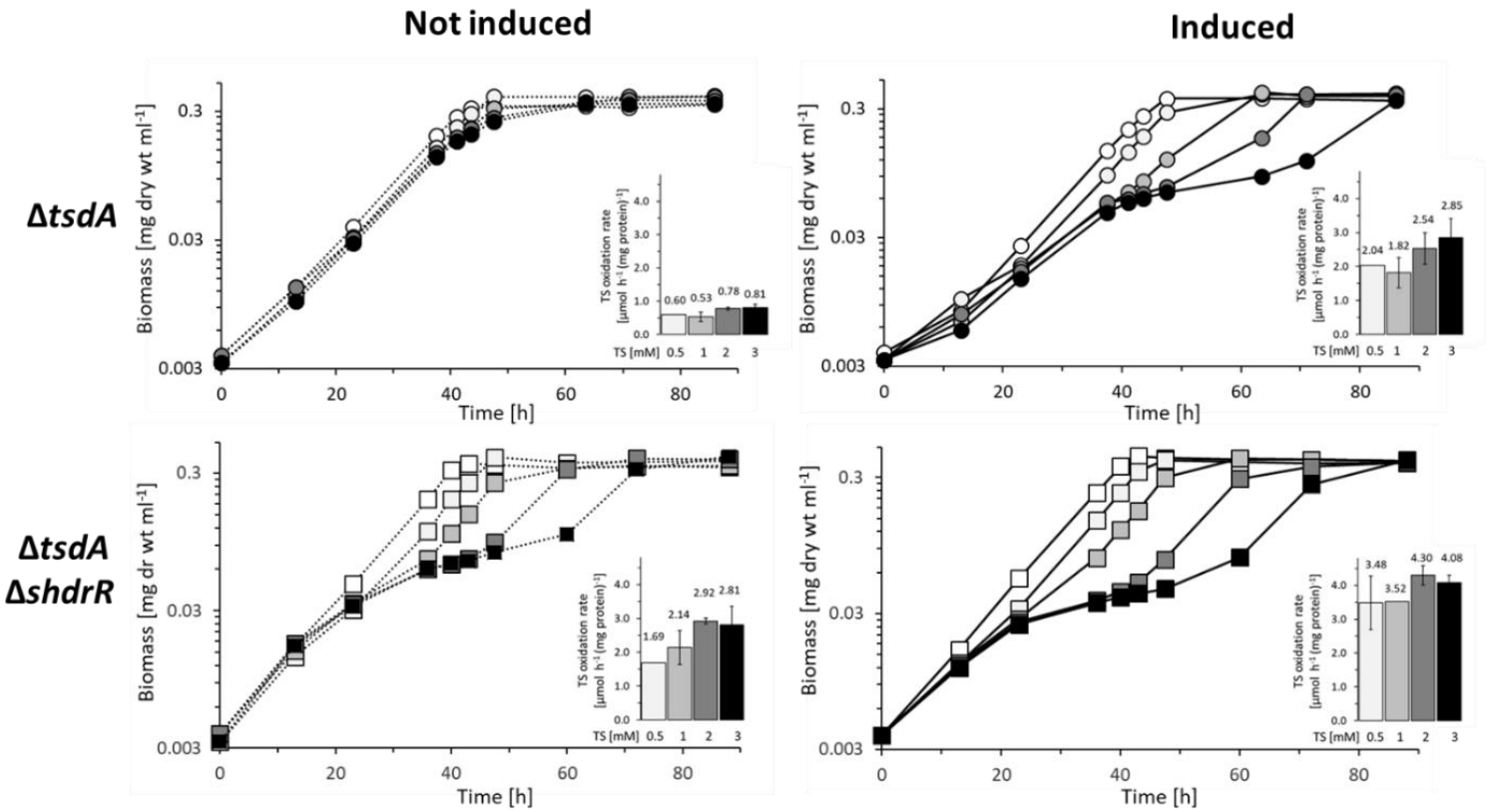


Fig. S5. Growth and thiosulfate consumption of *H. denitrificans* Δ*tsdA* Δ*shdrR* on 25 mM methanol (left panels) and 50 mM methanol (right panels). Cultures were grown without (filled boxes) or with 2 mM thiosulfate (open boxes). Thiosulfate was added to growing cultures as indicated by red arrows. Pre-cultures did not contain thiosulfate. In the lower panels, triangles indicate thiosulfate concentrations. Error bars indicating SD are too small to be visible for determination of biomass.

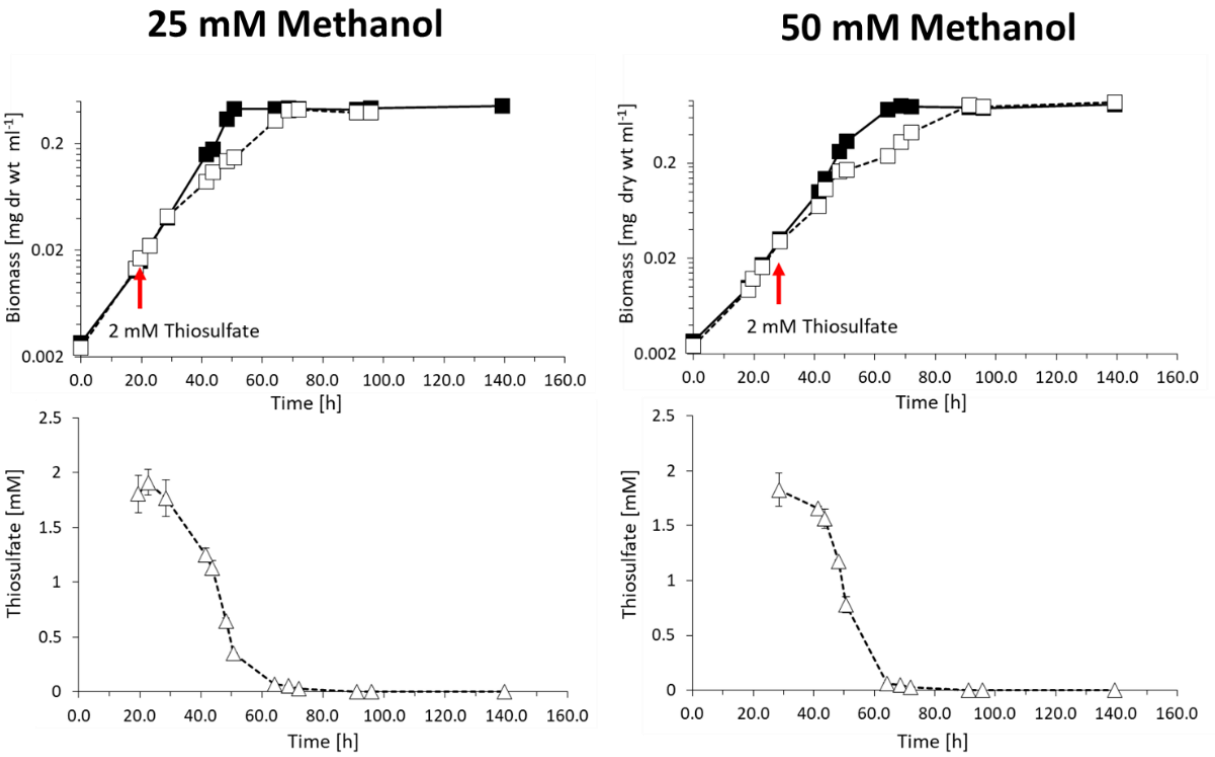


Fig. S6. Growth and thiosulfate consumption of *H. denitrificans ΔtsdA* (left panels, black boxes) and *ΔtsdA ΔshdrR* (right panels, red boxes). Cultures were grown on medium containing 25 mM methanol without (open symbols) or with 2 mM thiosulfate (filled black or red boxes) Pre-cultures did not contain thiosulfate. In the lower panels, triangles indicate thiosulfate concentrations for *H. denitrificans ΔtsdA* (black) and for *H. denitrificans ΔtsdA ΔshdrR* (red). Cultures were grown at 30°C and 200 rpm in 500 ml Erlenmeyer flasks containing either 100 ml (broken lines) or 200 ml (solid lines) medium. Specific thiosulfate oxidation rates are depicted as insets and follow the same code. Error bars indicating SD are too small to be visible for determination of biomass.

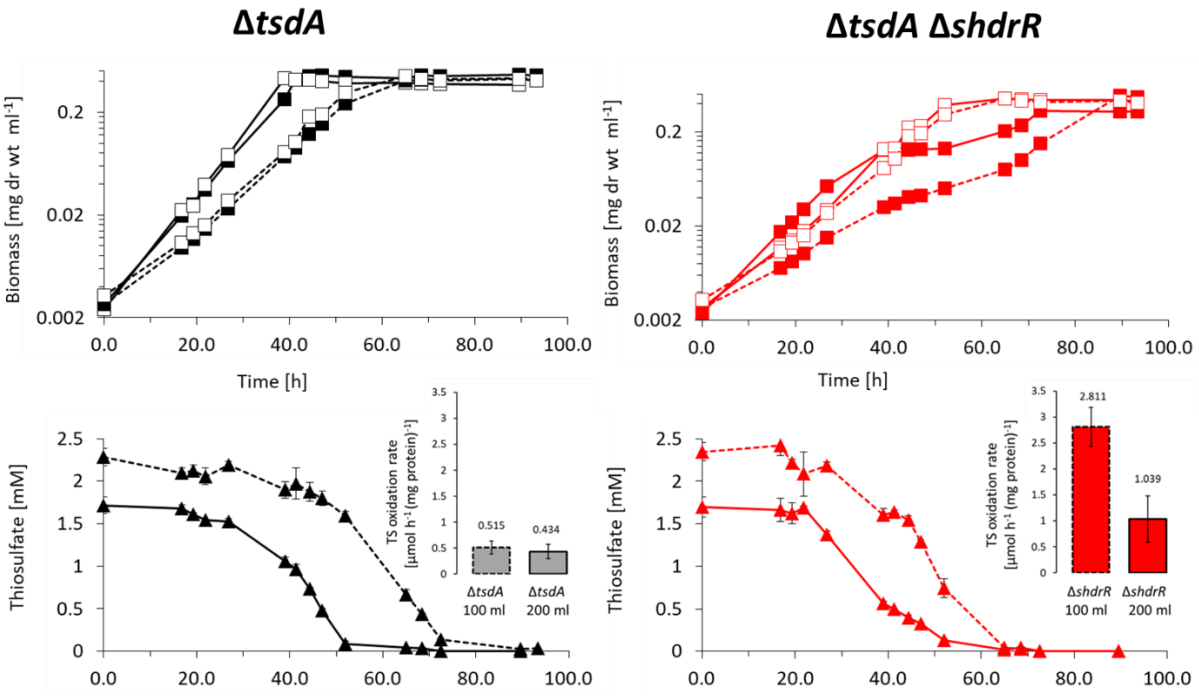


Fig. S7. Overview of electron input and output modules in *Hyphomicrobium denitrificans* ATCC 51888. Proteins that were not detected in a proteomic study during growth on dimethyl sulfide (DMS) at high oxygen concentration [1] are printed in light grey. DMS is transformed to methanethiol in a monoxygenase-catalyzed reaction and sulfide is released from methanethiol by a periplasmic oxidase. In both cases the electrons released from the organosulfur substrate are transferred directly onto oxygen and formaldehyde is formed as a product [1-3] which is then oxidized to formate along a tetrahydromethanopterin (THMPT)-based pathway yielding NADH as indicated in the figure. Methylamine is metabolized via *N*-methyl-glutamate (NMG) using an *N*-methyl-glutamate dehydrogenase [4]. Di- and trimethylamine dehydrogenases are located in the cytoplasm [5]. TMA/DMA, trimethyl-/dimethylamine; DH, dehydrogenase; MxaF and XoxF, methanol dehydrogenase. Locus tags are given for enzymes/pathways not listed in Table S2.

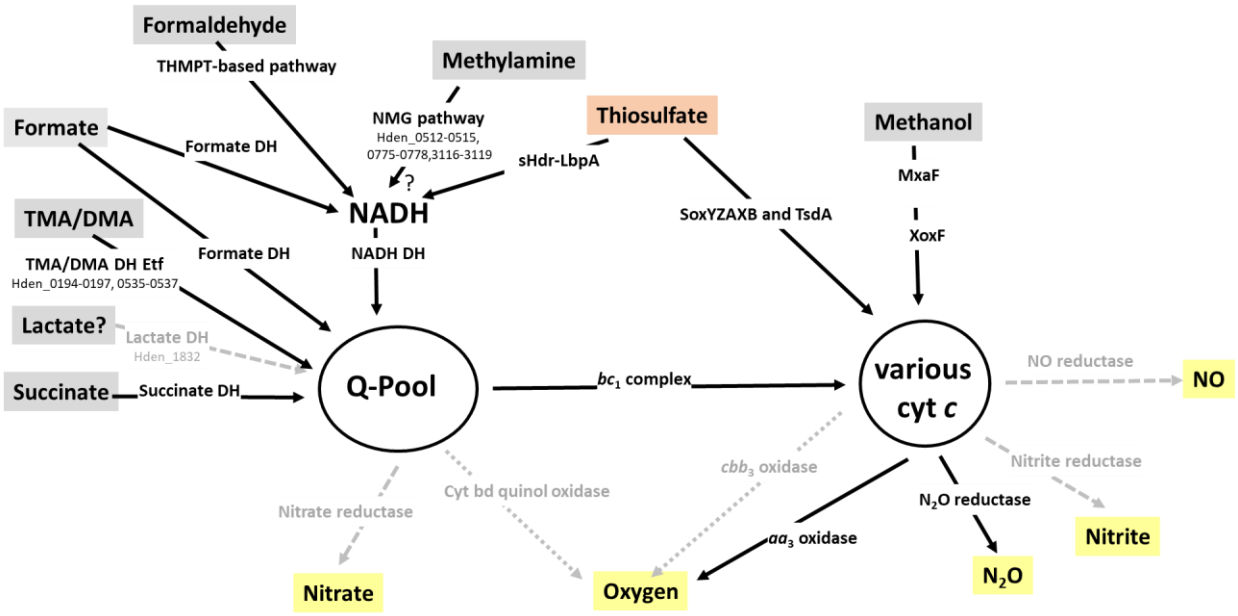
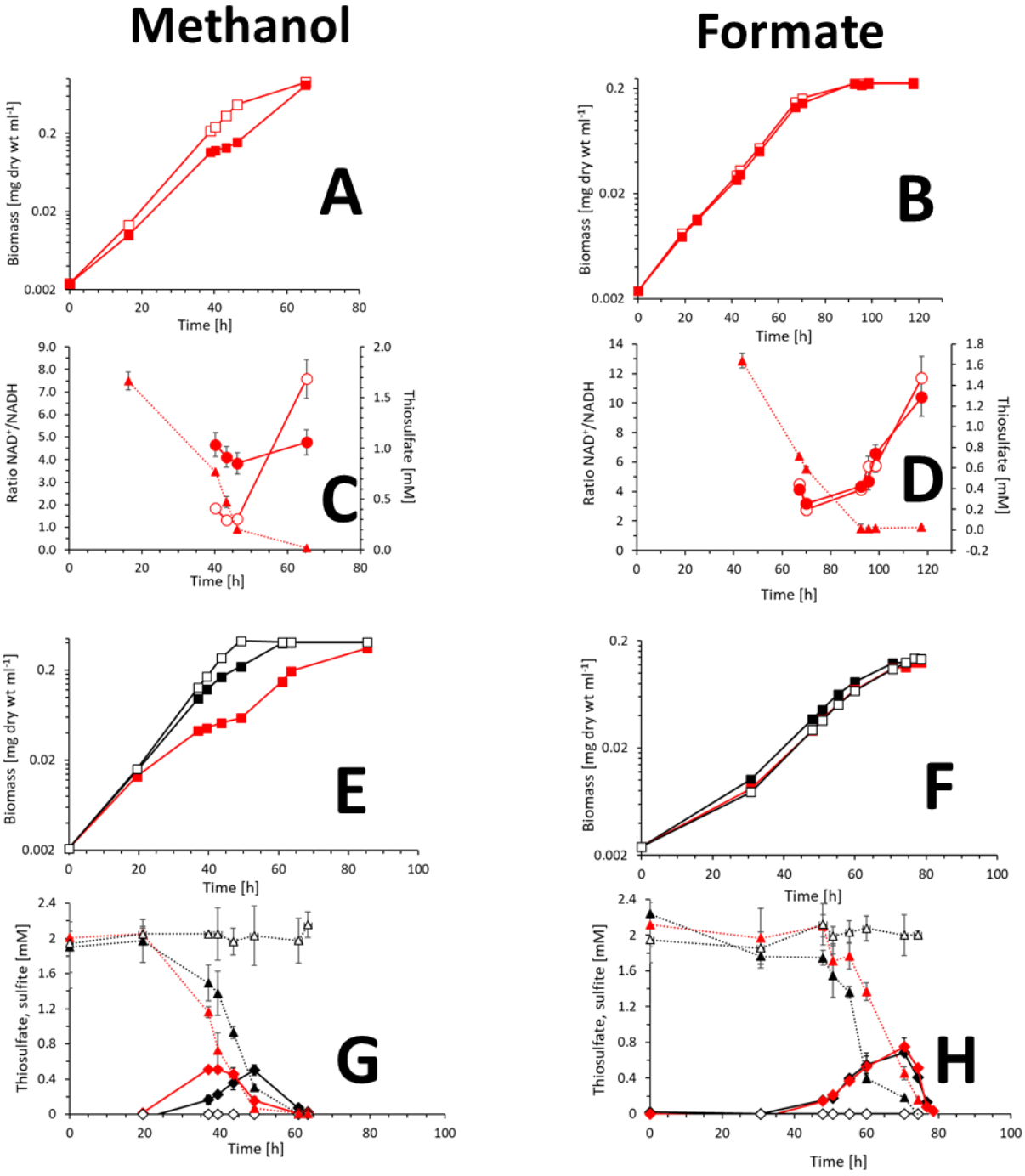


Fig. S8. Growth of *H. denitrificans* on 50 mM methanol (A) and 50 mM formate (B) in the absence (open boxes) and presence of thiosulfate (filled boxes). NAD^+/NADH ratios in *H. denitrificans ΔtsdA ΔshdrR* on methanol (C) and formate (D) are given for cells grown in the absence (open circles) or in the presence of thiosulfate (filled circles). Thiosulfate is indicated by red filled triangles and was added at a biomass of 0.033 mg dry wt ml^{-1}. Panels E and F show growth for *H. denitrificans ΔtsdA* (black), for *H. denitrificans ΔtsdA ΔshdrR* (red) and a thiosulfate-oxidation negative strain (open) in the presence of 2 mM thiosulfate on 25 mM methanol or 25 mM formate, respectively. Consumption of thiosulfate and formation of sulfite are shown in panels G and H. Thiosulfate is given as triangles, sulfite concentrations are shown by diamonds following the same color code as in panels E and F.



Supplementary Tables

Table S1. Strains, plasmids and primers

Strains primers or plasmids	Relevant genotype, description or sequence	Reference or source
Strains		
E. coli NEB 10β	*Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL* (Str^r) *rph spoT1 Δ(mrr-hsdRMS-mcrBC)*	New England Biolabs
E. coli BL21 (DE3)	F$^-$ *ompT hsdS*$_B$(r_B m_{B^-}) *gal dcm met*(DE3)	Novagen
H. denitrificans Δ*tsdA*	Sm^R, in-frame deletion of *tsdA* (Hden_2748) in *H. denitrificans* Sm200	[1]
H. denitrificans Δ*tsdA* Δ*shdrR*	In-frame deletion of *shdrR* (Hden_0682) in *H. denitrificans* Δ*tsdA*	This work
H. denitrificans Δ*tsdA* Δ*soxXA*	In-frame deletion of *soxX* (Hden_0702) and *soxA* (Hden_0703) in *H. denitrificans* Δ*tsdA*	This work
H. denitrificans Δ*tsdA* Δ*soxYZ*	In-frame deletion of *soxY* (Hden_0704) and *soxZ* (Hden_0705) in *H. denitrificans* Δ*tsdA*	This work
Primers		This work
Hden_0703_MaDel_upFW	ACG**TCTAGA**ATTGAAGGACGCGGTGAACTTATTG (XbaI)	This work
Hden_0703_MaDel_upRV	GCGTAGAACGGTCTTAGCGCATGGGTCACCAAATTCTGC	This work
Hden_0703_MaDel_dwnFW	GCAGAATTTGGTGACCCATGCGCTAAGACCGTTCTACGC	This work
Hden_0703_MaDel_dwnRV	GTAT**TCTAGA**CTAACGACGACGAAGGTGGGCGTG (XbaI)	This work
Hden_0704_MaDel_upFW	AGG**TCTAGA**GATGGGACATTGATCTCTCC (XbaI)	This work
Hden_0704_MaDel_upRV	GACGACGCCGAACCCGTCATTCCATCACGCCATCTCTC	This work
Hden_0704_MaDel_dwnFW	GAGAGATGGCGTGATGGAATGACGGGTTCGGCGTCGTC	This work
Hden_0704_MaDel_dwnRV	CAAC**TCTAGA**CTTGTATGGCGCACGCGAC (XbaI)	This work
Fwd_deltaHden0682_BamHI	GCAT**GGATC**CGCGAAAATGTGCACCGGAG (BamHI)	This work
Up_Rev_deltaHden0682	GCTGAAGACTTCGCTCTAATTAGCCATAGGAGTTGCATCCA	This work
Down_Fwd_deltaHden0682	TGGATGCAACTCCTATGGCTAATTAGAGCGAGTCTTCAGC	This work
Rev_deltaHden0682_XbaI	AAGC**TCTAGA**TATGCGGCAGCCGTTGACGC (XbaI)	This work
Fr-pet22b-Hden0682-NdeI	GGCA**CATATG**GCTGTCGTGAAGCCACG (NdeI)	This work
Rev-pet22b-Hden0682-NotI	TTTT**GCGGCCGC**ATTCGAGCGTTTTCCCGCAC (NotI)	This work
EMSA-Fr	TTCCCGCCCCGTCTTGGTTT	This work
EMSA-Rev	AGGAGTTGCATCCAAAAAAGCGTG	This work
Plasmids		
pET-22b (+)	Ap^R, T7 promoter, lac operator, C-terminal His tag, pelB leader	Novagen
pHP45Ω-Tc	Ap^R, Tc^R	[6]
pET-22bHdsHdrR	Ap^R, NdeI-NotI fragment of PCR-amplified *shdrR* (Hden_0682) in NdeI-NotI of pET-22b (+)	This work
pk18*mobsacB*	Km^R, Mob$^+$, *sacB*, *oriV*, *oriT*, *lacZα*	[7]
pk18*mobsacB*-Tc	pHP45ΩTc tetracycline cassette inserted into pk18*mobsacB* using SmaI	This work
pk18*mobsacB*ΔsoxYZ-Tc	Km^R, Tc^R, 2 kb XbaI fragment of PCR-amplified genome region around *soxYZ* with deletion of *soxYZ* cloned into XbaI of pk18*mobsacB*-Tc	This work
pk18*mobsacB*ΔsoxXA-Tc	Km^R, Tc^R, 1.82 kb XbaI fragment of PCR-amplified genome region around *soxXA* with deletion of *soxXA* cloned into XbaI of pk18*mobsacB*-Tc	This work
pk18*mobsacB*ΔshdrR	Km^R, 2.2 kb BamHI/XbaI fragment of PCR-amplified genome region around *shdrR* with deletion of *shdrR* cloned into BamHI/XbaI of pk18*mobsacB*	This work
pk18*mobsacB*ΔshdrR-Tc	Km^R, Tc^R, pHP45ΩTc tetracycline cassette inserted into pk18*mobsacB*ΔshdrR using SmaI	This work

Table S2. Respiratory enzyme complexes and enzymes of C_1 metabolism in *H. denitrificans* X^T

Enzyme	EC number	Subunits	Locus tags in *H. denitrificans* X^T	Reference enzyme activity/presence
Complex I: NADH:ubiquinone oxidoreductase	7.1.1.2	NuoABCDEFGHIJKLMN	Hden_1929-1946	
Complex II: succinate dehydrogenase	1.3.5.1	SdhABCD	Hden_3236, Hden_3238-3240	[8]
Complex III: ubiquinol: cytochrome *c* reductase	7.1.1.8	Cyt 1 CytB ISP	Hden_2526-2528	
Cytochrome c oxidase: cytochrome aa_3-type	7.1.1.9	CoxABC	Hden_2903, 2907, 2908	[9, 10]
Cytochrome c oxidase: cytochrome cbb_3-type	7.1.1.9	CcoNOQP	Hden_2047-2050	
Cytochrome *bd* ubiquinol oxidase	7.1.1.7	CydAB	Hden_3144-3145	
Nitrate reductase	1.7.5.1	NarGHI	Hden_0926, 0927, 0929	[11]
Nitrite reductase	1.7.2.1	NirK	Hden_0591	[12-14]
Nitric oxide (NO) reductase	1.7.2.5	cNorCBQD	Hden_0581-0584	[14]
Nitrous oxide (N_2O) reductase	1.7.2.4	NosZ	Hden_1882	[15]
Methanol dehydrogenase PQQ-dependent	1.1.2.7	MxaFIG	Hden_1320, 1321, 1323	[16, 17]
Methanol dehydrogenase, lanthanide-dependent	1.1.2.10	XoxF	Hden_1305 Hden_1869 Hden_1617 Hden_2848	
Formaldehyde-activating enzyme, NAD-linked glutathione-independent, 5.6.7.8-THMPT hydrolase	4.2.1.147	Fae	Hden_1474 Hden_1875 Hden_2126	[18]
Methylene-THMPT dehydrogenase	1.5.1.-	MtdB	Hden_1479	[19]
Methenyl-THMPT cyclohydrolase	3.5.4.27	Mch	Hden_1477	
Formylmethanofuran-THMPT N-formyltransferase, formyltransferase/hydrolase complex	2.3.1.101	FhcABCD	Hden_1608-1611	
Formate dehydrogenase	1.17.1.9	FdoGHI FdhD FdwAB	Hden_2464-2486 Hden_0607, 0527	[20]
Formate-tetrahydrofolate ligase	6.3.4.3	FtfL	Hden_0104 Hden_3136	[21]
Bifunctional methylene tetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase	1.5.1.5/ 3.5.49	FolD	Hden_3211 Hden_0103	[19, 22]
Glycine hydroxymethyltransferase	2.1.2.1	GlyA	Hden_0960	[23]

Table S3. Occurence of genes for enzymes involved in thiosulfate and cytoplasmic sulfur oxidation in the genus *Hyphomicrobium*. The gene *soxX* is put in brackets, because it is not always present. In classical heterodimeric SoxAX proteins, SoxX serves as the site of electron storage and transfer, while SoxA harbours the catalytically active site. It is therefore well conceivable that SoxA alone is active and transfers electrons directly to a separate cytochrome *c* acceptor encoded elsewhere in the genome.

***Hyphomicrobium* species**	***tsdA***	***soxA(X)BYZ***	***shdrC1B1AHC2B2***	**Genome assembly accession**	**Assembly level**	**Reference**
Hyphomicrobium album XQ2^T (KCTC 82378)	-	-	-	GCA_009708035.1	Contig	[24]
Hyphomicrobium denitrificans X^T (DSM1869)	Hden_2748	Hden_0703-0706	Hden_0689-0694	GCA_000143145.1	Complete	[14, 25]
Hyphomicrobium denitrificans 1NES1	-	-	-	GCA_000230975.3	Complete	[26]
Hyphomicobium denitrificans SCN18_30_10_14_R2_B_61_9	J0H36_02460	J0H36_02935 06390, 07480-5	J0H36_02865-02890	GCA_017304115.1	Scaffold	[27]
Hyphomicobium denitrificans SCN18_30_10_14_R3_B_61_7	J0H04_09105	-	-	GCA_017307215.1	Scaffold	[27]
Hyphomicobium denitrificans SCN18_30_10_14_R1_P_61_7	-	J0H37_09715-30	J0H37_09135-40* J0H37_09200-05	GCA_017305615.1	Scaffold	[27]
Hyphomicrobium facile subsp. *facile* DSM 1565^T	SAMN04488557_3656	-	-	GCA_900116175	Contig	[28, 29]
Hyphomicrobium methylovorum Bras1	DLM45_14295	DLM45_12165-85 DLM45_14665- 85	-	GCA_013626205.1	Contig	[30]
Hyphomicrobium nitrativorans NL23^T (ATCC BAA-2476)	-	-	-	GCA_000503895.1	Complete	[31, 32]
Hyphomicrobium sulfonivorans S1^T (DSM 13863)	-	-	-	GCA_013306565.1 GCA_016125985.1	Contig Contig	[33]
Hyphomicrobium sulfonivorans WDL6	-	-	-	GCA_001541235.1	Scaffold	Albers, P., unpublished
Hyphomicrobium zavarzinii ATCC 27496^T	-	F812_RS23075, 0107895, 930-940	-	GCA_000383415.1	Scaffold	[28]
Hyphomicrobium zavarzinii new MAG-140	-	JNN24_16950, 980-90	-	GCA_016793385.1	Contig	[34]
Hyphomicrobium sp. AWTP1-2	EKK30_01635	-	-	GCA_003987855.1	Scaffold	[35]
Hyphomicrobium sp. AWTP1-10	EKK38_19120	-	-	GCA_003987725.1	Scaffold	[35]
Hyphomicrobium sp. GJ21	HYPGJ_31387	HYPGJ_30398-402	HYPGJ_30410-15	GCA_001006785.1	Contig	[36]
Hyphomicrobium sp. MAG_27	-	-	-	GCA_019912585.1	Contig	[37]
Hyphomicrobium sp. MC1	HYPMC_2182	-	-	GCA_000253295.1	Complete	Ge-scope
Hyphomicrobium sp. 802	-	-	-	GCA_000526135.1	Contig	Chistoserdova, L. et al., unpublished

Hyphomicrobium sp. ghe19	HYPP_04503	-	-	LR743509	Complete	Cremers, G. unpublished
Hyphomicrobium sp. CS1BSMeth3	-	-	CS1BSM3_RS27400-45	GCA_900117415.1	Contig	Adelskov, J., Patel, K.C.B., unpublished
Hyphomicrobium sp. NBD2Meth4	-			GCA_900117445.1	Contig	Adelskov, J., Patel, K.C.B., unpublished
Hyphomicrobium sp. 99	G359_RS04385	-	-	GCA_000384335.2	Scaffold	Adelskov, J., Patel, K.C.B., unpublished
Hyphomicrobium sp. New MAG-139	-	-	-	GCA_016793445.1	Contig	[34]
Hyphomicrobium sp. RGIG5714	-	-	-	GCA_017512425.1	Contig	[38]
Hyphomicrobium sp. SB8	-	-	-	GCA_009026145.1	Contig	unpublished
Hyphomicrobium sp. Hjor_18-Q3-R7-51_BAT3C.262	-	-	-	GCA_016716675.1	Contig	[39]
Hyphomicrobium sp. DS3.3.23	-	-	-	GCA_002928735.1	Contig	[40]
Hyphomicrobium sp. FW.3.32	-	CTY20_06750- 65 CTY20_09450-65	-	GCA_002928955.1	Contig	[40]
Hyphomicrobium sp. PB1.3.35	-	-	-	GCA_002928515.1	Contig	[40]
Hyphomicrobium sp. WM.3.63	-	-	-	GCA_002928465.1	Contig	[40]
Hyphomicrobium sp. WM.3.50	-	C0511_04010-25 C0511_16275-85	-	GCA_013821915.1	Contig	[40]
Hyphomicrobium sp. WM.3.5	-	CTY40_030865-80 CTY40_10070-80	-	GCA_002928395.1	Contig	[40]
Hyphomicrobium sp. 12-62-95	-	-	-	GCA_002279935.1	Scaffold	Kantor, R.S. et al, unpublished
Hyphomicrobium sp. 32-62-53	-	-	-	GCA_002280885.1	Scaffold	Kantor, R.S. et al, unpublished
Hyphomicrobium sp. Time.spades.CONCOCT.2.5kb_061	-	-	-	GCA_019744875.1	Contig	[41]
Hyphomicrobium sp. co.spades.DASTOOL.2.5kb_064	-	-	-	GCA_019748215.1	Contig	[26]
Hyphomicrobium sp. co.spades.CONCOCT.2.5kb_134	-	-	-	GCA_019751875.1	Contig	[41]
Hyphomicrobium sp. co.spades.CONCOCT.1kb_066	K2Q04_10990	-	-	GCA_019753045.1	Contig	[41]
Hyphomicrobium sp. SJ665	-	-	-	GCA_020852195.1	Contig	[42]
Hyphomicrobium sp. SCN 65-11	-	-	ABS54_03385- 03410	GCA_001724295.1	Scaffold	[43]
Hyphomicrobium sp. P-RSF-NP-07	-	-	-	GCA_014379315.1	Contig	[44]
Hyphomicrobium sp. SCN18_10_11_15_R1_B_65_8	-	J0J14_11055-70* J0J14_14585*	J0J14_15020-040*	GCA_017306765.1	Scaffold	[27]
Hyphomicrobium sp. SCN18_10_11_15_R2_B_65_9	-	J0I57_14080-90*	J0I57_20835-50*	GCA_017306735.1	Scaffold	[27]
Hyphomicrobium sp. SCN18_30_10_14_R3_B_64_9	-	J0I75_12920-30*	J0I75_04305--030	GCA_017306765.1	Scaffold	[27]
Hyphomicrobium sp. SCN18_26_2_15_R2_B_61_8	J0I81_01825	-	-	GCA_017306805.1	Scaffold	[27]

*Partly present

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In the Alphaproteobacterium *Hyphomicrobium denitrificans* SoxR Serves a Sulfane Sulfur-Responsive Repressor of Sulfur Oxidation

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Abstract: In organisms that use reduced sulfur compounds as alternative or additional electron donors to organic compounds, transcriptional regulation of genes for enzymes involved in sulfur oxidation is needed to adjust metabolic flux to environmental conditions. However, little is known about the sensing and response to inorganic sulfur compounds such as thiosulfate in sulfur-oxidizing bacteria. In the Alphaproteobacterium *Hyphomicrobium denitrificans*, one strategy is the use of the ArsR–SmtB-type transcriptional regulator SoxR. We show that this homodimeric repressor senses sulfane sulfur and that it is crucial for the expression not only of *sox* genes encoding the components of a truncated periplasmic thiosulfate-oxidizing enzyme system but also of several other sets of genes for enzymes of sulfur oxidation. DNA binding and transcriptional regulatory activity of SoxR are controlled by polysulfide-dependent cysteine modification. The repressor uses the formation of a sulfur bridge between two conserved cysteines as a trigger to bind and release DNA and can also form a vicinal disulfide bond to orchestrate a response to oxidizing conditions. The importance of the sulfur bridge forming cysteines was confirmed by site-directed mutagenesis, mass spectrometry, and gel shift assays. In vivo, SoxR interacts directly or indirectly with a second closely related repressor, sHdrR.

Keywords: *Hyphomicrobium denitrificans*; sulfur oxidation; thiosulfate; SoxR; transcriptional regulation; reactive sulfur species; repressor

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1. Introduction

Thiosulfate ($S_2O_3^{2-}$) is a sulfur substrate that is oxidized by the majority of dissimilatory sulfur oxidizers. Its complete oxidation to sulfate is always initiated, and in many cases also completely performed, in the bacterial periplasm and involves the well-studied thiosulfate-oxidizing Sox multienzyme system [1–3] (Figure 1a). Three proteins, SoxYZ, SoxXA, and SoxB, are required for the initial steps. The *c*-type cytochrome SoxXA catalyzes the oxidative formation of a disulfide linkage between the sulfane sulfur of thiosulfate and the persulfurated active site cysteine residue of SoxY [4]. Then, SoxB catalyzes the hydrolytic release of the sulfone group as sulfate, leaving the original sulfane sulfur of thiosulfate bound to SoxY [5,6]. The reaction cycle can be fully completed in the periplasm of organisms containing the hemomolybdo-protein SoxCD, which catalyzes the oxidation of SoxY-bound sulfane sulfur to sulfone, followed again by SoxB-catalyzed hydrolytic release of sulfate [7].

Many sulfur oxidizers do not contain SoxCD and have a so-called "truncated" Sox system (Figure 1b) [2]. For complete oxidation to sulfate, truncated Sox systems can be combined with cytoplasmic sulfur oxidation systems. How the sulfur is transferred into the cytoplasm for further oxidation is still a mystery. The Alphaproteobacterium *Hyphomicrobium denitrificans* X^T (DSM 1869^T) is a representative of this group [8] (Figure 1b).

In this organism, two genes encoding predicted sulfur compound transporters (SoxT1A and SoxT1B) are located in close proximity to the *sox* genes and the genes for the cytoplasmic sulfane sulfur-oxidizing heterodisulfide reductase-like (sHdr) system (Figure 1b). While the *H. denitrificans* Sox and sHdr proteins have been shown experimentally to be essential for thiosulfate oxidation [8–10], evidence for the proposed sulfur transport has not been provided so far.

The obligately heterotrophic *H. denitrificans* oxidizes thiosulfate as an additional electron donor during growth on compounds like methanol [9]. In batch culture, substantial amounts of sulfite are excreted as the product of sHdr-catalyzed sulfur oxidation and accumulate because an enzyme catalyzing efficient sulfite oxidation is not present [9]. Accumulation of sulfite as an intermediate has also been described for some facultatively autotrophic sulfur oxidizing Alphaproteobacteria, e.g., *Rhodovulum* (previously *Rhodobacter*) *sulfidophilum* [16].

(Bi)sulfite (HSO_3^-), SO_3^{2-} is a highly reactive, strong nucleophile and has many toxic effects. Its strong reducing capacity (E_0' for the sulfate/sulfite couple is −515 mV) contributes to its toxicity and antimicrobial action, which have led to its widespread use as a food preservative [17,18]. Free sulfite can damage DNA through the formation of adducts [19–21]. Its toxic effect on mammalian cells has been attributed to the formation of sulfur- and oxygen-based free radicals [22,23] which can in turn react with lipids and proteins [24,25]. The full Sox pathway or the truncated Sox/sHdr combination may be advantageous, despite the intermediate release of sulfite, for organisms such as *H. denitrificans* or *R. sulfidophilum* at low thiosulfate concentrations if removal by other members of the community or chemical oxidation in oxygenated environments keeps sulfite concentrations below inhibitory levels. In any case, the formation of the toxic intermediate sulfite during the oxidation of sulfur compounds, as well as the switching between organic and inorganic electron donors, requires fine-tuning to the environmental conditions.

Accordingly, complex regulatory patterns have been reported for facultative sulfur oxidizers, with upregulation usually occurring only in the presence of metabolizable sulfur substrates, whereas the corresponding genes are thought to always be highly expressed in chemolithoautotrophs restricted to the oxidation of sulfur compounds. In *H. denitrificans* and other Alphaproteobacteria that are not restricted to sulfur oxidation, such as *R. sulfidophilum*, *Paracoccus pantotrophus* or *Pseudaminobacter salicylatoxidans*, the ability to oxidize thiosulfate and, depending on the organism, other reduced inorganic and organic sulfur compounds such as sulfide or dimethyl sulfide, is not constitutive but can be induced by the presence of oxidizable sulfur compounds [9,16,26,27]. While the transcriptional repressor sHdrR is involved in this process in *H. denitrificans* [9], genetic and biochemical studies have identified the related SoxR protein as a major regulator in *P. pantotrophus* and *P. salicylatoxidans* [26–28], both of which contain a complete Sox system and are unable to oxidize sulfane sulfur in the cytoplasm.

SoxR is a member of the arsenic repressor (ArsR–SmtB) family of prokaryotic repressors [29–32]. Members of the ArsR–SmtB family were originally recognized as metal-responsive transcriptional regulators, but there are also members in this family that have been shown to sense reactive oxygen or sulfur species [33]. SqrR from *Rhodobacter capsulatus* and BigR from *Xylella fastidiosa* belong to this group and control the transcription of genes involved in sulfide-dependent photosynthesis and the detoxification of H_2S derived from associated host plants, respectively [34–36]. Knowledge about SoxR is comparatively sparse. While binding regions for the transcriptional repressor have been identified in promoter–operator segments within the *sox* gene clusters of *P. denitrificans* and *P. salicylatoxidans* [26,27], no information is available on factors that control its DNA-binding capacity. It is therefore completely unclear how SoxR senses the presence of oxidizable sulfur compounds and how it then triggers the transcription of sulfur oxidation genes.

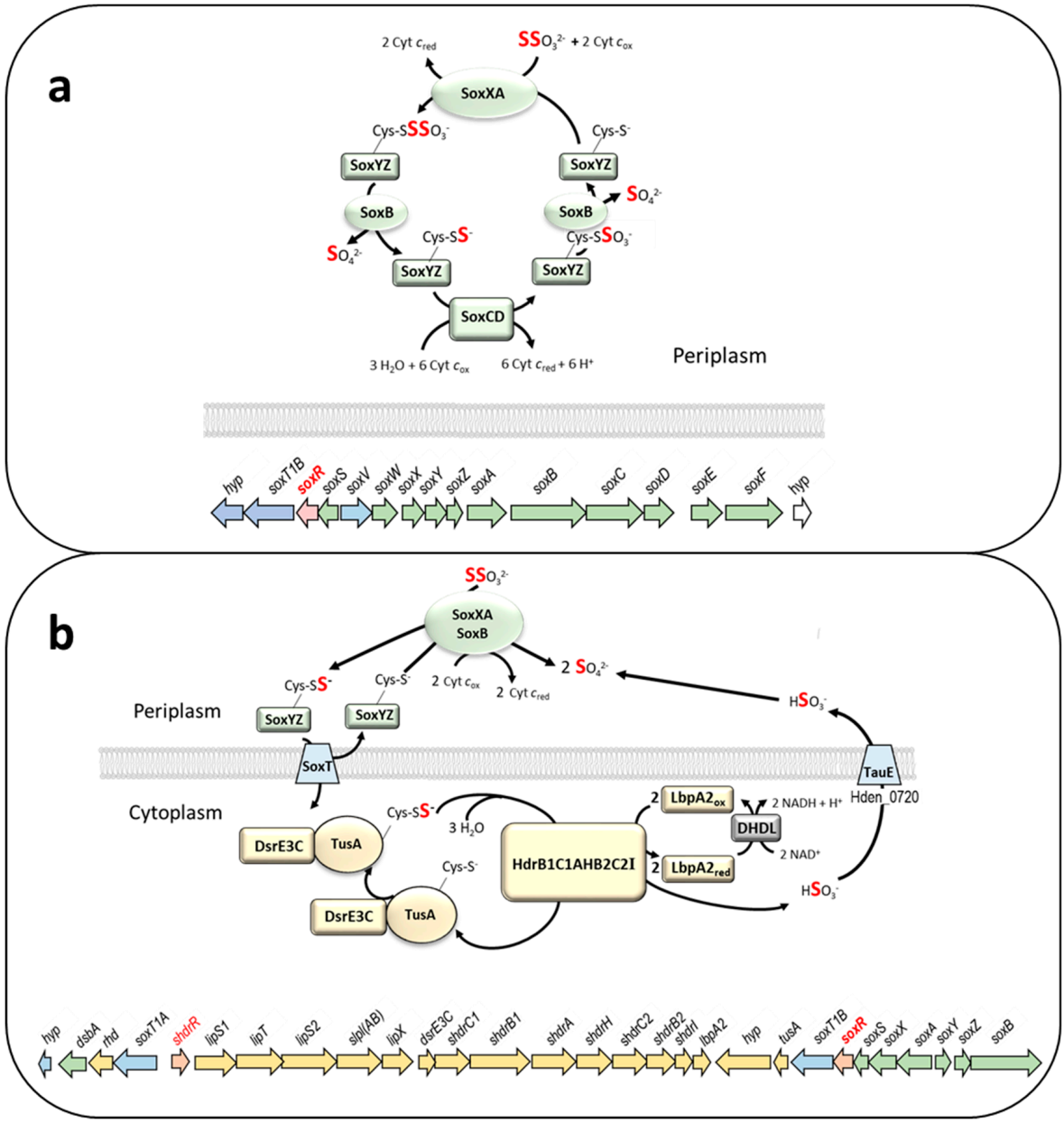

Figure 1. (**a**) Model of the complete periplasmic Sox pathway and exemplary *sox* gene cluster (A6W98_09510 to A6W98_09585) from the Alphaproteobacterium *Rhodovulum sulfidophilum* DSM 1374^T (Rhodobacterales, Rhodobacteraceae) [11,12]. SoxS is neither part of the Sox enzyme system nor involved in its regulation [13]. This periplasmic thiol–disulfide oxidoreductase of the Dsb family prevents SoxYZ inactivation by reducing false mixed disulfides [14,15]. (**b**) Model of thiosulfate oxidation and a genome region for sulfur oxidation (Hden_0678 to Hden_0706) in *Hyphomicrobium denitrificans* DSM 1869^T (Hyphomicrobiales, Hyphomicrobiaceae) [8]. The *lip* genes encode proteins involved in post-translational assembly of lipoate on the lipoate-binding LbpA2 protein. The truncated Sox system in the periplasm consists of SoxXY, SoxB, and SoxYZ. The sulfane sulfur stemming from thiosulfate and bound to SoxY is transferred to the cytoplasm, possibly via one (or both) of the transporters SoxT1A and Soxt1B, and oxidized to sulfite by the sHdr–LbpA2 system. Sulfite is excreted, probably via TauE, and cannot be effectively oxidized. In panels (**a**,**b**), periplasmic, membrane-bound, and cytoplasmic proteins and the encoding genes are shown in green, blue, and yellow, respectively. Regulator genes are highlighted in red. The *hyp* and *rhd* genes encode a predicted cytochrome P450 and a rhodanese-like protein, respectively. To make them easier to follow, the sulfur atoms that come from thiosulfate are highlighted in bold red.

Here, we start to close this knowledge gap by first providing information on the general distribution of complete and truncated Sox systems and their co-occurrence with SoxR. Furthermore, we present genetic information for SoxR function in *H. denitrificans*, identify target genes and map its binding sites. The DNA-binding properties of the homodimeric repressor and its response to bridging of the sulfur atoms of two conserved cysteine residues by one to three sulfur atoms are characterized via site-directed mutagenesis, mass spectrometry, MalPEG assays, and electrophoretic mobility shift assays (EMSA).

2. Materials and Methods

2.1. Bacterial Strains, Plasmids, Primers, and Growth Conditions

Table S1 lists the bacterial strains, primers and plasmids that were used for this study. *Escherichia coli* strains were grown on complex lysogeny broth (LB) medium [37] under aerobic conditions at 37 °C unless otherwise indicated. *Escherichia coli* BL21 (DE3) was used for recombinant protein production. *E. coli* strains 10 beta and DH5α were used for molecular cloning. *H. denitrificans* strains were cultivated in minimal media containing 24.4 mM methanol kept at pH 7.2 with 100 mM 3-(*N*-Morpholino)propanesulfonic acid (MOPS) buffer as described before [8]. Thiosulfate was added as needed. Antibiotics for *E. coli* and *H. denitrificans* were used at the following concentrations (in μg mL^{-1}): ampicillin (Ap), 100; kanamycin (Km), 50; streptomycin (Sm), 200; tetracycline (Tc), 15; and chloramphenicol (Cm), 25.

2.2. Recombinant DNA Techniques

DNA manipulation and cloning were performed using standard techniques, unless otherwise indicated [38]. Restriction enzymes, T4 ligase and Q5 polymerase were purchased from New England Biolabs (Frankfurt, Germany) and used according to the manufacturer's instructions. Oligonucleotides for cloning were obtained from Eurofins MWG (Ebersberg, Germany). The GenJET Plasmid Miniprep kit (Thermo Scientific, Waltham, MA, USA) and the First-DNA all-tissue Kit (GEN-IAL GmbH, Troisdorf, Germany) were used for the purification of plasmid DNA from *E. coli* and chromosomal DNA from *H. denitrificans* strains, respectively.

2.3. Construction of Plasmid for Deletion of soxR in H. denitrificans

For markerless deletion of the *H. denitrificans soxR* (Hden_0700) gene by splicing overlap extension (SOE) [39], PCR fragments were constructed using the primers P1 fwd up hden_0700, P2 rev up hden_0700, P3 fwd down hden 0700 and P4 rev down hden_0700 (Table S1). The resulting 1.04 kb SOE PCR fragment was cloned into the XbaI and PstI sites of pK18*mobsacB*-Tc [9]. The final construct, pK18*mobsacB*_Tc_Δ*soxR*, was electroporated into *H. denitrificans* Δ*tsdA* and transformants were selected using previously published procedures [8,10]. Single crossover recombinants were Cm^r and Tc^r. Double crossover recombinants were Tc^s and survived in the presence of sucrose due to the loss of the vector-encoded tetracyclin resistance and levansucrase (SacB) genes.

2.4. Characterization of Phenotypes, Quantification of Sulfur Compounds and Protein Content

Growth experiments with *H. denitrificans* were run in 200 mL medium with 24.4 mM methanol and varying concentrations of thiosulfate in 500 mL Erlenmeyer flasks, as described in [9]. Thiosulfate concentrations, protein content, and specific thiosulfate oxidation rates were determined by previously described methods [9,40]. All growth experiments were repeated three times. Representative experiments with two biological replicates for each strain are shown. All quantifications are based on at least three technical replicates.

2.5. RNA Preparation

Total RNA of *H. denitrificans* was isolated from cells harvested in mid-log phase. *H. denitrificans* strains Δ*tsdA* and Δ*tsdA* ΔsoxR were grown in 50 mL methanol-containing medium at 30 °C with shaking at 250 rpm in 100 mL Erlenmeyer flasks. Cells from 2 mL

were harvested by centrifugation at 16,000× *g* for 5 min. The cell pellet was incubated with 500 µL of 10% SDS (sodium dodecyl sulfate) containing 1 mg mL^{-1} lysozyme at room temperature for 5 min. Then 700 µL of TRIzol [41] was added and the mixture was incubated for another 5 min. This step was followed by the addition of 1 mL ROTI®Aqua-P/C/I reagent (Carl Roth GmbH, Karlsruhe, Germany), 10 min of incubation and centrifugation at 13,000× *g* for 5 min. RNA purification from the supernatant was achieved with the Monarch Total RNA Miniprep Kit (New England Biolabs, Frankfurt, Germany). gDNA was removed by treating 10 µL samples with an absorption at 260 nm corresponding to ~1 µg RNA with 1 U of RNase-free DNase I (ThermoFisher, Waltham, MA, USA) in the $MgCl_2$-containing reaction buffer provided by the manufacturer. RNA concentrations were measured with an NanoDrop Biospectrometer (Eppendorf, Hamburg, Germany). The absence of gDNA was verified using the primers rpoB-denitf and rpoB-denitr [42], which bind only to gDNA and not to the corresponding RNA.

2.6. Expression Studies Based on RT-qPCR

RNA samples of 100 ng were used for RT-qPCR analysis via the Luna Universal One-Step RT-qPCR Kit (New England Biolabs, Frankfurt, Germany) and the CFX Connect™ real-time detection system (Bio-Rad, Munich, Germany) according to the instructions of the manufacturers. The level of *rpoB* mRNA was used as an internal standard [42]. Approximately 200 bp fragments were amplified (see Table S1 in the supplemental material) with an annealing temperature of 60 °C. The RT-qPCR conditions were as follows: 10 min at 55 °C (reverse transcription using random nonamer primers), 1 min at 95 °C (inactivation of the reverse transcriptase and activation of the polymerase), 40 cycles of 15 s at 95 °C, 30 s at 60 °C, followed by melting curve analysis, in which the temperature was increased every 10 s by 1 °C, from a start at 60 °C to 95 °C. Analyses of melting curves and calculations of C_t (calculated threshold) values were automatically quantified with the Bio-Rad CFX Manager 3.1 (3.1.1517.0823) software. C_t values for each point in time were run in triplicate. Relative expression ratios were calculated by the $2^{-\Delta\Delta CT}$ method [43].

2.7. Cloning, Site-Directed Mutagenesis, Overproduction, and Purification of Recombinant SoxR Proteins

The *soxR* gene was amplified from *H. denitrificans* genomic DNA with primers adding a sequence for an N-terminal Strep-tag and cloned between the NdeI and HindIII sites of pET-22b (+), resulting in pET22b-SoxR-Strep. Cysteine-to-serine exchanges were implemented with the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Frankfurt, Germany) according to the manufacturer's instructions and using the primers listed in Table S1. Recombinant SoxR proteins were overproduced in *E. coli* BL21 (DE3) containing plasmids pET22b-SoxR-Strep, pET-22b-SoxR $C^{50}S$, pET-22b-SoxR $C^{116}S$, and pET-22b-SoxR $C^{50}S$ $C^{116}S$. The cells were grown in 1 L Erlenmeyer flasks at 37 °C in 400 mL LB medium containing ampicillin up to an OD_{600} of 0.5–0.6. Expression of *soxR* was induced by adding 0.5 mM IPTG (isopropyl-β-d-thiogalactopyranoside). IPTG-induced *E. coli* cells were grown overnight at 20 °C. Cells were harvested at 14,000× *g* for 30 min. Three mL of lysis buffer (100 mM Tris-HCl buffer pH 7.0 and 5 mM EDTA (ethylenediaminetetraacetic acid containing a spatula tip of deoxyribonuclease I and protease inhibitor) were added per g of wet weight for homogenization. Cell lysis was achieved by sonification and followed by centrifugation (16,100× *g*, 30 min, and 4 °C) and ultracentrifugation (145,000× *g*, 1 h, 4 °C). The supernatant was applied to a Strep–tactin affinity chromatography column equilibrated with buffer W (100 mM Tris-HCl, pH 8.0, 150 mM NaCl). The column was washed with six volumes of buffer W and eluted with buffer E (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM D-desthiobiotin). The protein was assessed for its purity by 12.5% SDS–PAGE (polyacrylamide gel electrophoresis). Pure SoxR proteins were stored on ice in buffer W. Buffer exchange was achieved with Amicon® Ultra-3K centrifugal filters (Merck Millipore, Darmstadt, Germany).

2.8. Electrophoretic Mobility Shift Assays (EMSA)

Interactions between proteins and nucleic acids are detected by gel electrophoretic mobility shift assays. In these, solutions of nucleic acid and protein are combined and analyzed for the distribution of nucleic acid species by native polyacrylamide gel electrophoresis. In general, the migration of protein–nucleic acid complexes is slower than that of the corresponding free nucleic acid. The binding reaction mixture (15 µL final volume) contained purified SoxR wild-type or variant protein in various concentrations (up to 700 nM), 2 µL 50% glycerol and 1.5 µL 10 × binding buffer (100 mM Tris-HCl, 500 mM KCl, 10 mM DTT, 5% glycerol, pH 8.0). Reaction mixtures were pre-incubated for 20 min at room temperature, followed by a further 30 min incubation at 30 °C after adding the DNA probe to a final concentration of 17 nM. The DNA probes consisted of a 362-bp fragment covering the entire intergenic region between the *shdrR* (Hden_0682) and the *soxT1A* (Hden_0681) genes, a 180-bp fragment representing the central part of the first product (created with primers EMSA-Fr2-Fr and EMSA_Fr3-Rev), a 177-bp fragment situated between the *shdrR* and the *lipS1* (Hden_0683) genes, a 173-bp fragment situated between the *lipX* (Hden_0687) and *dsrE3C* (Hden_0688) genes, a 176-bp fragment located between the *tusA* (Hden_0698) and *hyp* (Hden_0697) genes, and a 151-bp fragment situated between the *soxA* (Hden_0703) and *soxY* (Hden_0704) genes. All primers used are listed in Table S1. Native 6% polyacrylamide gels were loaded with the reaction mixtures after pre-running the gels at 100 V for 1 h at 4 °C with 0.25 × TBE buffer (25 mM Tris-borate, 0.5 mM EDTA). 0.25 × TBE with 0.5% glycerol was used as the buffer for running the loaded gels for 1 h at 180 V and 4 °C. Gels were subsequently stained for 20 min with SYBR green I. The bands corresponding to SoxR-bound and free DNAs were visualized with a ChemiDoc Imaging System (BioRad, Munich, Germany).

2.9. Gel Permeation Chromatography

The size exclusion chromatography column Superdex™ 75 Increase 10/300 GL (Cytiva, Freiburg, Germany) was calibrated using Blue dextran (2000 kDa), conalbumin (75 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), lactoglobulin (35 kDa), carbonic anhydrase (29 kDa), chymotrypsin (23 kDa), and ribonuclease (13.7 kDa). The calibration curve was plotted using the gel-phase distribution coefficient (k_{av}) versus the logarithm of molecular weight. $K_{av} = (V_e - V_0/V_c - V_0)$, where V_e = elution volume, V_0 = column void volume (7.94 mL based on Blue dextran elution volume) and V_c geometric column volume (24 mL). The column was run in 50 mM Tris-HCl, pH 8.0 and 150 mM NaCl at a flow rate of 0.8 mL min^{-1} using an Äkta FPLC system.

2.10. Preparation of Polysulfides

A polysulfide stock solution was prepared according to Ikeda et al. [44] by mixing 1.2 g NaHS × H_2O and 0.16 g sulfur powder with 3 mL oxygen-free water in a closed 10 mL serum bottle under a nitrogen atmosphere for 1 h at room temperature. Then, the volume was filled up to 10 mL with oxygen-free water. Based on the average length of the resulting polysulfides of four sulfur atoms, their concentration is 0.5 M in the final solution, which can be kept at room temperature for many months. If necessary, the polysulfide solution was diluted with oxygen-free water and immediately used for persulfuration reactions.

2.11. Redox Treatments, Persulfuration Reactions, MalPEG Gel-Shift Assays and Mass Spectrometry

A total of 5 µg protein was treated with dithiothreitol (DTT, 1 mM and 5 mM for samples analyzed by mass spectrometry and EMSA, respectively) for reduction, 5 mM $CuC1_2$ for oxidation, 0.5 mM polysulfide for persulfuration, 1 mM MalPEG (methoxy-polyethylene glycol maleimide, MW 10,000 g mol^{-1}) for PEGylation, or 5 mM iodoacetamide for carbamidomethylation in a final volume of 15 µL containing 100 mM Tris-HCl, pH 8.0 and 150 mM NaCl. When polysulfide, MalPEG, and DTT were applied consecutively, concentrations were 0.5 mM, 5 mM, and 1 mM, respectively. When polysulfide and DTT

were applied consecutively, concentrations were 0.5 mM and 10 mM, respectively. Protein samples used in EMSA experiments were reacted with the reagents for 20 min at 25 °C. Samples analyzed by SDS–PAGE were incubated with each reagent for 15 min at 30 °C. Reactions were either stopped by the addition of 5 µL of 4 × non-reducing Roti®-Load2 (Carl Roth GmbH, Karlsruhe, Germany) and subjected to 15% SDS–PAGE without boiling the sample or analyzed by mass spectrometry. Samples of 20 µL were desalted by ZiptipC4 Pipette tips (Merck Millipore, Darmstadt, Germany) and measured by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry at the Core Facility Protein Synthesis & BioAnalytics, Pharmaceutical Institute, University of Bonn.

2.12. Distribution of Sox Systems and SoxR: Dataset Generation and Analysis

Archaeal and bacterial genomes were downloaded from the Genome Taxonomy Database (GTDB, release R207). In the GTDB, all genomes are pre-validated, sorted according to validly published taxonomies and are of high quality (completeness minus 5 *contamination must be greater than 50%). One representative of each of the current 65,703 species clusters has been analyzed. The GTDB is based on recently standardized archaeal and bacterial taxonomies derived by normalizing the evolutionary distance between taxonomic levels [45,46]. For bacteria, the database currently lists 148 phyla. For the Archaea, GTDB distinguishes 16 phyla. Open reading frames were determined using Prodigal [47] and subsequently annotated for SoxR, other Sox proteins, and clustering of the respective genes via HMS-S-S with default conditions [48]. Chromatiaceae and Ectothiorhodospiraceae were treated as exceptions as they do not contain contiguous *sox* clusters, but the thiosulfate-oxidizing capabilities and functionality of the Sox proteins have been experimentally established for relevant species [49].

3. Results

3.1. Distribution of Sox Systems and the SoxR Regulator

We first asked how complete and truncated Sox systems (Figure 1) are distributed among the prokaryotes and analyzed the genomes available in the Genome Taxonomy Database (GTDB, release R207). In GTDB, all genomes are sorted according to validly published taxonomies. In addition, we asked which groups of these prokaryotes contain a *soxR* that is linked to the other *sox* genes. In order to accurately identify and discriminate between the Sox components, we used HMS-S-S, a tool that specifically finds sulfur metabolism-related proteins [48]. As shown in Figure 2 and Table S2, genes encoding Sox proteins are not found among the Archaea. They exclusively occur in 17 of the currently 169 bacterial phyla distinguished in the GTDB. The highest proportion of species with Sox in a phylum is observed for the Aquificota (54%), followed by the Campylobacterota (30.7%), the Deinococcota (24.3%), and the Proteobacteria (19.3%) (Table S2). The SoxR regulator is strictly confined to the Proteobacteria (Figure 2).

The Aquificota contain exclusively organisms with a truncated Sox system (Table S2), which are strictly chemolithoautotrophic sulfur oxidizers, with a few having additional organoheterotrophic capacity [50]. Among the Sox-containing Campylobacteria, about three quarters rely on a complete system. The type of Sox system varies within a family and even within a single genus. Many Campylobacterota species, e.g., members of the families Sulfurimonadaceae, Sulfurispirillaceae or Sulfurovoraceae, are established chemolithoautotrophic sulfur oxidizers [51–53]. In the Deinococcota, the complete Sox system is much more abundant than the truncated version (Table S2), with occurrences in *Thermus* and *Meiothermus* species known as sulfur-oxidizing mixotrophs [54,55]. Among the Bacteroidota, the general abundance of Sox is low, but here we find the obligately photolithoautrophic sulfur oxidizers of the order Chlorobiales [56], all of which encode the truncated set of Sox proteins.

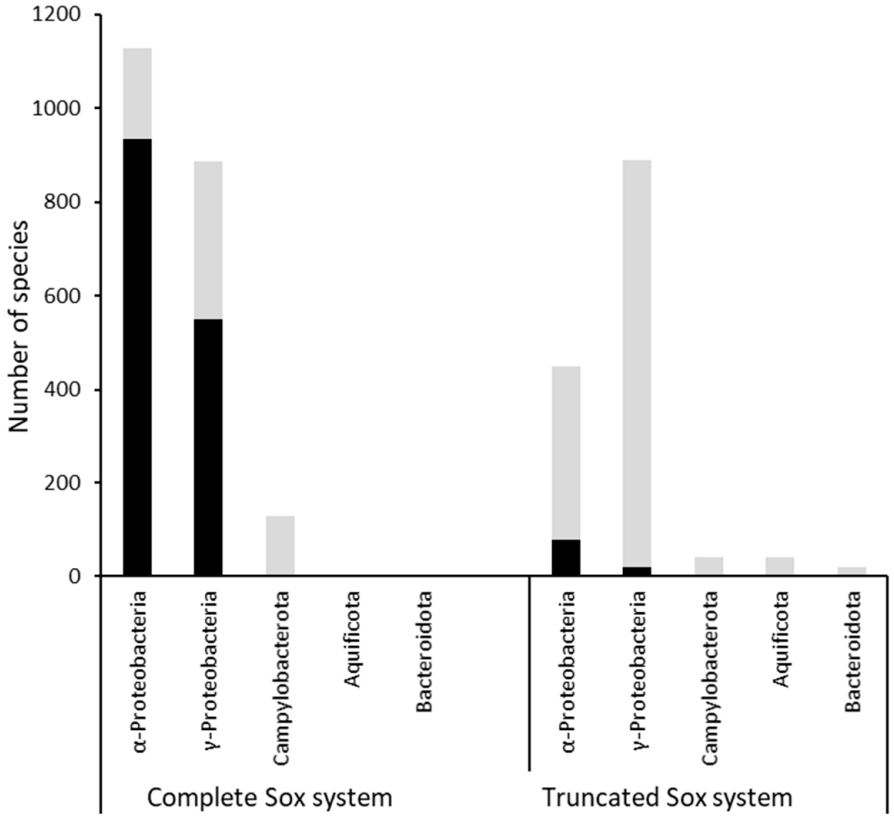

Figure 2. Occurrence of complete and truncated Sox systems among five bacterial phyla. The simultaneous presence of SoxR is indicated in black.

By far the highest absolute numbers of Sox-containing species are found among the Proteobacteria, here exclusively in the classes Alphaproteobacteria and Gammaproteobacteria. The complete Sox system appears more frequently than the truncated version in metabolically versatile members of the alphaproteobacterial families Rhizobiaceae [57] and Rhodobacteraceae [58], while the opposite is true for a number of gammaproteobacterial families, e.g., the Thioglobaceae, Chromatiaceae, Ectothiorhodospiraceae, Thiomicrospiraceae, and Thiotrichaceae (Table S2), all of which contain members with established chemo- or photolithotrophic sulfur-oxidizing capabilities [59–63]. On the other hand, families like the alphaproteobacterial Xanthobacteraceae or the gammaproteobacterial Burkholderiaceae contain species encoding complete or truncated Sox systems in almost equal numbers. The important general rule to emerge from our analysis is that the gene for the SoxR transcriptional regulator is more often linked to the genes for the complete Sox System than to those for the truncated Sox system (Figure 2).

3.2. Genetic Evidence for SoxR Function in H. denitrificans

Previously, we showed that the ArsR-type regulator encoded by the first gene of the *H. denitrificans shdr–lbpA* operon, sHdrR, functions as a repressor of *shdr* gene expression in the absence of oxidizable sulfur compounds [9]. The phenotypic characterization of a mutant strain lacking the *shdrR* gene indicated an additional regulator involved in the overall process. Indeed, a further candidate transcriptional repressor, SoxR, is encoded downstream of *soxXA* in *H. denitrificans* (Figure 1b). To assign a function for SoxR in transcriptional regulation of the hyphomicrobial *sox* and possibly also the *shdr* and associated genes, we constructed *H. denitrificans* Δ*tsdA* Δ*soxR*, a mutant strain with a markerless deletion of *soxR* in a Δ*tsdA* background. The reference strain *H. denitrificans* Δ*tsdA* lacks thiosulfate dehydrogenase and oxidizes thiosulfate exclusively via the pathway combining Sox and sHdr–LbpA [8,9] (Figure 1b). When grown in the presence of methanol as a carbon

source and thiosulfate as an additional electron source, the Δ*tsdA* reference strain excretes sulfite, which causes a growth retardation that is particularly impressive when cultures are inoculated with thiosulfate-induced cells ([9], also compare open and filled circles in the upper right panel of Figure 3). Like the *H. denitrificans* strain lacking the *shdrR* gene, the *soxR*-deficient strain exhibited a high specific thiosulfate oxidation rate and a significantly reduced growth rate even without the induction of pre-cultures (Figure 3). The growth rate increased significantly when thiosulfate was depleted. When pre-cultures were exposed to thiosulfate, both regulator-negative strains exhibited slightly higher specific thiosulfate consumption rates than in the non-induced case, fully in line with the finding that a second regulator is involved in the overall process.

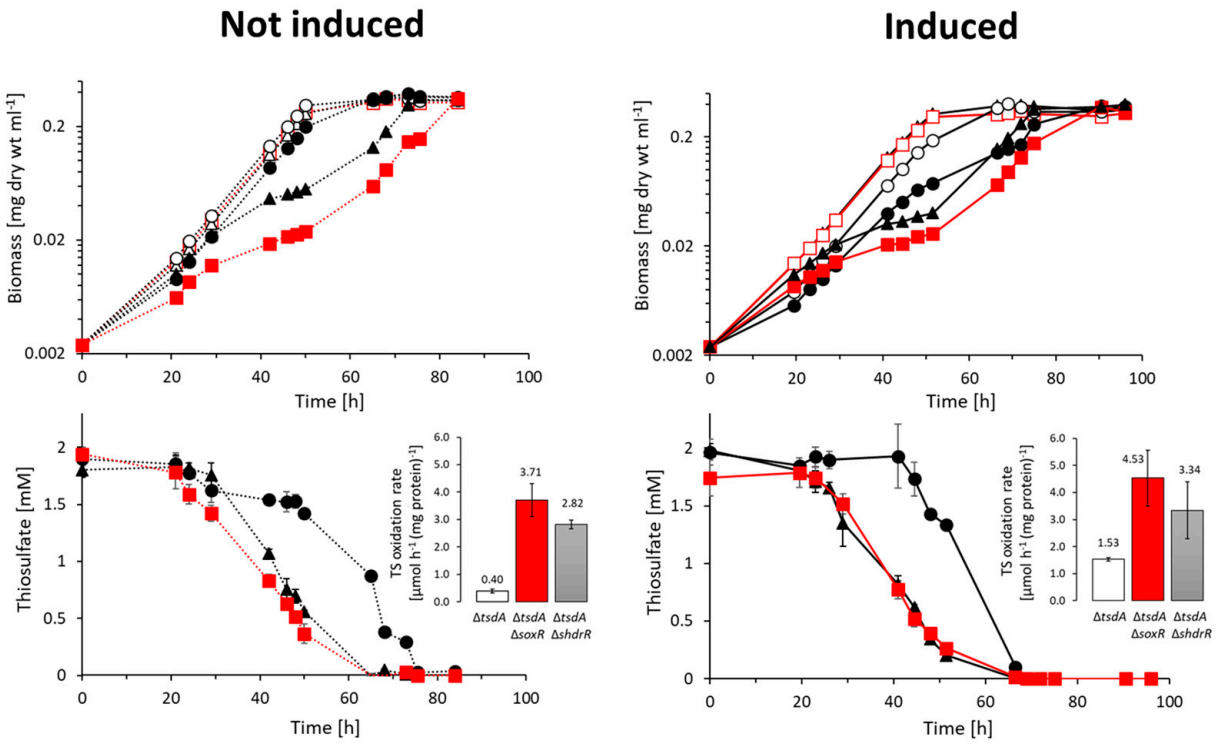

Figure 3. Growth and thiosulfate consumption of *H. denitrificans* Δ*tsdA* (black circles and lines), Δ*tsdA* Δ*shdrR* (black triangles and lines), and Δ*tsdA* Δ*soxR* (red boxes and lines). All strains were grown in medium containing 24.4 mM methanol, either in the absence (open symbols) or in the presence of 2 mM thiosulfate (filled symbols). Pre-cultures contained either no thiosulfate (not induced, broken lines) or 2 mM thiosulfate (induced, solid lines). Thiosulfate concentrations for the different cultures are depicted in the lower panels. Symbol assignments and the color code for specific thiosulfate (TS) oxidation rates are the same as in the upper panels. Error bars indicating SD are too small to be visible for the determination of biomass.

3.3. Identification of Genes Controlled by SoxR by RT-qPCR for Different H. denitrificans Strains

To examine which genes are affected by the SoxR regulator protein, RT-qPCR experiments were performed, and the transcription levels of twelve genes in the *H. denitrificans* sulfur oxidation genome region were compared in the absence and presence of thiosulfate for the Δ*tsdA* reference strain (Figure 4). In addition, transcription levels were determined for the same genes in the *H. denitrificans* Δ*tsdA* Δ*soxR* mutant in the absence of thiosulfate. All cultures were harvested in the exponential growth phase. The studied genes included *soxT1A*, the first of a set of genes transcribed in the opposite direction of *shdrR*, the gene for the sHdrR regulator, and two of the genes encoding proteins involved in LbpA2 assembly (*lipS1* and *slpl*(*AB*)). LbpA2 is a lipoate-binding protein essential for sulfur oxidation [10]. Four genes were chosen as examples for those encoding the *shdr–lbpA2* cytoplasmic sulfane sulfur oxidation system (*dsrE3C*, *shdrA*, *shdrB2*, and *lbpA2*). These genes are followed by

genes transcribed in the opposite direction and encoding part of the Sox system (SoxXA), the SoxR regulator, SoxS, which is a periplasmic thiol–disulfide oxidoreductase, as well as a second potential sulfur transporter, SoxT1B, the cytoplasmic sulfurtransferase TusA, and a predicted cytochrome P450 (Figures 1 and 4b). Except for *soxS*, all of these genes were included in the RT-qPCR analysis. Finally, the analysis was extended to *soxY* and *soxB*. These genes follow the previously described genes in the opposite direction in a *soxYZB* arrangement (Figures 1 and 4b).

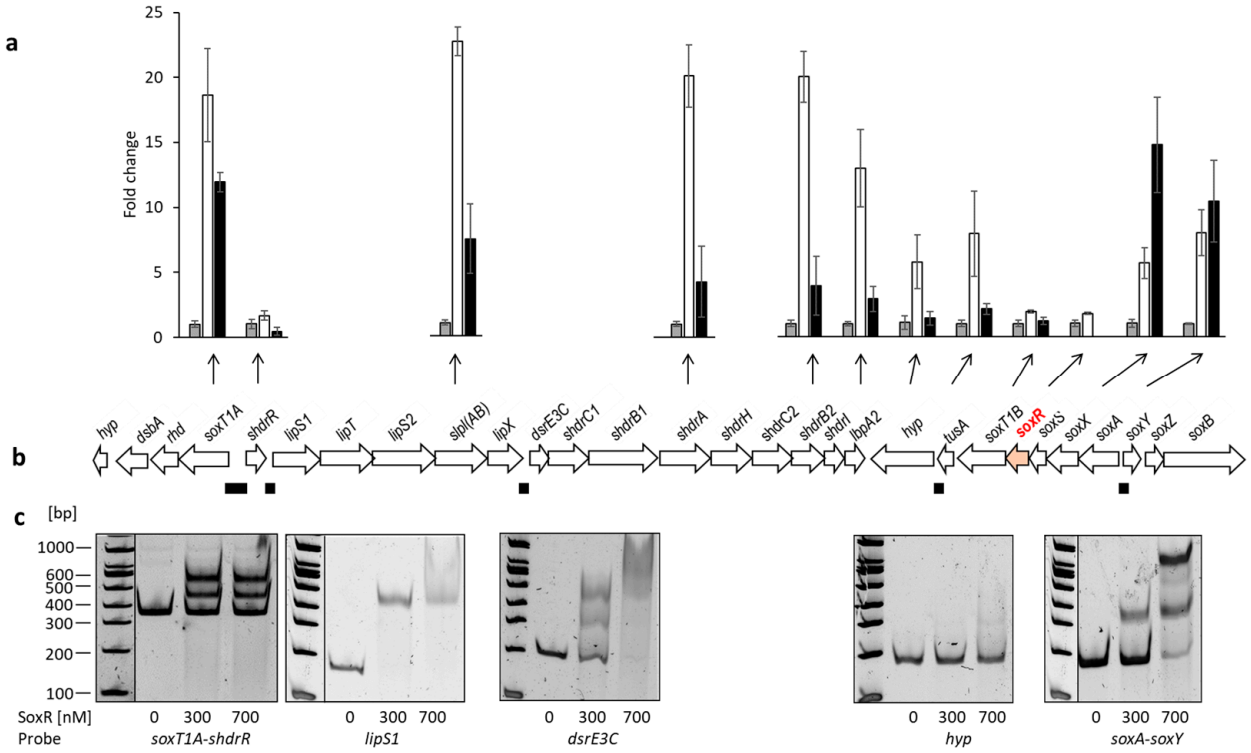

Figure 4. (**a**) Relative mRNA levels of twelve genes located in the *shdr–sox* genetic region (depicted in panel; (**b**)) from *H. denitrificans* for the Δ*tsdA* reference strain in the absence (gray columns) and presence of thiosulfate (white columns), as assessed by RT-qPCR. Results for *H. denitrificans* Δ*tsdA* Δ*soxR* are shown by black columns. Results were adjusted using *H. denitrifcans rpoB*, which encodes the β-subunit of RNA polymerase, as an endogenous reference, according to [42]. (**b**) DNA regions tested in EMSA assays for SoxR binding are indicated as black rectangles below the hyphomicrobial *shdr–sox* genes. The *soxR* gene is highlighted in red for easier orientation. Fragment sizes: 362 bp for the *soxT1A–shdrR* intergenic region, 177 bp and 173 bp for the regions upstream of *lipS1* and *dsrE3C*, respectively. The fragments downstream of *tusA* and between *soxA* and *soxY* had sizes of 176 bp and 151 bp, respectively; (**c**) EMSA analysis of Strep-tagged SoxR with upstream promoter sequence probes of sulfur oxidation-related genes as specified in (**b**). DNA probes of 17 nM were incubated with different amounts of SoxR (300 and 700 nM). Vertical lines separate samples that were run on the same gel but were not directly adjacent.

With the exception of the genes for the two transcriptional regulators, *shdrR* and *soxR*, and *soxT1B*, which is located just downstream of *soxR*, all the genes tested were upregulated at least five-fold when the reference strain was exposed to thiosulfate, with the strongest responses for *lpl(AB)*, *shdrA*, and *shdrB2* (Figure 4a). In the strain lacking SoxR, transcription of various *sox* and *shdr* genes was much higher than in the reference strain, even in the absence of thiosulfate. The lack of *soxR* most strongly affected transcription of *soxXA* and *soxY* but was also evident for *shdr* genes, *soxT1A*, *lipS1*, and *lpl*(*AB*) (Figure 4a). With the exception of the genuine *sox* genes tested, the reference strain showed a stronger response to the presence of thiosulfate than the Δ*tsdA* Δ*soxR* mutant in its absence. This observation

clearly points to the presence of at least one further regulatory element, most probably sHdrR [9]. On the other hand, the strong response of numerous genes, in addition to those for the genuine Sox system, shows that their transcription is either directly or indirectly affected by SoxR.

3.4. Identification of SoxR Target Sites by EMSA

The finding that SoxR affects transcription of genes outside the *sox* operons was unexpected and afforded a closer analysis. To that end, we inspected intergenic regions within the hyphomicrobial sulfur oxidation region and identified four with conspicuous inverted and direct repeats with the potential to serve as repressor binding sites and used them as probes for EMSA (Figure 4b). A 176-bp fragment located upstream of the hypothetical gene Hden_0697 served as a control (Figure 4b). Indeed, SoxR bound to four of the five tested probes (Figure 4c). Among these is the intergenic region between *soxT1A* and the gene for the SoxR-related repressor sHdrR. This region had already been shown to serve as a binding site for sHdrR [9], further emphasizing the notion that the two repressors work intimately together.

3.5. Properties of the SoxR protein

The *H. denitrificans* SoxR protein has a length of 124 amino acids, and a BlastP search (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 29 June 2023) identified *R. capsulatus* SqrR as the most similar structurally characterized protein. *H. denitrificans* SoxR shows 53%, 52%, 43%, and 42% amino acid identity to *R. capsulatus* SqrR, *P. salicylatoxidans* SoxR, *Xylella fastidiosa* BigR, and *H. denitrificans* sHdrR, respectively. All of these regulators share two conserved cysteine residues, Cys^{50} and Cys^{116}, in the hyphomicrobial protein (Figure 5). The equivalent residues in SqrR are required for sensing sulfide [35,64].

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H. denitrificans SoxR       MSGILPNEVIAALEADEEVSPELKRLVLRARKASDFLKALAHESRLLILCLLAEKE-RSA  59
H. denitrificans sHdrR      MAVVKPRTNRPAVRKARTRQPALHSTDASIEQATALLRALGSPHRLAILCLLLEGE-RTV  59
Pseudaminobacter SoxR       -------MNLPKLSADQS-PEEFEKLLEQARKASDLLKALSHEGRLLILCLLAEGE-KSV  51
Xylella fastidiosa BigR     --------MVNEMRDDTRPHMTREDMEKRANEVANLLKTLSHPVRLMLVCTLVEGE-FSV  51
Vibrio cholerae HluY        ---------MPYL---KGAPMNLQEMEKNSAKAVVLLKAMANERRLQILCMLLDNE-LSV  47
Rhodobacter capsulatus SqrR ---------MGSDTDERCAALDAEEMATRARAASNLLKALAHEGRLMIMCYLASGE-KSV  50
Escherichia coli YgaV       -------------------MTELAQLQASAEQAAALLKAMSHPKRLLILCMLSGSPGTSA  41
                                                  .  :*:::.   ** ::* *      :.

H. denitrificans SoxR       GELENLLSINQPTVSQQLARLRLDGLVQARREGKAVIYSLPDETTRRFIGAIYDKFCREE  119
H. denitrificans sHdrR      SEICDKIGARQSLVSQHLTRLRLDGLVKSDRNGYFVSYSLTSAPAQEIIATLHKYYCATS  119
Pseudaminobacter SoxR       SELESIMHMPQAAVSQQLARLRFDRLVNTRREGRVIYYSIASSEVSSVISTLYGLFCAPV  111
Xylella fastidiosa BigR     GELEQQIGIGQPTLSQQLGVLRESGIVETRRNIKQIFYRLTEAKAAQLVNALYTIFCAQE  111
Vibrio cholerae HluY        GELSSRLELSQSALSQHLAWLRRDGLVNTRKEAQTVFYTLSSTEVKAMIELLHRLYCQAN  107
Rhodobacter capsulatus SqrR TELETRLSTRQAAVSQQLARLRLEGLVQSRREGKTIYYSLSDPRAARVVQTVYEQFCSGD  110
Escherichia coli YgaV       GELTRITGLSASATSQHLARMRDEGLIDSQRDAQRILYSIKNEAVNAIIATLKNVYCP--  99
                              *:          **:*  :* . ::.: ::   : * : .  .  .:  :   :*
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Figure 5. Amino sequence alignment of SoxR homologs. Accession numbers/locus tags and references in the order of appearance: (Hden_0700 [8], Hden_0682 [8,9], WP_010893290 [34], HLYU_VIBCH [65], WP_019171658 [27], ADE85198 [35], and b2667 [66]). An * (asterisk) indicates positions which have a single, fully conserved residue. Conserved cysteine residues are highlighted in yellow. Colons (:) and single dots (.) indicate conserved and semi-conserved amino acids, respectively.

We sought to obtain information about the oligomerization state and conformation of SoxR as well as about the reactivity of the two cysteine residues. To this end, Strep-tagged SoxR as well as variants with serine in place of either one (SoxR $Cys^{50}Ser$ and SoxR $Cys^{116}Ser$) or both cysteines (SoxR $Cys^{50}Ser$ $Cys^{116}Ser$) were overexpressed in *E. coli*, purified by affinity chromatography, and subjected to reducing and non-reducing SDS–PAGE analysis in the as-isolated state, after reduction with DTT, and after oxidation with $CuCl_2$. The same single 15 kDa band was obtained in all cases under reducing conditions (not shown). The band for the oxidized wild-type protein migrated slightly further than those for the as-isolate and reduced proteins under non-reducing conditions (Figure 6a), indicating a more compact structure due to the formation of an intramolecular

disulfide bond between Cys^{50} and Cys^{116}. The oxidized SoxR Cys^{50}Ser and SoxR Cys^{116}Ser variants formed intermolecular dimers connected by the remaining cysteine on each of the monomers (Figure 6a). These observations indicated a homodimeric state for the native proteins that allows close contact between the Cys^{50} and Cys^{116} residues, respectively, of the monomers and thus the formation of disulfide bridges under oxidizing conditions.

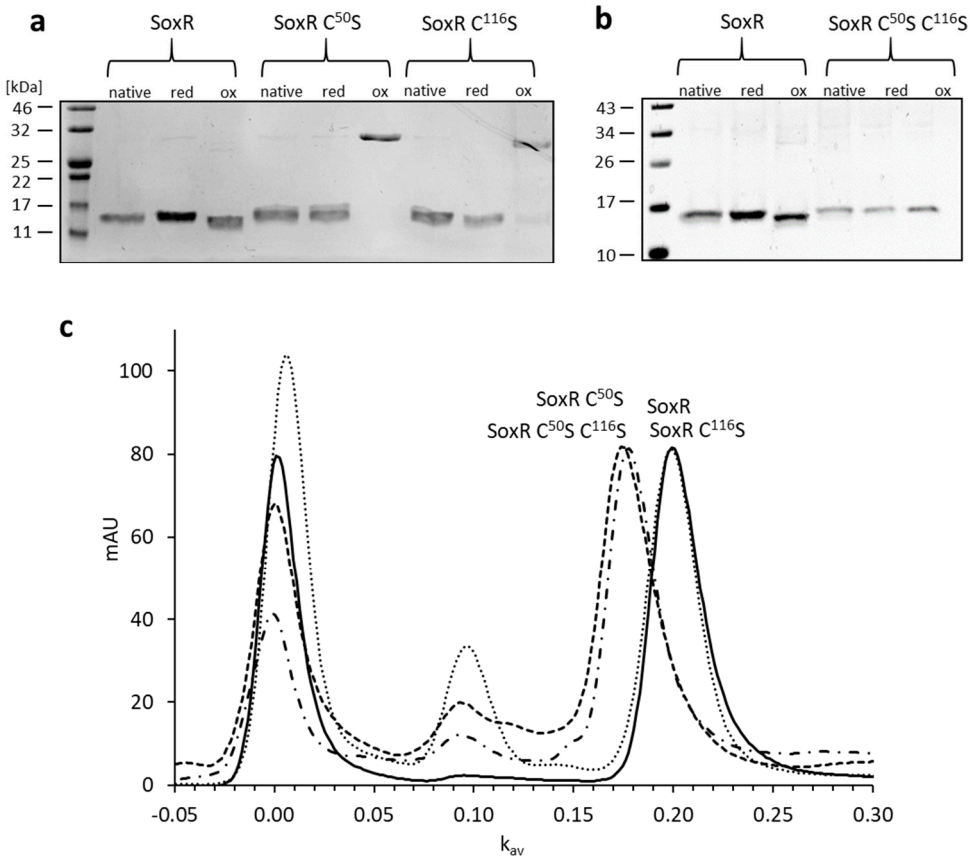

Figure 6. Conformation of SoxR and its variants as analyzed by non-reducing SDS–PAGE analysis (**a**,**b**) and gel permeation chromatography (**c**). For the experiments shown in (**a**) and (**b**), 5 µg of SoxR or its variants were incubated in 15 µL of 100 mM Tris-HCl, pH 8.0 and 150 mM NaCl with either 1 mM DTT or 5 mM $Cucl_2$ for 20 min at room temperature, mixed with 5 µL of non-reducing Roti®-Load2 (Carl Roth GmbH, Karlsruhe, Germany) and run on 15% SDS polyacrylamide gels. The wild-type SoxR protein is shown twice (**panels a**,**b**) to allow direct comparison with protein variants on different gels. In (**c**), the elution profiles upon gel filtration on Superdex 75 Increase 10/300 are depicted for SoxR, solid line; SoxR Cys^{50}Ser, dotted line; SoxR Cys^{116}Ser, dashed line; SoxR Cys^{50}Ser Cys^{116}Ser, dashed-dotted line. SoxR and SoxR Cys^{116}Ser dimers elute at a k_{av} of 0.2, corresponding to a molecular mass of 36.7 kDa, whereas SoxR Cys^{50}Ser and SoxR Cys^{50}Ser Cys^{116}Ser elute earlier (k_{av} = 0.174, 41.9 kDa), indicating a more open conformation. The resolution of the column does not allow clear separation of the different tetrameric conformations (k_{av} 0.086 to 0.093, corresponding to 65.9 to 63.6 kDa).

This conclusion was fully supported by size exclusion chromatography (Figure 6b). All variants, as well as wild-type SoxR, eluted with k_{av} values corresponding to molecular masses between 37.6 and 41.6 kDa, indicating dimerization of the 15.2 kDa monomers. Tetramers were also observed, with the highest abundance for the SoxR Cys^{116}Ser variant. All proteins showed a tendency for the formation of higher oligomers in the void volume (Figure 6b). Notably, the Sox Cys^{50}Ser single and the Cys^{50}Ser Cys^{116}Ser variant exchanges led to dimers eluting significantly earlier than those of wild-type SoxR and SoxR

Cys^{116}Ser, indicating that the loss of Cys^{50} but not that of Cys^{116} leads to a more open, space-demanding conformation of the regulator protein.

3.6. SoxR Binding Properties

In the next step, EMSA assays were performed that allowed more detailed insights into the binding capacity of SoxR to the intergenic region between the divergently oriented *soxXA* and *soxYZB* genes (Figure 7). SoxR binds to the DNA probe in a concentration-dependent manner and leads to the appearance of two shifted bands indicating two different binding sites (Figure 7a, upper panel). As related proteins respond to persulfuration [35,64], we tested the response of SoxR to treatment with polysulfide, oxidized and reduced glutathione (GSH and GSSG), tetrathionate, sulfite, and thiosulfate in various molar ratios of protein and additive. Whereas GSH, GSSG, tetrathionate, and sulfite had no effect even when present in 50-fold excess compared to the protein (not shown), treatment with polysulfide above a molar ratio of 1 completely prevented binding of SoxR to the target DNA, and a shift was no longer observed (Figure 7a, lower panel, and Figure 7b, upper panel). Thiosulfate also had an effect, albeit a much milder one (Figure 7b, lower panel). The second shifted band disappeared at a ratio thiosulfate/SoxR of 5, and the first band still persisted at a ratio of 50, corresponding to a thiosulfate concentration of 0.2 mM. As outlined in the introduction, the initial steps of thiosulfate degradation occur in the periplasm, and it is therefore unlikely that thiosulfate would ever reach concentrations in the cytoplasm that would be required to elicit a response from SoxR.

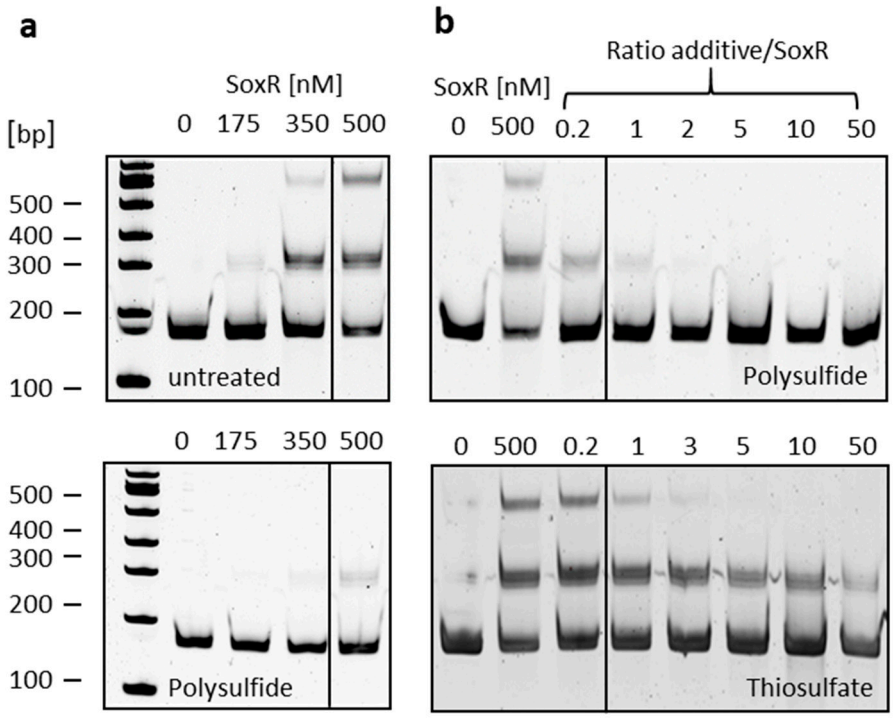

Figure 7. (**a**) EMSA of the 151-bp *soxA–soxY* intergenic fragment with increasing amounts of untreated SoxR (**upper panel**) or SoxR pre-incubated with polysulfide in a molar ratio of SoxR/polysulfide of 1:1 (**lower panel**); (**b**) EMSA of the 151-bp *soxA–soxY* intergenic fragment with SoxR pre-incubated with increasing amounts of polysulfide (**upper panel**) or thiosulfate (**lower panel**). Vertical lines separate samples that were run on the same gel but were not directly adjacent.

EMSA assays were also performed with the as-isolated, reduced, oxidized, and polysulfide-treated SoxR variants and two different DNA probes (Figure 8). Reduction with DTT led to the same results as those obtained for the untreated proteins, indicating that they are fully reduced upon isolation and remain in this state during storage. Oxidation of wild-

type SoxR prevented binding to both tested DNA probes. While the SoxR $Cys^{50}Ser$ variant completely lost its DNA binding ability, the $Cys^{116}Ser$ variant bound effectively to the DNA probes. When polysulfide-treated wild-type SoxR was reduced with DTT in a second step, the protein regained its DNA-binding capacity, demonstrating that the modification caused by polysulfide was fully reversible by reduction. A response to oxidation or incubation with polysulfide was still observed for the $Cys^{116}Ser$ variant, albeit weaker than that of the wild-type protein. This behavior differs significantly from that of the related SqrR from *R. capsulatus*, where variants lacking one of the two conserved cysteines bind to their target DNA but do not show a loss of affinity upon persulfuration [35]. The SoxR variant lacking both cysteines was unable to bind DNA, regardless of the treatments applied.

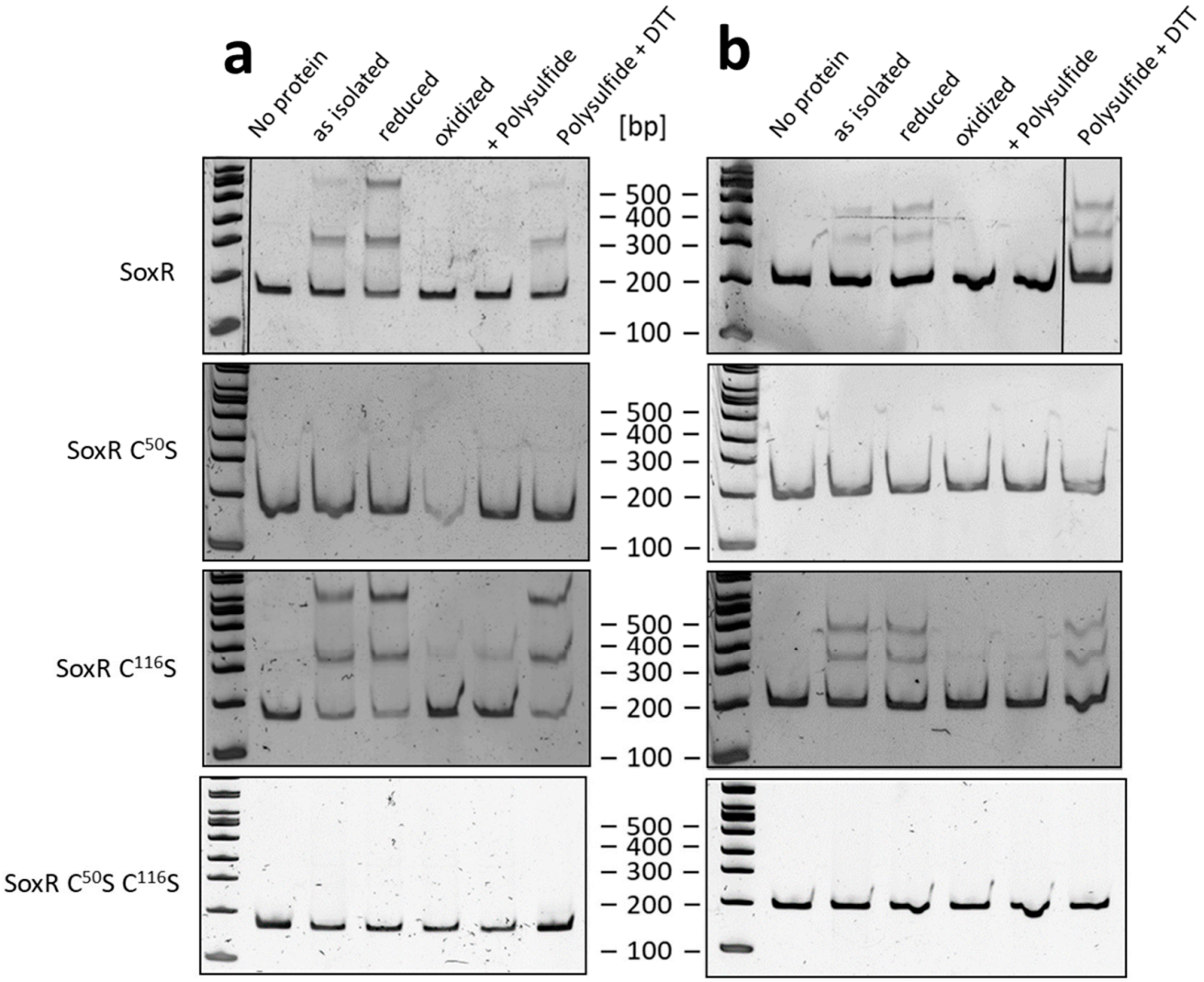

Figure 8. (**a**) EMSA of the 151-bp *soxA–soxY* intergenic fragment (17 nM) with 700 nM SoxR wild-type and variant proteins as isolated, reduced with DTT, oxidized with $CuCl_2$, treated with polysulfide, and sequentially treated with polysulfide and DTT; (**b**) EMSA of the 180 bp central part of the *soxT1A–shdrR* intergenic fragment (17 nM) with 300 nM SoxR wild-type and variant proteins as isolated, reduced with DTT, oxidized with $CuCl_2$, treated with polysulfide, and sequentially treated with polysulfide and DTT.

3.7. Redox State and Modification of SoxR

To clarify the chemical nature of the SoxR modifications by polysulfide and oxidation, gel-shift assays were performed using MalPEG, which selectively labels free thiol groups

covalently [67]. The modification can be detected by non-reducing SDS–PAGE since the molecular mass of the protein is increased by ~10 kDa per SH group modified. Treatment of the recombinant wild-type SoxR protein with MalPEG resulted in a single 20 kDa band shift, indicating that it contains two free cysteine residues, as expected (Figure 9a). In contrast, oxidized SoxR did not react with MalPEG (Figure 9a), demonstrating the existence of a disulfide bridge between Cys^{50} and Cys^{116}, as also suggested by non-reducing SDS–PAGE in the absence of MalPEG (Figure 6a). MalPEG labeling of the SoxR variants gave the expected results, with the variants carrying one cysteine showing a single 10 kDa shift (Figure 9b,c) and the double mutated variant not reacting with MalPEG as predicted (Figure 9d). After oxidation, the SoxR Cys^{50}Ser variant produced exclusively dimers connected by Cys^{116}–Cys^{116} disulfide bridges and not reacting with MalPEG (Figure 9b), whereas the Cys^{116}Ser variant showed incomplete dimerization. This observation is corroborated by the response of SoxR and its variants to polysulfide (Figure 9, right panels). While the wild-type protein stayed essentially monomeric, i.e., disulfide bonds between protein monomers were not formed, the SoxR Cys^{50}Ser variant completely transformed into a dimer stable under denaturing conditions (Figure 9b). The Cys^{116}Ser variant behaved differently, with a substantial fraction staying monomeric (Figure 9c). We note that the dimeric fraction of both variants obtained after treatment with polysulfide turned monomeric after incubation with MalPEG, possibly indicating a (poly)sulfur bridge between the remaining cysteine residues that is susceptible to cleavage by the thiol-binding agent. In conclusion, Cys^{50} residues appear less prone to reaction than Cys^{116} residues, just as has been reported for the corresponding cysteines in *R. capsulatus* SqrR [68], and/or they reside further apart from each other in the native SoxR dimer than Cys^{116} residues.

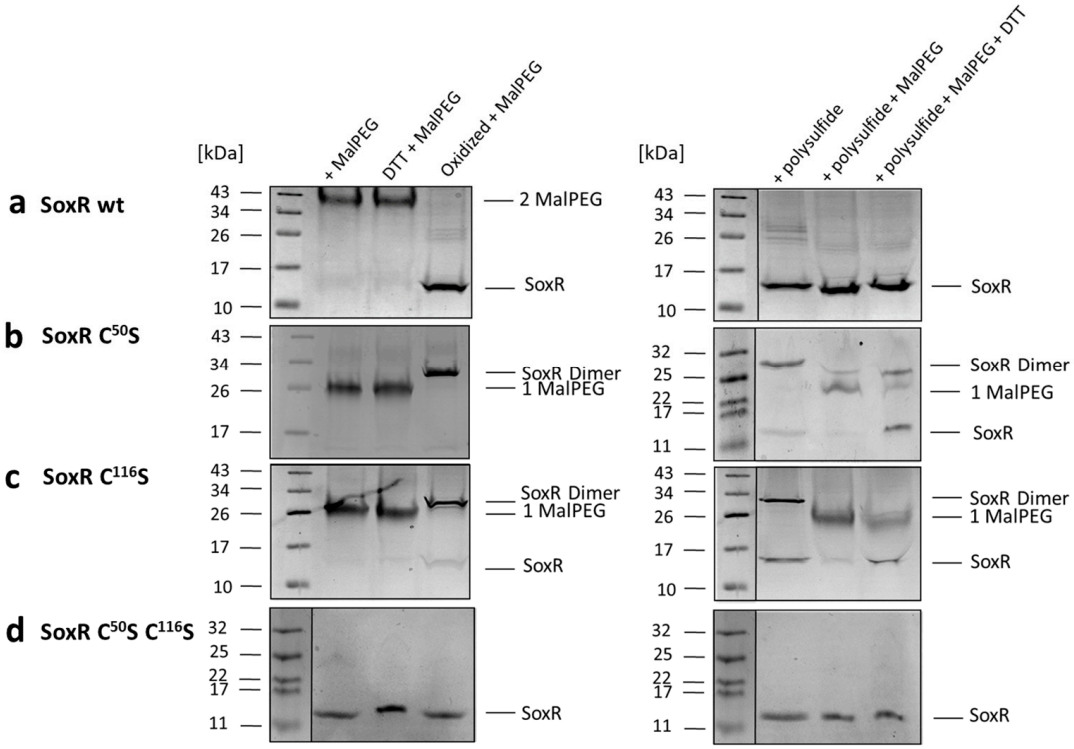

Figure 9. Analysis of *H. denitrificans* SoxR cysteines with MalPEG gel-shift assays in non-reducing SDS–PAGE. Results are shown for the SoxR wild-type (wt) protein (**a**), single (Cys^{50}Ser (**b**), and Cys^{116}Ser (**c**), and double (Cys^{50}Ser Cys^{116}Ser (**d**)) variants after MalPEG treatment of the as-isolated, reduced, and oxidized states (**left panels**) as well as after pre-incubation with polysulfide (**right panels**). Polysulfide and MalPEG-reacted samples were further reduced with DTT. Vertical lines separate samples that were run on the same gel but were not directly adjacent.

The next set of reactions was the most revealing. When wild-type polysulfide-treated SoxR was reacted with MalPEG, it behaved just like the oxidized protein, i.e., MalPEG was not bound, indicating the absence of free cysteines (Figure 9a). Instead, one MalPEG was bound to the polysulfide-treated single cysteine replacement variants and could be released upon reduction with DTT (Figure 9b,c). We conclude that in the two latter cases, polysulfide led to the persulfuration of the single remaining cysteines, which then bound MalPEG. In the final step, MalPEG-sulfide conjugates were released by treatment with DTT, and the single cysteine SoxR variants reappeared in their unmodified monomeric form. The situation for wild-type SoxR is completely different. Either polysulfide merely leads to the formation of a Cys^{50}–Cys^{116} bridge, or one or more sulfur atoms are enclosed by the two cysteines.

Mass spectrometric analyses finally allowed a clear differentiation between these two possibilities (Table 1, Supplementary Figure S1). For these experiments, MalPEG was replaced by the thiol-modifying agent iodoacetamide, which leads to carbamidomethylation of free Cys sulfhydryl groups and thus adds a mass of 57 Da. As expected, wild-type SoxR gained 57 Da twice after iodoacetamide treatment, whereas the single Cys replacement variants were modified with only one carbamido group. Notably, polysulfide treatment led to persulfuration of all SoxR proteins except for the cysteine-less double replacement variant, which was measured as a control. The SoxR wild-type protein was modified with up to three sulfur atoms (+32 Da each) and did not react with iodoacetamide, demonstrating the formation of an intramolecular tri-, tetra-, or penta-sulfide bond between Cys^{50} and Cys^{116}. Although mass spectra do not provide exact quantitative information, peak heights indicate that bridges by two additional sulfur atoms are more abundant than one or three atom bridges for the SoxR wild-type protein, while the majority of the SoxR Cys^{50}Ser and Cys^{116}Ser variant polypeptides are modified by only one sulfur atom (Supplementary Figure S1).

Table 1. Mass spectrometry of SoxR and variants after treatment with modifying agents. CAM, carbamidomethylation; S, sulfur. Calculated masses for Strep-tagged SoxR and SoxR Cys^{50}Ser, SoxR Cys^{116}Ser, and SoxR Cys^{50}Ser Cys^{116} without the initiator methionine are 15,212.54 Da, 15,197.54 Da, 15,197.54 Da, and 15,182.54 Da, respectively.

Treatment	SoxR Mass (Da) (Addition: [Da])	SoxR $C^{50}S$ Mass (Da) (Addition: [Da])	SoxR $C^{116}S$ Mass (Da) (Addition: [Da])	SoxR $C^{50}S$ $C^{116}S$ Mass (Da) (Addition: [Da])
Native	15,212.8	15,197.3	15,198.2	15,182.3
DTT reduced	15,212.5	15,199.3	15,198.8	nd
$CuCl_2$ oxidized	15,210.5	15,196.7	15,196.9	15,182.0
Iodoacetamide	15,328.0 (2 CAM: 2 × 57.07)	15,255.2 (1 CAM: 57.07)	15,255.22 (1 CAM: 57.07)	nd
Polysulfide	15,212.5 15,244.7 (1 S: 32) 15,276.4 (2 S: 64) 15,308.0 (3 S: 96)	15,198.9 15,230.0 (1 S: 32)	15,198.0 15,230.3 (1 S: 32) 15,261.1 (2 S: 64)	15,182.0
Polysulfide + Iodoacetamide	15,212.9 15,244.3 (1 S: 32) 15,275.2 (2 S: 64) 15,306.0 (3 S: 96)	15,198.0 15,286.0 (1 S + 1 CAM: 90)	15,197.6 15,228.5 (1 S: 32) 15,285.4 (1 S + 1 CAM: 90)	15,180.5

4. Discussion

In this study, we collected a wealth of new information on the transcriptional repressor SoxR. We show that among the more than 70,000 prokaryotic genomes investigated, bonafide *soxR* (i.e., genetically linked to the genes for the SoxYZ sulfur-binding protein and/or catalytic Sox components) occurs exclusively among the bacterial phylum Pro-

teobacteria, where it is more frequently found in gene clusters for complete Sox systems than for truncated Sox systems. Based on the available data, it is difficult to draw general conclusions from this observation. However, it appears that a number of bacteria that operate the truncated Sox system, such as the green and purple sulfur bacteria or members of the Aquificota, are dedicated sulfur-oxidizing chemolithoautrophs without much need for sophisticated transcriptional regulation of the sulfur oxidation machinery.

We show that in the model Alphaproteobacterium *H. denitrificans* SoxR is not only involved in the transcriptional regulation of true *sox* genes but that it also affects the transcription of a number of other genes. In particular, the *shdr* genes, which encode the cytoplasmic sulfur-oxidizing multi-enzyme system required for sulfane sulfur oxidation that cannot be achieved by the truncated hyphomicrobial Sox system, are co-controlled by SoxR. How it interacts with a second, related repressor, sHdrR, that affects the transcription of the same genes [9] is an important research question for the future.

The expression levels of *sox* as well as of *shdr* and associated genes are increased by thiosulfate in wild-type cells and elevated in the *soxR*-deficient *H. denitrificans* mutant, irrespective of the presence of thiosulfate (Figure 4). DNA binding in vitro and probably also transcriptional repression in living cells involve thiol modifications. This can be concluded from the observation that the DNA-binding activity of recombinant SoxR is strongly reduced upon incubation with polysulfide, which leads to persulfuration of the regulator, as proven by reaction with MalPEG (Figure 9) and mass spectrometry (Table 1, Supplementary Figure S1). In polysulfide-treated SoxR, the two conserved cysteine residues can neither be modified by MalPEG nor by iodoacetamide. In addition, polysulfide treatment increases the mass of wild-type SoxR by 32, 64, or 96 Da. These findings can be fully explained by the formation of an intramolecular tri-, tetra-, or penta-sulfide bond formed upon interaction with reactive sulfane sulfur species. Thus, SoxR clearly is not a simple redox sensor switching between dithiol and disulfide states but has been identified as a transcriptional regulator sensing reactive sulfane sulfur species (Figure 10), similar to the related SqrR protein from *R. capsulatus* [35,68]. Notably, the substitution of the two crucial conserved cysteine residues leads to a different outcome for SoxR as compared to SqrR: The lack of Cys^{50} causes complete loss of DNA binding in SoxR, whereas the lack of Cys^{116} creates a variant that tightly binds to its target DNA and is less sensitive to persulfuration than the wild-type protein. In SqrR, both equivalent Cys–Ser variants are DNA-binding competent and do not respond to persulfuration as a signal [35]. Clearly, this difference inspires future research that should also include a detailed inspection of the conformational changes triggered by the formation of a sulfur bridge and resulting in the detachment of SoxR from its target DNA (Figure 10).

The physiological processes involving the various sulfane sulfur-responsive regulators characterized so far [34,35,69–72] differ fundamentally from those controlled by SoxR. The former mainly regulate stress responses, sense intracellular and extracellular reactive sulfur species, and ensure upregulation of H_2S oxidation genes for the purpose of detoxification, i.e., they control the removal of excess sulfide and sulfane sulfur, thus contributing to cell survival in the presence of external reactive sulfur species. In contrast, SoxR regulates dissimilatory sulfur metabolism and enables the use of reduced sulfur compounds such as thiosulfate as electron donors for lithotrophic or mixotrophic growth.

As pointed out earlier, thiosulfate oxidation is initiated in the periplasm, and it is highly unlikely that thiosulfate itself serves as the signaling molecule. Instead, SoxR responds to the presence of low concentrations of sulfane sulfur, which was provided as polysulfide in our in vitro assays. A working hypothesis for how this signal reaches its destination, inspired by the arrangement of the respective genes in *H. denitrificans* (Figure 1b), is presented in Figure 10. It is conceivable that the sulfur bound to the sulfur carrier protein SoxYZ in the periplasm in the course of thiosulfate oxidation reaches the cytoplasm via a YedE-like SoxT transporter [73]. The periplasmic thiol–disulfide oxidoreductase SoxS [15] could be involved in the transfer of the sulfane sulfur to the transporter. Once in the cytoplasm, the sulfur transferase TusA [74] is a possible acceptor protein for the sulfur,

which could be passed on from there to SoxR, possibly involving the cytochrome P450 encoded by gene Hden_0697.

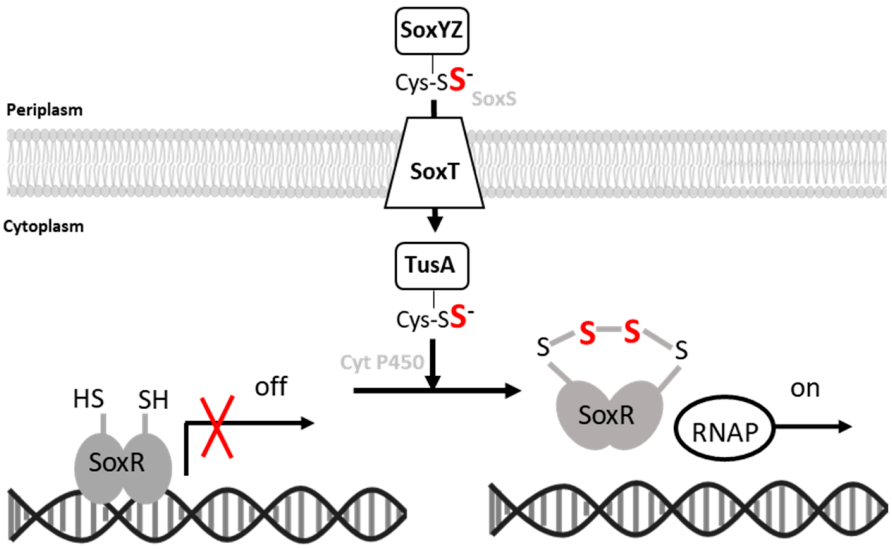

Figure 10. Proposed signal transduction pathway and mode of action of the homodimeric SoxR repressor protein. Established sulfur-binding proteins are printed in black. Sulfane sulfur atoms that come originally from thiosulfate (cf. Figure 1) are highlighted in bold red.

5. Conclusions

In conclusion, our study shows that SoxR allows *H. denitrificans* to adapt to changes in thiosulfate availability via thiol persulfidation chemistry and the formation of an intramolecular sulfur bridge, which may involve transporters and sulfurtransferases encoded in the same genetic region. Clearly, much remains to be learned about this regulator, not only in terms of signal transduction but also in terms of crosstalk with its counterpart, sHdrR.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox12081620/s1, Figure S1: Mass spectra for SoxR and variants with Cys-Ser exchanges; Table S1: Strains, plasmids, and primers; Table S2: Distribution of Sox systems and SoxR. References [8,9,42] are cited in Table S1.

Author Contributions: C.D. and J.L. conceptualized the study; J.L., K.T., J.K., T.S.T. and H.Y.H. performed experiments; T.S.T. performed bioinformatic analyses. C.D. supervised the work and acquired funding. C.D. and J.L. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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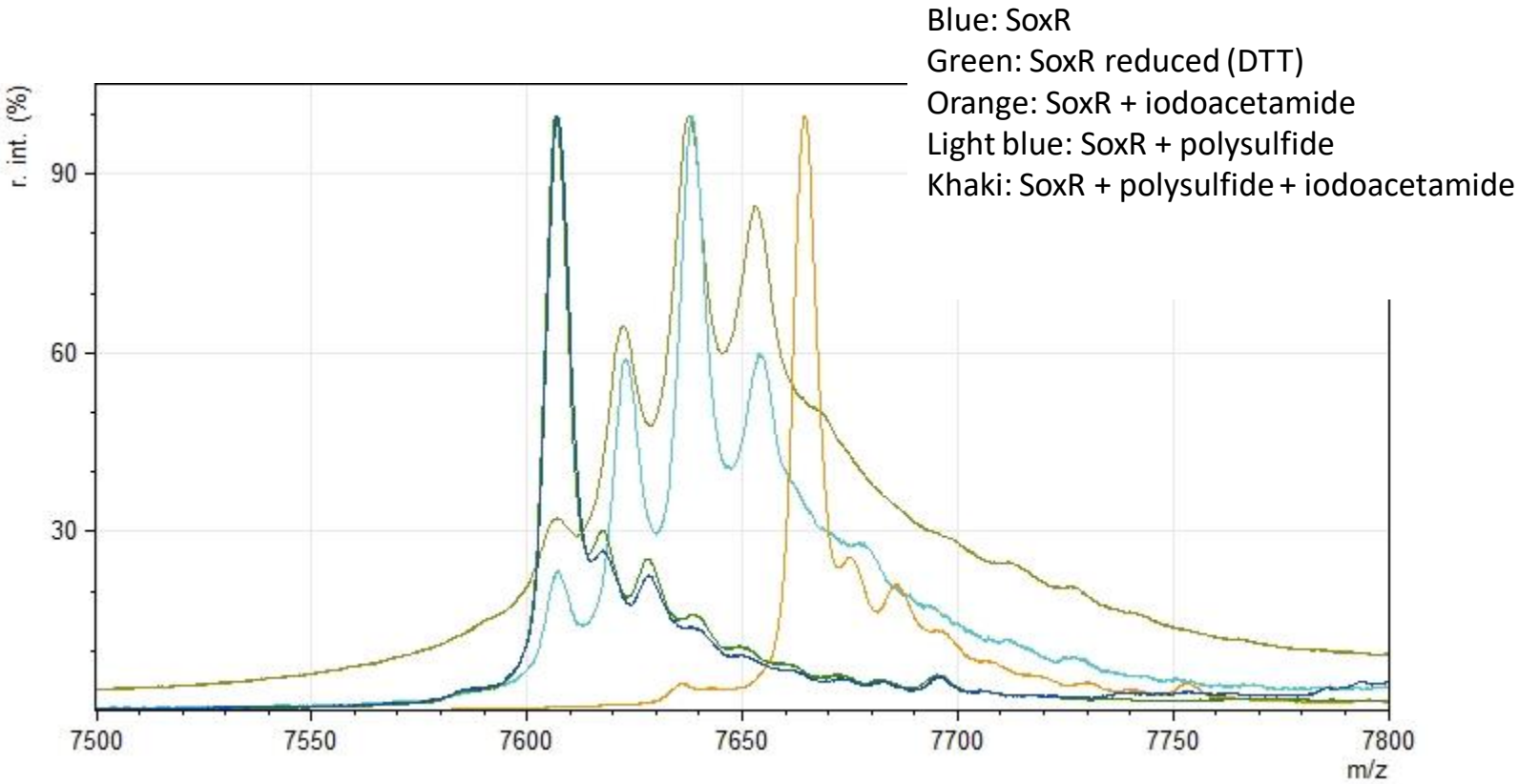
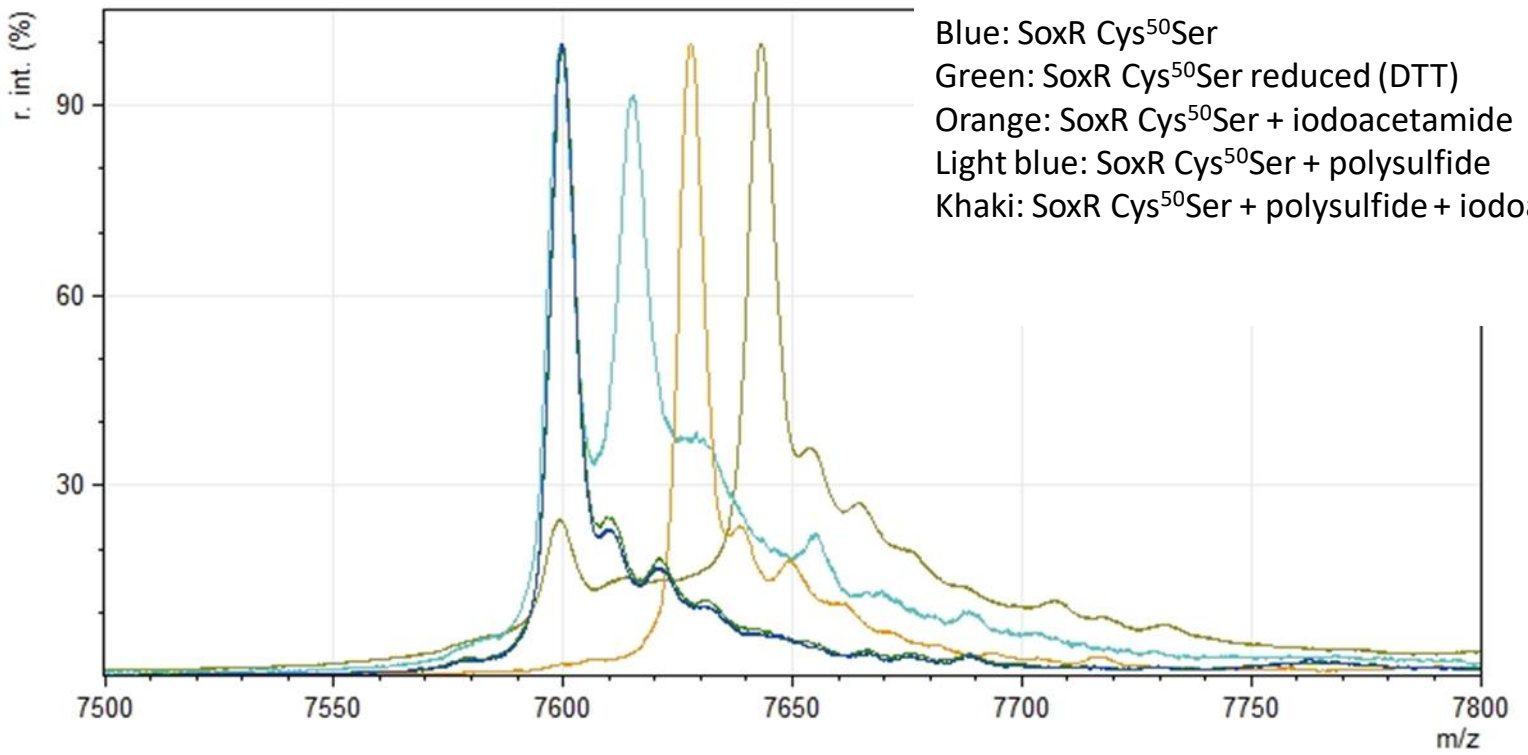
Figure S1. Mass spectra for SoxR and variants with Cys-Ser exchanges

SoxR wildtype



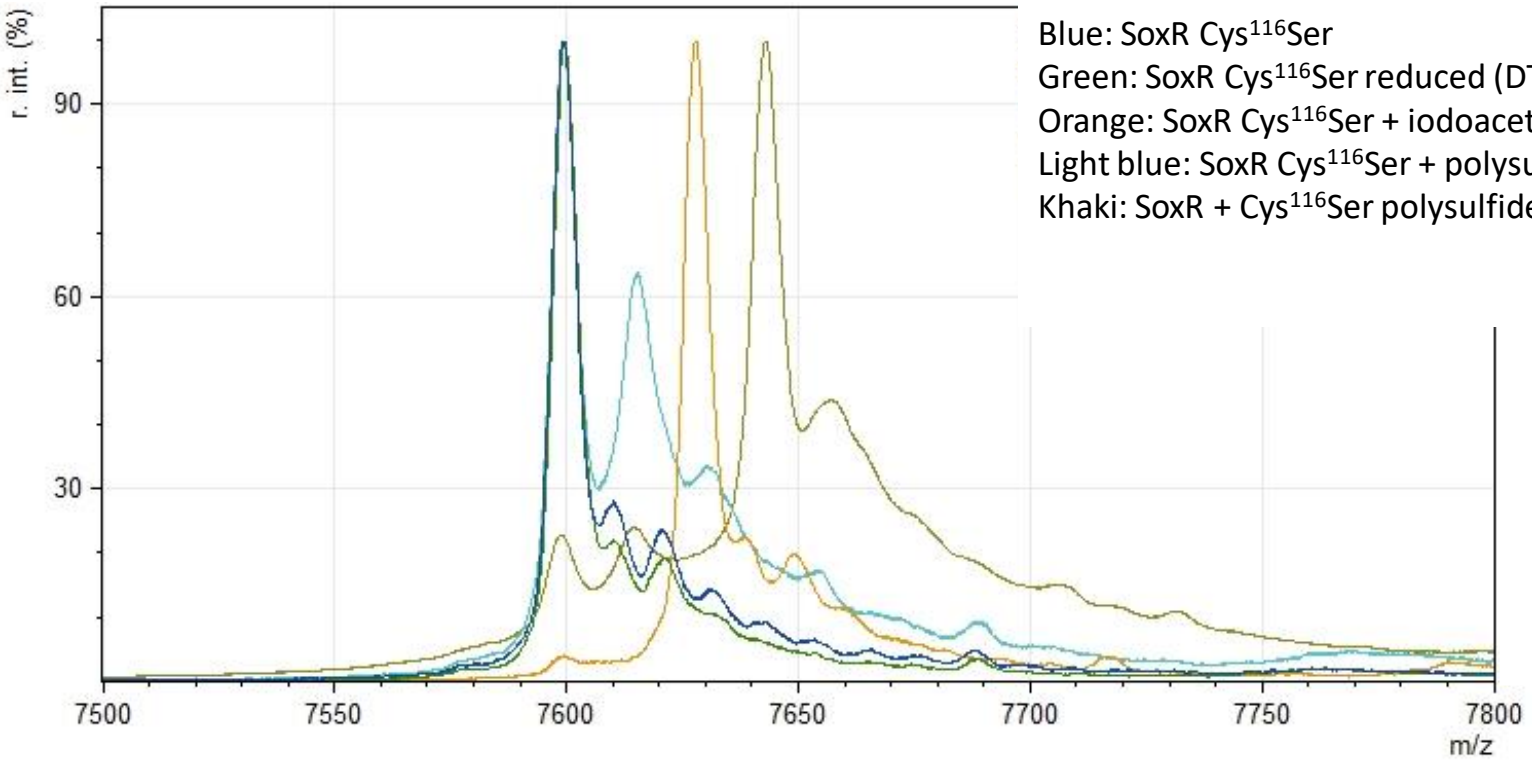
Masses are shown for the *mz*/2 species. Mass spectra obtained for the $CuCl_2$ oxidized protein were almost identical to those for the as isolated protein and are not shown for clarity.

SoxR Cys^{50}Ser



Masses are shown for the *mz*/2 species. Mass spectra obtained for the $CuCl_2$ oxidized protein were almost identical to those for the as isolated protein and are not shown for clarity.

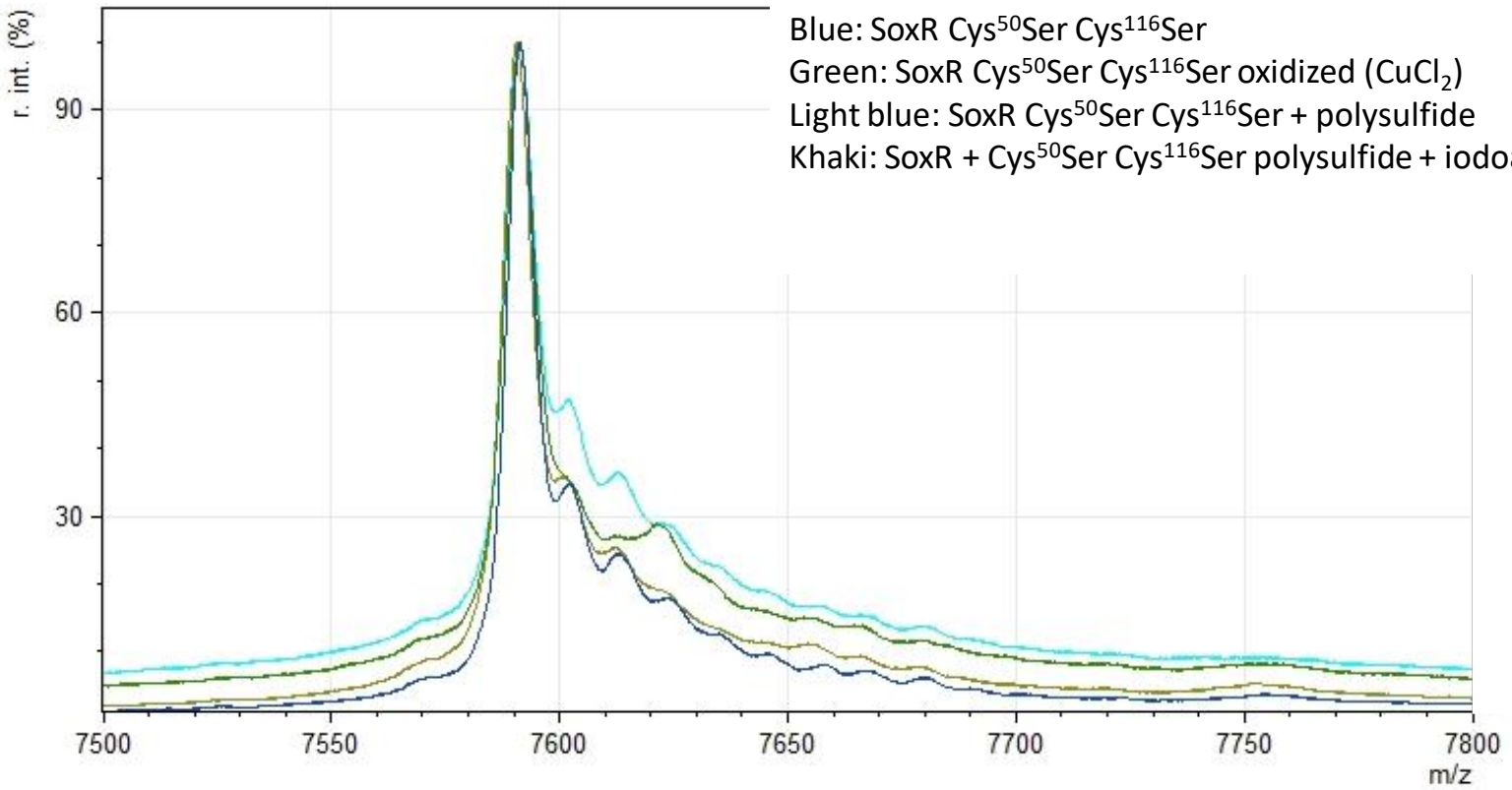
SoxR $Cys^{116}Ser$



Blue: SoxR $Cys^{116}Ser$
Green: SoxR $Cys^{116}Ser$ reduced (DTT)
Orange: SoxR $Cys^{116}Ser$ + iodoacetamide
Light blue: SoxR $Cys^{116}Ser$ + polysulfide
Khaki: SoxR + $Cys^{116}Ser$ polysulfide + iodoacetamide

Masses are shown for the *mz*/2 species. Mass spectra obtained for the $CuCl_2$ oxidized protein were almost identical to those for the as isolated protein and are not shown for clarity.

SoxR $Cys^{50}Ser$ $Cys^{116}Ser$



Blue: SoxR $Cys^{50}Ser$ $Cys^{116}Ser$
Green: SoxR $Cys^{50}Ser$ $Cys^{116}Ser$ oxidized ($CuCl_2$)
Light blue: SoxR $Cys^{50}Ser$ $Cys^{116}Ser$ + polysulfide
Khaki: SoxR + $Cys^{50}Ser$ $Cys^{116}Ser$ polysulfide + iodoacetamide

Masses are shown for the *mz*/2 species.

Table S1. Strains, plasmids and primers

Strains primers or plasmids	Relevant genotype, description or sequence	Reference or source
Strains		
E. coli 10-beta	*Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL* (Str^R) *rph spoT1 Δ(mrr-hsdRMS-mcrBC)*	New England Biolabs
E. coli DH5α	F$^-$ φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_{K^-}, m_{K^+}) *pho*A *sup*E44 λ$^-$*thi*-1 *gyr*A96 *rel*A1	New England Biolabs
E. coli BL21 (DE3)	F$^-$*omp*T *hsd*S_B (r_{B^-}, m_{B^-}) *gal dcm* (DE3)	Novagen
Hyphomicrobium denitrificans Δ*tsdA*	Sm^r, in-frame deletion of *tsdA* in *H. denitrificans* Sm200	[8]
Hyphomicrobium denitrificans Δ*tsdA* Δ*shdrR*	Sm^R, in-frame deletion of *shdrR* (Hden_0682) in *H. denitrificans* Δ*tsdA*	[9]
Hyphomicrobium denitrificans Δ*tsdA* Δ*soxR*	Sm^R, deletion of *soxR* (Hden_0700) in *H. denitrificans* Δ*tsdA*	This work
Primers		This work
EMSA-Fr	TTCCCGCCCCGTCTTGGTTT	[9]
EMSA_Fr2_Fr	TCAGCGCTCGCCTGGAAGTC	This work
EMSA_Fr3_Rev	TCTAAGCATCAACATATTCATATCTTTATATATTTTCG	This work
EMSA-Rev	AGGAGTTGCATCCAAAAAAGCGTG	[9]
EMSA-Hden_0703/04-fw	GGGTCACCAAATTCTGCAGGTCTC	This work
EMSA-Hden_0703/04-rev	ATCACGCCATCTCTCCCGGAA	This work
EMSA-Hden_0699/0698-fw	AATTCCACGGCTCCGCC	This work
EMSA-Hden_0699/0698-rev	TCGACAGCTTGCGGAAATCC	This work
EMSA-sHdrR-LipS1_F	TAGAGCGAGTCTTCAGC	This work
EMSA-sHdrR-LipS1_R	CGGCCCTCTGAGAAAAG	This work
EMSA-LipX-DsrE_F	GACTTCGCCGATCAATCGATC	This work
EMSA-LipX-DsrE_R	TGCCACCTCCCCGATATG	This work
rpoB-denitf	AGGACGTGTTCACCTCGATT	[42]
rpoB-denitr	CGGCTTCGTCAAGGTTCTTC	[42]
SoxT1A 0681_qPCR-Fr	CCCGAGTGATACGATTCGCA	This work
SoxT1A 0681_qPCR-Rev	CTAAAATGCCGCCGGTGATG	This work
LplA_qPCR-Fr	GGCCATGATCGATTTGCACC	This work
LplA_qPCR-Rev	CGAGATAAATTGCACCGCCG	This work
sHdrA_qPCR-Fr	CCGATCACCATTCCGTTCGA	This work
sHdrA_qPCR-Rev	CAATTGTTTCCGGGCCGATC	This work

sHdrB2_qPCR-Fr	GACGTGGCCTACTATTCCGG	This work
sHdrB2_qPCR-Rev	CCGCGACGACAGATAGGTTT	This work
LbpA2_qPCR-Fr	GGTTCCAAGAGCAGCCTGAT	This work
LbpA2_qPCR-Rev	TCGTTGATCTCCAGAACCGC	This work
SoxXA_qPCR-Fr	CGGCGCTCATTACCTATCTC	This work
SoxXA_qPCR-Rev	TCGGGGTGTCTTTTTCAGTC	This work
TusA_qPCR-Fr	TCTGACAGTTGATGCCAAGG	This work
TusA_qPCR-Rev	CGTTTCCTCATGTTCAAGCA	This work
CytP450_qPCR-Fr	CAATACGGTTCTCGGACGTT	This work
CytP450_qPCR-Rev	CATTCGTTTCCTGACGAGGT	This work
SoxT1B (0699)_qPCR-Fr	GCCGCCGTCTCAGTAAATAA	This work
SoxT1B (0699)_qPCR-Rev	AGCAGAAGACGGCAGATGAT	This work
SoxR_qPCR-Fr	TGAAGCGGACGAGGAAGTAT	This work
SoxR_qPCR-Rev	GAGACTGTGGGCTGGTTGAT	This work
sHdrR_qPCR-Fr	TTAGGAAGTCCGCATCGTCT	This work
sHdrR_qPCR-Rev	GCACTCGTTGCGCAATAATA	This work
SoxY_qPCR-Fr	GTTCAGCTTGCGGACTTTTC	This work
SoxY_qPCR-Rev	GCCAATCGTCACCTTCACTT	This work
P1 fwd up hden_0700	TATA**CTGCAG**GATCAAGGACGTGGTGGCG (PstI)	This work
P2 rev up hden_0700	CTCTCTATCGTTTGCGGCTCCATTCCTATCCCTCGGTCGC	This work
P3 fwd down hden_0700	GCGCACCGAGGGATAGGAATGGAGCCGCAAACGATAGAGAG	This work
P4 rev down hden_0700	GTAC**TCTAGA**ACGAACGCTGCCAGAAGCCC (XbaI)	This work
pET22 SoxR-Strep fw	TATA**CATATG**TGGAGCCACCCGCAGTTCGAGAAAGCTAGCTCGGGCATCTTGCCAAAC (NdeI)	This work
pET22 SoxR-Strep rev	TGCT**AAGCTT**CTATCGTTTGCGGCTCGGTT (HindIII)	This work
SoxR C(50)S_fwd	CTGATCCTCTCCCTGCTCGCTG	This work
SoxR C(50)S_rev	CAGGCGGGATTCGTGAGC	This work
SoxR C(116)S_fwd	GATAAGTTTTCCCGCGAGGAAC	This work
SoxR C(116)S_rev	GTAGATGGCGCCGATGAA	This work
Plasmids		
pET-22b(+)	Ap^r	Novagen
pET-22b-SoxR-N-Strep	Ap^r, NdeI/HindIII fragment of amplified SoxR in Nde/HindIII of pET	This work
pET-22b-SoxR $C^{50}S$	Ap^r, pET-22b-SoxR-N-Strep with a $Cys^{50}Ser$ exchange	This work
pET-22b-SoxR $C^{116}S$	Ap^r, pET-22b-SoxR-N-Strep with a $Cys^{116}Ser$ exchange	This work
pET-22b-SoxR $C^{50}S$ $C^{116}S$	Ap^r, pET-22b-SoxR-N-Strep with $Cys^{50}Ser$ ans $Cys^{116}Ser$ exchanges	This work

pK18*mobsacB*-Tc	Km^r, Tc^r pHP45ΩTc tetracycline cassette inserted into pk18*mobsacB* using SmaI	[9]
pK18*mobsacB*_Tc_Δ*soxR*	Km^r, Tc^r, 1.04 kb SOE PCR fragment implementing deletion of nucleotides 4 to 362 of *soxR* cloned into pk18mobsacB-Tc using PstI and XbaI restriction sites	This work

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Article

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YeeE-like bacterial SoxT proteins mediate sulfur import for oxidation and signal transduction

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Many sulfur-oxidizing prokaryotes oxidize sulfur compounds through a combination of initial extracytoplasmic and downstream cytoplasmic reactions. Facultative sulfur oxidizers adjust transcription to sulfur availability. While sulfur-oxidizing enzymes and transcriptional repressors have been extensively studied, sulfur import into the cytoplasm and how regulators sense external sulfur are poorly understood. Addressing this gap, we show that SoxT1A and SoxT1B, which resemble YeeE/YedE-family thiosulfate transporters and are encoded alongside sulfur oxidation and transcriptional regulation genes, fulfill these roles in the Alphaproteobacterium *Hyphomicrobium denitrificans*. SoxT1A mutants are sulfur oxidation-negative despite high transcription levels of sulfur oxidation genes, showing that SoxT1A delivers sulfur to the cytoplasm for its further oxidation. SoxT1B serves as a signal transduction unit for the transcriptional repressor SoxR, as SoxT1B mutants are sulfur oxidation-negative due to low transcription unless SoxR is also absent. Thus, SoxT1A and SoxT1B play essential but distinct roles in oxidative sulfur metabolism and its regulation.

The biogeochemical cycle of sulfur is primarily driven by prokaryotes, which reduce sulfate or sulfite in an anaerobic respiratory process to conserve energy[1]. Dissimilatory sulfur oxidizers maintain the cycle by oxidizing reduced sulfur compounds and using them as electron donors for energy conservation through respiration or photosynthesis[2,3]. Sulfide and thiosulfate ($S_2O_3^{2-}$) are common sulfur substrates in these organisms and in many cases their oxidation is initiated outside of the cytoplasm (if present in the bacterial periplasm). Further oxidative steps take place in the cytoplasm. This requires the import of sulfur into this cellular compartment[2,4–6]. In organisms that use reduced sulfur compounds as alternative or additional electron donors to organic compounds, transcriptional regulation of sulfur oxidation allows adaptation of metabolic flux to environmental conditions[7,8]. Sulfur transport across the cytoplasmic membrane is likely involved in the sensing and response to externally available reduced sulfur compounds. While intensive experimental work has been dedicated to elucidating the wide variety of redox reactions involved in prokaryotic sulfur oxidation[2,3], less effort has been devoted to clarifying the mechanisms of sulfur transport required for its use as an electron source or in the course of signal transduction. Uptake of sulfur compounds for assimilatory purposes, i.e., for the biosynthesis of sulfur-containing cell constituents, has been much better investigated and provides starting points for answering the many open questions.

Assimilation of sulfur is required for the growth of all living beings and prokaryotes obtain it either from inorganic sulfate or from organosulfur compounds such as sulfonates, sulfate esters, or sulfur-containing amino acids[9–13]. Transporters mediating the import of such precursors include a variety of ABC-type systems with solute-binding proteins as the primary determinants of transporter specificity[11]. The *Escherichia coli* CysUWA complex is a prime example for this concept. It takes up sulfate and thiosulfate as a sulfur source and acts in combination with periplasmic Sbp and CysP, respectively[9,12]. Recent work has shown that *E. coli* has an additional transporter, TsuA, which imports thiosulfate as a source of sulfur[14–16]. The protein belongs to the YeeE/YedE family (COG2391; DUF395) and has nine transmembrane helices. The structurally characterized protein from *Spirochaeta thermophila* contains three conserved cysteine residues that play a role in transport, probably through transient hydrogen bond mediated interaction with thiosulfate ions[14]. In *E. coli*, the soluble cytoplasmic protein TsuB (YeeD), that is encoded immediately adjacent to *tsuA*, is essential for thiosulfate uptake via TsuA[15,16]. TsuB is similar to, but cannot replace, TusA[17], which is a central sulfur hub in bacterial cells[18]. In the archaeon

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Methanococcus maripaludis a YedE-like protein is involved in transport of selenium, which is chemically similar to sulfur[19,20]. PmpA and PmpB from *Serratia* sp. ATCC39006 are other members of the YeeE/YedE family that have been predicted to transport sulfur-containing ions, albeit not for assimilatory purposes[21]. Similar proteins facilitate the uptake of extracellular zero-valent sulfur across the cytoplasmic membrane, thereby increasing cellular sulfane sulfur levels in bacterial cells[22].

Genes encoding YeeE/YedE-like proteins also occur together with genes for sulfur-metabolizing enzymes in sulfur-oxidizing prokaryotes. In the Alphaproteobacteria *Paracoccus pantotrophus* GB17^T (DSM2944^T), *Pseudaminobacter salicylatoxidans* KCT001, and *Hyphomicrobium denitrificans* X^T (DSM 1869^T), large *sox* gene clusters encoding the thiosulfate-oxidizing periplasmic Sox multienzyme system are accompanied by *soxT* genes encoding YeeE-like transporters[8,23–25]. In these organisms, *soxT* is located in a *soxSRT* arrangement. SoxR, a repressor protein, binds to the promoter-operator region of the *sox* operon and prevents transcription when sulfur compounds are absent[8,23,24]. This suggests a potential role in signal transduction for the membrane protein. In *Paracoccus denitrificans* PD1222 (DSM 104981), *Cereibacter sphaeroides* (formely *Rhodobacter sphaeroides*[26]), and *Roseovarius* sp. 217, the *sox* genes are flanked by two *soxT* genes[27]. We denote the one in the *soxRST* arrangement *soxT1* and term the other *soxT2*.

Two *soxT* genes are also found in *H. denitrificans*. This genetically tractable bacterium serves as a model for the elucidation of the cytoplasmic sulfur-oxidizing sHdr-LpbA pathway[5,7,28–31]. In *H. denitrificans*, thiosulfate oxidation starts in the periplasm, where the SoxXAB proteins work together to oxidatively conjugate thiosulfate to a conserved cysteine of the substrate-binding protein SoxYZ and release a sulfate molecule[2,7,32,33]. The second sulfur atom of the original thiosulfate molecule is by unknown means transferred to the cytoplasm, where it is oxidized to sulfite by the sHdr-LbpA system[5,6]. In *H. denitrificans*, the typical *soxSRT* arrangement resides immediately upstream of the genes for a TusA-like sulfur carrier protein and a putative cytochrome P450[7,8]. A second *soxT* gene is located downstream of the large set of genes that encode the enzymes for cytoplasmic sulfite formation and is transcribed divergently from them.

Here, we set out to decipher the function of the two different potential SoxT transporters in *H. denitrificans*. To this end, we collected information on the distribution and phylogeny of related transporters in sulfur-oxidizing prokaryotes and constructed a set of informative mutant strains lacking the transporter genes, the genes for two different transcriptional regulators, *soxR* and *shdrR*, and combinations thereof. Phenotypic characterization of the mutants and comparative analysis of transcription levels for relevant sulfur-oxidizing proteins finally allow functional assignments.

Results

Occurrence and phylogeny of YeeE/YedE-like proteins

Members of the YeeE/YedD family are found in organisms across a wide variety of metabolic pathways and prokaryotic phyla, both within the Archaea and the Bacteria[14,21,22,28]. As of March 2022, the Database of Clusters of Orthologous groups included complete genomes from 1187 bacteria and 122 archaea. Among the latter, YeeE-type proteins occur in *Saccharolobus* and *Sulfolobus* (Thermoproteota) and some representatives from the Thermoplasmatota. Among the bacteria, some YeeE-containing representatives are found in the phyla Actinobacteriota, Bacteroidota, Cyanobacteriota, Deinococcota, Bacillota, Spirochaetota, Verrucomicrobiota and Thermotogota, while there are many organisms with YeeE among the Pseudomonadota and the Desulfobacterota.

Conspicuously, the proteins of the YeeE family vary greatly in length. The structurally characterized *S. termophila* TsuA and relatives, as well as the SoxT proteins, share lengths of 330 to 350 aa, nine transmembrane helices and three conserved cysteines. In contrast, PmpA and PmpB, as well as their relatives[21,22], are much shorter, approximately 130 amino acids in length. They share four predicted transmembrane helices and one conserved cysteine residue. We re-evaluated the relationship between the long and short members of the family and found that PmpB and related proteins align perfectly with the N-terminal half of the full-length YeeE family members, while PmpA and relatives match with their carboxy-terminal half (Supplementary Fig. 1). PmpB contains one cysteine that is in the same position as the second conserved cysteine of *S. termophila* TsuA (Cys^{91}) and a PmpA cysteine matches the third conserved cysteine (Cys^{293}). Cys^{91} and Cys^{293} are indispensable for proper function of the *S. termophila* transporter[14]. The central transmembrane helix (H7 in *S. thermophila* TsuA) is not covered by the PmpAB sequences. We propose that PmpA and PmpB form a heterodimer and that together they perform functions similar to those of the YeeE proteins. The similarity of PmpA to PmpB suggests that they arose from a gene duplication. The two genes may then have fused and acquired an element encoding an additional transmembrane helix, resulting in the full-length YeeE family proteins.

As a first step towards a sequence-based grouping of YeeE-like proteins from dissimilatory sulfur-oxidizing bacteria, we created a phylogenetic tree including all YeeE-like transporters encoded in organisms containing the full set of *soxXABYZ* genes. The functionally characterized TsuA transporters from *E. coli* and *S. thermophila* were also included (Fig. 1). The tree reveals multiple paralogous groups with the TsuA proteins residing on a well separated branch. The most closely related group consists of SoxT2 proteins such as those encoded in close proximity to the *sox* genes in *C. sphaeroides, P. denitrificans* PD1222, and *P. salicylatoxydans*. SoxT1 proteins form another coherent clade, distant from the SoxT2 group. Both *soxT* genes from *H. denitrificans* are of the SoxT1 type and we term them SoxT1A (Hden_0681) and SoxT1B (Hden_0699).

Further information was obtained by screening all sulfur-oxidizing prokaryotes containing *shdr* genes for the presence of *soxT1*, *soxT2, sox* genes, genes for the sHdr-LbpA sulfur-oxidizing system and genes for the transcriptional repressors sHdrR[7] and SoxR[8] by HMSS2[34]. Clusters of genes encoding the sHdr-LbpA pathway for sulfane sulfur oxidation in the cytoplasm fall into two distinct categories. Type I and type II sHdr systems share the Fe/S flavoprotein sHdrA, the electron carrier protein sHdrC1 and the proposed catalytic subunit sHdrB1. The type I sHdrC2 and sHdrB2 polypeptides are encoded by a fused gene, *shdrB3* in the type II-containing organisms[5,6]. As evident from Fig. 2, SoxT transporters are only rarely present in genomes with the type I *shdr* genes and completely absent in genomes with type II sHdr, even though some of these organisms harbor the capacity for Sox-driven thiosulfate oxidation (see also Supplementary Data 1). In all genomes encoding the regulator SoxR, either SoxT1 or SoxT2 is present. The same is not true for the related repressor sHdrR. It does not always co-occur with SoxT1 or SoxT2. This is in line with a possible function for SoxT1 and/or SoxT2 in SoxR-dependent gene regulation, but contradicts a general role for the transporters in sulfur compound import. Nevertheless, sulfur import may be facilitated by either one of the transporters in a subset of sulfur oxidizers.

Regulation of *yeeE*-like genes in *Hyphomicrobium denitrificans*

H. denitrificans has the genetic potential for two distinct pathways of thiosulfate oxidation, both of which occur or begin in the periplasm. At 5 mM, all thiosulfate is converted to tetrathionate[28]. This reaction is catalyzed by thiosulfate dehydrogenase (TsdA), a periplasmic diheme cytochrome *c*. At 2.5 mM or less, most thiosulfate is oxidized to sulfite and eventually to sulfate. However, the formation of tetrathionate is not completely suppressed in wildtype *H. denitrificans* even at low substrate concentrations. This makes phenotypic characterization of mutant strains difficult[28,29]. Strains lacking thiosulfate dehydrogenase (Δ*tsdA*) are unable to form tetrathionate and are ideal for studying the processes involved in the complete oxidation of the substrate to sulfate[6–8,30]. Therefore, all experiments reported in this and also our previous studies on the transcriptional regulation of sulfur oxidation[7,8] were performed with a *H. denitrificans* Δ*tsdA* reference strain.

RT-qPCR provided initial evidence that SoxT1A and SoxT1B from *H. denitrificans* may be intricate components of the oxidation pathway and/or involved in its transcriptional regulation[8]. The transcript abundance for *soxT1A* increased more than tenfold upon addition of thiosulfate in the

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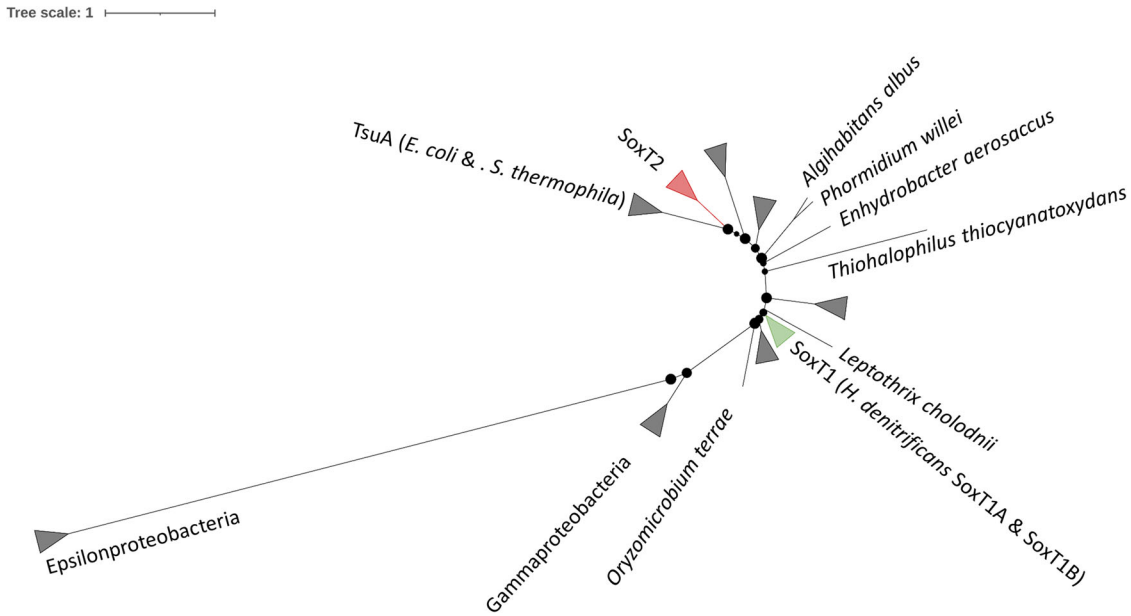

Fig. 1 | Unrooted phylogenetic tree for YeeE-like proteins in Sox-containing sulfur oxidizers. Groups of proteins encoded in or immediately adjacent to *sox* clusters (SoxT1 and SoxT2) are highlighted. SoxT1 includes both studied transporters from *H. denitrificans*. The TsuA group includes the thiosulfate uptake proteins from *E. coli* and *S. thermophila*[14,16]. The tree was calculated with 1000 bootstrap resamplings using Ultrafast Bootstrap[55] and IQ-Tree[57,58]. Bootstrap values between 50% and 100% are displayed as scaled circles at the branching points. Protein accession numbers and species names are available at https://github.com/WandaFlegler/Masterarbeit/blob/main/Galaxy3-%5BBMGE_Cleaned_sequences_Fasta%5D.fasta.treefile.

H. denitrificans Δ*tsdA* reference strain, while *soxT1B* expression remained essentially unaffected and thus similar to the expression of the genes for the transcriptional repressors *soxR* and *shdrR*[8]. Here, we extend these analyses with genome-wide mRNA-Seq data for the reference strain, comparing transcription in the absence and presence of 2 mM thiosulfate. Of the 3529 predicted genes, 3379 mRNAs (95.7%) were identified. The availability of thiosulfate affected the abundance of a total of 136 (4.1%) of the detected mRNAs (Supplementary Fig. 2, Supplementary Tables 1 and 2). In the presence of thiosulfate, mRNA transcripts of 47 genes showed lower mRNA abundance than in its presence (Supplementary Table 1), among them several genes for enzymes of fatty acid biosynthesis (acyl carrier protein (ACP), β-hydroxyacyl-ACP dehydratase, β-ketoacyl-ACP synthase) and assimilatory sulfate reduction (assimilatory sulfite reductase, sulfate adenylyltransferase).

mRNA transcripts of 89 genes (18 genes for hypothetical proteins) were more abundant in the presence of thiosulfate (Supplementary Table 2). The most affected gene (Hden_0834, YeiH) with a fold change of +526 (Supplementary Fig. 3a) encodes a putative efflux pump belonging to the PSE (Putative Sulfate Exporter) family (entry 2.A.98, Transporter Classification Database). The classification as a putative sulfate exporter is based on a study in *Paracoccus pantotrophus*, where 3-sulfolactate is converted to pyruvate and sulfite during the dissimilation of cysteate. It has been proposed that sulfite is oxidized to sulfate in the cytoplasm and then exported by the transporter, designated SuyZ in *P. pantotrophus*[35]. However, sulfite dehydrogenases of *Paracoccus* species are periplasmic enzymes and it is more likely that the transporter extrudes sulfite from the cytoplasm into the periplasm where it is then detoxified by oxidation to sulfate. A similar role may be played by YeiH in *H. denitrificans*, which not only closely resembles *P. pantotrophus* SuyZ at the amino acid sequence level (30% identity, 56.4% similarity), but also shares the same structural features (Supplementary Fig. 4). The second most affected gene (Hden_0835) encodes a LysR-type transcriptional activator and resides next to *yeiH* (Supplementary Table 3 and Supplementary Fig. 3a). Strong increases, up to 20-fold, were also observed for the transcripts from the *shdr-lbpA2-sox* locus. Those for *soxT1A* were among the top three (Fig. 3a). In full agreement with RT-qPCR analysis (Fig. 3b,c), the transcription of only three genes in the genomic sulfur oxidation region shown in Fig. 3a proved unaffected in the mRNA-Seq experiment, and these were the genes for the two transcriptional repressors, sHdrR and SoxR, and *soxT1B*. These results are consistent with previously published RT-qPCR analyses showing that transcription of these genes was barely affected by thiosulfate[8]. We thus state with confidence that in the Δ*tsdA* reference strain *soxT1A* expression increases substantially during thiosulfate oxidation, while *soxT1B* expression along with that of *soxR* and also *shdrR* does not change significantly. These findings are corroborated by RT-qPCR analysis for *H. denitrificans* strains Δ*tsdA* Δ*soxR*[8] and Δ*tsdA* Δ*shdrR*[7], which lack the individual repressor genes. In the repressor-negative strains, *soxT1A* expression is high even in the absence of thiosulfate, while *soxT1B* transcript abundance is not affected (Fig. 3b). When the strains deficient in one or the other transcriptional repressor are grown in the presence of thiosulfate, transcript abundance for *soxT1A* along with that for *shdrA* raises substantially above the level observed in the absence of the sulfur compound (Fig. 3c). This can be explained by the action of the remaining regulator protein in these strains, which requires the presence of an oxidizable substrate for full release of transcriptional repression. Note that the increase in transcript abundance for *soxT1B* was almost negligible (from 1.2 to 4.5 fold in the Δ*tsdA* Δ*soxR* and from 0.6 to 2.8 fold in the Δ*tsdA* Δ*shdrR* strain). Also note that the transcript levels for the tested genes are lower in the Δ*tsdA* Δ*shdrR* strain in the absence of thiosulfate (Fig. 3b) than in the *H. denitrificans* Δ*tsdA* reference strain in the presence of the sulfur substrate (Fig. 3c). This observation cannot be fully explained yet but is certainly related to the second regulator SoxR. This protein is still present in the Δ*tsdA* Δ*shdR* strain, and transcriptional repression is apparently not fully relieved by the absence of sHdrR alone. Experiments described further below confirm SoxR as the major regulator of sulfur oxidation in *H. denitrificans*. Clearly, the interplay of the two regulators needs to be investigated in more detail in the future.

Our mRNA-Seq analyses also yielded insight into the transcription of further *yeeE*-like genes in *H. denitrificans*, i.e., *pmpA* and *pmpB*. In contrast to *soxT1A*, the expression of *pmpA* and *pmpB* is not affected by the availability of thiosulfate (Supplementary Fig. 5). These genes attracted our

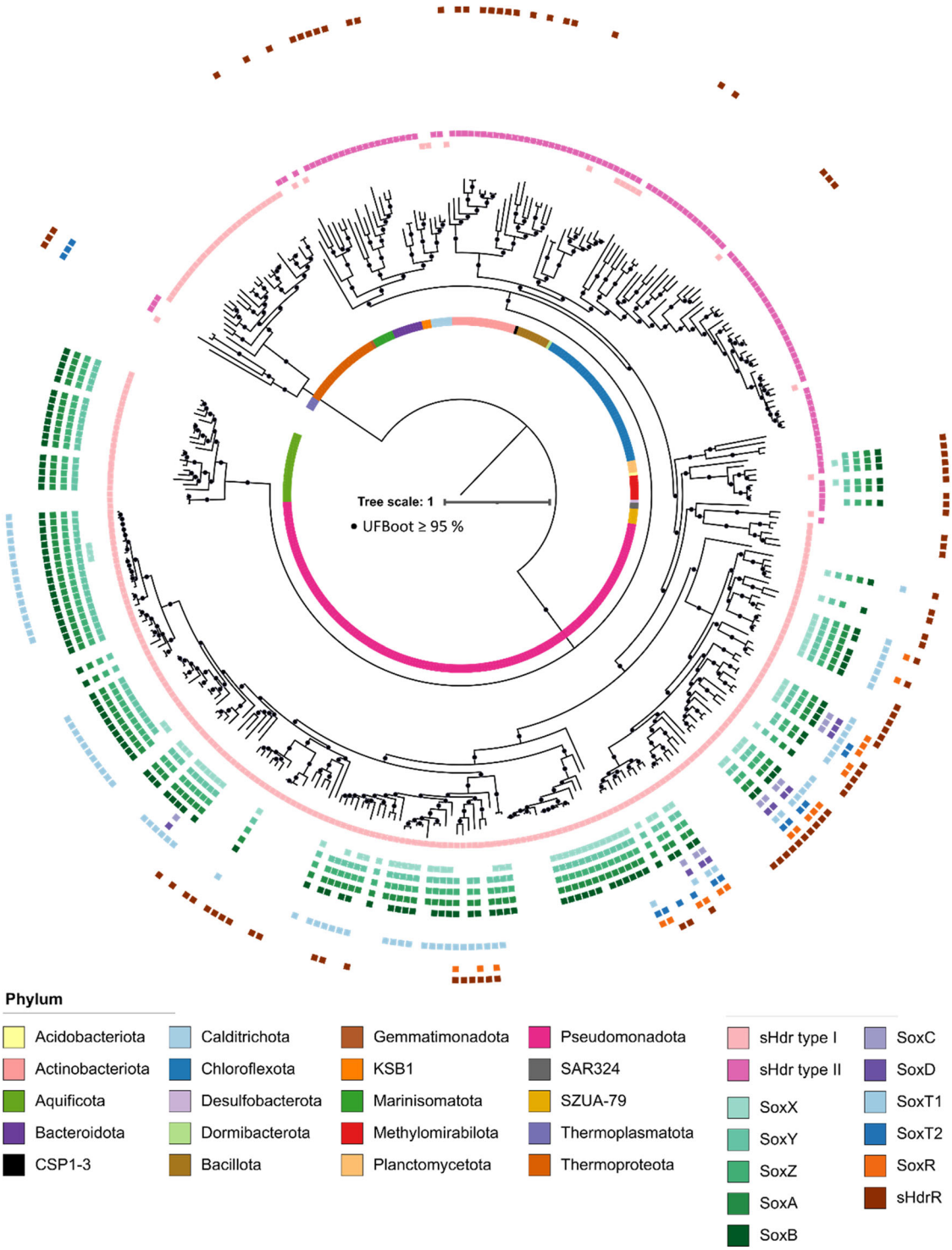

Fig. 2 | Distribution of SoxT transporters in sulfur oxidizers with sHdr systems. The distribution of genes for type I and type II sHdr systems, full Sox systems enabling complete oxidation of thiosulfate in the periplasm (SoxAXBYZCD), truncated Sox systems requiring formation of sulfite in the cytoplasm (SoxXABYZ), SoxT and two different transcriptional repressors, SoxR and sHdrR, is shown. In order to be classified as present, at least the proteins SoxA, SoxB, SoxY, and SoxZ had to be encoded in a syntenic gene cluster. The *sox* genes of species of the order Ectothiorhodospirales are not syntenic and were therefore assigned manually as described before[59]. A type I sHdr system was marked positive when the core genes *shdrC1B1AHC2B2* were present in a syntenic gene cluster. For assignment of a type II sHdr system, *shdrC1B1AHB3* and *etfAB* had to be present in a single syntenic gene cluster[6] The gene for the regulator sHdrR was only considered positive when located in or immediately next to a *shdr* gene cluster. The species tree was calculated as described before[6]. The data underlying the figure is provided in Supplementary Data 1.

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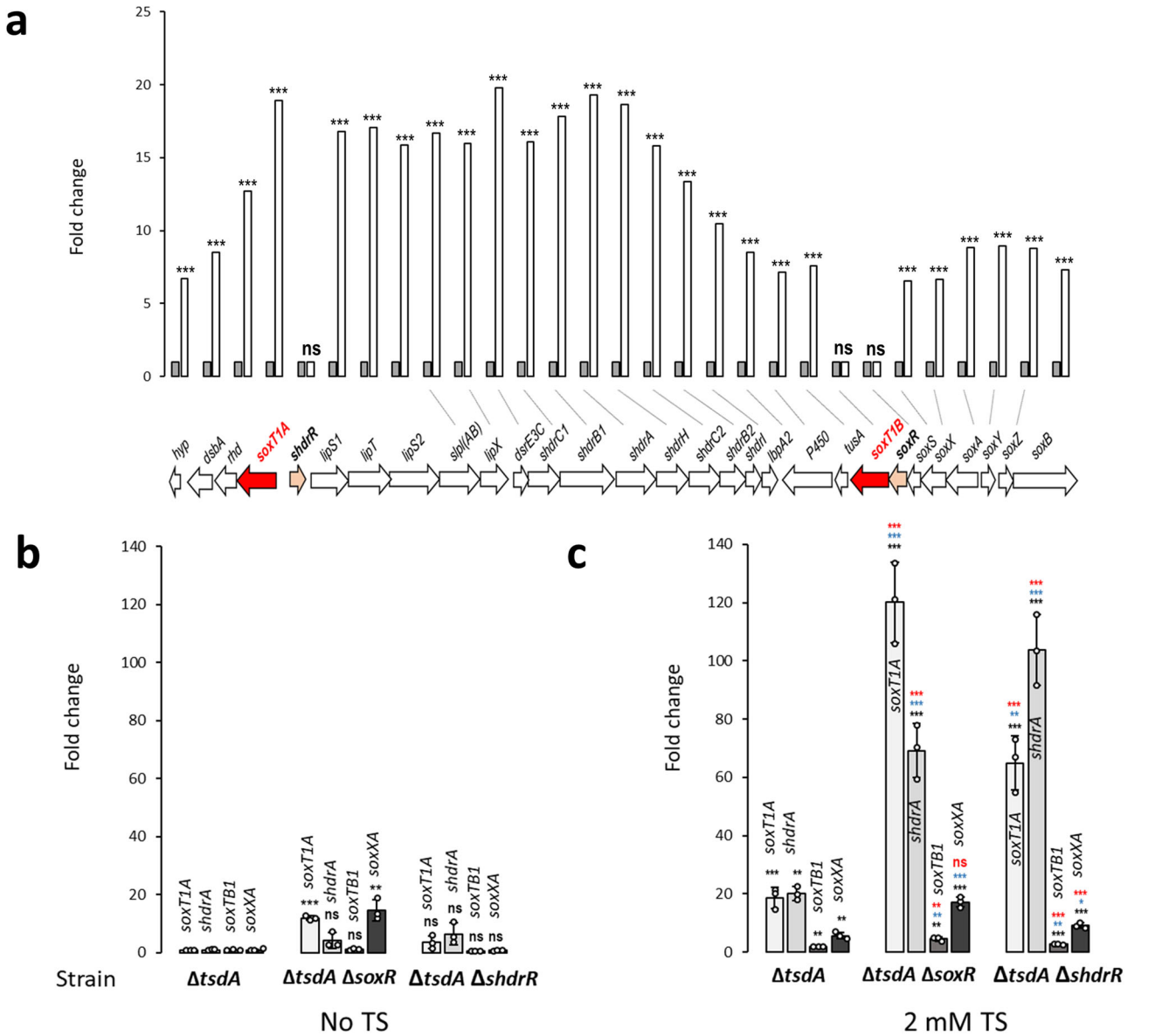

Fig. 3 | Thiosulfate-dependent regulation of gene expression in the *lip-shdr-lbpA-sox* locus in *H. denitrificans*. **a** Transcript abundance changes of genes encoding enzymes involved in thiosulfate oxidation in the *H. denitrificans* Δ*tsdA* reference strain as assessed by mRNA-Seq analysis. Columns in gray show the reference values for cells grown in the absence of thiosulfate, white columns apply to cells grown with 2 mM thiosulfate. The experiment was conducted in duplicate, each time using mRNA preparations from two different cultures. Adjusted *p* values for statistically significant changes were all below 0.001 (Supplementary Table 2) and are indicated by three asterisks (*** $p < 0.001$); ns, not significant. Relative mRNA levels of four indicative genes/combinations of genes located in the *shdr-sox* genetic region (depicted in **a**) from *H. denitrificans* for the Δ*tsdA* reference strain and the regulator deficient strains Δ*tsdA* Δ*soxR* and Δ*tsdA* Δ*shdrR* grown in the absence (**b**) or in the presence of 2 mM thiosulfate (**c**). All changes are compared to *H. denitrificans* Δ*tsdA* in the absence of thiosulfate. Results were adjusted using *H. denitrificans rpoB*, which encodes the β-subunit of RNA polymerase, according to[60]. All three strains grow equally well on methanol and are capable of thiosulfate oxidation[7,8]. Three parallel experiments were performed to obtain the averages and standard deviation. Data are presented as means ± SD. Individual data points are indicated. One-way ANOVA was performed to calculate *p*-values. Asterisks indicate statistically significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = ($p > 0.05$)). Color coding: black: differences compared to strain Δ*tsdA* in the absence of thiosulfate; blue, differences compared to Δ*tsdA* in the presence of thiosulfate; red, differences compared to the same strain in the absence of thiosulfate. TS thiosulfate.

attention because they are located in close proximity to genes encoding proteins that may be related to sulfur metabolism, such as a Sox(YZ) fusion and SoxH[36]. However, close inspection revealed that the gene ensemble rather encodes a PQQ-dependent enzyme for alcohol catabolism, its electron acceptor and an associated transport system (Supplementary Fig. 5). Since there is no evidence that PmpAB is involved in transport processes relevant to oxidative sulfur metabolism in *H. denitrificans*, they were not analyzed further.

Role of SoxT1B in *H. denitrificans*: gene inactivation, complementation, and cysteine exchanges

To clarify the role of SoxT1B, an *H. denitrificans* strain carrying an *in frame* deletion of the gene was constructed and phenotypically characterized. In addition, a complemented strain was investigated. Both strains grew equally well on methanol in the absence of thiosulfate (Supplementary Fig. 6a). While the deletion mutant proved negative with regard to thiosulfate oxidation, the complemented strain *H. denitrificans* Δ*tsdA soxT1Bcomp*

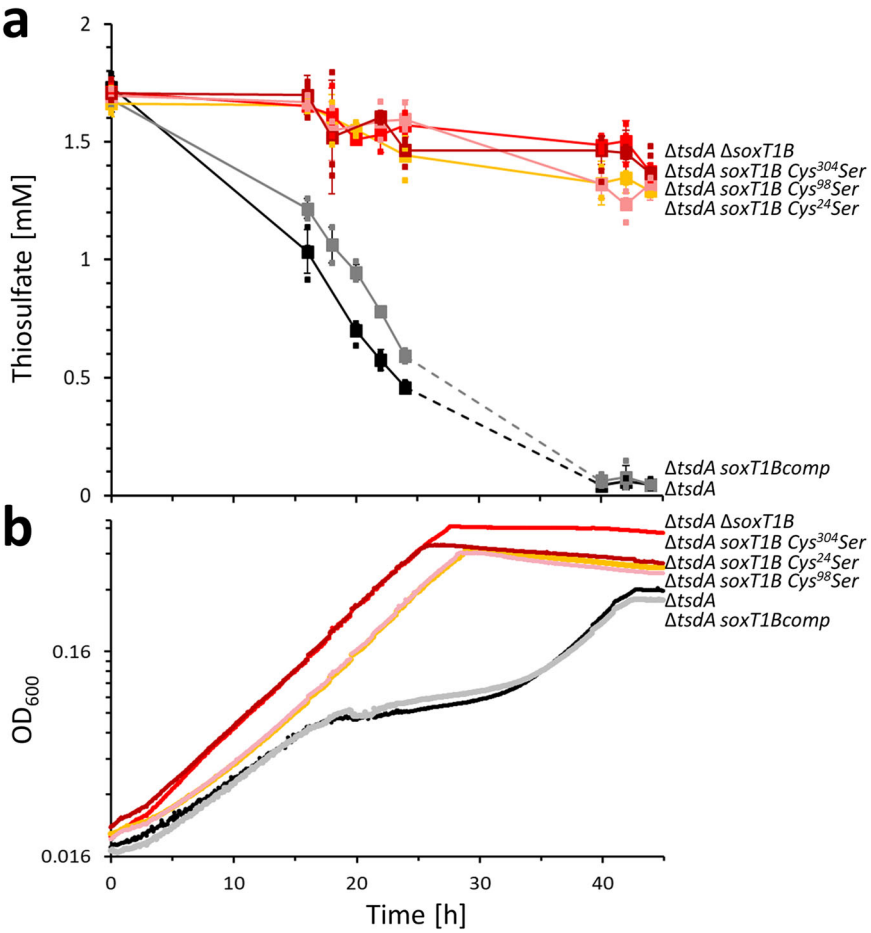

Fig. 4 | Growth and thiosulfate consumption for the *H. denitrificans* Δ*tsdA* reference strain compared with strains lacking *soxT1B* or producing SoxT1B with cysteine to serine exchanges. Thiosulfate consumption (**a**) and growth (**b**) of *H. denitrificans* Δ*tsdA* (black), Δ*tsdA* Δ*soxT1B* (bright red), Δ*tsdA soxT1Bcomp* (gray), Δ*tsdA soxT1B* $C^{24}S$ (orange), Δ*tsdA soxT1B* $C^{98}S$ (pink) and Δ*tsdA soxT1B* $C^{304}S$ (dark red). All strains were grown in 48-well microtiter plates as previously described[7] in medium containing 24.4 mM methanol and 2 mM thiosulfate. Precultures contained 2 mM thiosulfate. In (**a**), data were measured using $n = 3$ experiments and are presented with the individual measurements (small symbols) and as the mean value of these measurements ± SD (big symbols). In (**b**), representative experiments of biological duplicates are shown. Source data are provided as Source Data 2.

oxidized thiosulfate with the same rate as the reference strain (Fig. 4a). When the *H. denitrificans* Δ*tsdA* reference strain is grown with thiosulfate as an additional electron source, it excretes toxic sulfite[7], which causes growth retardation (Fig. 4b). In line with the thiosulfate oxidation capabilities of the studied strains, growth retardation was not observed for *H. denitrificans* strain Δ*tsdA* Δ*soxT1B* and returned upon complementation *in cis* of the Δ*soxT1B* deletion strain with an intact copy of the *soxT1B* gene (Fig. 4b).

As a proof of principle, the three cysteine residues conserved in the YeeE and SoxT proteins (Supplementary Fig. 1) were individually replaced by serine through site directed mutagenesis of the chromosomal *H. denitrificans soxT1B* gene. All three strains encoding variants of SoxT1B with cysteine to serine substitutions showed no growth retardation in the presence of thiosulfate and were unable to oxidize the sulfur compound, confirming the essentiality of these residues (Fig. 4).

Role of SoxT1B in *H. denitrificans*: interaction with transcriptional regulators

In principle, the thiosulfate oxidation-negative phenotype of the *H. denitrificans* Δ*tsdA* Δ*soxT1B* strain can be explained by two fundamentally different functions of the membrane protein: (1) Either it is essential for import of oxidizable sulfur into the cytoplasm or (2) it is essential for signal transduction, informing one or both transcriptional repressors about the presence of external thiosulfate. In the latter case, simultaneous removal of the genes for the signal transducing membrane protein and the transcriptional repressor should allow thiosulfate oxidation, because transcription of the relevant genes would no longer be blocked. A signal-transducing unit would be dispensable in this case. On the other hand, if SoxT1B were responsible for import of oxidizable sulfur, it should be essential for thiosulfate oxidation even when the genes for other components of the sulfur-oxidizing machinery are constitutively expressed.

To differentiate between these possibilities, the transcription of indicator genes was compared by RT-qPCR in the absence versus the presence of thiosulfate. We chose the genes *soxXA* and *shdrA* because they encode central components of thiosulfate oxidation in the periplasm and sulfane sulfur oxidation in the cytoplasm, respectively. The transcription of *soxT1A* and *soxT1B* was also followed. While thiosulfate increases transcript abundance for *soxT1A*, *shdrA* and *soxXA* in the *H. denitrificans* reference strain[8], this is not the case for the strain lacking the *soxT1B* gene (Fig. 5). The thiosulfate oxidation-negative phenotype of this strain is therefore explained by a lack of enzymes required for the degradation of the sulfur substrate. When the gene for the SoxR regulator was deleted together with Δ*soxT1B* from *H. denitrificans* Δ*tsdA*, this resulted in a thiosulfate oxidation-positive phenotype. Transcription was high for *soxT1A, shdrA* and *soxXA* irrespective of the presence of thiosulfate (Fig. 5). The constitutive expression of these genes is caused by the lack of the transcriptional repressor. In the next step, a strain was constructed that lacks genes *soxT1B* and *shdrR*. This strain behaves differently from *H. denitrificans* Δ*tsdA* Δ*soxT1B* Δ*soxR*. It cannot oxidize thiosulfate and the substrate does not induce substantial increase of transcript abundance of the tested sulfur oxidation genes (Fig. 5). SoxR is present in this strain and we suggest that it acts as the major regulator, that prevents transcription even in the presence of thiosulfate when the signal-transducing SoxT1B is not available. This conclusion is particularly true for the bona fide *sox* genes that encode the enzymes that initiate thiosulfate oxidation in the periplasm, i.e., SoxXA, SoxB, and SoxYZ. Note that the *soxXA* transcript abundance in the Δ*tsdA* Δ*soxT1B* Δ*shdrR* strain is low (on the same level as in the reference strain grown in the absence of thiosulfate) and not affected by thiosulfate, as also observed for the Δ*tsdA* Δ*soxT1B* strain. This not only fully explains the inability of the strains to grow on thiosulfate but also indicates that sHdrR plays a subordinate role in the transcription of the *sox* genes.

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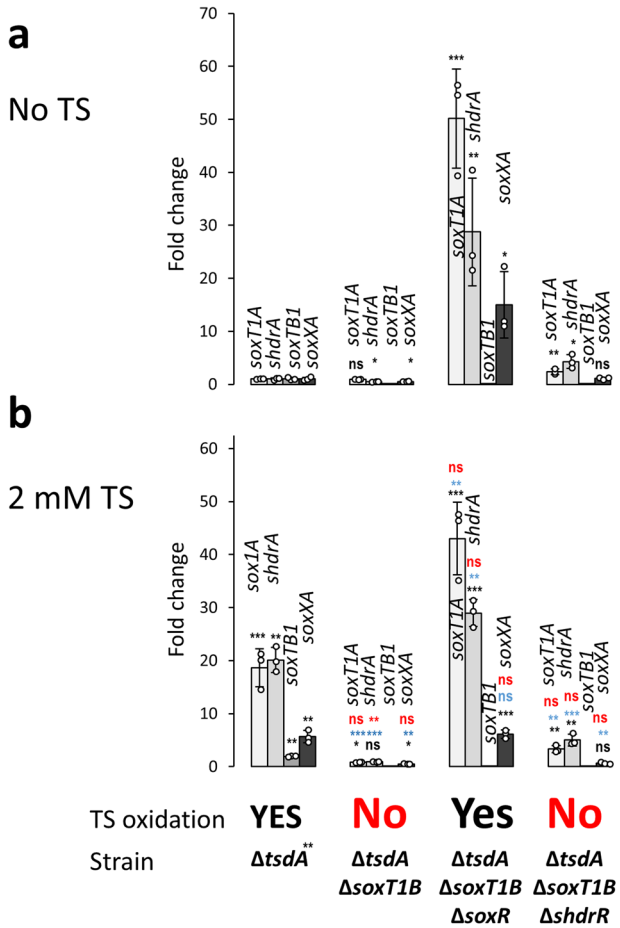

Fig. 5 | Transcription of sulfur oxidation genes in *H. denitrificans* strains lacking *soxT1B*. RT-qPCR analysis is shown for four indicative genes in three different *H. denitrificans* Δ*soxT1B* strains in the absence (**a**) and in the presence of 2 mM thiosulfate (**b**). The ability of the strains to oxidize thiosulfate is indicated. The growth experiments are shown in full in Supplementary Fig. 7. All strains grew equally well on methanol in the absence of thiosulfate (Supplementary Fig. 6a). Three parallel experiments were performed to obtain the averages and standard deviation. Data are presented as means ± SD. Individual data points are indicated. One-way ANOVA was performed to calculate *p*-values. Asterisks indicate statistically significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = ($p > 0.05$)). Color coding: black: differences compared to strain Δ*tsdA* in the absence of thiosulfate; blue, differences compared to Δ*tsdA* in the presence of thiosulfate; red, differences compared to the same strain in the absence of thiosulfate. TS thiosulfate, ns not significant.

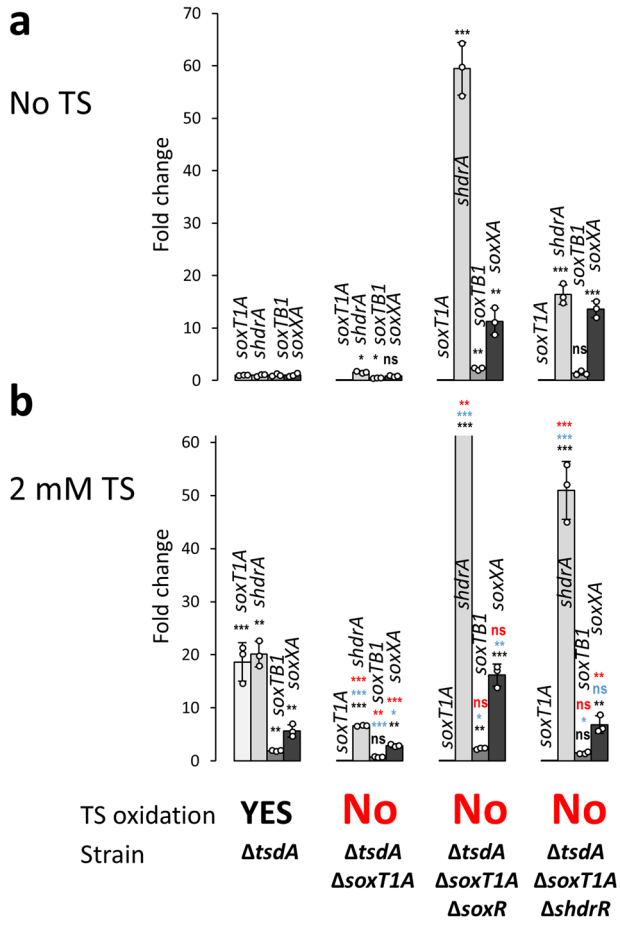

Fig. 6 | Transcription of sulfur oxidation genes in *H.denitrificans* strains lacking *soxT1A*. RT-qPCR analysis for four indicative genes in three different *H. denitrificans* strains in the absence (**a**) and in the presence of 2 mM thiosulfate (**b**). Note that the fold change for *shdrA* transcript abundance was 201.7 ± 35.5 and thus far exceeds the y-axis range appropriate for all other cases. The ability of the strains to oxidize thiosulfate is indicated. The growth experiments are shown in full in Supplementary Fig. 7. All strains grew equally well on methanol in the absence of thiosulfate (Supplementary Fig. 5b). Three parallel experiments were performed to obtain the averages and standard deviation. Data are presented as means ± SD. Individual data points are indicated. One-way ANOVA was performed to calculate *p*-values. Asterisks indicate statistically significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = ($p > 0.05$)). Color coding: black: differences compared to strain Δ*tsdA* in the absence of thiosulfate; blue, differences compared to Δ*tsdA* in the presence of thiosulfate; red, differences compared to the same strain in the absence of thiosulfate. TS thiosulfate, ns not significant.

In conclusion, the described experiments show that SoxT1B is dispensable for thiosulfate oxidation and that the import of sulfur for further oxidation is not its primary function. Instead, all results are consistent with a signal transduction function.

Role of SoxT1A in *H. denitrificans*

Like SoxT1B, the related membrane protein SoxT1A could in principle act either as an importer of sulfur for further oxidation in the cytoplasm or as a means of transmitting the information that oxidizable sulfur is available externally. To decide between the two possibilities, the strain *H. denitrificans* Δ*tsdA* Δ*soxT1A* was constructed, phenotypically characterized and studied concerning *shdr* and *sox* gene transcription (Fig. 6). The strain proved to be thiosulfate oxidation negative and accordingly did not show any growth retardation in the presence of thiosulfate (Supplementary Fig. 8), although transcript abundance for *shdrA* and *soxXA* increased significantly in the presence of thiosulfate (Fig. 6).

Further insights were obtained when *H. denitrificans* strains Δ*tsdA* Δ*soxT1A* Δ*soxR* and Δ*tsdA* Δ*soxT1A* Δ*shdrR* were studied. Both strains show a very high transcript abundance for *soxXA* and *shdrA* in the absence as well as in the presence of thiosulfate. Thus, in principle, sufficient Sox and sHdr proteins are present in SoxT1A-deficient strains to allow thiosulfate oxidation. Still, all SoxT1A-deficient strains are unable to oxidize thiosulfate, suggesting an essential function of SoxT1A in the overall sulfur oxidation pathway.

Remarkably, transcript level increases for *shdrA* and *soxXA* in *H. denitrificans* Δ*tsdA* Δ*soxT1A* caused by the presence of thiosulfate appear low, 6.62 ± 0.13 and 2.92 ± 0.36 fold, respectively, when compared to the *soxT1A*-deficient strains that additionally lack either one of the studied regulator genes (Fig. 6). It is important to note that the increases determined for the Δ*tsdA* Δ*soxT1A* strain are in a similar range though not exactly the same as observed for the Δ*tsdA* reference strain by RT-qPCR (20.11 ± 2.39 fold and 5.68 ± 1.18 fold for *shdrA* and *soxXA*, respectively, Fig. 3c) and in our mRNA-Seq experiments (19.3 and 6.7–8.9 fold, Supplementary Table 2). Increasing transcript abundance in this moderate range allows

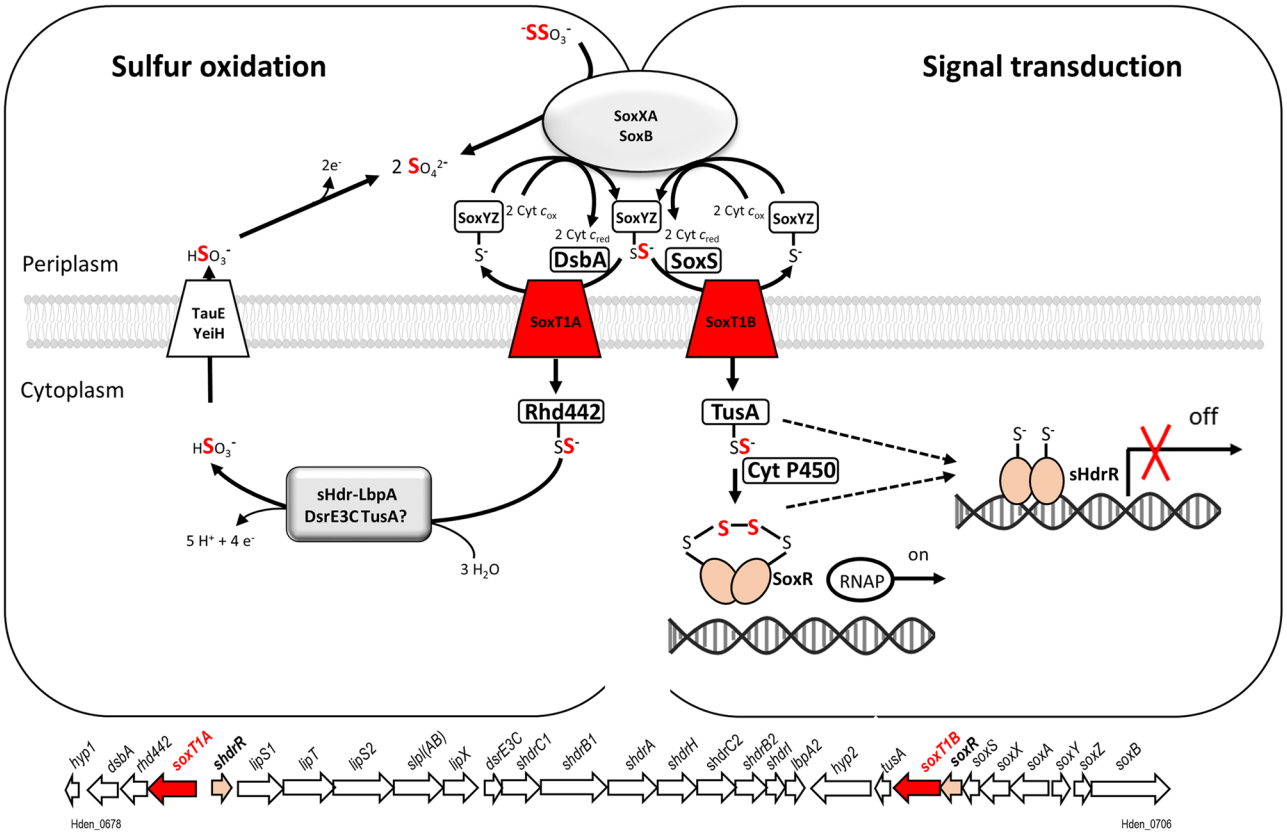

Fig. 7 | Model of thiosulfate oxidation in *H.denitrificans* integrating the sulfur transport and signal transduction functions of SoxT1A and SoxT1B, respectively. TauE, putative sulfite exporter encoded by Hden_0720[28]. Transcripts are 2.6-fold more abundant when thiosulfate is present (Supplementary Table 1). YeiH (Hden_0834) is another candidate for sulfite export, with increased transcript abundance in the presence of oxidizable sulfur. The *lipS1,lipT, lipS2, slpl(AB)* and *lipX* genes encode enzymes that assemble the cofactor on the lipoate-binding protein LbpA2[30]. RNAP, RNA polymerase. Sulfur atoms printed in red stem from the sulfane sulfur atom of thiosulfate, the oxidation of which is initiated in the periplasm. sHdrH and sHdrI are soluble, cytoplasmic proteins of unknown function. *hyp1*, encodes a56-aa transmembrane protein; *hyp2* encodes a putative cytochrome P450.

thiosulfate oxidation. However, if one of the repressors is missing, the whole system apparently loses its balance resulting in extremely high transcription levels (Fig. 6) and thus energy-intensive biosynthesis of the sulfur-oxidizing system including a complete assembly machinery for its specific lipoate-binding proteins. Further studies are needed to elucidate how exactly the fine-tuning of transcription rates to metabolic needs is achieved in the reference strain, whether SoxT1A has a share in the signaling process, even if sulfur import appears to be its major function, and whether transcriptional regulators other than SoxR and sHdrR are involved in this process. The latter seems likely since the transcription of genes for LuxR, LysR, and Fur-type regulators is significantly affected by the presence of thiosulfate (Supplementary Table 2 and Supplementary Fig. 3).

Discussion

Here, we provide information on the distribution and phylogeny of YeeE-like transporters in sulfur-oxidizing prokaryotes and even more importantly, we assign fundamentally different functions to two of these proteins, SoxT1A and SoxT1B, that co-occur in the same Alphaproteobacterium, *H. denitrificans*. The completely different regulation of the respective genes upon exposure of the organism to thiosulfate is the first milestone for functional assignment. Expression of *soxT1A* is highly increased, while *soxT1B* expression is hardly affected at all by the presence of the reduced sulfur compound. All of our observations are consistent with the central role of SoxT1A in sulfur oxidation. The amount of SoxT1A molecules in the cells is increased to ensure efficient import of sulfur into the cytoplasm where it is further processed by the sHdr-LbpA system (Fig. 7). To the best of our knowledge, *H. denitrificans* SoxT1A is the only experimentally demonstrated sulfur importer in dissimilatory sulfur-oxidizing prokaryotes. However, it does not provide a general solution because it not even occurs in all sulfur oxidizers using the cytoplasmic sHdr pathway. SoxT transporters are completely absent genomes with type II sHdr, even though some of these organisms harbor the capacity for Sox-driven thiosulfate oxidation (Fig. 2).

SoxT1B functions as a signal transducing module. The same function can be assumed for the SoxT proteins in Alphaproteobacteria with complete Sox systems. In these organisms, thiosulfate is completely oxidized to sulfate in the periplasm and accordingly they lack cytoplasmic sulfur-oxidizing enzymes. As a consequence, there is no need for mass import of sulfur as carried out by SoxT1A. In full agreement with these conclusions, a function of SoxT from *Pseudaminobacter salicylatoxidans* in the transport of an inducer to the cytosol to activate the transcriptional regulator SoxR has been suggested[37].

The genetic neighborhood of the *soxT1A* and *soxT1B* genes provides a basis for a model of how sulfur might be presented to the transporters, transported through them, and delivered to their final targets (Fig. 7). In immediate vicinity to and in the same direction of transcription with *soxT1A*, a gene (Hden_0679) is located that encodes a periplasmic DsbA-like thioredoxin with two thioredoxin-like cysteine motifs (Cys-X_2-Cys), one of which resides at the very carboxy-terminal end of the protein. Thioredoxins serve as general protein disulfide oxidoreductases that interact with a broad range of proteins by a redox mechanism based on reversible oxidation of two cysteine thiol groups to a disulfide, accompanied by the transfer of two electrons and two protons (IPR013766). We consider the possibility that the *H. denitrificans* DsbA is involved in the release of sulfane sulfur from the persulfidated periplasmic sulfur carrier SoxYZ and

that the sulfur is then transferred into the cytoplasm through SoxT1A. In the cytoplasm, the sulfur is further handled by cytoplasmic Rhd442 (Hden_680), a protein that we recently characterized as a rhodanese-like sulfur transferase[6]. From there, the sulfur is delivered to the sulfur transferase DsrE3C and finally oxidized to sulfite by the sHdr-LbpA system, possibly involving TusA[5,6]. Hden_0678 encodes short 56 aa membrane protein, lacking cysteine residues and consisting of one central transmembrane helix (aa 12 to 27) with the N-terminus predicted to reside in the cytoplasm. Functional assignment is currently not possible.

The genes in the vicinity of *soxT1B* appear to encode a second module dedicated to the transport of sulfur, albeit for a different purpose. As suggested earlier, it is conceivable that sulfur bound to the sulfur carrier protein SoxYZ is in this case presented to the transporter by the periplasmic thiol–disulfide oxidoreductase SoxS[38]. In fact, SoxS from *P. denitrificans* specifically binds SoxY[38]. Once in the cytoplasm, the sulfur transferase TusA[6,18] is a likely acceptor protein for the sulfur. This idea is corroborated by recent findings for the *E. coli* thiosulfate transporter TsuA[14,16]. TsuA belongs to same family as the SoxT transporters and the TusA-like TsuB protein was shown to be essential for TsuA mediated thiosulfate uptake in vivo. TsuB can cleave thiosulfate resulting in persulfidation of its conserved cysteine and the release of sulfite. In *H. denitrificans*, sulfur atoms could be passed on from TusA to either one or both of the transcriptional repressors encoded in the *shr-lbpA-sox* genomic region. For SoxR, we showed that it forms an intramolecular sulfur bridge between two conserved cysteines[8]. The formation of this bridge is the trigger to detach from its target DNA and thus to enable transcription. We assume that sHdrR, which closely resembles SoxR[8], functions accordingly. Whether SoxR and/or sHdrR are indeed loaded with sulfur in a reaction mediated by TusA or rather directly by the sulfur species transported through SoxT1B, cannot be answered on the current data basis.

We believe that it is advantageous for *H. denitrificans* to have the genetic potential for two different SoxT1 modules, one dedicated to signal transduction and one dedicated to import sulfur for further oxidation. Only a few SoxT1B protein molecules have to be present to serve their purpose in a signaling cascade. On the other hand, cells probably have to be richly equipped with SoxT1A molecules that have to ensure mass transport of sulfur as part of energy conservation. Separating the two functions has the advantage that large quantities of SoxT1A are only synthesized when they are really needed.

The exact chemical nature of the sulfur species transported by SoxT1A and SoxT1B requires further investigation, although both transporters are structurally similar to the characterized *S. thermophila* thiosulfate transporter TsuA (YeeE) and share the substrate binding pocket (Supplementary Fig. 9). The substrate for TsuA (YeeE) is thiosulfate. At present, we cannot rule out the possibility that in *H. denitrificans* a small fraction of the thiosulfate available for oxidation is itself used as a signal molecule, channeled through SoxT1B and then cleaved by TusA, as proposed for TsuA. In mutant strains lacking SoxT1A, sulfane sulfur oxidation in the cytoplasm is halted because there is no supply of substrate, thus causing inability to oxidize thiosulfate altogether. Notably, in *ΔsoxT1A* strains higher transcription is observed for *shdrA* and *soxXA* in the presence than in the absence of thiosulfate (Fig. 6), pointing at thiosulfate itself as the signal molecule. However, other results presented in Fig. 6 are not fully in line with this possibility: Transcript levels of *shdrA* and *soxXA* in the *H. denitrificans ΔsoxT1A* strain in the presence of thiosulfate are lower than those in the Δ*soxT1A* Δ*soxR* strain, although the signal transduction pathway is intact in that mutant. If the signaling molecule transported by SoxT1B were thiosulfate, full release of transcriptional repression would be expected. On the other hand, unknown layers of regulation may be involved in the fine tuning of *shdr* and *sox* gene transcription and it appears premature to draw conclusions about the transported compounds based on the results thus far available.

Concerning SoxT1A, thiosulfate can be excluded as its substrate. In *H. denitrificans*, thiosulfate degradation is initiated in the periplasm, where it is attacked by the periplasmic proteins of the truncated Sox system. SoxXA and SoxB release sulfate and SoxYZ-bound sulfane sulfur, which then has to be further processed in the cytoplasm. Thiosulfate oxidation is not possible in the absence of SoxXA and SoxYZ[7], proving that thiosulfate cannot be taken up and processed in the cytoplasm.

In fact, a transport by passing sulfur from the SoxY cysteine along the three cysteines lining the central channel of the SoxT1 proteins is conceivable. Ikei and coworkers suggest that interaction of thiosulfate with the cysteine residues occurs via S—H—S hydrogen bonds[16]. The three cysteine residues in TsuA (YeeE) are linearly located at intervals of ~7 Å, while disulfide bonds are usually about 2.05 Å in length, and 3.0 Å is taken as the cutoff for disulfides in the PDB database. It is therefore questionable whether sulfur atoms can be directly transferred from one cysteine sulfur to the next. Free HS^- ions or short polysulfides ($^-S\text{-}S_n\text{-}S^-$), that are possibly formed by the action of the periplasmic protein disulfide oxidoreductases DsbA and SoxS, are alternatives and conceivable substrates for cytoplasmic sulfur transferases such as Rhd442 or TusA. Even a direct reaction of polysulfides with the transcriptional repressors, as occurs in vitro[8], is conceivable.

Methods

Bacterial strains, plasmids, primers, and growth conditions

Supplementary Table 3 lists the bacterial strains, and plasmids that were used for this study. *Escherichia coli* strains were grown on complex lysogeny broth (LB) medium[39]. *E. coli* 10β was used for molecular cloning. *H. denitrificans* strains were cultured in minimal medium kept at pH 7.2 with 100 mM 3-(*N*-Morpholino)propanesulfonic acid (MOPS) buffer as previously described[28]. Media contained 24.4 mM methanol. Antibiotics for *E. coli* and *H. denitrificans* were used at the following concentrations (in μg ml^{-1}): ampicillin, 100; kanamycin, 50; streptomycin, 200; chloramphenicol, 25.

Recombinant DNA techniques

Standard techniques for DNA manipulation and cloning were used unless otherwise indicated[40]. Restriction enzymes, T4 ligase and Q5 polymerase were obtained from New England Biolabs (Ipswich, UK) and used according to the manufacturer's instructions. Oligonucleotides were obtained from Eurofins Genomics Germany GmbH (Ebersberg, Germany). Plasmid DNA from *E. coli* was purified using the GenJET Plasmid Miniprep kit (Thermo Scientific, Waltham, USA). Chromosomal DNA from *H. denitrificans* strains was prepared using the Simplex Easy DNA Extract Kit (GEN-IAL GmbH, Troisdorf, Germany). DNA fragments were extracted from agarose gels using the GeneJET Gel Extraction Kit (Thermo Scientific, Waltham, USA).

Construction of *H. denitrificans* mutant strains

Plasmids for reverse genetics in *H. denitrificans* were constructed using the suicide plasmid pk18*mobsacB*[41] and the tetracycline cassette from pHP45Ω-Tc[42] on the basis of previously published procedures[28,29]. For markerless *in frame* deletion of the individual *H. denitrificans soxT1A* and *soxT1B* genes by splicing overlap extension (SOE)[43], PCR fragments were constructed using the primers listed in Supplementary Table 3. The *soxT1A* or *soxT1B* fragments were inserted into pk18*mobsacB* using XbaI and SaII or XbaI and PstI restriction sites, respectively. The SmaI-excised tetracycline cassette from pHP45Ω-Tc[42] was inserted into the SmaI site, resulting in plasmids pK18*mobsacB*-Δ*soxT1A*-Tc and pK18*mobsacB*-Δ*soxT1B*-Tc. Another plasmid was constructed for concomitant deletion of *soxR* and *soxT1B* by SOE PCR with primers P1 fwd up hden_0700, P5 fwd down hden_soxR/soxT1B, P6 rev down hden_ soxR/soxT1B and P7 rev up hden_ soxR/soxT1B (Supplementary Table 3). The PCR fragment was cloned into the XbaI and PstI sites of pk18*mobsacB*-Tc[7]. For chromosomal complementation of the *H. denitrificans* Δ*tsdA* Δ*soxT1B* strain, the *soxT1B* gene was amplified together with upstream and downstream regions using primers SoxT1B_Del_Up_Fw and SoxT1B_Del_Down_Rev and cloned into the XbaI/PstI sites of pk18*mobsacB*-Tc. For chromosomal integration of the genes encoding SoxT1B $Cys^{24}Ser$, SoxT1B $Cys^{98}Ser$ and SoxT1B $Cys^{304}Ser$, the modified genes and upstream and downstream sequences were

amplified by SOE PCR using the appropriate primers listed in Supplementary Table 3.

All final constructs were electroporated into the desired *H. denitrificans* strains and transformants were selected using previously published procedures[28,29]. *H. denitrificans* Δ*tsdA* served as acceptor for plasmids pK18*mobsacB*-Δ*soxT1B*-Tc, pK18*mobsacB*-Δ*soxT1A*-Tc and pk18*mobsacB*_Tc_Δ*soxR/soxT1B*. *H. denitrificans* Δ*tsdA* Δ*shdrR* and *H. denitrificans* Δ*tsdA* Δ*soxR* served as strain backgrounds for deletion of *soxT1A*. The *soxT1B* deletion was also established in the *H. denitrificans* Δ*tsdA* Δ*shdrR* strain. The plasmids for complementation and cysteine exchanges of SoxT1B were transferred into *H. denitrificans* Δ*tsdA* Δ*soxT1B* in all cases, single crossover recombinants were Cm^r and Tc^r. Double crossover recombinants were Tc^s and survived in the presence of sucrose due to loss of both, the vector-encoded levansucrase (SacB) and the tetracyclin resistance gene. The genotype of the *H. denitrificans* strains generated in this study was confirmed by PCR.

Characterization of phenotypes, quantification of sulfur compounds, and biomass content

Growth experiments with *H. denitrificans* were run in medium with 24.4 mM methanol in Erlenmeyer flasks or 96-well microtiter plates as described earlier[7]. 2 mM thiosulfate was added when needed. Biomass content, thiosulfate, and sulfite concentrations were determined by previously described methods[7,44]. All growth experiments were repeated three to five times. Representative experiments with two biological replicates for each strain are shown. All quantifications are based on at least three technical replicates.

Expression studies based on RT-qPCR

Total RNA of the relevant *H. denitrificans* strains was isolated from cells harvested in mid-log phase according to an established procedure[8]. RNA samples of 100 ng were used for RT-qPCR analysis which was performed with the primers listed in Supplementary Table 3 following the method described in ref. 8.

Genome-wide transcriptomic analysis of *H. denitrificans* Δ*tsdA* in the absence and presence of thiosulfate

For transcriptome sequencing (RNA-Seq), *H. denitrificans* Δ*tsdA* was cultured in 50 ml minimal medium containing either 24.4 mM methanol or 24.4 mM methanol plus 2 mM thiosulfate in 200 ml Erlenmeyer flasks at 30°C with shaking at 200 rpm to early log phase. Cells from 20 ml culture were harvested and flash frozen in liquid N_2 and stored at −70 °C. From the frozen pellets, the RNA was purified with the FastGene RNA Premium Kit (NIPPON Genetics EUROPE, Düren, Germany) according to the manufacturer's instructions. A modification was introduced regarding the cell lysis step. After the addition of the lysis buffer that contained 1% (v/v) 2-mercaptoethanol, cells were disrupted by bead beating (Bead Ruptor 12 Bead Mill Homogenizer, Omni International, Kennesaw, GA, USA) for three cycles of 30 s at maximum speed and incubation on ice for 1 min. RNA quality was checked on 1% agarose gels and its concentration was measured using NanoPhotometer NP80 (IMPLEN, Munich, Germany). The RNA was shipped on dry ice to Eurofins Genomics GmbH (Ebersberg, Germany). The subsequent analysis pipeline included rRNA depletion, library preparation (mRNA fragmentation, strand-specific cDNA synthesis), Illumina paired-end sequencing (2 ×150 bp, minimum 10 MB reads)), and bioinformatic analysis (mapping against the reference genome, identification and quantification of transcripts, pairwise comparison of expression levels and determination of significant fold differences) and was conducted by the company.

Generation of datasets for phylogenetic analyses

Archaeal and bacterial genomes were downloaded from Genome Taxonomy Database (GTDB, release R207). In GTDB, all genomes are sorted according to validly published taxonomies, they are pre-validated and have high quality (completeness minus 5*contamination must be higher than 50%). One representative of each of the current 65,703 species clusters was analyzed. Open reading frames were determined using Prodigal[45] and subsequently annotated for sulfur-related proteins via HMSS2[34]. Annotation was extended by HMMs from TIGRFAMs[46] and Pfam[47] databases representing the 16 syntenic ribosomal proteins RpL2, 3, 4, 5, 6, 14, 15, 16, 18, 22, and 24, and RpS3, 8, 10, 17, and 19. A type I sHdr system was considered to be present if the core genes *shdrC1B1AHC2B2* were present in a syntenic gene cluster. For a type II sHdr system gene cluster *shdrC1B1AHB3* and *etfAB* had to be present in a single syntenic gene cluster[29,48].

Phylogenetic tree inference

For species tree inference, results for each ribosomal protein were individually aligned, trimmed and subsequently concatenated before they were used for phylogenetic tree construction. Proteins were aligned using MAFFT[49] and trimmed with BMGE[50] (entropy threshold = 0.95, minimum length = 1, matrix = BLOSUM30). Alignments were then used for maximum likelihood phylogeny inference using IQ-TREE v1.6.12[51] implemented on the "bonna" high-performance clusters of the University of Bonn. The best-fitting model of sequence evolution was selected using ModelFinder[52]. Branch support was then calculated by SH-aLRT (2000 replicates)[53], aBayes (2000 replicates)[54], and ultrafast bootstrap (2000 replicates)[55]. Finally, trees were displayed using iTol[56].

Statistics and reproducibility

Experimental data are expressed as the mean ± standard deviation of the mean of the number of tests stated for each experiment. All analysis was reproduced in at least three independent experiments. One-way ANOVA was performed to calculate *p*-values, with values < 0.05 indicating statistical significance.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

RNA-Seq raw data files and processed data files are available via the NCBI GEO repository (accession number GSE278992). Protein accession numbers and species names for Fig. 1 are available at https://github.com/WandaFlegler/Masterarbeit/blob/main/Galaxy3-%5BMGE_Cleaned_sequences_Fasta%5D.fasta.treefile. The authors declare that all other data supporting the findings of this study are available within the article (and its supplementary information files). The source data underlying Fig. 2 are provided as a source data file (Supplementary data 1). The source data underlying Figs. 3, 4, 5, 6 and Supplementary Figs. 6, 7, and 8 are provided as a source data file (Supplementary data 2).

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Author contributions

Conceptualization, J.L. and C.D.; investigation, J.L., F.G., H.Y.H., J.N.K., N.H., W.A.F., and T.S.T.; validation, J.L., T.S.T., and C.D.; writing-original draft preparation, J.L. and C.D.; writing-review and editing, J.L. and C.D., visualization, J.L., T.S.T., and C.D.; supervision, C.D.; project administration, C.D.; funding acquisition, C.D. All authors have read and agreed to the published version of the manuscript.

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Competing interests

The authors declare no competing interests.

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Appendix 3

YeeE-like bacterial SoxT proteins mediate sulfur import for oxidation and signal transduction

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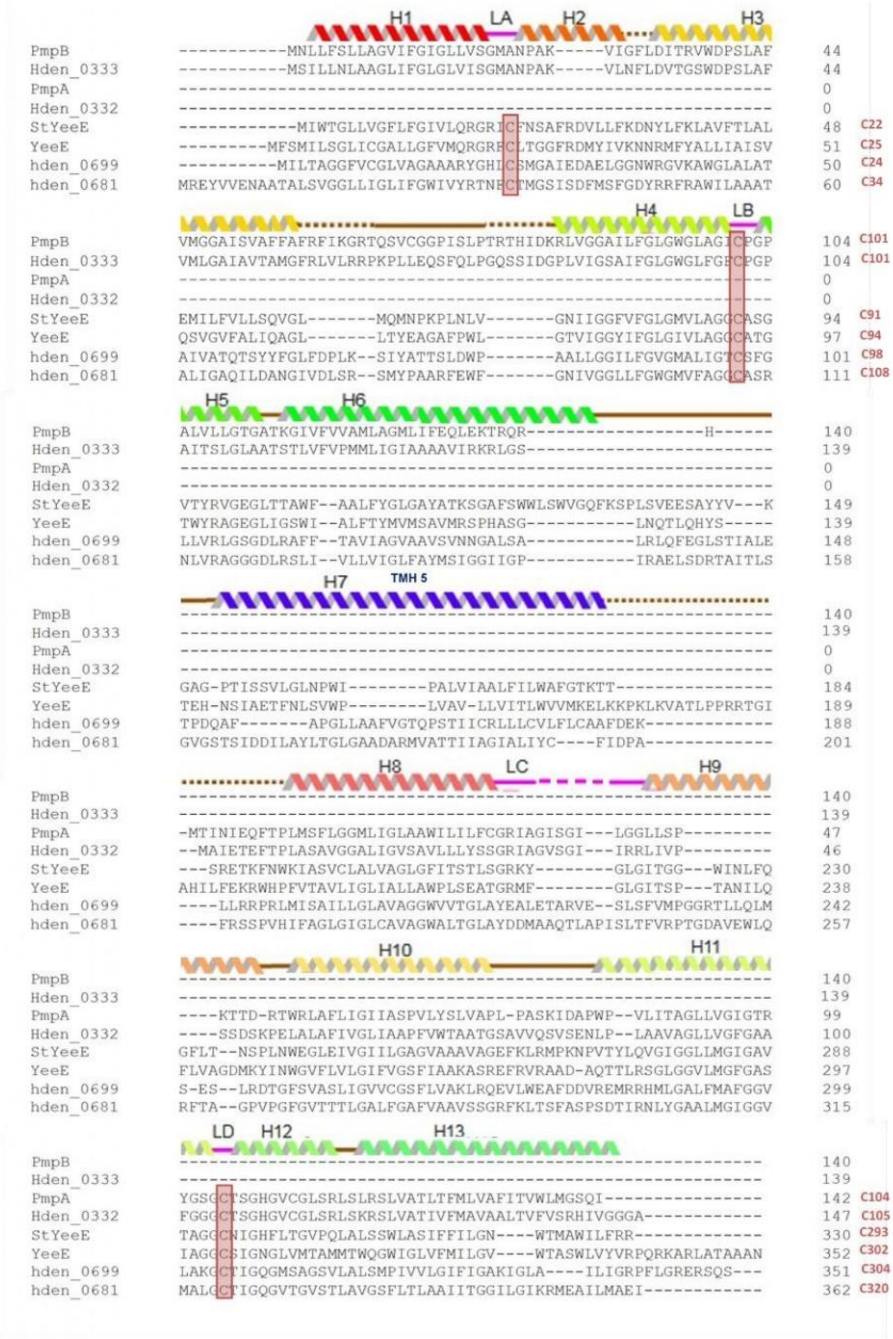
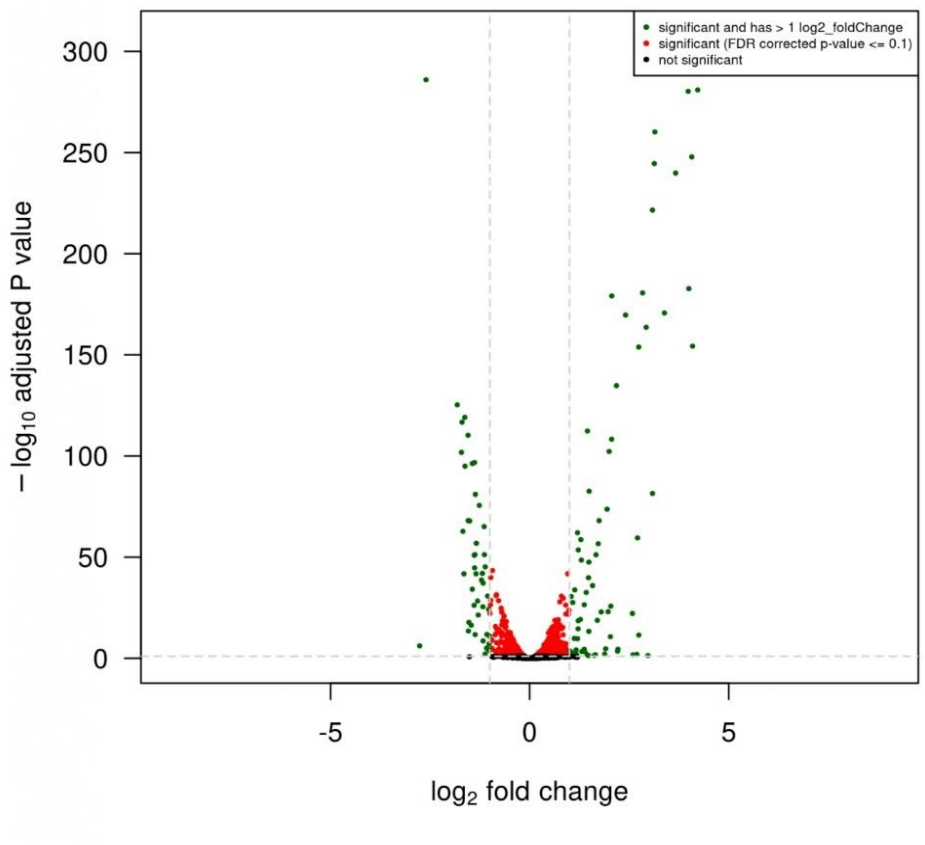
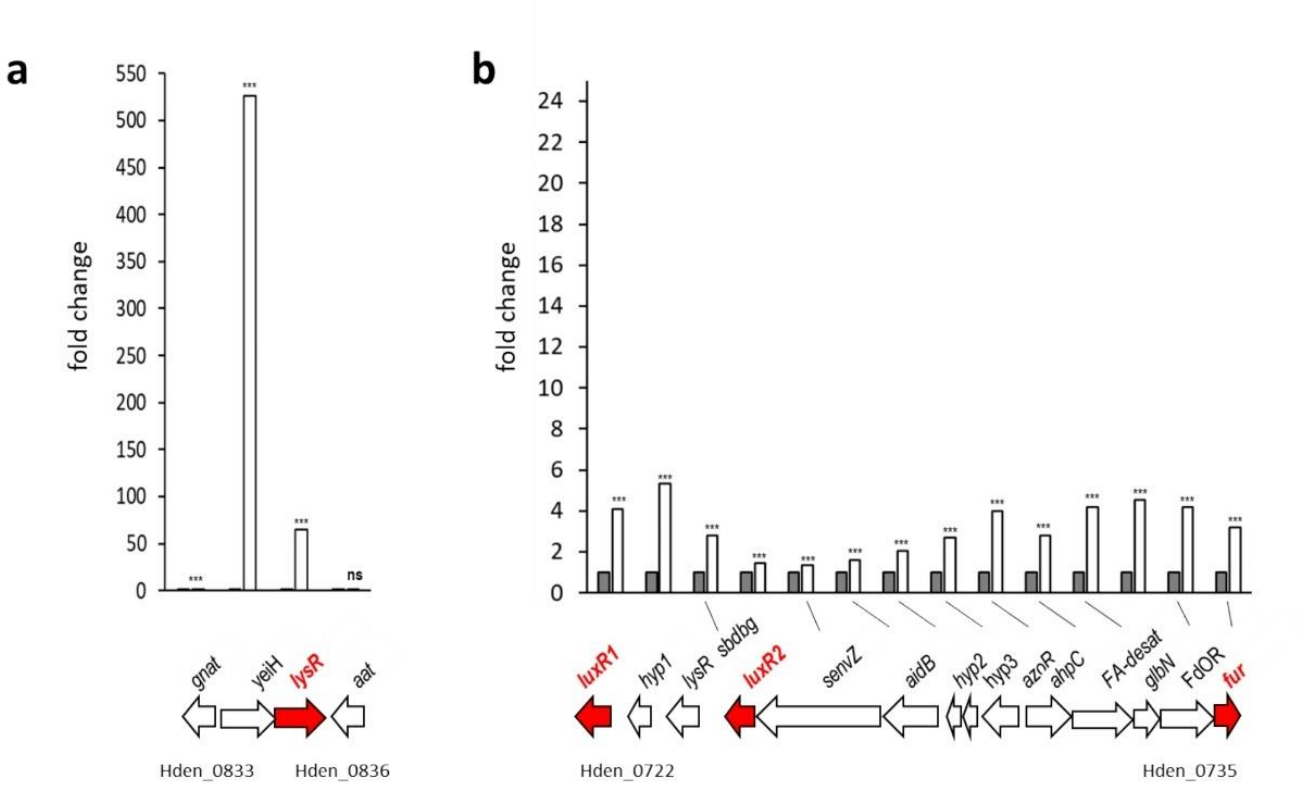
Supplementary Figures and Tables:



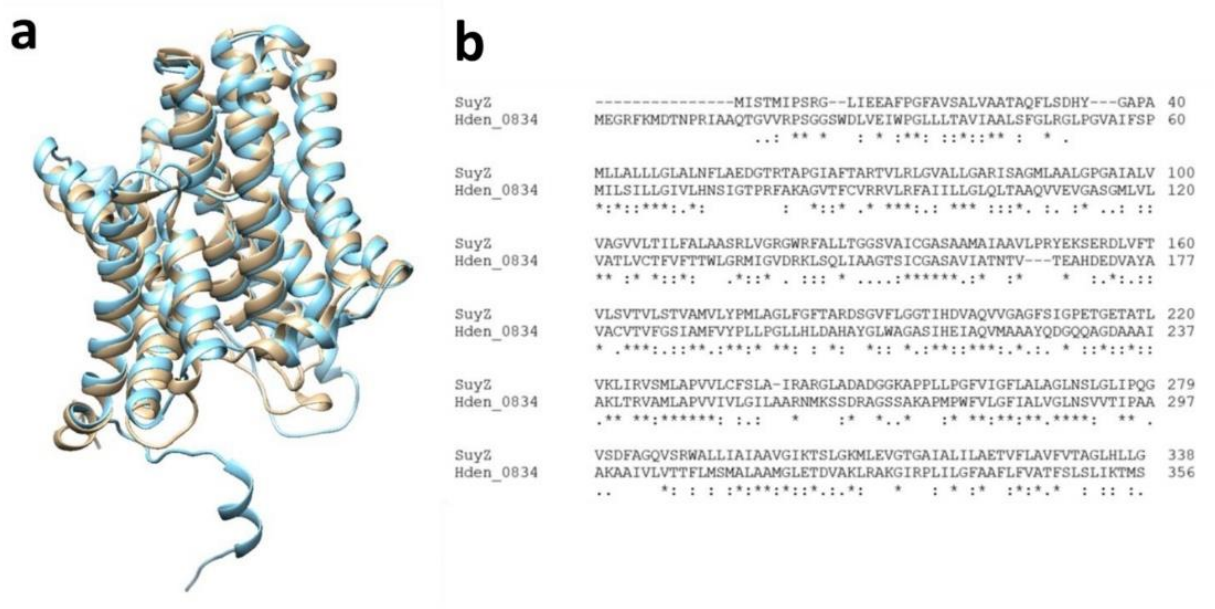
Supplementary Fig. 1. Alignment of YeeE/YedE family proteins. Amino acid sequences of PmpA and PmpB from *Serratia* sp. (Ser39006_020715 and Ser39006_02071520) and *Hyphomicrobium denitrificans* (Hden_0332 and Hden_0333), SoxT1A (Hden_0681) and SoxT1B (Hden_0699) from *H. denitrificans*, YeeE (TsuA, b2013) from *E. coli* K12 and StYeeE (Spith_0734) from *Spirochaeta thermophila* DSM 6578 were aligned with Clustal Omega (EMBL-EBI). Conserved cysteines are marked in red. The secondary structure of StYeeE with loops LA to LD and α-helices H1 to H13 was taken from Tanaka *et al.,* 2020 [1]. Helices 1, 3, 4, 6,7, 8, 10, 11 and 13 are membrane-spanning.



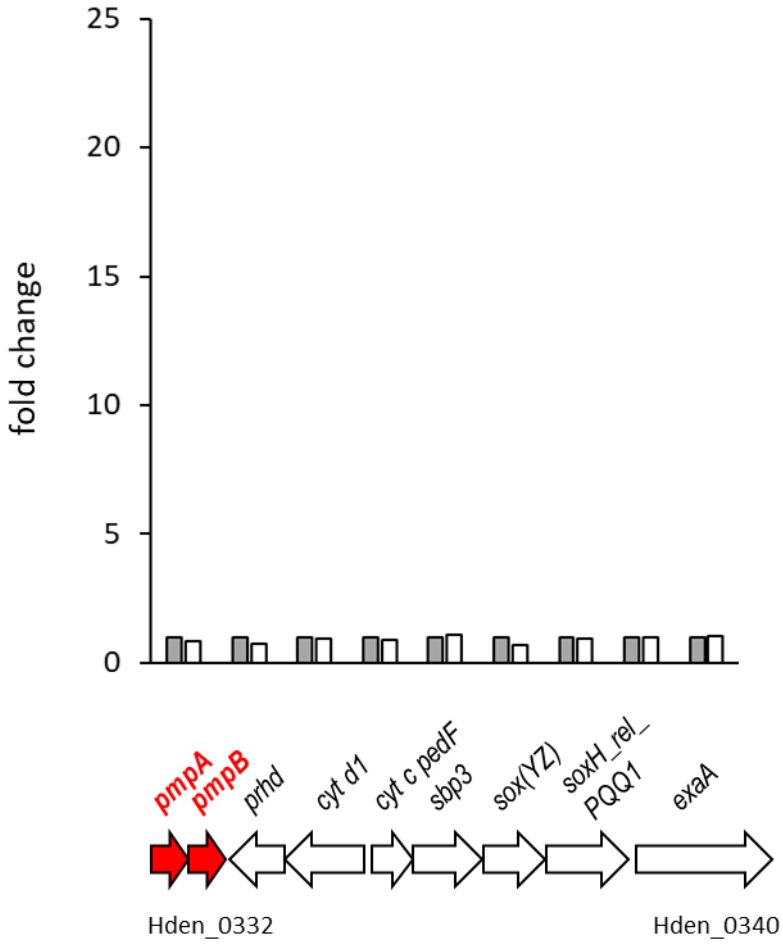
Supplementary Fig. 2. Volcano plot of differentially expressed genes for the *H. denitrificans* Δ*tsdA* reference strain in the absence versus the presence of thiosulfate. log2-fold change threshold =1, Benjamini-Hochberg corrected p-value = 0.1.



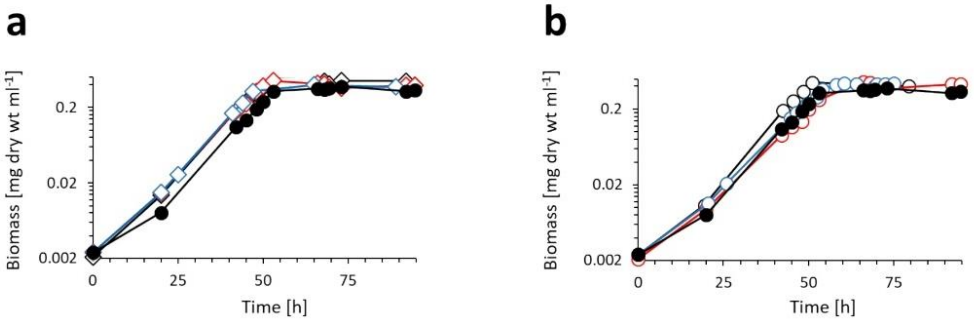
Supplementary Fig. 3. Transcript abundance changes of genes encoding transcriptional regulators and neighboring genes from *Hyphomicrobium denitrificans* Δ*tsdA*. Columns in gray show the reference value for cells grown in the absence of thiosulfate, white columns apply to cells grown with 2 mM thiosulfate. The experiment was conducted in duplicate (n=2), each time using mRNA preparations from two different cultures. Adjusted *p* values for statistically significant changes were all below 0.001 (Supplementary Table 2) and are indicated by three asterisks (***p < 0.001); ns, not significant. **a,** *gnat*, acyl-CoA N-acetyltransferase domain; *yeiH,* proposed sulfate/sulfite exporter; *lysR*, transcriptional regulator LysR family; the majority of these proteins appear to be transcription activators and most are known to negatively regulate their own expression; *aat*, leucyl/phenylalanyl-t-RNA/protein transferase. **b,** *luxR1*, LuxR family transcriptional regulator; most luxR-type regulators act as transcription activators, but some can be repressors or have a dual role for different sites; *hyp1*, hypothetical periplasmic protein; *lysR sbdg*, substrate binding domain LysR family of prokaryotic transcriptional regulatory proteins; *luxR2*, LuxR family response regulator; *envZ*, multi-sensor signal transduction histidine kinase; *aidB,* acyl-CoA dehydrogenase domain related to the alkylation response protein AidB; *hyp2* and *hyp3*, hypothetical proteins; *azoR*, FMN-dependent NADH:quinone oxidoreductase; *ahpC*, peroxiredoxin, alkyl hydroperoxide reductase subunit C; *FA-desat*, fatty acid desaturase; *glbN*, globin, truncated bacterial like; *FdOR*, 2Fe-2S ferredoxin domain containing FAD/NAD(P)-binding oxidoreductase; *fur*, ferric uptake regulator, Fur family, iron-responsive DNA-binding repressor protein;



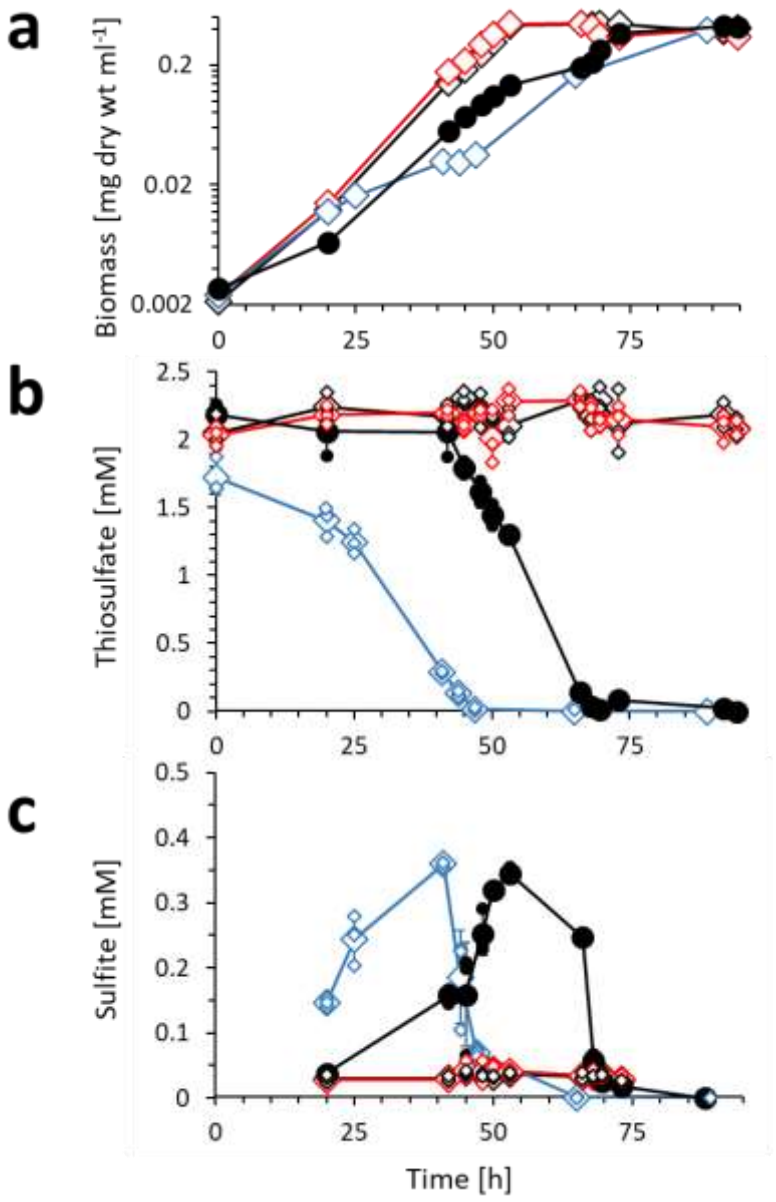
Supplementary Fig. 4. Comparison of *Hyphomicrobium denitrificans* YeiH (Hden_0834, UniProt D8JU61) with SuyZ from *Paracoccus pantotrophus* NKNCYSA (beige, GenBank accession AY704413). a. Alphafold[2] structures of HdYeiH (light blue) and PpSuyZ (beige). Structures were overlayed and visua-lized by Chimera 1.14[3]. **b.** Sequence alignment of SuyZ and HdYeiH (Hden_0834) created by Clustal Omega[4].



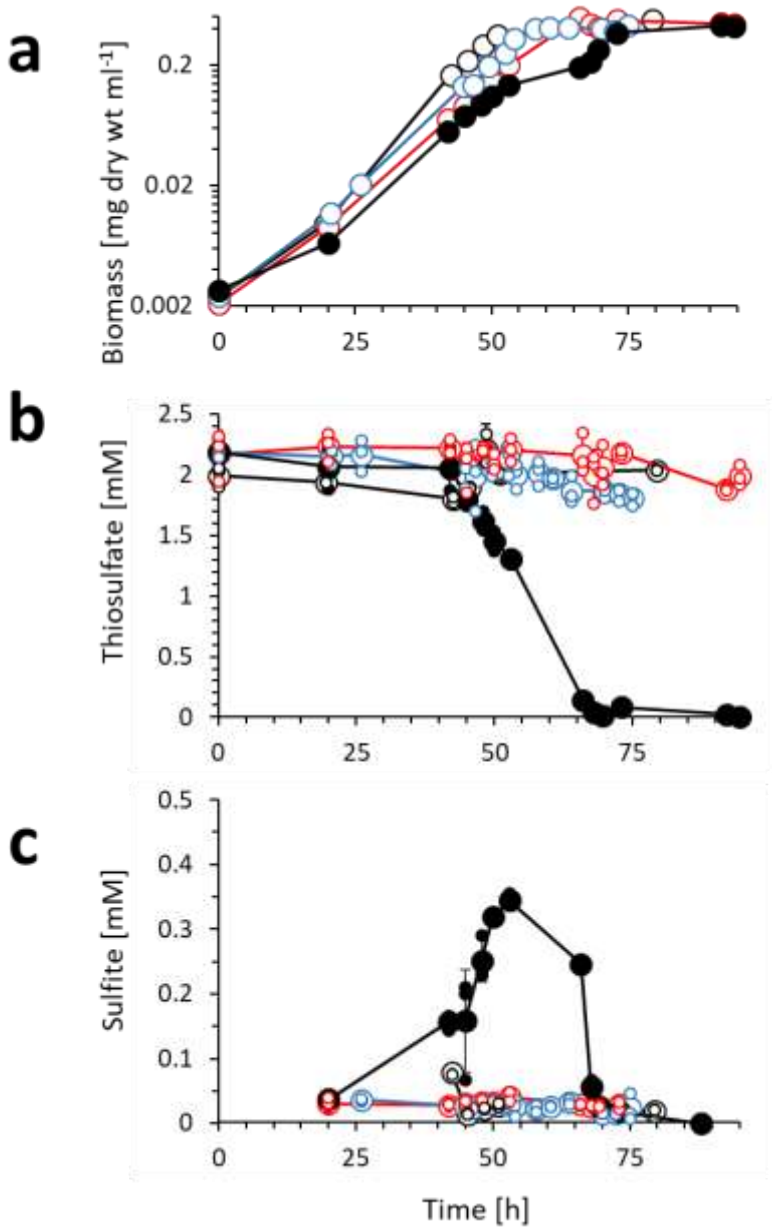
Supplementary Fig. 5. Transcript abundance changes of genes encoding PmpA and PmpB and neighboring genes from *Hyphomicrobium denitrificans* Δ*tsdA*. Columns in gray show the reference value for cells grown in the absence of thiosulfate, white columns apply to cells grown with 2 mM thiosulfate. The experiment was conducted in duplicate, each time using mRNA preparations from two different cultures. Adjusted *p* values were all above 0.05, indicating no significant changes. *prhd*, periplasmic rhodanese-like domain-containing sulfurtransferase with a CXXXCW motif, consistent with a possible role in redox cofactor binding (IPR001763 and IPR022376); *cyt d1*, periplasmic protein with a cytochrome *d1* heme domain and a YVTN beta-propeller repeat (TIGR03866); *pedF*, cytochrome c_{550} (TIGR04494), periplasmic electron carrier; *sbp3*, extracellular substrate-binding protein family 3 (IPR001638). *sox(YZ),* Sox(YZ) fusion protein (TIGR04557); *soxH_rel_PQQ1*, encodes a potential Zn metallohydrolase of the same family as the SoxH protein (TIGR04558) associated with thiosulfate oxidation[5]. *prhd*, *cyt d1*, *cyt c550*, *sox(YZ)*, and *soxH_rel_PQQ1* show relationships by phylogenetic profiling and conserved gene neighborhoods with transport systems for alcohols metabolized by PQQ-dependent enzymes (here probably the enzyme encoded by *exaA*), that have a Cys-Cys motif (TIGR03075) for electron transfer to c_{550} family cytochromes.



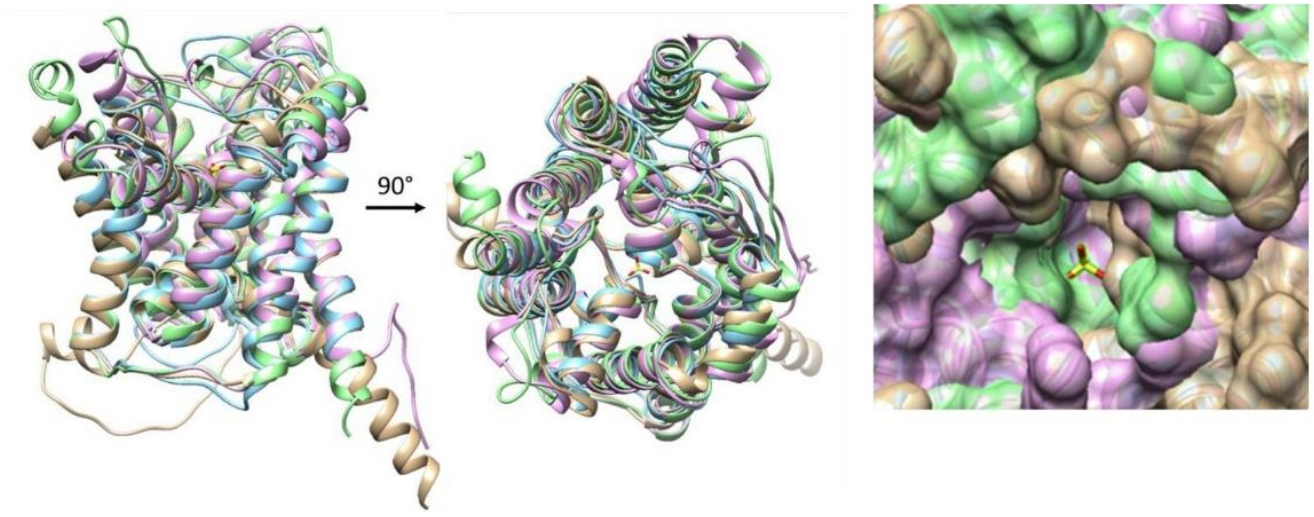
Supplementary Fig. 6. Growth of *H. denitrificans* reference and mutant strains on methanol. a. Growth curves are compared for the reference strain *H. denitrificans* Δ*tsdA* (black filled circles) and strains lacking gene *soxT1B*: *H. denitrificans* Δ*tsdA* Δ*soxT1B* (black open diamonds), *H. denitrificans* Δ*tsdA* Δ*soxT1B* Δ*soxR* (blue open diamonds) and *H. denitrificans* Δ*tsdA* Δ*soxT1B* Δ*shdrR* (red open diamonds). **b.** Growth curves are compared for the reference strain *H. denitrificans* Δ*tsdA* (black filled circles) and strains lacking gene *soxT1A*: *H. denitrificans* Δ*tsdA* Δ*soxT1A* (black open circles), *H. denitrificans* Δ*tsdA* Δ*soxT1A* Δ*soxR* (blue open circles) and *H. denitrificans* Δ*tsdA* Δ*soxT1A* Δ*shdrR* (red open circles). Error bars indicating SD for three replicates are too small to be visible for the determination of biomass.



Supplementary Fig. 7. Growth and thiosulfate consumption of *H. denitrificans* reference and mutant strains lacking *soxT1B*. a. Growth curves on medium containing 2 mM thiosulfate. Error bars indicating SD for three replicates are too small to be visible for the determination of biomass. **b.** Thiosulfate consumption **c**. Sulfite production. Symbols identifying strains: *H. denitrificans* Δ*tsdA* (black filled circles), *H. denitrificans* Δ*tsdA* Δ*soxT1B* (black open diamonds), *H. denitrificans* Δ*tsdA* Δ*soxT1B* Δ*soxR* (blue open diamonds) and *H. denitrificans* Δ*tsdA* Δ*soxT1B* Δ*shdrR* (red open diamonds). In **b** and **c**, data was measured using *n* = 3 experiments and is presented with the individual measurements (small symbols) and as the mean value of these measurements ± SD (big symbols). Precultures contained 2 mM thiosulfate.



Supplementary Fig. 8. Growth and thiosulfate consumption of *H. denitrificans* reference and mutant strains lacking *soxT1A*. a. Growth curves on medium containing 2 mM thiosulfate. Error bars indicating SD for three replicates are too small to be visible for the determination of biomass. **b.** Thiosulfate consumption **c**. Sulfite production. Symbols identifying strains: *H. denitrificans* Δ*tsdA* (black filled circles), *H. denitrificans* Δ*tsdA* Δ*soxT1A* (black open circles), *H. denitrificans* Δ*tsdA* Δ*soxT1A* Δ*soxR* (blue open circles) and *H. denitrificans* Δ*tsdA* Δ*soxT1B* Δ*shdrR* (red open circles). In **b** and **c**, data was measured using *n* = 3 experiments and is presented with the individual measurements (small symbols) and as the mean value of these measurements ± SD (big symbols). Precultures contained 2 mM thiosulfate.



Supplementary Fig. 9. Overlay of YeeE-like protein structures. The crystal structure of *Spirochaeta thermophila* TsuA (YeeE) with thiosulfate bound (6LEO[1], light blue) was matched with the structures for *E. coli* YeeE (beige), *H. denitrificans* SoxT1A (green) and *H. denitrificans* SoxT1B (violet) predicted by Alphafold[2]. The image on the right displays the protein surfaces with 30% transparency and reveals free access for thiosulfate and/or other potential substrates to the (proposed) binding site for all cases. Structures were matched and visualized by Chimera 1.14[3].

Supplementary Table 1. mRNAseq analysis of *H. denitrificans ΔtsdA*, part 1. Genes with lower mRNA abundances in thiosulfate containing medium in comparison to thiosulfate free medium.

Locus tag NCBI	Annotation	Fold change	log_2_fold change	p_value	adj_p_value
Hden_0086	group II truncated hemoglobin	0.34	-1.54	1.36E-15	3.34E-14
Hden_0095	HPF/RaiA family ribosome-associated protein	0.31	-1.68	2.70E-65	1.79E-63
Hden_0096	zinc-dependent alcohol dehydrogenase family protein	0.31	-1.71	1.75E-104	1.56E-102
Hden_0097	flavin reductase family protein	0.32	-1.65	3.84E-44	1.85E-42
Hden_0099	PAS domain-containing protein	0.47	-1.07	7.24E-14	1.60E-12
Hden_0138	hypothetical protein	0.46	-1.12	2.13E-03	9.53E-03
Hden_0432	hypothetical protein	0.35	-1.51	7.50E-02	1.72E-01
Hden_0554	beta-ketoacyl-ACP synthase	0.39	-1.37	8.25E-14	1.81E-12
Hden_0555	beta-ketoacyl-ACP synthase	0.38	-1.39	1.84E-28	6.70E-27
Hden_0556	zinc-binding dehydrogenase	0.37	-1.44	1.58E-36	7.02E-35
Hden_0557	beta-ketoacyl-ACP synthase	0.44	-1.17	9.49E-28	3.38E-26
Hden_0558	beta-ketoacyl-ACP synthase	0.38	-1.39	2.03E-53	1.12E-51
Hden_0559	beta-hydroxyacyl-ACP dehydratase	0.34	-1.54	1.21E-70	8.68E-69
Hden_0560	acyl carrier protein	0.31	-1.70	1.88E-119	1.92E-117
Hden_0561	SDR family oxidoreductase	0.28	-1.82	4.85E-128	5.29E-126
Hden_0562	HAD-IIIC family phosphatase	0.39	-1.36	1.22E-83	9.35E-82
Hden_0567	U32 family peptidase	0.49	-1.02	5.71E-09	8.10E-08
Hden_0568	U32 family peptidase	0.47	-1.08	9.51E-07	9.79E-06
Hden_0570	cyclic nucleotide-binding domain-containing protein	0.39	-1.35	3.58E-44	1.76E-42
Hden_0572	hypothetical protein	0.35	-1.53	5.98E-20	1.73E-18
Hden_0573	4Fe-4S binding protein	0.49	-1.03	2.07E-26	7.21E-25
Hden_0574	cupredoxin domain-containing protein	0.38	-1.38	3.70E-47	1.92E-45
Hden_0575	iron transporter	0.37	-1.44	6.51E-99	5.50E-97
Hden_0576	oxygen-independent coproporphyrinogen III oxidase	0.32	-1.62	1.38E-97	1.13E-95
Hden_0581	cytochrome c	0.36	-1.47	1.69E-18	4.68E-17
Hden_0589	hypothetical protein	0.44	-1.17	1.49E-10	2.47E-09
Hden_0590	NnrS family protein	0.50	-1.01	1.98E-05	1.55E-04
Hden_0591	copper-containing nitrite reductase	0.39	-1.37	1.02E-53	5.92E-52
Hden_0592	host attachment family protein	0.40	-1.31	1.26E-30	4.91E-29
Hden_0595	helix-turn-helix domain-containing protein	0.38	-1.38	2.00E-99	1.73E-97
Hden_0925	MFS transporter	0.40	-1.34	2.23E-59	1.37E-57
Hden_0976	gamma-glutamyl-gamma-aminobutyrate hydrolase family protein	0.44	-1.17	1.44E-39	6.59E-38
Hden_1046	GTP-binding protein	0.16	-2.60	4.68E-289	1.13E-286
Hden_1047	sulfate adenylyltransferase subunit CysD	0.35	-1.51	1.81E-70	1.25E-68
Hden_1055	hypothetical protein	0.44	-1.19	2.16E-44	1.09E-42
Hden_1491	NADPH-dependent assimilatory sulfite reductase hemoprotein subunit	0.32	-1.63	7.95E-122	8.40E-120
Hden_1773	radical SAM protein	0.50	-1.01	5.37E-13	1.10E-11
Hden_1841	universal stress protein	0.48	-1.06	4.24E-33	1.77E-31
Hden_2047	cytochrome-c oxidase 2C cbb3-type subunit III	0.46	-1.14	1.05E-53	6.03E-52
Hden_2048	cbb3-type cytochrome c oxidase subunit 3	0.43	-1.21	4.63E-41	2.14E-39
Hden_2049	cytochrome-c oxidase 2C cbb3-type subunit II	0.45	-1.14	1.21E-67	8.15E-66
Hden_2050	cytochrome-c oxidase%2C cbb3-type subunit I	0.42	-1.26	3.47E-78	2.61E-76
Hden_2136	DHA2 family efflux MFS transporter permease subunit	0.41	-1.29	1.26E-23	3.97E-22
Hden_2177	Crp/Fnr family transcriptional regulator	0.46	-1.12	1.21E-47	6.37E-46
Hden_2272	membrane protein	0.49	-1.04	9.08E-06	7.75E-05
Hden_2394	lysozyme inhibitor LprI family protein	0.15	-2.76	5.63E-08	7.02E-07
Hden_2827	ferric reductase-like transmembrane domain-containing protein	0.34	-1.55	5.87E-113	5.66E-111

Supplementary Table 2. mRNAseq analysis of *H. denitrificans ΔtsdA*, part 2. Genes with higher mRNA abundances in thiosulfate containing medium in comparison to thiosulfate free medium

Locus tag NCBI	Annotation	Fold change	log_2_fold change	p_value	adj_p_value
Hden_0441	glycosyltransferase	6.04	2.59	4.91E-03	1.91E-02
Hden_0444	glycosyltransferase family 4 protein	2.09	1.07	6.44E-03	2.40E-02
Hden_0457	hypothetical protein	4.65	2.22	3.21E-06	2.99E-05
Hden_0460	DUF983 domain-containing protein	2.42	1.27	1.97E-21	6.04E-20
Hden_0523	zf-HC2 domain-containing protein	2.61	1.38	7.21E-05	4.99E-04
Hden_0525	catalase family peroxidase	2.24	1.17	8.70E-06	7.46E-05
Hden_0678	hypothetical protein	6.69	2.74	1.58E-13	3.38E-12
Hden_0679	DsbA family protein	8.49	3.09	4.28E-84	3.36E-82
Hden_0680	sulfur transferase domain-containing protein	12.69	3.67	8.60E-243	1.45E-240
Hden_0681	YeeE/YedE family protein	18.94	4.24	0.00E+00	0.00E+00
Hden_0683	radical SAM protein LipS1	16.81	4.07	7.46E-251	1.40E-248
Hden_0684	NAD(P)/FAD-dependent oxidoreductase LipT	17.04	4.09	4.28E-157	5.17E-155
Hden_0685	radical SAM protein LipS2	15.85	3.99	0.00E+00	0.00E+00
Hden_0686	lipoate--protein ligase family protein sLpl(AB)	16.65	4.06	7.66E-319	1.99E-316
Hden_0687	GMP synthase - glutamine amidotransferase domain-like protein LipX	15.98	4.00	1.23E-185	1.89E-183
Hden_0689	sHdrC1	16.09	4.01	0.00E+00	0.00E+00
Hden_0690	sHdrB	17.82	4.16	0.00E+00	0.00E+00
Hden_0691	sHdrA	19.29	4.27	0.00E+00	0.00E+00
Hden_0692	sHdrH	18.64	4.22	5.23E-284	1.18E-281
Hden_0693	sHdrC2	15.79	3.98	2.81E-283	5.93E-281
Hden_0694	sHdrB2	13.34	3.74	0.00E+00	0.00E+00
Hden_0695	sHdrI	10.45	3.39	1.48E-173	2.00E-171
Hden_0696	LbpA2	8.49	3.09	1.81E-224	2.91E-222
Hden_0697	cytochrome P450	7.14	2.84	1.62E-183	2.38E-181
Hden_0698	sulfurtransferase TusA family protein	7.60	2.93	1.86E-166	2.32E-164
Hden_0701	SoxS	6.54	2.71	4.74E-62	3.02E-60
Hden_0702	sulfur oxidation c-type cytochrome SoxX	6.68	2.74	1.21E-156	1.41E-154
Hden_0703	sulfur oxidation c-type cytochrome SoxA	8.85	3.15	3.34E-263	6.63E-261
Hden_0704	thiosulfate oxidation carrier protein SoxY	8.97	3.16	0.00E+00	0.00E+00
Hden_0705	thiosulfate oxidation carrier complex protein SoxZ	8.76	3.13	1.60E-247	2.84E-245
Hden_0706	thiosulfohydrolase SoxB	7.29	2.86	0.00E+00	0.00E+00
Hden_0719	TIGR01244 family sulfur transferase	2.79	1.48	3.31E-42	1.55E-40
Hden_0720	sulfite exporter TauE/SafE family protein	2.59	1.37	9.37E-29	3.52E-27
Hden_0721	MBL fold metallo-hydrolase	2.99	1.58	2.59E-38	1.16E-36
Hden_0722	response regulator transcription factor	4.08	2.03	1.03E-12	2.04E-11
Hden_0723	hypothetical protein	5.31	2.41	1.61E-172	2.09E-170
Hden_0724	substrate-binding domain-containing protein	2.80	1.49	2.09E-15	5.03E-14
Hden_0728	hypothetical protein	2.06	1.04	6.78E-33	2.76E-31
Hden_0729	hypothetical protein	2.68	1.42	6.37E-35	2.76E-33
Hden_0730	NAD(P)H-dependent oxidoreductase	3.99	2.00	6.42E-105	5.86E-103
Hden_0731	peroxiredoxin	2.82	1.49	3.20E-85	2.58E-83
Hden_0732	fatty acid desaturase	4.18	2.06	5.43E-182	7.65E-180
Hden_0733	group 1 truncated hemoglobin	4.53	2.18	1.53E-137	1.72E-135
Hden_0734	2Fe-2S iron-sulfur cluster binding domain-containing protein	4.17	2.06	5.70E-111	5.35E-109
Hden_0735	transcriptional repressor	3.17	1.67	1.19E-53	6.72E-52
Hden_0737	alpha/beta fold hydrolase	3.30	1.72	3.97E-59	2.40E-57
Hden_0738	hypothetical protein	5.98	2.58	2.10E-24	6.83E-23
Hden_0739	flavin reductase family protein	3.25	1.70	5.67E-21	1.69E-19
Hden_0742	SCO family protein	3.85	1.95	2.72E-76	2.00E-74
Hden_0743	selenium-binding protein	7.36	2.88	0.00E+00	0.00E+00
Hden_0748	cell envelope integrity protein TolA	2.03	1.02	3.91E-05	2.85E-04
Hden_0783	PQQ-binding-like beta-propeller repeat protein	2.46	1.30	4.87E-51	2.65E-49
Hden_0784	thiamine pyrophosphate-dependent dehydrogenase E1 component subunit alpha	2.81	1.49	5.40E-50	2.90E-48
Hden_0785	alpha-ketoacid dehydrogenase subunit beta	2.44	1.29	3.49E-61	2.19E-59
Hden_0786	acetoin dehydrogenase dihydrolipoyllysine-residue acetyltransferase subunit	2.33	1.22	4.78E-56	2.84E-54
Hden_0788	thiazole synthase	2.59	1.37	3.40E-06	3.13E-05
Hden_0791	dihydrolipoyl dehydrogenase	2.33	1.22	9.79E-21	2.88E-19
Hden_0792	hypothetical protein	2.30	1.20	1.29E-64	8.40E-63
Hden_0796	methanol/ethanol family PQQ-dependent dehydrogenase	2.12	1.08	6.90E-30	2.62E-28
Hden_0834	YeiH family protein	525.97	9.04	0.00E+00	0.00E+00
Hden_0835	LysR family transcriptional regulator	65.57	6.03	0.00E+00	0.00E+00
Hden_0914	hypothetical protein	6.46	2.69	2.83E-03	1.20E-02

Hden_1114	hypothetical protein	3.08	1.62	1.14E-02	3.84E-02
Hden_1133	Do family serine endopeptidase	2.74	1.45	4.60E-115	4.58E-113
Hden_1509	metal-sensitive transcriptional regulator	2.16	1.11	6.08E-02	1.47E-01
Hden_1967	hypothetical protein	2.72	1.45	3.95E-03	1.59E-02
Hden_2058	isocitrate lyase	2.24	1.17	2.35E-02	6.90E-02
Hden_2164	AraC family transcriptional regulator	2.01	1.01	3.10E-02	8.60E-02
Hden_2373	DUF4118 domain-containing protein	2.26	1.18	3.47E-03	1.42E-02
Hden_2458	hypothetical protein	7.83	2.97	1.25E-02	4.16E-02
Hden_2475	PsiF family protein	3.92	1.97	2.80E-25	9.57E-24
Hden_2542	class I SAM-dependent methyltransferase	4.62	2.21	4.34E-05	3.14E-04
Hden_2565	Spy/CpxP family protein refolding chaperone	2.19	1.13	3.46E-36	1.52E-34
Hden_2684	hypothetical protein	2.33	1.22	1.06E-16	2.70E-15
Hden_2786	hypothetical protein	4.11	2.04	4.69E-28	1.69E-26
Hden_2822	efflux RND transporter periplasmic adaptor subunit	3.69	1.88	2.01E-03	9.02E-03
Hden_2931	potassium-transporting ATPase subunit KdpA	3.76	1.91	2.11E-06	2.03E-05
Hden_2933	potassium-transporting ATPase subunit KdpC	2.10	1.07	3.98E-02	1.05E-01
Hden_2938	formylglycine-generating enzyme family protein	2.14	1.09	9.57E-04	4.77E-03
Hden_2956	hypothetical protein	2.79	1.48	1.57E-02	5.02E-02
Hden_2958	glycoside hydrolase family 108 protein	2.06	1.04	2.02E-02	6.14E-02
Hden_2965	hypothetical protein	2.26	1.18	7.04E-02	1.64E-01
Hden_3023	hypothetical protein	3.14	1.65	4.24E-03	1.69E-02
Hden_3040	hypothetical protein	2.30	1.20	1.04E-11	1.99E-10
Hden_3139	hypothetical protein	3.35	1.74	1.30E-70	9.15E-69
Hden_3465	hypothetical protein	2.18	1.12	8.00E-12	1.54E-10
Hden_R0052		2.48	1.31	6.69E-05	4.66E-04
none_0000	DUF1488 family protein	3.48	1.80	3.67E-25	1.24E-23

Supplementary Table 3. Strains, primers and plasmids

Strains primers or plasmids	Relevant genotype, description or sequence	Reference or source
Strains		
Escherichia coli 10-beta	*Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL* (Str^R) *rph spoT1 Δ(mrr-hsdRMS-mcrBC)*	New England Biolabs
E. coli BL21 DE3	*B dcm ompT hsdS($r_B^-m_B^-$) gal*	Novagen
Hyphomicrobium denitrificans Δ*tsdA*	Sm^r, in-frame deletion of *tsdA* in *H. denitrificans* Sm200	[6]
H. denitrificans Δ*tsdA* Δ*shdrR*	Sm^R, in-frame deletion of *shdrR* (Hden_0682) in *H. denitrificans* Δ*tsdA*	[7]
H. denitrificans Δ*tsdA* Δ*soxR*	Sm^R, deletion of *soxR* (Hden_0700) in *H. denitrificans* Δ*tsdA*	[8]
H. denitrificans Δ*tsdA* Δ*soxT1B*	Sm^R, in-frame deletion of *soxT1B* (Hden_0699) in *H. denitrificans* Δ*tsdA*	This work
Hyphomicrobium denitrificans Δ*tsdA soxT1Bcomp*	Sm^R, cis complementation of *H. denitrificans* Δ*tsdA* Δ*soxT1B* with *soxT1B*	This work
H. denitrificans Δ*tsdA soxT1B-Cys^{24}Ser*	Exchange of SoxT1B-Cys^{24} to Ser in *H. denitrificans* Δ*tsdA*	This work
H. denitrificans Δ*tsdA soxT1B-Cys^{98}Ser*	Exchange of SoxT1B-Cys^{98} to Ser in *H. denitrificans* Δ*tsdA*	This work
H. denitrificans Δ*tsdA soxT1B-Cys^{304}Ser*	Exchange of SoxT1B-Cys^{304} to Ser in *H. denitrificans* Δ*tsdA*	This work
H. denitrificans Δ*tsdA* Δ*soxT1B* Δ*soxR*	Sm^R, simultaneous deletion of *soxR* (Hden_0700) and *soxT1B* (Hden_0699) in *H. denitrificans* Δ*tsdA*	This work
H. denitrificans Δ*tsdA* Δ*soxT1B* Δ*shdrR*	Sm^R, simultaneous deletion of *shdrR* (Hden_0682) and *soxT1B* (Hden_0699) in *H. denitrificans* Δ*tsdA*	This work
H. denitrificans Δ*tsdA* Δ*soxT1A*	Sm^R, in-frame deletion of *soxT1A* (Hden_0681) in *H. denitrificans* Δ*tsdA*	This work
H. denitrificans Δ*tsdA* Δ*soxT1A* Δ*soxR*	Sm^R, deletion of *soxR* in *H. denitrificans* Δ*tsdA* Δ*soxT1A*	This work
H. denitrificans Δ*tsdA* Δ*soxT1A* Δ*shdrR*	Sm^R, deletion of *shdrR* in *H. denitrificans* Δ*tsdA* Δ*soxT1A*	This work
Primers		
SoxT1B_Del_Up_Fw	AATA**TCTAGA**CGAGCGATCGCCATCGCGAG (XbaI)	This work
SoxT1B_Del_Up_Rev	CGCCCGCATGCCAATCAGCTGATCATCGGAATTCGCTCTCT	This work
SoxT1B_Del_Down_Fw	AGAGAGCGATTCCGATGATCAGCTGATTGGCATGCGGGCG	This work
SoxT1B_Del_Down_Rev	ATCT**CTGCAG**TTCGAACCTGGACGCCGCG (PstI)	This work
SoxT1A_del_Up_Fw	GTGG**TCTAGA**TGTTCAAGCTCCTCGACAAG (XbaI)	This work
SoxT1A _del_Up_Rev	GCCGCCTGTCGTTCTTAAATCCGCATTTCCCGCCCCGTCT	This work
SoxT1A_del_Down_Fw	AGACGGGGCGGGAAATGCGGATTTAAGAACGACAGGCGGC	This work
SoxT1A_del_Down_Rev	GTGG**GTCGAC**CCTTTCTGGTCCATCAATGC (SalI)	This work
SoxT1B _C24S_Up_Rev	GGCGCCGCCGCCCGCTACGGACATCTCTCCTCCATGGGAG	This work
SoxT1B_C24S_Down_Fw	CTCCCATGGAGGAGAGATGTCCGTAGCGGGCGGCGGCGCC	This work
SoxT1B C98S_Up_rev	GGCACATCCAGCTTCGGGTTGCTCGTGCGCCTC	This work
SoxT1B_C98S_Down_Fw	GAGGCGCACGAGCAACCCGAAGCTGGATGTGCC	This work
SoxT1B_C304S_Up_rev	AAGGGCTCCACCATCGGCCAAGGCATGAGCGCCGGC	This work
SoxT1B_C304S_Down_Fw	GCCGGCGCTCATGCCTTGGCCGATGGTGGAGCCCTT	This work
P1 fwd up hden_0700	TATA**CTGCAG**GATCAAGGACGTGGTGGCG (PstI)	[8]
P5 fwd down hden_soxR/soxT1B	CCAGGGATAGGAATGTCAGCTGATTGGCATGCGGGC	This work
P6 rev down hden_ soxR/soxT1B	TTGC**TCTAGA**TCCGGCGCGACGATCGATG (XbaI)	This work
P7 rev up hden_ soxR/soxT1B	GCCCGCATGCCAATCAGCTGACATTCCTATCCCTCGG	This work
rpoB-denitf	AGGACGTGTTCACCTCGATT	[9]
rpoB-denitr	CGGCTTCGTCAAGGTTCTTC	[9]
SoxT1A 0681_qPCR-Fr	CCCGAGTGATACGATTCGCA	[8]
SoxT1A 0681_qPCR-Rev	CTAAAATGCCGCCGGTGATG	[8]
sHdrA_qPCR-Fr	CCGATCACCATTCCGTTCGA	[8]
sHdrA_qPCR-Rev	CAATTGTTTCCGGGCCGATC	[8]
SoxXA_qPCR-Fr	CGGCGCTCATTACCTATCTC	[8]
SoxXA_qPCR-Rev	TCGGGGTGTCTTTTTCAGTC	[8]
SoxT1B (0699)_qPCR-Fr	GCCGCCGTCTCAGTAAATAA	[8]
SoxT1B (0699)_qPCR-Rev	AGCAGAAGACGGCAGATGAT	[8]

Plasmids

pHP45Ω-Tc	Ap^r, Tc^r	10
pk18*mobsacB*	Km^r, Mob^+, *sacB*, *oriV*, *oriT*, *lacZα*	11
pk18*mobsacB*-Tc	Km^r, Tc^r pHP45Ω-Tc tetracycline cassette inserted into pk18*mobsacB* using SmaI	7
pk18*mobsacB*ΔtsdATc	Km^r, Tc^r, 2.01 kb fragment implementing deletion of a 996 bp *tsdA* fragment in pK18*mobsacB* with additional tetracycline resistance	6
pk18*mobsacB*-Δs*oxT1A*	Km^r, 2.07 kb SOE PCR fragment implementing *in-frame* deletion of nucleotides encoding amino acids 3 to 361 of SoxT1A cloned into XbaI and SalI of pk18*mobsacB*	This work
pk18*mobsacB*-Δs*oxT1A*-Tc	Km^r, Tc^r, pk18*mobsacB*-Δs*oxT1A* with tetracycline resistance gene from pHP45Ω cloned into SmaI site	This work
pk18*mobsacB*-Δs*oxT1B*	Km^r, 2.07 kb SOE PCR fragment implementing *in-frame* deletion of nucleotides encoding amino acids 3 to 350 of SoxT1B cloned into XbaI and PstI of pk18*mobsacB*	This work
pk18*mobsacB*-Δs*oxT1B*-Tc	Km^r, Tc^r, pHP45Ω-Tc tetracycline cassette inserted into pk18*mobsacB*-Δs*oxT1B* using SmaI	This work
pk18*mobsacB-soxT1Bcomp-Tc*	Km^r, Tc^r, SOE PCR fragment implementing chromosomal integration of *soxT1B* cloned into pk18*mobsacB*-Tc using XbaI and PstI restriction sites	This work
pk18*mobsacB-soxT1B-C24S*-Tc	Km^r, Tc^r, SOE PCR fragment implementing chromosomal integration of *soxT1B* encoding a $Cys^{24}Ser$ exchange cloned into pk18*mobsacB*-Tc using XbaI and PstI restriction sites	This work
pk18*mobsacB-soxT1B-C98S*-Tc	Km^r, Tc^r, SOE PCR fragment implementing chromosomal integration of *soxT1B* encoding a $Cys^{98}Ser$ exchange cloned into pk18*mobsacB*-Tc using XbaI and PstI restriction sites	This work
pk18*mobsacB-soxT1B-C304S*-Tc	Km^r, Tc^r, SOE PCR fragment implementing chromosomal integration of *soxT1B* encoding a $Cys^{304}Ser$ exchange cloned into pk18*mobsacB*-Tc using XbaI and PstI restriction sites	This work
pk18*mobsacB*_Tc_Δ*soxR* (Hden0700)	Km^r, Tc^r, 1.04 kb SOE PCR fragment implementing deletion of nucleotides 4 to 362 of *soxR* to cloned into pk18*mobsacB*-Tc using XbaI and PstI restriction sites	8
pk18*mobsacB*ΔshdR-Tc	Km^r, Tc^r, 2.2 kb BamHI/XbaI fragment of PCR-amplified genome region around *shdrR* with deletion of *shdrR* cloned into BamHI/XbaI of pk18*mobsacB*	7
pk18*mobsacB*_Tc_Δ*soxR/soxT1B*	Km^r, Tc^r, 1.04 kb SOE PCR fragment implementing deletion of nucleotides 4 to of SoxR to nucleotide 1050 of *soxT1B* cloned into pk18*mobsacB*-Tc using XbaI and PstI restriction sites	This work

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In *Hyphomicrobium denitrificans* two related sulfane-sulfur responsive transcriptional repressors regulate thiosulfate oxidation and have a deep impact on nitrate respiration and anaerobic biosyntheses

Running title: Sulfur-responsive repressors from *Hyphomicrobium*

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Abstract

Bacteria have evolved multiple strategies to sense and respond to the availability of inorganic reduced sulfur compounds such as thiosulfate. In *Hyphomicrobium denitrificans*, an obligately chemoorganoheterotrophic Alphaproteobacterium, the use of thiosulfate as a supplemental electron donor is regulated by two homologous sulfane-sulfur-responsive ArsR-type transcriptional repressors, sHdrR and SoxR. Here, we provide information on the distribution and phylogeny of sHdrR, the relevance of its two conserved cysteines i*n vivo*, and identify the genes controlled by SoxR and sHdrR not only by targeted qRT-PCR but also by global RNA-Seq-based analyses of regulator-deficient mutant strains.The absence of sHdrR and SoxR affected 165 and 170 genes, respectively, with 138 genes overlapping. SoxR affects the *sox* genes for periplasmic thiosulfate oxidation and sulfane sulfur import into the cytoplasm, as well as the *lip-shdr-lbpA* genes encoding the cytoplasmic enzymes essential for sulfite formation. sHdrR affects only a subset of these genes. The transcription of *sox* genes remains unaltered in its absence. sHdrR and SoxR act cooperatively, possibly involving heterodimer formation, and their activity also involves interaction with other transcriptional regulators. Most importantly, sHdrR/SoxR regulation extends far beyond sulfur oxidation and deeply affects anaerobic metabolism, particularly denitrification in *H. denitrificans*.

Keywords: *Hyphomicrobium denitrificans*; thiosulfate oxidation; transcriptional regulation; denitrification; PQQ; iron acquisition; ubiquinone biosynthesis

INTRODUCTION

Most dissimilatory sulfur oxidizing prokaryotes oxidize thiosulfate ($S_2O_3^{2-}$), an inorganic sulfur compound of intermediary redox state. Accordingly, microbial thiosulfate oxidation has a major impact on global sulfur cycling. Thiosulfate oxidation under aerobic conditions is very widespread, particularly in marine environments (Podgorsek and Imhoff, 1999; Marshall and Morris, 2013; Watsuji et al., 2016). Anaerobic thiosulfate oxidizers have also been reported from a large range of environments, including anoxic marine basins (Menezes et al., 2020), oceanic oxygen minimum zones (Callbeck et al., 2021), and hydrothermal vents (Teske et al., 2000). In fact, it is likely that anaerobic oxidation, primarily through nitrate and nitrite reduction, is responsible for much of the thiosulfate removal in the marine environment (Ding et al., 2023). Thiosulfate-dependent denitrification to N_2 is best known for obligately autotrophic species such as *Thiobacillus denitrificans*, *Thiomicrospira denitrificans* or *Sulfurovum lithotrophicum* (Inagaki et al., 2004), has been found to be important in marine chemosynthetic symbioses (Paredes et al., 2021), and has also been reported for the facultatively autotrophic *Paracoccus pantotrophus* (Robertson and Kuenen, 1983).

Sulfur oxidizers include not only autotrophic prokaryotes from various groups, but also a large number of obligately organoheterotrophic bacteria that oxidize thiosulfate as an additional electron donor and are widely distributed in soil and natural waters (Trudinger, 1967; Tuttle and Jannasch, 1972; Sorokin et al., 1999; Ding et al., 2023). These include species of the genus *Hyphomicrobium*, Alphaproteobacteria that can be isolated from virtually any freshwater or soil sample, where they can constitute up to 0.2% of the total bacteria (Hirsch and Conti, 1964; Gliesche et al., 2015; Li et al., 2023b). They are also prevalent in temporary puddles and in activated sludge, even under anaerobic conditions. Denitrifying hyphomicrobia such as *H. denitrificans*, are of particular interest because of the need to remove nitrate in drinking water and sewage treatment plants, where these organisms are indeed highly abundant (Holm 1996). *Hyphomicrobium* spp. are typically restricted to C_1 and C_2 compounds as carbon sources and are commonly identified as major players in denitrification systems supplied with methanol (Martineau et al., 2015). While enzymes required for denitrification have been purified and characterized from *H. denitrificans* (Deligeer et al., 2002; Yamaguchi et al., 2003; Yamaguchi et al., 2004), and genetic and physiological aspects of its denitrification are beginning to emerge (Martineau et al., 2015), these studies have not yet included any aspects of oxidative sulfur metabolism.

In *H. denitrificans* thiosulfate oxidation commences in the periplasm. Here, two thiosulfate molecules can be oxidatively linked to form the dead-end product tetrathionate, a reaction catalyzed by thiosulfate dehydrogenase (TsdA) (Koch and Dahl, 2018; Li et al., 2023b). Alternatively, thiosulfate can be completely oxidized to sulfate. This pathway is preferred at lower substrate concentrations (<2.5 mM) and involves the periplasmic SoxYZ carrier protein to which thiosulfate is oxidatively bound by the action of the *c*-type cytochrome SoxXA (Li et al., 2023b). Sulfate is then hydrolyzed off by SoxB and the sulfane sulfur remaining on SoxYZ is transferred to the cytoplasm via the membrane transporter SoxT1A (Li et al., 2024). Once inside, the sulfur is delivered through a cascade of sulfur transfer reactions to the sulfur-oxi-

dizing heterodisulfide-reductase-like enzyme complex, sHdr (Tanabe et al., 2024), which releases sulfite as a product. Sulfite is excreted and, in the absence of efficient sulfite-oxidizing enyzmes in *H. denitrificans*, chemically oxidized to sulfate in the presence of oxygen or may be transformed by other community members under environmental conditions (Li et al., 2023b; Li et al., 2024).

In the environment, *H. denitrificans* must not only cope with constantly changing concentrations of respiratory electron acceptors (oxygen, nitrate), but may also encounter varying concentrations of reduced sulfur compounds such as thiosulfate. To make the most of these additional electron sources, the organism must have strategies for sensing their presence. Indeed, we have recently identified two distinct but closely related ArsR-SmtB family transcriptional repressors, SoxR and sHdrR, that are responsible for the transcriptional regulation of genes encoding Sox, sHdr and associated proteins in *H. denitrificans* (Li et al., 2023b; Li et al., 2023a; Li et al., 2024). SoxR has been extensively characterized at the molecular level. Its sensing mechanism involves the formation of a sulfane sulfur bridge between two conserved cysteine residues in the presence of thiosulfate. In the sulfur-bridged form, the repressor can no longer bind to its operator region(s) on the DNA and transcription is released (Li et al., 2023a). Much less is known about sHdrR. It appears to regulate the sHdr system by modulating sHdrA levels, as seen in Western blot experiments, and to bind to the DNA region upstream of its own gene (Li et al., 2023b). Whether and how the two repressors interact in regulating the overall sulfur oxidation process and possibly other parts of hyphomicrobial energy conservation has not been studied in detail.

Here, we address these knowledge gaps by first providing insights into the relationship between SoxR and sHdrR, the distribution and phylogeny of sHdrR, and the relevance of the conserved sHdrR cysteines i*n vivo*, and then collecting information on the SoxR and sHdrR regulons not only by targeted qRT-PCR but also by a global RNA-Seq-based analysis of regulator-deficient mutant strains, the latter revealing a profound effect on anaerobic metabolism, in particular denitrification.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, primers, and growth conditions

H. denitrificans strains were cultured in minimal medium kept at pH 7.2 with 100 mM 3-(*N*-Morpholino)propanesulfonic acid (MOPS) buffer as previously described (Koch and Dahl, 2018). Media contained 24.4 mM methanol. Thiosulfate was added as needed. *Escherichia coli* strains were grown on complex lysogeny broth (LB) medium (Bertani, 2004) under aerobic conditions at 37°C unless otherwise indicated. *Escherichia coli*. BL21 (DE3) was used for recombinant protein production. *E. coli* strains 10-beta and DH5α were used for molecular cloning. Antibiotics for *E. coli* and *H. denitrificans* were used at the following concentrations (in µg ml^{-1}): ampicillin, 100; kanamycin, 50; streptomycin, 200; chloramphenicol, 25. Supplementary Table 1 lists the bacterial strains, and plasmids that were used for this study.

Recombinant DNA techniques

Restriction enzymes, T4 ligase and Q5 polymerase were obtained from New England Biolabs (Ipswich, UK) and used according to the manusfacturer's instructions. Oligonucleotides were obtained from Eurofins Genomics Germany GmbH (Ebersberg, Germany). Standard techniques for DNA manipulation and cloning were used unless otherwise indicated (Ausubel et al., 1997). Plasmid DNA from *E. coli* was purified using the GenJET Plasmid Miniprep kit (Thermo Scientific, Waltham, USA). Chromosomal DNA from *H. denitrificans* strains was prepared using the Simplex Easy DNA Extract Kit (GEN-IAL GmbH, Troisdorf, Germany). DNA fragments were extracted from agarose gels using the GeneJET Gel Extraction Kit (Thermo Scientific, Waltham, USA).

Overproduction and purification of recombinant truncated sHdrR

A truncated version of the *H. denitrificans shdrR* gene not encoding the first 25 amino acids of the wildtype protein was amplified from *H. denitrificans* genomic DNA using primers Fr-pET22b-sHdrR-trun-NdeI and Rev-pET22b-0682-NotI and (Supplementary Table 1) and cloned between the NdeI and NotI sites of pET22b (+), resulting in pET22bHdsHdrR-trunc. Recombinant sHdrR was overproduced in *E. coli* BL21(DE3). The cells were grown at 37°C in 200 ml LB medium containing ampicillin up to an OD600 of 0.6. Expression of *shdrR-trunc* was induced by adding 0.5 mM IPTG. IPTG-induced *E. coli* cells were grown over night at 20°C. The carboxy-terminally His-tagged protein was purified by affinity chromatography on Ni^{2+}-NTA using the same conditions as described for the full length protein (Li et al., 2023b)

Construction of *H. denitrificans* mutant strains

The suicide plasmid pk18*mobsacB* (Schäfer et al., 1994) and the tetracycline cassette from pHP45Ω-Tc (Fellay et al., 1987) were used for reverse genetics in *H. denitrificans*. Derivatives were constructed using on the basis of previously published procedures (Cao et al., 2018; Koch and Dahl, 2018). For chromosomal complementation of the *H. denitrificans* Δ*tsdA* Δ*shdrR* strain, the *shdrR* gene was amplified together with upstream and downstream regions using primers Fwd_deltaHden0682_BamHI and Rev_deltaHden0682_XbaI and cloned into the XbaI/BamHI sites of pk18*mobsacB* . For chromosomal integration of the genes encoding sHdrR $Cys^{50}Ser$, sHdrR $Cys^{116}Ser$ and sHdrR $Cys^{50}Ser$ $Cys^{116}Ser$, the modified genes and upstream and downstream sequences were amplified by SOE PCR using the appropriate primers listed in Supplementary Table 1. Finally, the tetracycline resistance cassette from pHP45ΩTc was inserted into each of the plasmids using SmaI. The final constructs were electroporated into *H. denitrificans* Δ*tsdA* Δ*shdrR* and transformants were selected using previously published procedures(Cao et al., 2018; Koch and Dahl, 2018). Single crossover recombinants were Cm^r and Tc^r. Double crossover recombinants were Tc^s and survived in the presence of sucrose due to loss of both, the vector-encoded levansucrase (SacB) and the tetracyclin resistance gene. The genotype of the *H. denitrificans* strains generated in this study were confirmed by PCR and sequencing.

Characterization of phenotypes, quantification of sulfur compounds and biomass content

Growth experiments with *H. denitrificans* were run in medium with 24.4 mM methanol in Erlenmeyer as described earlier(Li et al., 2023b). 2 mM thiosulfate were added when needed. Biomass content, thiosulfate and sulfite concentrations were determined by previously described methods(Dahl, 1996; Li et al., 2023b). All growth experiments were repeated three to five times. Representative experiments with two biological replicates for each strain are shown. All quantifications are based on at least three technical replicates.

Electrophoretic mobility shift assays (EMSA)

The binding reaction mixtures for EMSA assays (15 µl final volume), contained purified truncated sHdrR protein in various concentrations (up to 400 nM), 2 µl 50 % glycerol and 1.5 µl 10 × binding buffer (100 mM Tris-HCl, 500 mM KCl, 10 mM DTT, 5 % glycerol, pH 8.0). Reaction mixtures were pre-incubated for 20 min at room temperature followed by a further 30 min incubation at 30°C after adding the DNA probe to a final concentration of 17 nM. DNA probes were prepared using the primers listed in Supplementary Table S1. The DNA probes were the same as described in (Li et al., 2023a). After pre-running 6% native polyacrylamide gels at 100 V for 1 h at 4 °C with 0.25 × TBE buffer (25 mM Tris-borate, 0.5 mM EDTA), they were loaded with the reaction mixtures. 0.25 × TBE with 0.5 % glycerol was used as running buffer. The gels were run at 180 V for 1h at 4 °C. Gels were subsequently stained for 20 min with SYBR green I. The bands corresponding to sHdrR-bound and free DNAs were visualized with a ChemiDoc Imaging System (BioRad).

Expression studies based on RT-qPCR

Total RNA of the relevant *H. denitrificans* strains was isolated from cells harvested in mid-log phase according to an established procedure (Li et al., 2023a). RNA samples of 100 ng were used for RT-qPCR analysis which was performed with the primers listed in Supplementary Table 1 following the method described in (Li et al., 2023a).

Genome-wide transcriptomic analysis of *H. denitrificans* strains Δ*tsdA* Δ*shdrR* and Δ*tsdA* Δ*soxR* in the absence of thiosulfate

For transcriptome sequencing (RNA-Seq), *H. denitrificans* strains Δ*tsdA* Δ*shdrR* and Δ*tsdA* Δ*soxR* were cultured in 50 ml minimal medium containing 24.4 mM methanol in 200 ml Erlenmeyer flasks at 30°C with shaking at 200 rpm to early log phase. Cells from 20 ml culture were harvested and flash frozen in liquid N_2 and stored at -70°C. As described in (Li et al., 2024), the RNA was purified with the FastGene RNA Premium Kit (NIPPON Genetics Europe, Düren, Germany) according to the manufacturer's instructions and with introduction of a modified cell lysis step by bead beating. RNA quality was checked on 1% agarose gels and its concentration was measured using NanoPhotometer NP80 (IMPLEN, Munich, Germany). The RNA was shipped on dry ice to Eurofins Genomics GmbH (Ebersberg, Germany). The subsequent analysis pipeline included rRNA depletion, library preparation (mRNA fragmentation, strand specific cDNA synthesis), Illumina paired end sequencing (2 x 150 bp, minimum 10 MB reads)), and

bioinformatic analysis (mapping against the reference genome, identification and quantification of transcripts, pairwise comparison of expression levels and determination of significant fold differences) and was conducted by the company.

Phylogenetic tree inference

For inference of a phylogenetic tree for sHdrR proteins and relatives, proteins were aligned using MAFFT (Katoh and Standley, 2013) and the alignment was manually trimmed. A maximum likelihood phylogeny was inferred iusing IQ-TREE v1.6.12 (Nguyen et al., 2015). Branch support was calculated by ultrafast bootstrap (2000 replicates) (Hoang et al., 2018). Finally, the tree was displayed using iTol (Letunic and Bork, 2021).

Statistics and reproducibility

Experimental data are expressed as the mean ±standard deviation of the mean (SEM) of the number of tests stated for each experiment. All analysis was reproduced in at least three independent experiments. The significant difference between the two groups was analyzed using an independent student's t-test; the p-value < 0.05 indicated statistical significance.

RESULTS

Distribution and phylogeny of sHdrR-related proteins

The *H. denitrificans* sHdrR (*Hd*sHdrR) protein is 125 amino acids in length, and a BlastP search identified *R. capsulatus* SqrR (Shimizu et al., 2017) as the most similar structurally characterized protein. *Hd*sHdrR is also similar to several other characterized transcriptional regulators, all of which belong to the same ArsR subfamily and include SoxR from *Pseudoaminobacter salicylatoxidans* (Mandal et al., 2007), *Paracoccus denitrificans* (Rother et al., 2005) and *H. denitrificans* (Li et al., 2023a), BigR from *Xylella fastidiosa* (Guimarães et al., 2011) and *Acinetobacter baumannii* (Walsh et al., 2020), YgaV from *E. coli* (Gueuné et al., 2008; Balasubramanian et al., 2022) and HlyU from *Vibrio cholerae* (Pis Diez et al., 2023) (Figure 1). All of these regulators share two conserved cysteine residues, Cys^{50} and Cys^{116} in the hyphomicrobial protein (Figure 1). The characterized proteins sense reactive sulfur species (RSS) and form an intraprotomer tetrasulfide bridge between the conserved cysteines when exposed to sulfane sulfur transpersulfidation donors (Giedroc et al., 2023; Li et al., 2023a).

In a previous publication, we performed a survey of the occurrence of genes for sHdrR-like proteins based on an automatically generated hidden Markov model (HMM) with the additional condition that the respective gene is located in the vicinity of a *shdr*-like gene cluster (Li et al., 2024). This revealed related transcriptional regulators in a wide variety of prokaryotes bearing the genetic potential for sulfur oxidation in the cytoplasm via the sHdr system. However, close examination of the amino acid sequences showed that many of the identified proteins do not contain the two conserved cysteine residues typical of sulfane-sulfur-responsive transcriptional repressors. This means not only that the HMM for sHdrR does not emphasize the conserved cysteines and must therefore be interpreted with caution, but also that the expression of *shdr* genes in many organisms involves ArsR-type regulators that rely on different modes of action.

In bacteria, transcriptional regulators often control the expression of genes located close to their own (Martinez-Antonio and Collado-Vides, 2003; Browning and Busby, 2004; Seshasayee et al., 2006). Therefore, we linked the proteins retrieved by a BlastP search using *Hds*HdrR as a bait and the presence of the two conserved cysteines as a constraint with information about their genetic environment, and indeed we obtained revealing information (Supplementary Table 2, Figure 2). The general picture that emerged from our analysis is that sulfane sulfur-responsive ArsR-type regulators appear to act not only on the expression of genes for enzymes involved in the oxidation and/or detoxification of sulfur compounds, such as *sqr* for sulfide:quinone oxidoreductase, *sox* for thiosulfate oxidation in the periplasm, *pdo* for persulfide dioxygenase, *rhd* for rhodanese-type sulfur transferases or *shdr* for sulfite production in the cytoplasm, but also on target genes encoding membrane proteins that have been shown or are likely to be involved in the transport of sulfur compounds across the cytoplasmic membrane, such as efflux pumps of the resistance-nodulation-division (RND) family (Nikaido, 2011), sulfite exporters of the TauE family (Weinitschke et al., 2007), or SoxT and PmpAB, YeeE family transporters involved in the import of sulfur-containing ions (Gristwood et al., 2011; Tanaka et al., 2020; Ikei et al., 2024; Li et al., 2024) (Figure 2, Supplementary Table 2).

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X. fastidiosa BigR          NEVANLLKTLSHPVRLMLVCTLVEGEFSV-GELEQQIGIGQPTLSQQLGVLRESGIVETR    81
V. cholera HlyU             AKAVVLLKAMANERRLQILCMLLDNELSV-GELSSRLELSQSALSQHLAWLRRDGLVNTR    77
T. mangrovi sHdrR           TQATGLLKSMANESRLMILCLLSQQEMSV-GELAQRIELSQSALSQHLSILRREKLVKTR    80
H. denitrificans 1NES1      DESSALLKALSNRHRLLVLCQLIDGEKSV-GQLADFLGVRDSTASQHLALLRRDRIIASR    64
Rp. globiformis             EQASELLKSLANRHRLLILCQLVDGERSV-GDLAAFLKTRDSTVSQHLALLRKDGLVQAR    64
Rp. globiformis sHdrR       GRAAGFLKSLANEHRLMILCSLIEGEKSV-GDLQEELNMRQPHLSQQLSRLRLGGLVETR    64
D. nanyangense              RRTTDFLKSLAHEGRLVILCRLSEGPATV-TELEQVLGARQSSVSQQLARLRSEGLVDYE    81
Ps. salicylatoxidans SoxR   RKASDLLKALSHEGRLLILCLLAEGEKSV-SELESIMHMPQAAVSQQLARLRFDRLVNTR    81
Pc. denitrificans SoxR      NDASTFLKALGHDGRLMILCYLMSGPKSV-TELENLLSSRQAVVSQQLARLRHEGLVSAR    119
H. denitrificans X SoxR     RKASDFLKALAHESRLLILCLLAEKERSA-GELENLLSINQPTVSQQLARLRLDGLVQAR    89
Rb. capsulatus SqrR         RAASNLLKALAHEGRLMIMCYLASGEKSV-TELETRLSTRQAAVSQQLARLRLEGLVQSR    80
E. coli YgaV                EQAAALLKAMSHPKRLLILCMLSGSPGTSAGELTRITGLSASATSQHLARMRDEGLIDSQ    71
H. denitrificans X sHdrR    EQATALLRALGSPHRLAILCLLLEGERTV-SEICDKIGARQSLVSQHLTRLRLDGLVKSD    89
                                 :*:::.   ** ::* *     :   ::           **:*  :*   ::

X. fastidiosa BigR          RNIKQIFYRLTEAKAAQLVNA---LYTIFCAQEKQA-------------------------   114
V. cholera HlyU             KEAQTVFYTLSSTEVKAMIEL---LHRLYCQANQ----------------------------   108
T. mangrovi sHdrR           RESQFVWYSLASEEAERVVHT---LYDIYCADCEID-------------------------   113
H. denitrificans 1NES1      RDGQTIWYRIASEPALAVMQV---LNEVYCAGSKSQPGRPRK-------------------   103
Rp. globiformis             REAQTIYYSIASCPAQQVLET---LFAIYCSPTPICGMVPAPAESKPPLREPAAAQRTPT   121
Rp. globiformis sHdrR       REAQTIYYSLTDETATEVIGV---LHRRFCKAKPRQRTRQAATRATSAAR------RADP   115
D. nanyangense              RDGRILRYSIADDRARKVVAM---LYDLFCD-------------------------------   109
Ps. salicylatoxidans SoxR   REGRVIYYSIASSEVSSVIST---LYGLFCAPVRKKE-------------------------   115
Pc. denitrificans SoxR      RDGQTIFYSILDPKVVDLLAVLKKLFETDC---------------------------------   149
H. denitrificans X SoxR     REGKAVIYSLPDETTRRFIGA---IYDKFCREEPSRKR----------------------   124
Rb. capsulatus SqrR         REGKTIYYSLSDPRAARVVQT---VYEQFCSGD-----------------------------   110
E. coli YgaV                RDAQRILYSIKNEAVNAIIAT---LKNVYCP--------------------------------   99
H. denitrificans X sHdrR    RNGYFVSYSLTSAPAQEIIAT---LHKYYCATSAGKRSN----------------------   125
                            ::   : * : .  .  .:     :    *
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FIGURE 1. Amino sequence alignment of the central region of selected sHdrR homologs. Organism names, accession numbers/locus tags and references (if available) in the order of appearance: *Xylella fastidiosa* BigR, XF_0767 (Guimarães et al., 2011), *Vibrio cholera* HlyU, VC_A0642 (Mukherjee et al., 2014; Pis Diez et al., 2023); *Tsuneonella mangrovi*, CJO11_RS12710; *Hyphomicrobium denitrificans* 1NES1, HYPDE_25308 (Venkatramanan et al., 2013); *Rhodopila globiformis*, CCS01_RS26760 and *Rp. globiformis* sHdrR, CCS01_RS13140 (Imhoff et al., 2018); *Devosia nanyangense* HY834_20740 (He et al., 2021), *Pseudaminobacter salicylatoxidans* SoxR, WP_019171658 (Mandal et al., 2007); *Paracoccus denitrificans* SoxR, CAB94376 (Rother et al., 2005), *H. denitrificans* X^T SoxR, Hden_0700 (Li et al., 2023a); *Rhodobacter capsulatus* SqrR, ADE85198 (Shimizu et al., 2017); *Escherichia coli* YgaV, b2667 (Gueuné et al., 2008); *H. denitrificans* X^T sHdrR, Hden_0682 (Li et al., 2023b; Li et al., 2024). An * (asterisk) indicates positions with identical residues. Cysteines are highlighted in yellow. Colons (:) and single dots (.) indicate conserved and semi-conserved amino acids, respectively.

Remarkably, *Hds*Hdr-related regulators with two conserved cysteines are rarely encoded in the vicinity of *shdr* genes and the few that fall into this category do not form a coherent phylogenetic clade (Supplementary Table 2, Figure 2). *Hds*HdrR has a third cysteine, Cys^{63} (Figure 1). An equivalent of *Hds*HdrR Cys^{63} is only present in a few cases (e.g. in the proteins from *Hyphomicrobium* sp. strains GJ21 and SCN 65-11). Neither is the absence of the third cysteine an indicator that the regulator is likely unable to act on *shdr* genes (due to its absence in the proteins from *Devosia nanyangense*, *Tsuneonella mangrovi* and *Rhodopila globiformis*, all of which are encoded in close proximity to *shdr* gene clusters), nor is its presence typical for ArsR-type proteins, which are likely to regulate *shdrR* genes, as it is present in the proteins of the cyanobacterium *Sodalimona willei* and the Gram-positive *Hathewaya proteolytica*. Both cannot oxidize sulfur compounds for dissimilatory purposes and do not contain *shdr* or *sox* genes (Figure 1, Supplementary Table 2).

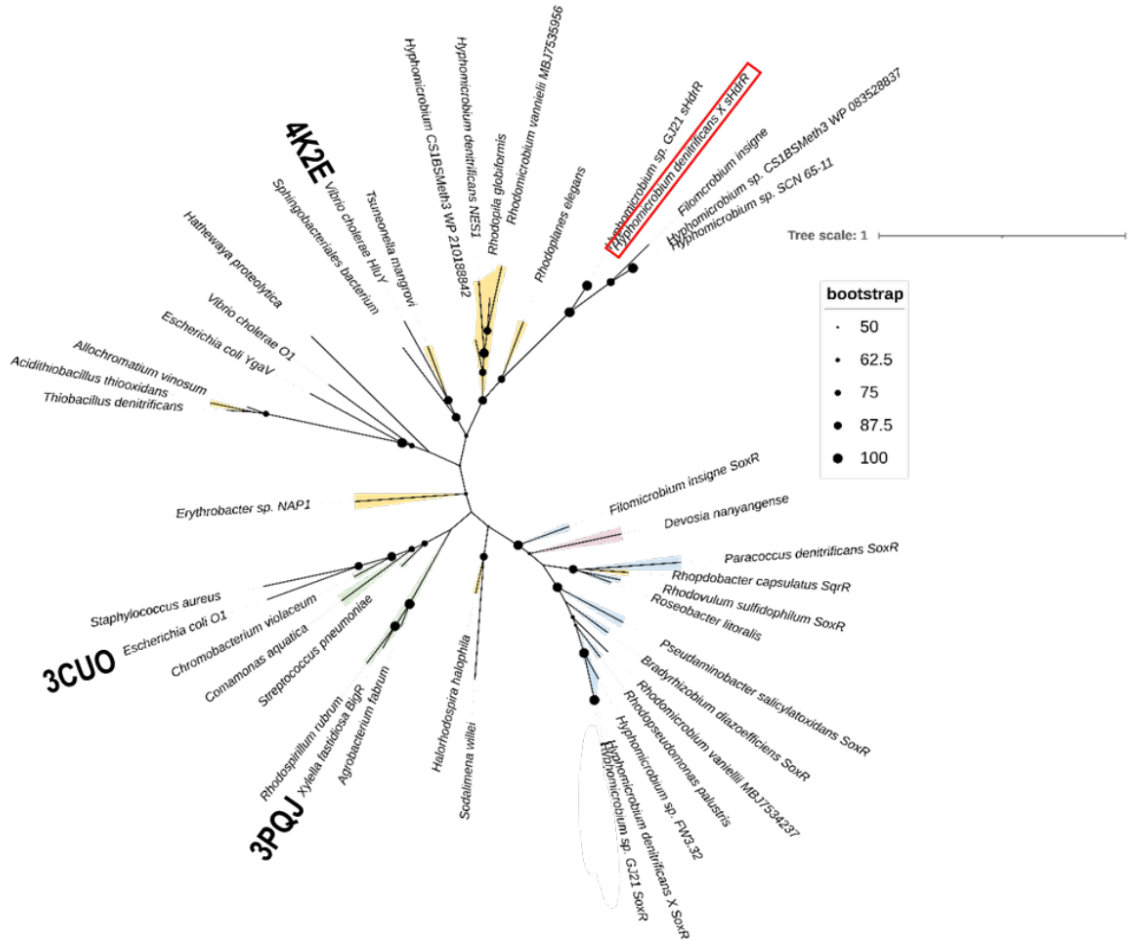

FIGURE 2. Unrooted phylogenetic tree for sulfane sulfur-responsive ArsR-type transcriptional regulators containing two conserved cysteine residues. Colored ranges indicate the occurrence of the respective gene in immediate vicinity of genes for resistance-nodulation-division (RND) transporters (beige), PmpAB-like YeeE family transporters (green), Sox proteins for thiosulfate oxidation (blue) or sHdr proteins for sulfur oxidation in the cytoplasm (purple). Note that in *Tsuneonella mangrovi* the *shdrR*-like gen is situated next to *shdr* genes and to genes for a RND transporter and that SoxR is encoded between sets of *sox* and RND-encoding genes in *Rhodopseuomonas palustris*. In both cases, only the vicinity to RND-encoding genes is indicated. The sHdrR protein from *H. denitrificans* X^T is highlighted by a red box. PDB codes are given for structurally characterized proteins. The tree was calculated with 2000 bootstrap resamplings using Ultrafast Bootstrap (Hoang et al., 2018) and IQ-Tree

(Trifinopoulos et al., 2016; Minh et al., 2020). Bootstrap values between 50% and 100% are displayed as scaled circles at the branching points. Protein accession numbers, information on adjacent genes and references are available in Supplementary Table 2. The tree is available in Newick format as Supporting information (Supplementary data sHdrR and relatives tree.nwk).

Importance of conserved sHdrR cysteines *in vivo*

In order to build a firm basis for the identification and characterization of sHdrR, we clarified whether the conserved cysteines (Cys^{50} and Cys^{116} in *Hd*sHdrR, Figure 1) are indeed necessary for proper function of the regulator *in vivo.* This was achieved by phenotypic characterization of *H. denitrificans* mutant strains that contained chromosomal replacements of the wild-type *shdrR* gene with variants encoding cysteine to serine exchanges of either one or both of the two conserved cysteines. All experiments reported in this and also previous studies (Li et al., 2023b; Li et al., 2023a) were performed using *H. denitrificans* Δ*tsdA* as the reference strain. This strain lacks the gene for thiosulfate dehydrogenase (TsdA). This enzyme catalyzes the oxidative formation of tetrathionate from two thiosulfate molecules. Thiosulfate dehydrogenase-deficient strains are unable to form tetrathionate and thus are perfectly suited for analyzing oxidation of the sulfur substrate to sulfite and eventually sulfate (Li et al., 2023b; Li et al., 2023a; Tanabe et al., 2023; Li et al., 2024; Tanabe et al., 2024). The *H. denitrificans* Δ*tsdA* reference strain excretes sulfite as an intermediate *en route* to sulfate, when methanol-grown cultures are provided with thiosulfate as an additional electron source. Sulfite is toxic and leads to growth retardation that is particularly impressive when cultures are inoculated with thiosulfate-induced cells ((Li et al., 2023b), also compare curves with filled circles in the upper panels of Figure 3). A Δ*tsdA* strain, that additionally lacks the transcriptional repressor *shdrR*, oxidizes thiosulfate without induction and accordingly, its growth rate slows down almost immediately when it is exposed to the sulfur compound ((Li et al., 2023b; Li et al., 2024), also compare curves with open boxes in the upper panels of Figure 3). When the *shdrR* gene is complemented *in cis*, the wild-type phenotype is reconstituted (Supplementary Figure 2).

Like the *H. denitrificans* Δ*tsdA* Δ*shdrR* strain, the strain with sHdrR bearing a Cys^{116}Ser exchange exhibited a high specific thiosulfate oxidation rate and a significantly reduced growth rate even without induction of pre-cultures (curves with filled triangles in Figure 3). The growth rate increased significantly after thiosulfate was consumed. This appears to mean that *in vivo* the sHdrR Cys^{116}Ser variant protein has a decreased DNA binding and thus transcription repressing activity. In contrast, both, the mutant strain encoding the sHdrR Cys^{50}Ser exchange and the mutant strain with both sHdrR cysteines replaced by serine, were unable to oxidize thiosulfate (curves with open circles and filled boxes in Figure 3). The most obvious conclusion here would be that these sHdrR repressor variants constitutively repress transcription, i.e. are always attached to their binding sites, and cannot react to the presence of oxidizable sulfur *in vivo*. However, we must take into account that the mutant strains studied still contain the fully functional SoxR regulatory protein, which we already know is the master regulator of sulfur oxidation in *H. denitrificans* (Li et al., 2024). Interactions with this protein *in vivo* may complicate conclusions about DNA binding capacity of the sHdrR variants. Irrespective of this, however, our experiments clearly demonstrate the relevance of the two conserved cysteines.

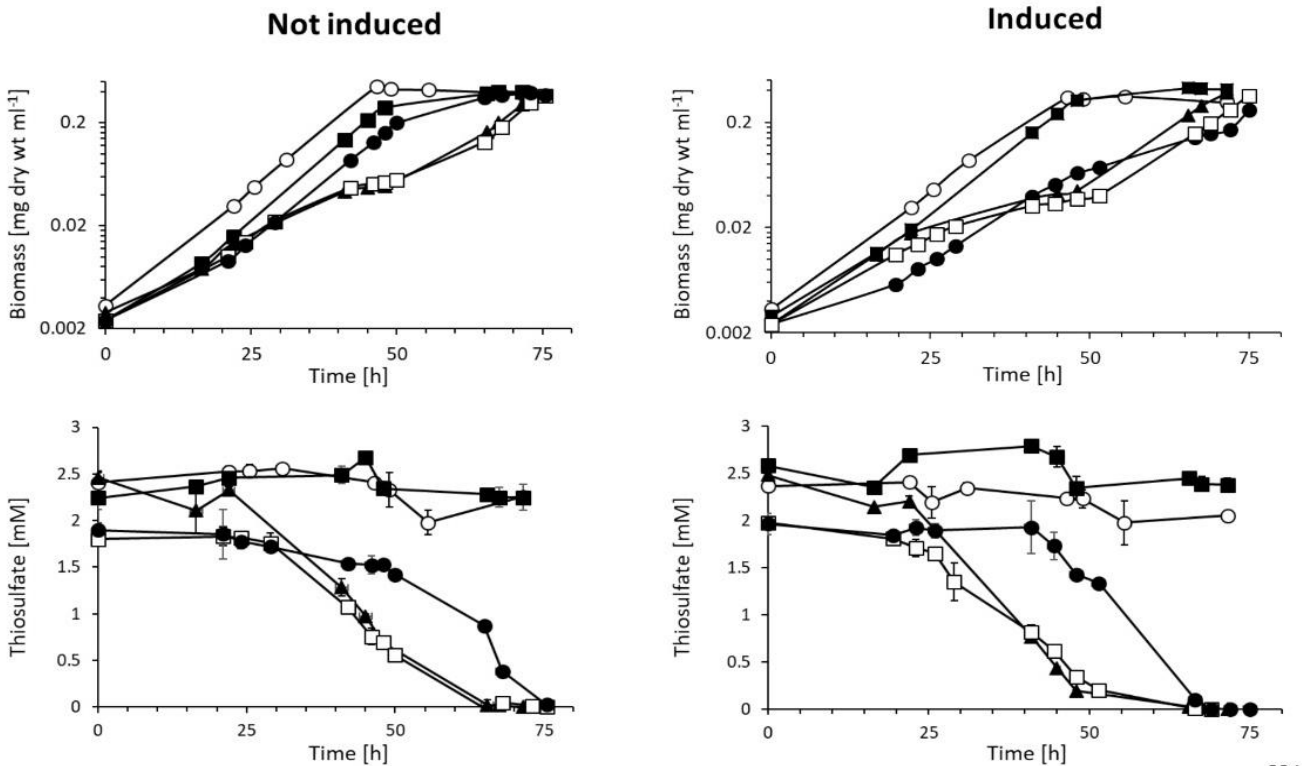

FIGURE 3. Growth and thiosulfate consumption of the *H. denitrificans* reference strain, a strain lacking sHdrR and strains producing sHdrR variants with cysteine to serine exchanges. *H. denitrificans* Δ*tsdA* (filled black circles) Δ*tsdA* Δ*shdrR* (open boxes), Δ*tsdA* sHdrR $Cys^{50}Ser$ filled boxes), Δ*tsdA* sHdrR $Cys^{116}Ser$ (filled triangles) and Δ*tsdA* sHdrR $Cys^{50}Ser$ $Cys^{116}Ser$ (open circles). All strains were grown in medium containing 24.4 mM methanol. In the "not induced" case, pre-cultures were grown without thiosulfate. In the "induced" case, pre cultures contained 2 mM thiosulfate. Thiosulfate concentrations for the different cultures are depicted in the lower panels. Symbol assignments are the same as in the upper panels. Error bars indicating SD are too small to be visible for determination of biomass. All studied strains grew equally well on methanol in the absence of thiosulfate (Supplementary Figure 1).

Identification of genes controlled by sHdrR by RT-qPCR for different *H. denitrificans* strains

As a first step into the description of the sHdrR regulon, we performed RT-qPCR and determined the transcription levels of twelve signature genes in the *H. denitrificans* sulfur oxidation locus for the Δ*tsdA* Δ*shdrR* mutant in the absence of thiosulfate. The results were compared with previous results for the Δ*tsdA* reference strain in the absence and presence of thiosulfate, and with results for a Δ*tsdA* Δ*soxR* mutant in the absence of thiosulfate (Li et al., 2023a) (Figure 4a). In accordance with comparative mRNA-Seq analysis (Li et al., 2023a), our work with the reference strain had already shown a strong positive effect of thiosulfate on the expression of all genes residing in the respective region (Figure 4b), including two divergently transcribed *sox* operons for the periplasmic thiosulfate-oxidizing Sox system, the *soxT1A* operon which encodes a sulfane sulfur import machinery, the *dsrE3C-shdr-lbpA* genes responsible for cytoplasmic sulfane sulfur transfer and its oxidation to sulfite and the *lip* genes for assembly of lipoate on the LbpA proteins. The only unaffected genes were those for the transcriptional repressors, sHdrR and SoxR, and the transporter SoxT1B, that acts as a signal transduction unit for SoxR (Li et al., 2024). For the hyphomicrobial sulfur oxidation signature genes, the absence

of SoxR had similar effects as the presence of thiosulfate, resulting in, but not limited to, high constitutive transcription rates for the *sox* and *shdr* genes (Figure 4a). As observed for SoxR, the absence of sHdrR led to constitutive transcription of genes relevant for oxidative sulfur metabolism. However, our qRT-PCR analyses showed that sHdrR affects only a subset of the genes affected by SoxR and that this sHdrR-controlled subset does not include the genes in the divergently transcribed *soxYZ* and *soxAX* loci (Figure 4a).

The finding that sHdrR does not exert a significant effect on *sox* gene expression *in vivo*, prompted us to analyse probable DNA binding regions. To that end, we inspected the same intergenic regions within the hyphomicrobial sulfur oxidation locus that had already been tested as probes for SoxR (Li et al., 2023b) and used them in electrophoretic mobility shift assays (EMSA) with sHdrR (Figure 4c,d). These experiments were performed with a carboxy-terminally His-tagged and amino-terminally truncated version of sHdrR produced in *E. coli*, that lacked the first 25 amino acids of the wild-type protein. The truncation was necessary because the full-length sHdrR proved to be very unstable, as documented by mass spectrometry, whereas the truncated variant could be used for a period of seven days when kept on ice. The truncated sHdrR variant fully encompasses the central conserved region shown in Figure 1 and binds effectively to all but the control DNA probe (Figure 4d). The latter is a 176-bp fragment located upstream of Hden_0697, encoding a putative cytochrome P450 that, unlike the other fragments, does not contain prominent inverted or direct repeats (Figure 4c). All four of the DNA fragments that were bound by sHdrR had previously been shown to contain binding sites for SoxR (Li et al., 2023b). Surprisingly, the intergenic region between the divergently oriented *soxA* and *soxY* genes is recognized by sHdrR *in vitro* (Figure 4d), although a lack of sHdrR has no significant effect on the transcription of these genes (Figure 4a). Together, our observations point at an intimate interplay of the two related repressor proteins *in vivo* that might be governed by different binding affinities and/or include heterocomplex formation.

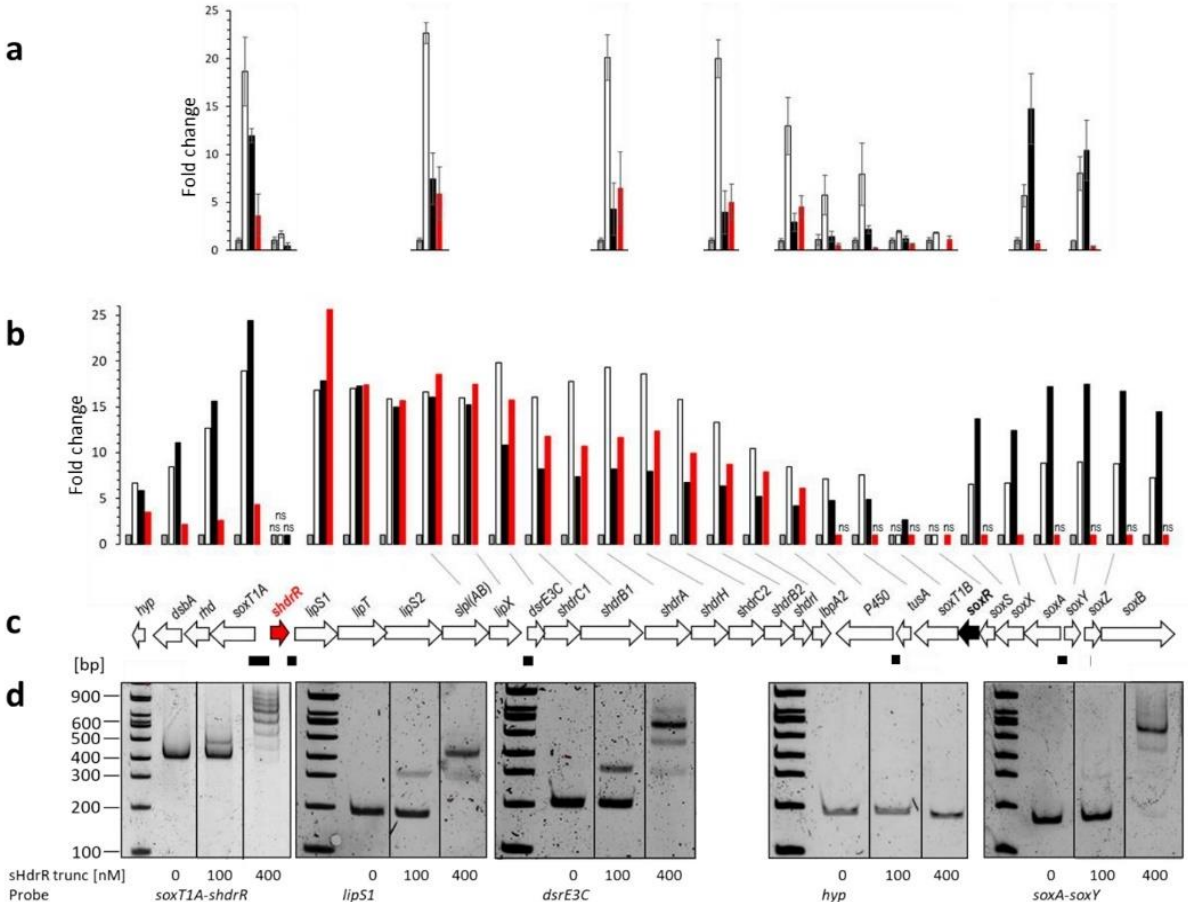

FIGURE 4. Regulation of the *shdr-sox* region in *H. denitrificans* (a). Relative mRNA levels of twelve genes located in the *shdr-sox* region (depicted in panel **(c)**) from *H. denitrificans* for the Δ*tsdA* reference strain in the absence (gray columns) and presence of thiosulfate (white columns), as assessed by RT-qPCR. Results for *H. denitrificans* Δ*tsdA* Δ*soxR* and *H. denitrificans* Δ*tsdA* Δ*shdrR* are shown by black and red columns, respectively. Results were adjusted using *H. denitrifcans rpoB*, which encodes the β-subunit of RNA polymerase, as an endogenous reference according to (Martineau et al., 2015). All cultures were harvested in the exponential growth phase. Three parallel experiments were performed to obtain averages and standard deviation. **(b)** Transcript abundance changes of genes encoding enzymes involved in thiosulfate oxidation in the same strains as in **(a)** as assessed by mRNA-Seq analysis. The same color coding applies. The experiments were conducted in duplicate, each time using mRNA preparations from two different cultures. Adjusted *p* values for statistically significant changes were all below 0.001 (Supplementary Table 3). ns, not significant. **(c)** DNA regions tested in EMSA assays for sHdrR binding are indicated as black rectangles below the hyphomicrobial *shdr-sox* genetic island. Fragment sizes: 362 bp for the *soxT1A-shdrR* intergenic region, 177 bp and 173 bp for the regions upstream of *lipS1* and *dsrE3C*, respectively. The fragments downstream of *tusA* and between *soxA* and *soxY* had sizes of 176 bp and 151 bp, respectively. **(d)** EMSA analysis of Strep-tagged sHdr-trunc with upstream promoter sequence probes of sulfur oxidation related genes as specified in **(c)**. 17 nM DNA probes were incubated with different amounts of sHdr-trunc (100 and 400 nM). Vertical lines separate samples that were run on the same gel but were not directly adjacent.

Global analysis of the sHdrR and SoxR regulons by RNA-seq analysis of different *H. denitrificans* strains

In the next step, we sought to identify the regulons of the intertwined transcriptional repressors SoxR and sHdrR on a global basis and extended previous genome-wide mRNA-Seq data for the Δ*tsdA* reference strain to *H. denitrificans* strains lacking either the genes for sHdrR or SoxR. We compared the mRNA abundance in these two strains to the mRNA abundance in

the reference strain in the absence of thiosulfate. In addition, the data set for the reference strain in the presence of thiosulfate (Li et al., 2024) was integrated in the analyses. The number of identified mRNAs was virtually identical in all cases and ranged between 95 to 96% of the 3529 predicted genes.

Volcano plots (Supplementary Figure 3) offer a comparative visual assessment of total gene expression patterns for each mutant relative to the wild type in the absence and in the presence of thiosulfate. The lack of the repressors sHdrR and SoxR affected the abundance of a total of 165 (4.8%) and 170 (5.0%) of the detected mRNAs, respectively, while the presence of thiosulfate affected the transcription of 136 (4.1%) genes in the reference strain. The genes affected by the lack of the two repressors exhibit a high overlap of 138 genes (83.6% and 81.2% of all genes affected by sHdrR and SoxR, respectively) (Figure 5a). Furthermore, 85 genes (65%) that are affected by the lack of either one or both of the repressors overlap with the genes affected by thiosulfate in the reference strain. We can therefore confidently state that SoxR and sHdrR are intimately involved in the cells' response to the availability of the oxidizable sulfur substrate thiosulfate.

Based on current models of how ArsR-type repressors control gene expression (Busenlehner et al., 2003; Ren et al., 2017; Roy et al., 2018), removing the repressor proteins (i.e. deletion mutations as in the current study) should result in constitutive expression of genes that are normally repressed by that protein in the absence of the de-repressing effector molecule. Decreased expression would indicate that the gene is activated (directly or indirectly) by the respective transcriptional regulator. The presence of thiosulfate positively influences (fold change >2) the transcription of 89 genes in the reference strain, while a lower number of genes (47) is negatively affected (Li et al., 2024). Both positive and negative changes are also observed for the SoxR and the sHdrR-deficient mutant strains (Figure 5b,c). In the *H. denitrificans* Δ*tsdA* Δ*soxR* and Δ*tsdA* Δ*shdrR* strains, 69 genes are significantly overexpressed respectively, with an overlap of 69.6% of the genes (Figure 5b). The overlap with transcripts of higher abundance in the presence of thiosulfate in the reference strain is 37.1% and 36.0%, respectively (Figure 5b,d, Supplementary Table 3). Conspicuously, there is a much higher overlap between the transcription of genes negatively affected by thiosulfate or the absence of either one of the repressors than seen for the genes with transcription increases (compare Figures 5b and 5c). While for the upregulated cases, 47 (52.8%) are induced by thiosulfate in the reference strain but do not show consequences upon deletion of the repressor genes, this is observed for only 4 (8.5%) of the downregulated cases. In addition, there are very few genes for which transcriptional downregulation is caused by the absence of only one of the regulators (Figure 5c). Only in 6.7% and 12.2% of cases, respectively, is the effect exclusively due to the absence of sHdrR or SoxR, while for genes with increased transcription the proportion amounts to 30.4% in both cases.

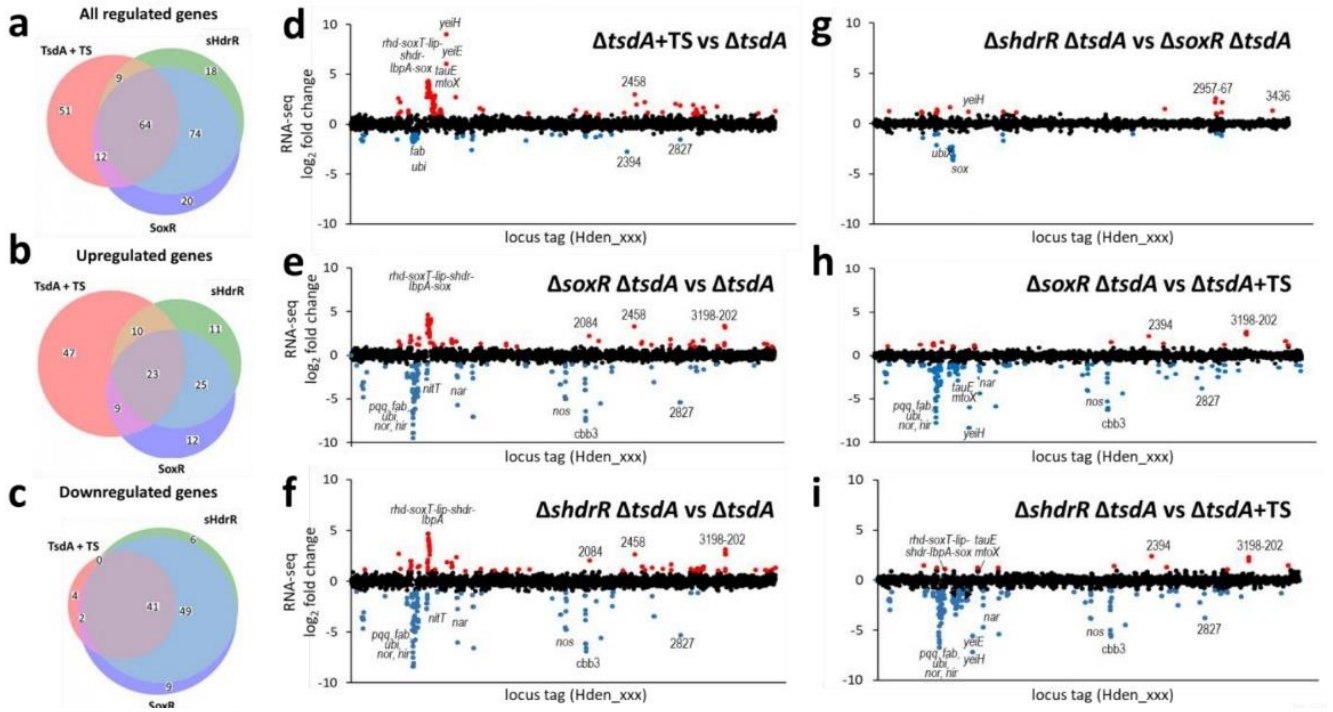

FIGURE 5. Global analysis of the sHdrR and SoxR regulons in *H denitrificans* by RNA-seq analysis. (a to c) Scaled Venn diagrams displaying the number differentially expressed genes from mRNA-Seq analysis of *H. denitrificans* strain Δ*tsdA* in the presence of thiosulfate (TsdA + TS, red) , and strains Δ*tsdA* Δ*soxR* (SoxR, blue) and Δ*tsdA* Δ*shdrR* (sHdrR, green) in the absence of thiosulfate compared to *H. denitrificans* strain Δ*tsdA* in the absence of thiosulfate. The number of differentially expressed genes in each group is represented by the size of each circle, and overlapping areas indicate genes shared between the strains/conditions. Genes meeting a log2-fold change of >2 and an adjusted p-value of <0.001 were considered differentially expressed. Numbers in each area are given for all regulated genes **(a),** all upregulated genes **(b)** and all downrgulated genes **(c)**. **(d to i)** Fold change in transcription for *H. denitrificans* Δ*tsdA* + thiosulfate (TS) versus Δ*tsdA* untreated **(d)**. Δ*tsdA* Δ*soxR* untreated versus Δ*tsdA* untreated **(e)**. Δ*tsdA* Δ*shdrR* untreated versus Δ*tsdA* untreated **(f)**. Δ*shdrR* Δ*tsdA* versus Δ*tsdA* Δ*soxR* **(g)**. Δ*tsdA* Δ*soxR* untreated versus Δ*tsdA* treated **(h)**. Δ*shdrR* Δ*tsdA* untreated versus Δ*tsdA* treated **(i)**. Genes with a significant fold change (p<0.001) in two biological replicates are highlighted (red for a >2-fold increase or blue for a >2-fold decrease.

Here, we focus first on the genes whose transcription is strongly increased in the absence of one or both of the two transcriptional regulators as well as in the presence of thiosulfate in the reference strain (Figure 5d-f). Most of these genes encode enzymes involved in oxidative sulfur metabolism (Supplementary Table 3, Figure 6a). In full agreement with the RT-qPCR analysis (Figure 4a), the transcription of the genes of the *soxT1A* operon, the *dsrE3C-shdr-lbpA* and the *lip* genes, which together encode proteins for sulfane sulfur import and its oxidation to sulfite, was strongly affected by the absence of either one, the sHdrR or the SoxR repressor (Figures 4b, 5e,f). The situation was completely different for the *sox-tusA-p450* genes. Here, sHdrR deficiency had only a marginal effect, if any, which is again fully consistent with our RT-qPCR results (Figures 4a,b, 5f). The genes for the transcriptional repressors themselves and that for the signal transducing SoxT1B membrane protein were barely affected by thiosulfate or the absence of the other repressor. There is a further conspicuous gene, Hden_2458, the transcription of which is increased in the presence of thiosulfate as well as in

the absence of both repressor proteins (Figure 5d-f). It encodes a small, soluble, cytoplasmic hypothetical protein of 46 amino acids with no known function.

When we look at the genes with increased transcript abundance only in the regulator-deficient mutants, several sets of genes stand out that form a functionally coherent group (Figure 5d-f, Supplementary Table 3, Figure 6c). The encoded enzymes all play a role in iron acquisition via uptake and subsequent degradation of hemin (Wandersman and Delepelaire, 2004) and include Hden_0541 and Hden_0875, two putative oxygen-dependent heme-degrading HmoA monoxygenases (Frankenberg-Dinkel, 2004). Key subunits of the sulfur-oxidizing Sox and sHdr systems contain either heme or iron-sulfur sulfur clusters, and it is therefore not surprising that cells prepare for thiosulfate oxidation by taking steps to ensure that these enzymes are equipped with the necessary prosthetic groups.

Other conspicuous positive changes in abundance seen only in the repressor-deficient mutants include genes for proteins involved in electron transport/aerobic respiration (Supplementary Table 3, Figure 6b). Among these are Hden_2084 and Hden_3539, both of which encode periplasmic pseudoazurins/cupredoxins. While the small copper-binding cupredoxins are best known as electron donors to the denitrification pathway (Kataoka et al., 2004; Impagliazzo et al., 2005; Fujita et al., 2012), members of the protein family can also be required for cytochrome *c* oxidase respiratory function under aerobic conditions (Castelle et al., 2010).

The two genes that show the highest abundance changes (526- and 66-fold, respectively) in the reference strain upon addition of thiosulfate, encode a sulfite exporter (Hden_0834, YeiH) and a LysR family transcriptional regulator (Hden_0835) (Li et al., 2024). Surprisingly, the transcription of these two genes is not affected by the removal of sHdrR or SoxR. Closer inspection of the LysR-type protein revealed that its most closely related structurally and biochemically characterized homologs are NdhR (or CcmR) from *Synechocystis* PCC6803 (5Y2V (Jiang et al., 2018) and YeiE from *Cronobacter sakazaki* (7ERQ_A (Hong et al., 2022)). While 2-phosphoglycolate is an inducer for NdhR, YeiE serves as a global virulence regulator in *C. sakazakii*, binds sulfite as an effector and has a central role in defending against sulfite toxicity (Hong et al., 2022). The Hden_0835 derived protein shares five of seven sulfite-interacting residues with *C. sakazakii* YeiH. Notably, these include Glu^{145}, which is responsible for discriminating between sulfite and sulfate (Supplementary Figure 4). In contrast, of the seven residues in NdhR that interact with 2-phosphoglycolate, only three are present in the *H. denitrificans* protein. Toxic sulfite is formed as an intermediate during thiosulfate oxidation by *H. denitrificans* (Li et al., 2023b) and is effectively excreted into the medium, probably mainly by the action of the YeiH exporter (Li et al., 2024). It is likely that the YeiE protein derived from Hden_0835-recognizes sulfite and activates transcription of the neighboring *yeiH* gene upon binding of the inducer. This is only seen in the *H. denitrificans* Δ*tsdA* reference strain because the sHdrR- and SoxR-deficient strains were cultivated in the absence of thiosulfate. There is another set of genes, Hden_0719 to Hden_0748, for which drastic changes in abundance occur when the reference strain is exposed to thiosulfate, but whose transcription is not triggered by the absence of sHdrR or SoxR, i.e. these genes do not belong to the regulons of these two repressors. The affected genes include those for a putative dimethylsulfide (DMS)

monooxygenase and for methanethiol oxidase, MtoX (Eyice et al., 2017). In *H. denitrificans* thiosulfate is formed during DMS degradation, which occurs with methanethiol as an intermediate product (Koch and Dahl, 2018). We conclude that cells that sense thiosulfate are stimulated to prepare for degradation of the environmentally abundant organosulfur compound.

We have previously reported that transcription of a number of genes decreases when the *H. denitrificans* Δ*tsdA* reference strain is exposed to thiosulfate and that the encoded proteins include those of assimilatory sulfate reduction, since thiosulfate can serve as a source of reduced sulfur (Li et al., 2024). A lower transcript abundance for the genes for sulfate adenylyltransferase CysDN, which catalyzes the activation of sulfate to adenosine-5'-phosphosulfate, is also observed in the repressor-deficient *H. denitrificans* strains (Supplemenmtary Table 4, Figure 6a), but the response to the lack of the repressors extends far beyond sulfur assimilation to central energy metabolism (Figure 5d-I, Figure 6a-e).

First, transcript abundance of the genes for cytochrome *c* oxidase of type cbb_3 is greatly reduced (Supplementary Table4, Figure 6b). This oxidase is adapted to low oxygen concentrations and plays a crucial role in microaerobic respiration (de Gier et al., 1996; Pitcher and Watmough, 2004). It has a higher affinity to oxygen and is less efficient in proton pumping compared to the aa_3-type cytochrome *c* oxidase, the transcription of which remains unaffected. Second, transcript abundance for virtually all genes underlying nitrate respiration is drastically reduced when either sHdr or SoxR are absent. Affected units include nitrate and nitrite transporters/antiporters (NitT, Nark), nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), nitrous oxide reductase (Nos), electron carriers involved in denitrification and redox balance (cytochromes *c*, cupredoxin, NosR/NirI) as well as nitrate/nitrite and NO responsive regulators (NarQL, NnrS) (Figure 5e,f, Supplementary Table 4, Figure 6b). It should be noted that the same differences are seen when the regulator-deficient mutant strains are compared with the reference strain in the presence ot thiosulfate (Figure 5h,i).

The diminished transcript abundance of genes for enzymes involved in ubiquinone (UQ) biosynthesis (Hden_0564 to 0568, UbiXDTUV) is directly related to denitrification, since ubiquinol serves as an electron donor for nitrate reduction. The first step in ubiquinone biosynthesis is prenylation of 4-hydroxy benzoic acid by the membrane-bound enzyme UbiA (Hden_2584), followed by decarboxylation (catalyzed by the UbiX-UbiD system), three hydroxylations, two *O*-methylations (catalyzed by UbiG) and one *C*-methylation (catalyzed by UbiE) (Figure 6d) (Aussel et al., 2014). Under anaerobic conditions, the hydroxylation reactions are achieved by the oxygen-independent UbiT, UbiV and UbiU proteins (Pelosi et al., 2019), all of which are essential for denitrification in *Pseudomonas aeruginosa* (Vo et al., 2020). It thus appears that oxygen-independent UQ synthesis is co-regulated with denitrification in *H. denitrificans*. It is important to note that the organism contains only UQ_9, an ubiquinone with a side-chain containing nine prenyl residues (Urakami and Komagata, 1979, 1987). To the best of our knowledge, menaquinone has not been detected in any *Hyphomicrobium* species. Accordingly, neither homologs of the *E. coli* menaquinone biosynthetic pathway nor enzymes of the alternative so-called futasoline pathway (Dairi, 2012) are encoded in *H. denitrificans*. Since UQ_9 is the only quinone, it must also be made available for aerobic respiration. In fact, two UbiH/UibF-like monooxygenases (Hden_1669, Hden_3309) are suitable for the catalysis of the

hydroxylation reactions on the aromatic ring in the presence of oxygen. Other proteobacteria also contain only two or even only one of these enzymes with relatively broad regioselectivity instead of the three specifically acting prototypical *E. coli* enyzmes (Pelosi et al., 2016). *H. denitrificans* has only one copy each of *ubiD* and *ubiX* and these are hardly transcribed in our sHdrR- and SoxR deficient mutant strains even when grown under full oxygen tension (Supplementary Table 4, Figure 6d), suggesting that UbiD and UbiX may be replaced by so far unidentified non-orthologous proteins under aerobic conditions. The existence of alternative decarboxylases is supported by the fact that some ubiquinone-containing Alphaproteobacteria lack *ubiD* and *ubiX* completely. In addition, the decarboxylating enzyme has not yet been identified in mitochondria (Guerra and Pagliarini, 2023).

The clustered genes for oxygen-independent UQ_9 biosynthesis (Hden_0564-571) and nitric oxide reductase plus accessory and regulatory proteins (Hden_0572-0595) are preceded by two other gene sets for which transcript abundance decreases significantly in the absence of either SoxR or sHdrR. The affected genes appear to be part of two divergently transcribed operons, Hden_0561-0563 and Hden_ 0560-0546, with the latter encoding a NnrS family protein (Gaimster et al., 2018). NnrS was initially described in *Rhodobacter* as a heme- and copper containing transmembrane protein (Kwiatkowski et al., 1997) and contributes to nitrosative stress tolerance (Stern et al., 2013). The proteins encoded by Hden_0554 to Hden_0563 all appear to be related to fatty acid biosynthesis and include four genes for 3-oxoacyl-ACP-[acyl-carrier protein]-synthase II or possibly 3-oxoacyl-ACPsynthase I, FabF, one each for FabG (3-oxoacyl-ACP reductase) and FabZ (beta-hydroxyacyl-ACP dehydratase) and two for acyl carrier proteins (Hden_0560, 0563). For all of these genes, *H. denitrificans* has at least one additional copy residing somewhere else on the genome. The last step of the elongation cycle during fatty acid synthesis is catalyzed by enoyl-ACP reductase (FabI). However, the canonical hyphomicrobial FabI enzyme, Hden_1970, which is a member of the short-chain dehydrogenase/reductase superfamily member, does not have a counterpart in the gene cluster underlying SoxR/sHdrR control. We speculate that the product of Hden_0556, annotated as alcohol dehydrogenase, performs this function under anaerobic/denitrifying conditions. It is well known that some bacterial species contain additional enoyl-ACP reductases (Massengo-Tiasse and Cronan, 2009; Hopf et al., 2022). In summary, we suggest that the mentioned gene products work together in a fatty acid biosynthesis pathway that is especially efficient in/designed for anaerobic/denitrifying conditions.

Hden_0551-0553 encode proteins dedicated to biosynthesis of pyrroloquinoline quinone (PQQ). PQQ is a cofactor of periplasmic quinoprotein dehydrogenases such as cytochrome *c*-dependent methanol dehydrogenase, an enzyme of major importance to *H. denitrificans* when it grows on methanol (Duine and Frank, 1980b, a; Dijkstra et al., 1989). PQQ is synthesized from a precursor peptide, PqqA, with the conserved sequence motif E-X_3-Y (Cordell and Daley, 2022) (Figure 6e). PqqA is bound by PqqD (Latham et al., 2015), and bond formation between the glutamate and tyrosine C9 atoms is catalyzed by PqqE. The next step involves the cleavage of the structure from PqqA, which is catalyzed by PqqF/PqqG /PqqH and/or other proteases (Cordell and Daley, 2022). PqqB probably hydroxylates and oxidizes

the Glu-Tyr dipeptide yielding 3a-(2-amino-2-carboxyethyl)-4,5-dioxo-4,5,6,7,8,9-hexahydro-quinoline-7,9-di-carboxylic acid (AHQQ). The last step is ring cyclization and eight-electron oxidation catalyzed by PqqC. The reaction includes four oxidative steps requiring molecular O_2 and hydrogen peroxide (H_2O_2) (Bonnot et al., 2013). *H. denitrificans* X^T contains the full gene set for PQQ synthesis under aerobic conditions (PqqABCDE, Hden_1484-1488). Genes for PqqF/G/H are not present, but their function could be taken over by PqqL (Hden_2899) or other peptidases (Grinter et al., 2019; Cordell and Daley, 2022). Second copies for *pqqA*, *pqqD*, and *pqqE* are located in the gene cluster that is under SoxR/sHdrR control (Hden_0552 to 0553), and it is tempting to speculate that the final steps of PQQ biosynthesis in the absence of oxygen may be encoded in the same transcriptional unit and undergo the same transcriptional regulation. There is some circumstantial support for this suggestion: The products of Hden_0550 and Hden_0548 are similar to the N-terminal domain with four transmembrane helices of cytochrome *c* urate oxidase, PuuD (Doniselli et al., 2015), and to dihydroorotate oxidase, PyrD (Larsen and Jensen, 1985), respectively, both of which catalyze reactions on carbon- and nitrogen-containing heterocycles with certain structural similarities to precursors of PQQ. Hden_0549 encodes a putative phosphoserine aminotransferase, SerC (Duncan and Coggins, 1986). Clusters of a *pqqAED-puuD-serC-pyrD* are not only present in addition to classical *pqqABCDE* clusters in other *Hyphomicrobium* species, i.e. *H. nitrativorans*, but also in the Gammaproteobacteria *Halomonas sulfidovorans* strain MCCC 1A13718 (locus tags for the two *pqqE* genes: HNO53_16555 and HNO53_16620), *Stutzerimonas* (former *Pseudomonas*) *stutzeri* (*pqqE* genes in strain CGMCC 22915: NPN27_09805 and NPN27_22915) and *Methylophaga nitratireducenticrescens* JAM1 (*pqqE* genes: Q7A_453 and Q7A_868), and the Betaproteobacterium *Azoarcus* sp. DN11 (*pqqE* genes: CDA09_04515 and CDA09_08755). All of these organisms are capable of a denitrifying metabolism (Kasai et al., 2007; García-Valdés et al., 2010; Martineau et al., 2013; Wang and Shao, 2021) and may therefore be prepared for PQQ synthesis under these conditions. *Klebsiella pneumonia* and *Pseudomonas aeruginosa* are counterexamples. They contain only the canonical *pqqABCDEF/H* genes, and PQQ is not synthesized during anaerobic growth, although the *pqq* gene set is transcribed (Velterop et al., 1995; Gliese et al., 2010).

The last set of genes that deserves attention due to strong decreases in transcript abundance in the sHdrR and SoxR-deficient mutant strains, are those encoding proteins needed for heme degradation and iron acquisition under anaerobic conditions. FtrA (Hden_0575) is a periplasmic iron protein, HemN (Hden_0576) serves as an oxygen-independent coproporphyrinogen III oxidase (Layer et al., 2002) and ChuW (Hden_0599) is a radical S-adenosylmethionine methyltransferase that catalyzes a radical-mediated mechanism facilitating iron liberation and the production of a tetrapyrrole product called “anaerobilin” (LaMattina et al., 2016). It serves as a substrate for ChuY, an anaerobilin reductase (Hden_0601), possibly also involving ChuX, a putative heme binding protein (Hden_0600). The transcript abundance for these two genes is not affected in the repressor-negative mutants.

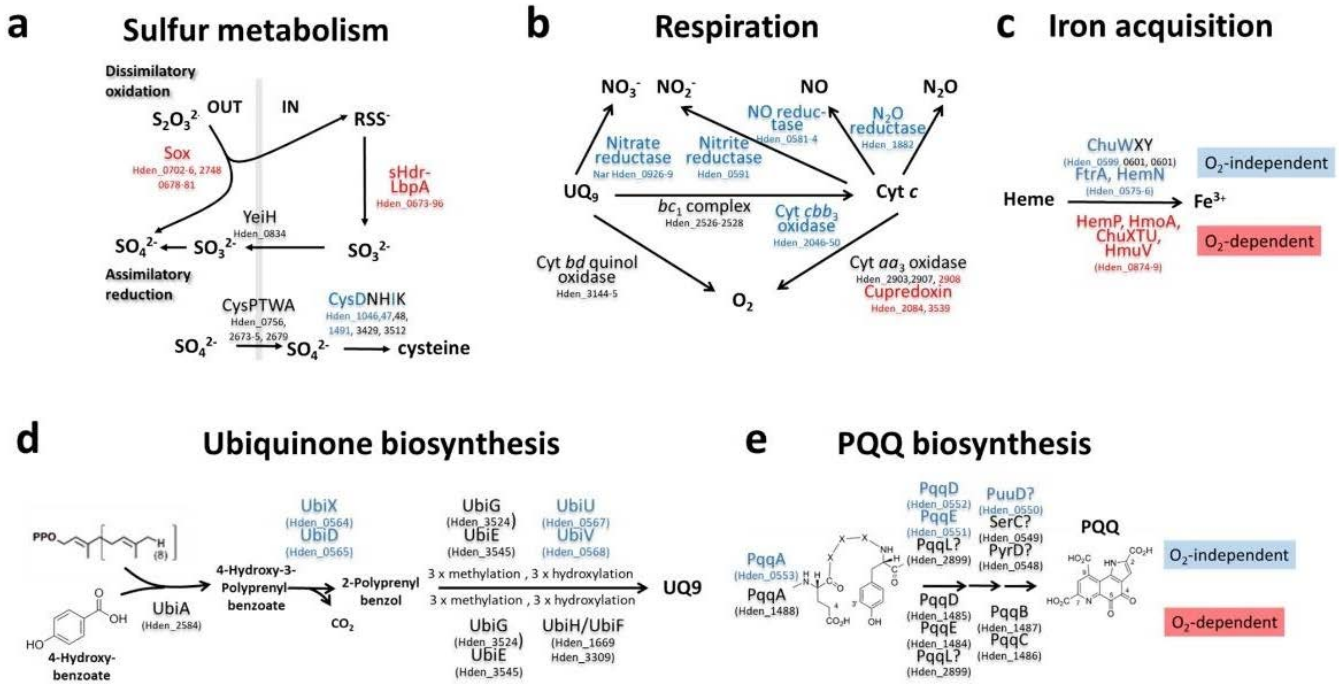

Figure 6. Effects of the absence of sHdrR or SoxR on central metabolic pathways in *H. denitrificans* X^T. Proteins whose genes showed increased (>2fold) transcript abundance either in *H. denitrificans* strain Δ*tsdA* Δ*shdrR* or in strain Δ*tsdA* Δ*soxR* or in both when compared to the *H. denitrificans* Δ*tsdA* reference strain in the absence of thiosulfate are printed in red. Proteins whose genes showed decreased (<0.5fold) transcript abundance in either of the three strains are printed in blue. Black letters indicate that significant changes in transcript abundance were not observed. **(a)** Dissimilatory oxidative and assimilatory reductive sulfur metabolism. **(b)** Electron delivery pathways to respiratory electron acceptors **(c)** Iron acquisition by heme degradation and iron release **(d)** Oxygen-dependent and oxygen independent biquinone biosynthesis. In *E. coli,* ubiquinone biosynthesis starts from 4-hydroxybenzoate, that is produced from chorismate (Abby et al., 2020), a step catalyzed by chorismate-pyruvate lyase (UbiC) (Pelosi et al., 2016). We are currently unable to explain how 4-hydroxybenzoic acid is synthesized in *H. denitrificans* and whether it is a mandatory precursor at all. *H. denitrificans* does not encode either a UbiC homolog or a homolog of XanB2, an unrelated chorismatase that fills the role of UbiC in *Xanthomonas campestris* (Zhou et al., 2013). In addition, it has been reported that alphaproteobacterial UbiA can accept *p*-amino-benzoic acid as a substrate for prenylation (Xie et al., 2015; Degli Esposti, 2017). **(e)** (Proposed) pathways of PQQ synthesis in the absence or presence of oxygen.

DISCUSSION

Here, we collected information on sHdrR, an ArsR-type regulator that functions as a transcriptional repressor of genes encoding enzymes involved in the oxidation of thiosulfate as a supplemental electron donor in *H. denitrificans*. Hyphomicrobial sHdrR belongs to a family of sulfane-sensitive regulators characterized by two conserved and essential cysteines, which also includes SoxR from the same organism. SoxR and sHdrR are homologous proteins. DNA-binding studies and expression analyses using RT-qPCR and RNA-Seq techniques show that both are directly involved in the control of sulfur oxidation. While both proteins bind to the same DNA fragments upstream of sulfur oxidation-related genes *in vitro*, the removal of each individual regulator has overlapping but non-identical effects on the transcription of these genes

in vivo. sHdrR regulates only a subset of SoxR-dependent genes, and this subset does not include the *sox* genes. These encode the enzymes that catalyze the initial steps of thiosulfate oxidation in the periplasm (Fig. 6a). Rather, in conjunction with SoxR, sHdrR is responsible for releasing transcriptional repression of genes for enzymes further downstream in thiosulfate degradation, i.e., import into the cytoplasm and oxidation to sulfite in this compartment (Fig. 6a). Given the close sequence similarity of SoxR and sHdrR, it is likely that their mechanism of action is similar, which would imply that the formation of a bridge of one to three sulfur atoms between the sulfur atoms of two conserved cysteine residues leads to a conformational change and unbinding of DNA (Li et al., 2023a). Whether and how the two transcriptional repressors compete for their binding sites or whether they even form heterocomplexes, cannot be answered on the basis of the available data. One possible model would be that SoxR and sHdrR co-repress their target promoters by binding as two separate homodimers. An alternative model would be that these two proteins repress their target promoters as a functional SoxR-sHdrR heterodimer. Heterodimerization of transcription factors is prevalent as a regulatory mechanism in eukaryotes (Remenyi et al., 2004) but is rare in bacteria. It has for example been reported for the LuxR-type transcription factor RcsB that forms heterodimers with several different auxiliary proteins. Like RcsB all of these carry a FixJ/NarL-type helix-turn-helix DNA binding motif (Kelm et al., 1997; Wehland and Bernhard, 2000; Venkatesh et al., 2010; Pannen et al., 2016). Another example is the interaction of BldM and WhiI from *Streptomyces*. Here, a BldM homodimer activates transcription of genes for early stages of development, while a BldM-WhiI heterodimer activates genes required for later stages (Al-Bassam et al., 2014).

Our global RNA-seq analyses of the *H. denitrificans* Δ*tsdA* reference strain in comparison with sHdrR- and SoxR-deficient strains have yielded detailed insights into the regulons of these sulfur-responsive transcriptional regulators. These regulons encompass not only genes for sulfur metabolism, but go considerably further than expected. This includes a drastic increase in the transcription of genes whose products are responsible for the availability of iron under aerobic conditions in the absence of one of the two repressor proteins. This finding underscores the critical need for a collaborative action of both proteins to maintain the transcription of the relevant genes at the optimal level. Comparable increases in abundance are not triggered by thiosulfate in the reference strain if it grows on the same defined medium with sufficient trace elements. It is thus evident that mutants lacking a single repressor are incapable of responding to iron availability. A logical explanation for this phenomenon is that the two repressors, functioning in conjunction, control the production of an iron-responsive regulator.

The most surprising result of our study is the unexpectedly large group of genes whose transcription is reduced by factors of up to 1000 in the absence of either sHdrR or SoxR (Figs. 5 and 6, Supplementary Table 4). While a comprehensive mechanistic explanation remains elusive, it is evident that the presence of both repressor proteins is imperative to prevent the near-total cessation of transcription of these genes. It is irrelevant which of the two repressor proteins is missing. In this context, we therefore observe a seemingly paradoxical phenomenon: repressor proteins are necessary for maintaining normal transcription levels. This effect is not observed in the *H. denitrificans ΔtsdA* reference strain when growing on thiosulfate, i.e.,

under conditions where neither of the two regulators can bind to its DNA target structures. On this basis, we once again conclude that cooperation between the two repressor proteins is indispensable for proper function *in vivo*. Furthermore, we suggest that, as with the regulation of iron availability, this is probably not a direct effect but rather an indirect one exerted via the control of other transcription factors. One explanation would be that the simultaneous presence of both repressor proteins is necessary to maintain the suppression of a stronger repressor. In the simultaneous presence of SoxR and sHdrR, the transcription of target genes would be sustained at a normal level due to the absence of the stronger repressor. However, if the repression of the controlled repressor is compromised due to the absence of either SoxR or sHdrR, then larger amounts of the stronger repressor protein can be formed, potentially leading to significant repression of the transcription of target genes. Alternative explanations include the indirect joint influence of SoxR and sHdrR on the transcription of an activator protein or participation in a feedback loop within a larger regulatory network.

The products of virtually all genes whose transcription is negatively affected in the absence of SoxR or sHdrR share a common overarching feature: their involvement in anaerobic metabolic pathways, particularly energy conservation in the absence of oxygen. It has long been known that *H. denitrificans* can grow on methanol as a carbon and electron source with respiration on nitrate as an electron acceptor. During aerobic growth of our regulator-deficient mutants, we observe a negative development of the transcription of all components involved, from the enzymes that drive denitrification to the anaerobic synthesis of the electron carrier ubiquinone and the O_2-independent synthesis of the cofactor PQQ, which is essential for methanol dehydrogenase. The regulation of sulfur oxidation and anaerobic respiration are thus deeply intertwined, an aspect that, to our knowledge, has not yet been reported for chemoorganoheterotrophic sulfur oxidizers and has only become clear through the investigation of our *H. denitrificans* mutant strains.

In 1999, Sorokin provided the first direct evidence of the ability of obligately heterotrophic bacteria to oxidize thiosulfate under anaerobic conditions (Sorokin et al., 1999). The organisms investigated in that study cannot oxidize thiosulfate completely to sulfate but form tetrathionate as an end product, while the *H. denitrificans* X^T wildtype strain can pursue both pathways. The reference strain we investigated in the current study exclusively produces sulfate. The free energy released by reaction of thiosulfate with oxygen or nitrate is in a very similar range, with the process being sightly less favorable under anaerobic conditions [equations 1 and 2], especially when considering the more reduced state of the respiratory chain in the absence of oxygen (Sorokin et al., 1999).

[equation 1] $S_2O_3^{2-} + 2\ O_2 + H_2O \rightarrow 2\ SO_4^{2-} + 2\ H^+$ $\Delta G^{0\prime}$ -818 kJ mol^{-1}

[equation 2] $S_2O_3^{2-} + 1.6\ NO_3^- + 0.2\ H_2O \rightarrow 2\ SO_4^{2-} + 0.8\ N_2 + H^+$ $\Delta G^{0\prime}$ −766 kJ mol^{-1}

Heterotrophic thiosulfate-oxidizing nitrate reducers are vital in ecosystems where oxygen is limited, such as in hypoxic or anoxic zones. In marine sediments, wastewater treatment systems, and other anoxic environments, these bacteria contribute to the cycling of nitrogen

and sulfur, affecting the overall health and functioning of these systems. Their ability to oxidize sulfur compounds and reduce nitrate makes them important contributors to the nitrogen and sulfur cycles. Among inorganic electron donors for nitrate reduction that may typically occur in subsurface environments, thiosulfate is a both readily available and non-toxic (Cardoso et al., 2006; Zhu and Getting, 2012; Kumar et al., 2018). In addition, several studies have investigated thiosulfate as an electron donor for nitrate removal in wastewater treatment but in depth studies of control mechanisms have not been performed (Cardoso et al., 2006). Knowledge concerning growth of *H. denitrificans* in the simultaneous presence of thiosulfate and nitrate is not available. It has been reported that the organism is able of aerobic denitrification in the presence of methylated amines (Meiberg et al., 1980), whereas on methanol nitrate reduction was not observed when cultures were incubated with oxygen and nitrate at the same time (Martineau et al., 2015).

CONCLUSIONS

Our work much expands the role of the sulfane-sulfur responsive regulators sHdr and SoxR, provides evidence that their cooperative action, possibly even heterodimer formation, is indispensable for proper function *in vivo*, and underscores the notion that their activity also includes interaction with other transcriptional regulators. Most importantly, our work expands the role of the sHdrR/SoxR regulatory system far beyond sulfur oxidation and shows that a profound effect is exerted on anaerobic metabolism, in particular denitrification in *H. denitrificans*. The present study has thus set the stage for future research, which will further elucidate the intricate relationship between oxidative sulfur metabolism and denitrification. Enhanced understanding of these processes promises significant insights into the biology of these bacteria, particularly their role in environmental contexts, such as their contribution to greenhouse gas emissions (e.g., N_2O).

ETHICS STATEMENT

This study adheres to the ethical standards set by *Environmental Microbiology*. All experimental protocols involving environmental samples, microbial cultures, and data collection were conducted following ethical guidelines and with proper approval from relevant institutional or governmental bodies. The research respects the principles of scientific integrity, transparency, and reproducibility. No conflicts of interest were declared by the authors. The study ensures that all findings are presented truthfully, with proper citations to prior works, and all sources of funding are disclosed.

DATA AVAILABILITY STATEMENT

RNA-Seq raw data files and processed data files are available via the NCBI GEO repository (accession number GSE282994)

AUTHOR CONTRIBUTIONS

Jinjing Li: investigation, formal analysis; writing – review and editing. **Nora Schmitte:** investigation. **Kaya Törkel:** investigation. **Christiane Dahl:** Conceptualization, writing – review and editing, supervision, funding acquisition

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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In *Hyphomicrobium denitrificans* two related sulfane-sulfur responsive transcriptional repressors regulate thiosulfate oxidation and have a deep impact on nitrate respiration and anaerobic biosyntheses

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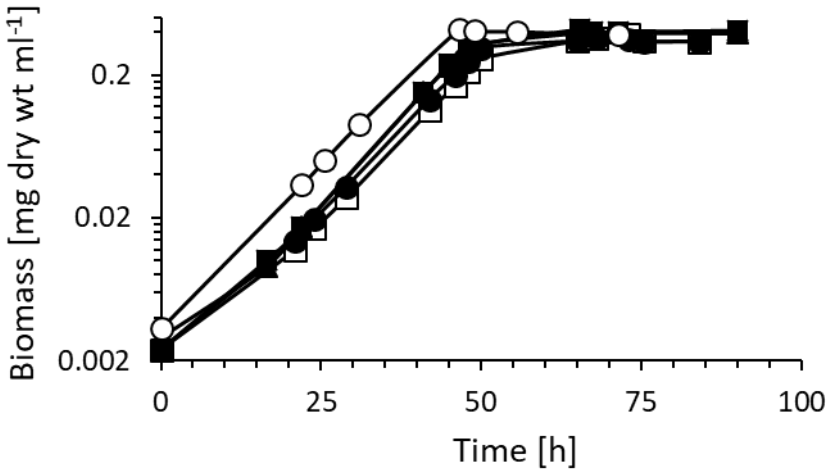
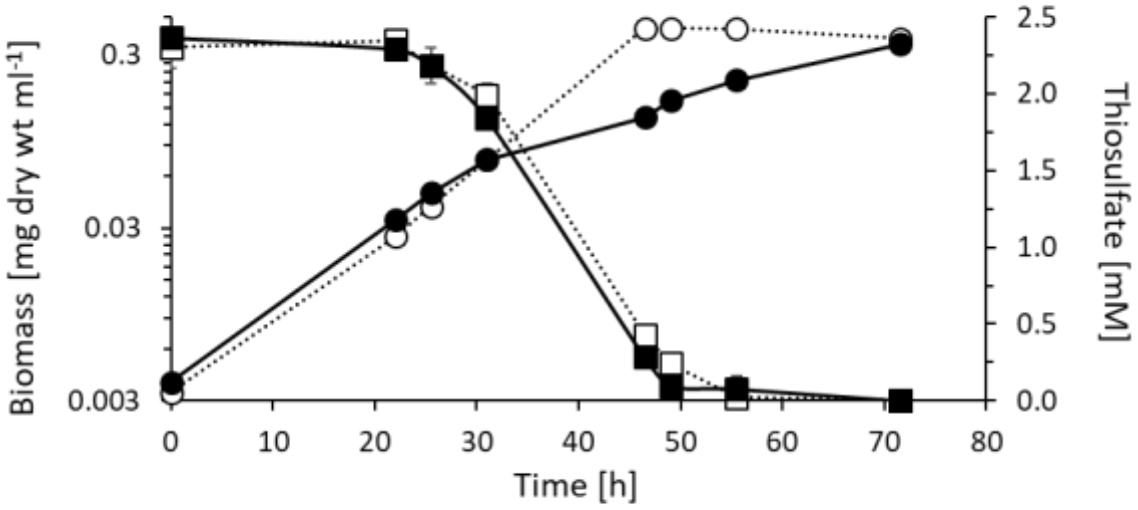
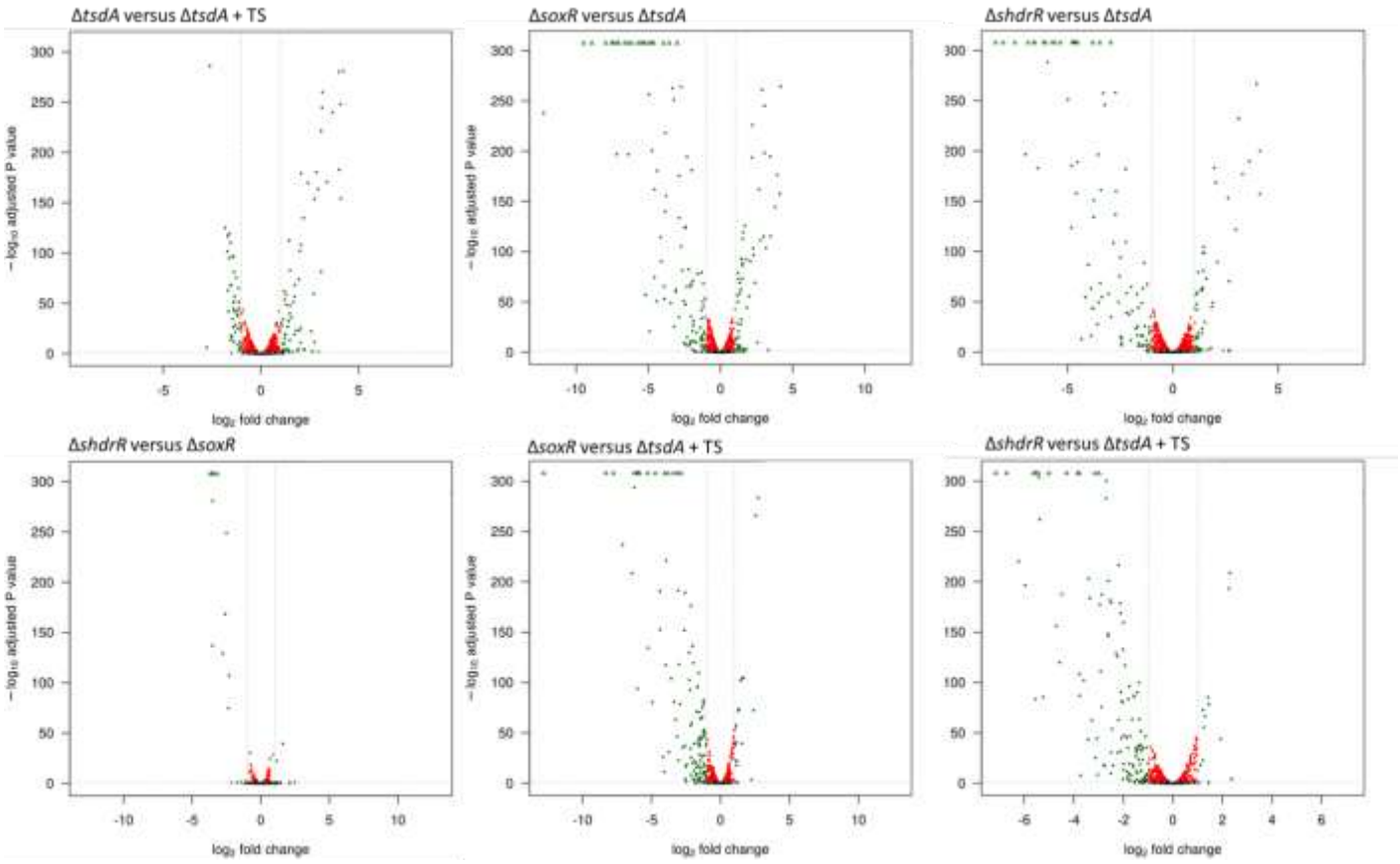
Supplementary Figures and Tables:



Supplementary Fig. 1. Growth of *H. denitrificans* reference and mutant strains on methanol. a. Growth curves are compared for the reference strain *H. denitrificans* Δ*tsdA* (filled circles), Δ*tsdA* Δ*shdrR* (open boxes), Δ*tsdA* sHdrR $C^{50}S$ (filled boxes), Δ*tsdA* sHdrR $C^{116}S$ (filled triangles), and Δ*tsdA* sHdrR $C^{50}S$ $C^{116}S$ (open circles). Error bars indicating SD for three replicates are too small to be visible for the determination of biomass.



Supplementary Fig. 2. Growth and thiosulfate consumption of *H. denitrificans* Δ*tsdA* carrying a *shdrR* complementation *in cis*. Growth curves are shown for medium containing 2 mM thiosulfate. Pre-cultures were either thiosulfate-free (broken lines, open symbols) or were pre-induced and contained 2 mM thiosulfate (solid lines, filled symbols). Values for biomass and thiosulfate are given as circles and boxes, respectively. Error bars indicating SD for three replicates are too small to be visible for the determination of biomass.



Supplementary Fig. 3. Volcano plots of differentially expressed genes for the *H. denitrificans* strains Δ*tsdA*, Δ*tsdA* Δ*shdrR* and Δ*tsdA* Δ*soxR*. The strains/conditions compared are given on top of each panel. TS, thiosulfate. • significant and has >1 log2-fold change, • significiant (FDR corrected p-value <= 0.1 • not significant.

```
CcmR           MGHHHHHHMQATLHQLKVFEATARHGSFTRAAEELYITQPTVSSQIKQLSKTVGLPLFEQ  60
YeiE           -----------TLRQLEVFAEVLKSGSTTQASQMLSLSQSAVSAALTDLEGQLGVQLFDR  49
Hden_0835      ----------MTLEQLRIFVAVAEREHVTQAAKELNLTQSATSAAVSALEARYATKLFDR  50
                         **.**.:*  . .    *:*:: * ::* :.*: :. *.   .  **::

CcmR           IGKRLYLTEAGQELLVTCQDIFQRLDNFAMKVADIKGTKQGRLRLAVI-TTAKYFIPRLL  119
YeiE           VGKRLVVNEHGRLLYPRTVALLEQAGEIERL----FRNDNGAIRVYASSTIGNYILPEII  105
Hden_0835      IGRRIVLTQAGKLFLVEAKSVLARAAAAEKVLADLAGLERGSLRIGASQTAGNYWLPEII  110
               :*:*: :.: *: :      :: :             ..* :*: .  * .:* :*.::

CcmR           GEFIQKYPGIEVSLKVTNHEQIRHRMQNNEDDLYIVSEPPEEIDLNYQPFLDNPLVVIAR  179
YeiE           ARYRRDFPDLPLEMSVGNSLDVVQAVCDFRVDIGLIEGPCHMAEIVAQPWLEDELVVFAS  165
Hden_0835      HRYQSLFPGISIALKIGNTETVAADVEDGVADLGFIEGEIDNPVLSVTPVADDDMVLVVA  170
                .:   :*.: : :.: *   :   : :   *: ::.   .   :   *  :: :*:..

CcmR           RDHPLAGKSNIPITALNDEAFIMREKGSGTRLAVQNLFHR---HYVDVRVRLELGSNEAI  236
YeiE           PASPLLEGEV-TLERLAAMPWILREKGSGTREIVDYLLLS---HLPQFRLSMELGNSEAI  221
Hden_0835      PNNPLAKQPLRALSQIAQARWVVREAGSGTRAILEADVAKLGIDPKSLDIALELPSNEAV  230
                  **       :  :    ::::** *****  ::  .     .  .. : :** ..**:

CcmR           KQAIAGGMGISVLSQHTLVSEGARSELTILDIDEFPIKRRWYVANLAGKQLSVITQTFLD  296
YeiE           KHAVRHGLGVSCLSRRVIAEQLETGSLVEVKVPLPPLVRTLYRIHHRQKHLSSALARFLR  281
Hden_0835      RGAVVAGSGITILSRLVVAAPLKAKTLVALDVPLPAR--KFFALRHKERYFTRAERTFID  288
               : *:  * *:: **: .:.       *. :.:         :  .   : ::     *:

CcmR           YLMAVTKNMPAPFAEQLTTQQTPVKLVL        324
YeiE           YCEL-------------------------       285
Hden_0835      VATGKQSSRAPG-----------------       300
```

Supplementary Fig. 4. Amino acid sequence alignment of selected LysR-type regulators. The protein deduced from Hden_0635 is aligned with CcmR/NdhR from *Synechocystis* PCC6803 (PDB 5Y2V) and YeiE from *Cronobacter sakazakii* (GenBank accession number ELY4740156). For *Synechocystis* CcmR/NdhR the residues interacting with 2-phosphoglycolate, which is an inducer (Jiang et al., 2018), are highlighted in green. For YeiE the residues interacting with sulfite (Hong et al., 2022) are marked yellow. An * (asterisk) indicates positions with identical residues. Cysteines are highlighted in yellow. Colons (:) and single dots (.) indicate conserved and semi-conserved amino acids, respectively.

Supplementary Table 1. Strains, primers and plasmids

Strains primers or plasmids	Relevant genotype, description or sequence	Reference or source
Strains		
Escherichia coli 10-beta	Δ*(ara-leu) 7697 araD139 fhuA* Δ*lacX74 galK16 galE15 e14-* Φ*80*d*lacZ*Δ*M15 recA1 relA1 endA1 nupG rpsL* (Str^R) *rph spoT1* Δ*(mrr-hsdRMS-mcrBC)*	New England Biolabs
E. coli DH5α	F$^-$ Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_{K^-}, m_{K^+}) *pho*A *sup*E44 λ$^-$*thi*-1 *gyr*A96 *rel*A1	New England Biolabs
E. coli BL21(DE3)	F$^-$*omp*T *hsd*S_B (r_{B^-}, m_{B^-}) *gal dcm* (DE3)	Novagen
Hyphomicrobium denitrificans Δ*tsdA*	Sm^R, in-frame deletion of *tsdA* in *H. denitrificans* Sm200	(Koch and Dahl, 2018)
H. denitrificans Δ*tsdA* Δ*shdrR*	Sm^R, in-frame deletion of *shdrR* (Hden_0682) in *H. denitrificans* Δ*tsdA*	(Li et al., 2023b)
H. denitrificans Δ*tsdA* Δ*soxR*	Sm^R, deletion of *soxR* (Hden_0700) in *H. denitrificans* Δ*tsdA*	(Li et al., 2023a)
H. denitrificans Δ*tsdA shdrRcomp*	Sm^R, *cis* complementation of *H. denitrificans* Δ*tsdA* Δ*shdrR* with *shdrR*	This work
H. denitrificans Δ*tsdA shdrR-Cys^{50}Ser*	Exchange of sHdrR-Cys^{50} to Ser in *H. denitrificans* Δ*tsdA*	This work
H. denitrificans Δ*tsdA shdrR-Cys^{116}Ser*	Exchange of sHdrR-Cys^{116} to Ser in *H. denitrificans* Δ*tsdA*	This work
H. denitrificans Δ*tsdA shdrR- Cys^{50}Ser-Cys^{116}Ser*	Exchange of sHdrR- Cys^{50} and Cys^{116} to Ser in *H. denitrificans* Δ*tsdA*	This work
Primers		
Fr-pET22b-sHdrR-trun-NdeI	GGCA**CATATG**ACCGACGCGTCGATCGAACAG (NdeI)	This work
Rev-pET22b-0682-NotI	TTTT**GCGGCCGC**ATTCGAGCGTTTTCCCGCAC (NotI)	(Li et al., 2023b)
sHdrR _C50S_Up_Rev	GGTTCTTTCTCCCTCGAGCAGGAGGGACAAAATCGCGAGA	This work
sHdrR _C50S_Down_Fw	TCTCGCGATTTTGTCCCTCCTGCTCGAGGGAGAAAGAACC	This work
sHdrR _C116S _Up_rev	GTTTTCCCGCACTCGTTGCACTATAATACTTATGCAGCGT	This work
sHdrR_C116S_Down_Fw	ACGCTGCATAAGTATTATAGTGCAACGAGTGCGGGAAAAC	This work
Fwd_deltaHden0682_BamHI	GCAT**GGATCC**GCGAAAATGTGCACCGGAG (BamHI)	(Li et al., 2023b)
Rev_deltaHden0682_XbaI	AAGC**TCTAGA**TATGCGGCAGCCGTTGACGC (XbaI)	(Li et al., 2023b)
EMSA-Fr	TTCCCGCCCCGTCTTGGTTT	(Li et al., 2023b)
EMSA_Fr2_Fr	TCAGCGCTCGCCTGGAAGTC	(Li et al., 2024)
EMSA_Fr3_Rev	TCTAAGCATCAACATATTCATATCTTTATATATTTTCG	(Li et al., 2024)
EMSA-Rev	AGGAGTTGCATCCAAAAAAGCGTG	(Li et al., 2023b)
EMSA-Hden_0703/04-fw	GGGTCACCAAATTCTGCAGGTCTC	(Li et al., 2024)
EMSA-Hden_0703/04-rev	ATCACGCCATCTCTCCCGGAA	(Li et al., 2024)
EMSA-Hden_0699/0698-fw	AATTCCACGGCTCCGCC	(Li et al., 2024)
EMSA-Hden_0699/0698-rev	TCGACAGCTTGCGGAAATCC	(Li et al., 2024)
EMSA-sHdrR-LipS1_F	TAGAGCGAGTCTTCAGC	(Li et al., 2024)
EMSA-sHdrR-LipS1_R	CGGCCCTCTGAGAAAAG	(Li et al., 2024)
EMSA-LipX-DsrE_F	GACTTCGCCGATCAATCGATC	(Li et al., 2024)
EMSA-LipX-DsrE_R	TGCCACCTCCCCGATATG	(Li et al., 2024)
EMSA-Hden_0703/04-fw	GGGTCACCAAATTCTGCAGGTCTC	(Li et al., 2024)
rpoB-denitf	AGGACGTGTTCACCTCGATT	(Martineau et al., 2015)
rpoB-denitr	CGGCTTCGTCAAGGTTCTTC	(Martineau et al., 2015)
SoxT1A 0681_qPCR-Fr	CCCGAGTGATACGATTCGCA	(Li et al., 2023a)
SoxT1A 0681_qPCR-Rev	CTAAAATGCCGCCGGTGATG	(Li et al., 2023a)
LplA_qPCR-Fr	GGCCATGATCGATTTGCACC	(Li et al., 2024)
LplA_qPCR-Rev	CGAGATAAATTGCACCGCCG	(Li et al., 2024)
sHdrA_qPCR-Fr	CCGATCACCATTCCGTTCGA	(Li et al., 2023a)
sHdrA_qPCR-Rev	CAATTGTTTCCGGGCCGATC	(Li et al., 2023a)
sHdrB2_qPCR-Fr	GACGTGGCCTACTATTCCGG	(Li et al., 2024)
sHdrB2_qPCR-Rev	CCGCGACGACAGATAGGTTT	(Li et al., 2024)
LbpA2_qPCR-Fr	GGTTCCAAGAGCAGCCTGAT	(Li et al., 2024)
LbpA2_qPCR-Rev	TCGTTGATCTCCAGAACCGC	(Li et al., 2024)
SoxXA_qPCR-Fr	CGGCGCTCATTACCTATCTC	(Li et al., 2024)

SoxXA_qPCR-Rev	TCGGGGTGTCTTTTTCAGTC	(Li et al., 2024)
TusA_qPCR-Fr	TCTGACAGTTGATGCCAAGG	(Li et al., 2024)
TusA_qPCR-Rev	CGTTTCCTCATGTTCAAGCA	(Li et al., 2024)
CytP450_qPCR-Fr	CAATACGGTTCTCGGACGTT	(Li et al., 2024)
CytP450_qPCR-Rev	CATTCGTTTCCTGACGAGGT	(Li et al., 2024)
SoxT1B (0699)_qPCR-Fr	GCCGCCGTCTCAGTAAATAA	(Li et al., 2024)
SoxT1B (0699)_qPCR-Rev	AGCAGAAGACGGCAGATGAT	(Li et al., 2024)
SoxR_qPCR-Fr	TGAAGCGGACGAGGAAGTAT	(Li et al., 2024)
SoxR_qPCR-Rev	GAGACTGTGGGCTGGTTGAT	(Li et al., 2024)
sHdrR_qPCR-Fr	TTAGGAAGTCCGCATCGTCT	(Li et al., 2024)
sHdrR_qPCR-Rev	GCACTCGTTGCGCAATAATA	(Li et al., 2024)
SoxY_qPCR-Fr	GTTCAGCTTGCGGACTTTTC	(Li et al., 2024)
SoxY_qPCR-Rev	GCCAATCGTCACCTTCACTT	(Li et al., 2024)
Plasmids		
pHP45Ω-Tc	Ap^r, Tc^r	(Fellay et al., 1987)
pk18*mobsacB*	Km^r, Mob^+, *sacB*, *oriV*, *oriT*, *lacZα*	(Schäfer et al., 1994)
pET-22b (+)	ApR, T7 promoter, lac operator, C-terminal His tag, pelB leader	Novagen
pET-22bHdsHdrR-trunc	Ap^R, NdeI-NotI fragment of PCR amplified truncated *shdrR* in NdeI-NotI of p ET-22b (+)	This work
pk18*mobsacB-shdrR*	Km^r, 2379 bp PCR fragment for chromosomal complementation of *shdrR* cloned int pk18*mobsacB* using XbaI and BamHI sites	This work
pk18*mobsacB-shdrR*-Tc	Km^r, Tc^r, pHP45ΩTc tetracycline cassette inserted into pk18*mobsacB-shdrR* using SmaI	This work
pk18*mobsacB-shdrR-C50S*	Km^r, SOE PCR fragment implementing chromosomal integration of *shdrR* encoding a $Cys^{50}Ser$ exchange cloned into pk18*mobsacB* using XbaI and BamHI restriction sites	This work
pk18*mobsacB-shdrR-C50S*-Tc	Km^r, Tc^r, pHP45ΩTc tetracycline cassette inserted into pk18*mobsacB-shdrR-C50S* using SmaI	This work
pk18*mobsacB-shdrR-C116S*	Km^r, SOE PCR fragment implementing chromosomal integration of *shdrR* encoding a $Cys^{116}Ser$ exchange cloned into pk18*mobsacB* using XbaI and BamHI restriction sites	This work
pk18*mobsacB-shdrR-C116S*-Tc	Km^r, Tc^r, pHP45ΩTc tetracycline cassette inserted into pk18*mobsacB-shdrR-C116S* using SmaI	This work
pk18*mobsacB-shdrR-C50S-C116S*	Km^r, SOE PCR fragment implementing chromosomal integration of *shdrR* encoding $Cys^{50}Ser$ and $Cys^{116}Ser$ exchange cloned into pk18*mobsacB* using XbaI and BamHI restriction sites	This work
pk18*mobsacB-shdrR-C50S-C116S*-Tc	Km^r, Tc^r, pHP45ΩTc tetracycline cassette inserted into pk18*mobsacB-shdrR-C50S-C116S* using SmaI	This work

Supplementary Table 2. Occurrence of sHdrR-related proteins with two conserved cysteines (Cys^{50} and Cys^{116} in *Hds*HdrR). Accession number and/or locus tags are provided. Linked genes were manually analyzed. Furthermore, genomes were checked via HMSS2 (Tanabe and Dahl, 2023) for the presence of genes encoding Sox-dependent thiosulfate oxidation in the periplasm (set positive when *soxYZAXB* were detected, thus covering complete and truncated Sox systems (Li et al., 2023a)) and sHdr-driven sulfane sulfur oxidation in the cytoplasm (set positive when at least 70 % of the genes *shdrC1B1AHC2B2* or *shdrC1B1AHB3etfAB* were present in a syntenic block, respectively (Kümpel et al., 2024)).

Organism	Accession, locus tag	Linked genes	sHdr system	Sox system	References
Pseudomonadota					
Alphaproteobacteria					
Hyphomicrobiales					
Hyphomicrobiaceae					
Hyphomicrobium denitrificans X^{T} (ATCC 51888^{T})	sHdrR: Hden_0682 SoxR: Hden_0700	*shdr-lbpA* *sox*	Yes	Yes	
Hyphomicrobium denitrificans 1NES1	HYPDE_25308	RND transporter	No	No	(Venkatramanan et al., 2013)
Hyphomicrobium sp. GJ21	sHdrR: HYPGJ_30422 SoxR: HYPGJ_30404	*shdr-lbpA* *sox*	Yes	Yes	(Tatusova et al., 2014)
Hyphomicrobium sp. SCN 65-11	ABS54_17655	Short fragment	No	No	(Kantor et al., 2015)
Hyphomicrobium sp. CS1BSMeth3	WP_083528837: CS1BSM3_04686 WP_210188842: CS1BSM3_RS16485	TauE, *ccm* genes RND transporter	Yes	Yes	Adelskov and Patel, unpublished
Hyphomicrobium sp. FW.3.32	CTY20_06775	*soxBZYAX*	No	Yes	(Zhang et al., 2017)
Filomicrobium insigne CGMCC 1.6497^{T}	SAMN04488061_2704 SoxR: SAMN04488061_1979	Only *soxYZ* *soxCBZY*	No	Yes	(Wu et al., 2009)
Rhodomicrobium vaniielii ATCC 17100^{T}	MBJ7534237 JDN40_08990 MBJ7535956 JDN40_17755	*tauE* RND transporter	No	SoxXA present	Conners et al, unpublished
Devosiaceae					
Devosia nanyangense NC_groundwater_1586_Pr3_B-0.1um_66_15	HY834_20740	*shdr* gene cluster, *tusA*	Yes	No	(He et al., 2021)
Acetobacterales					
Acetobacteriaceae					
Rhodopila globiformis DSM 161^{T}	CCS01_RS26760 CCS01_RS13140	RND transporter, Rhd *shdr* genes	Yes	No	(Imhoff et al., 2018)
Rhizobiales					
Rhizobiaceae					
Agrobacterium fabrum (tumefaciens) C58^{T}	BIGR_AGRFC, Atu3466	Rhd-PDO fusion-*bigR-pmpBA*	No	No	(Goodner et al., 2001; Guimarães et al., 2011)
Pseudaminobacter salicylatoxidans KCT001	WP_019171658	*sox*	No	Yes	(Mandal et al., 2007)

Xanthobacteraceae					
Bradyrhizobium diazoefficiens USDA10^T	BAC48771	*sox*	No	Yes	(Kaneko et al., 2002)
Rhodopseudomonas palustris TIE-1	Rpal_4967	Between *sox* genes and genes for RND transporter	No	Yes	(Larimer et al., 2004)
Rhodoplanes elegans DSM 11907^T	RAI38494, CH338_12525	RND transporter, sulfurtransferase	No	Yes	(LaSarre et al., 2018)
Rhodospirillales					
Rhodospirillaceae					
Rhodospirillum rubrum ATCC 11170^T	WP_011389407, ABC22517, Rru_A1717	*pmpB-like*	No	No	(Munk et al., 2011)
Rhodobacterales					
Paracoccaceae					
Paracoccus denitrificans GB17	CAB94376	*sox* genes	No	Yes	(Wodara et al., 1997; Rother et al., 2005)
Rhodobacteraceae					
Roseobacter litoralis Och 149^T	AEI95148	*sox* genes	No	Yes	(Kalhoefer et al., 2011)
Rhodobacter capsulatus SB 1003	ADE85198	RND transporter	No	No	(Shimizu et al., 2017; Capdevila et al., 2021)
Rhodovulum sulfidophilum DSM 1374^T	AAO11780	*sox* genes	No	Yes	(Appia-Ayme et al., 2001)
Sphingomonadales					
Sphingomonadaceae					
Tsuneonella (Altererythrobacter) mangrovi CD9-11^T	WP_240504499: CJO11_RS12710	close to *shdr* genes and genes for RND transporter	Yes	No	(Tatusova et al., 2014)
Erythrobacter sp. NAP1	EAQ29854, NAP1_03740	RND efflux system	No		(Koblizek et al., 2011)
Gammaproteobacteria					
Chromatiales					
Chromatiaceae					
Allochromatium vinosum DSM 180^T	Alvin_3027	Rhd	No	Yes	(Weissgerber et al., 2011)
Nitrococcales					
Ectothiorhodospiraceae					
Halorhodospira halophila DSM244^T	Hhal_1425	RND transporter	No	Yes	(Challacombe et al., 2013)
Enterobacterales					
Enterobacteriaceae					
Escherichia coli O1 strain PSU-0611	EEZ6061186, DCO30_005030	Short fragment	No	No	(Lacher et al., 2020)
Escherichia coli K12 substr. MG1655	b2667; YgaV PDB: 3CUO	YgaP: membrane-associated protein with rhodanese activity	No	No	(Paul and Larson, 2006; Riley et al., 2006; Gueuné et al., 2008)

Vibrionaceae					
Vibrio cholera O1 biovar El Tor N16961	HlyU, VC_0678, HLYU_VIBCH, PDB: 4K2E VC_A0642, AAF96543	Transcriptional activator of hemolysin,	No	No	(Williams et al., 1993; Heidelberg et al., 2000; Mukherjee et al., 2014)
Xanthomonadales					
Xanthomonadaceae					
Xylella fastidiosa 9a5c	WP_010893290, XF_0767, PDB: 3PQJ	Blh: DUF442-PDO fusion (XF_0768) *pmpBA* (XF_0765, 0766)	No	No	(Simpson et al., 2000; Barbosa and Benedetti, 2007; Guimarães et al., 2011)
Burkholderiales					
Chromobacteriaceae					
Chromobacterium violaceum ATCC 12472^{T}	CV_0084	*pmpAB,* Cyt c4	No	No	(Brazilian National Genome Project, 2003)
Burkholderiaceae					
Comamonas aquatica CJG	WP_045267543	*pmpAB,* DUF599 family	No	No	(Dai et al., 2016)
Thiobacillaceae					
Thiobacillus denitrificans ATCC 25No250	AAZ98348, Tbd_2395	alone	No	Yes	(Beller et al., 2006)
Acidithioacillales					
Acidithiobacillaceae					
Acidithiobacillus thiooxidans ATCC 19377^{T}	WP_024893036.1, GCD22_RS14465	RND transporter	Yes	Yes	(Valdes et al., 2011)
Bacteroidota					
Bacteroidia					
Chitinophagales					
Sphingobacteriales bacterium PMG_127	RYD90138	PmpA or B (short contig)	-	-	(Crombie et al., 2018)
Bacillota					
Bacilli					
Lactobacillales					
Streptpcoccaceae					
Streptococcus pneumonia SMRU2535	CJK49847, ERS022045_00348	*pmpBA*, uncharacterized, Rhd, sulfate permease, sulfide dehydrogenase	No	No	(Chewapreecha et al., 2014)
Staphylococcales					
Staphylococcaceae					
Staphylococcus aureus VB1919	RTY94661	Short contig	No	No	Balaji and Yamuna unpublished

Clostridia					
Clostridiales					
Clostridiaceae					
Hathewaya proteolytica DSM 3090^T	SHJ53940, SAMN02745248_00335	Upstream two genes for FeS containing proteins, then two for sulfur carrier protein ThiS	No	No	Joint Genome Institute
Cyanobacteriota					
Cyanobacteriia					
Cyanobacteriales					
Geitlerinemaceae					
Sodalimena (former *Phormidium*) *willei* BDU 130791	OAB56254, AY600_15135	All hypothetical	No	No	Peter. et al unpublished

Ccm, cytochrome c maturation (Thöny-Meyer, 2002); PDO, persulfide dioxygenase; PmpAB, members of the YeeE/YedE family of transporters that have been predicted to transport sulfur-containing ions (Gristwood et al., 2011); Rhd, rhodanese; RND, Resistance-nodulation-division family transporters, a category of bacterial efflux pumps, especially identified in Gram-negative bacteria (Nikaido, 2011); TauE, sulfite exporter (Weinitschke et al., 2007)

Supplementary Table 3. mRNAseq analysis of *H. denitrificans* strains *ΔtsdA ΔsoxR* and *ΔtsdA ΔshdrR*, part 1. Genes with higher mRNA abundance in the regulator-deficient mutants than in the reference strain in the absence of thiosulfate.

Locus tag	Annotation[a]	**Δ*tsdA* Δ*soxR* vs Δ*tsdA*** Fold change**	**Δ*tsdA* Δ*shdrR* vs Δ*tsdA*** Fold change**
Sulfur metabolism			
Hden_0678	hypothetical protein	5.82	3.51
Hden_0679	DsbA family protein	11.06	2.17
Hden_0680	Rhd, sulfur transferase domain-containing protein	15.61	2.60
Hden_0681	SoxT1A, YeeE/YedE family protein	24.43	4.32
Hden_0683	LipS1, radical SAM protein	17.88	25.64
Hden_0684	LipT, NAD(P)/FAD-dependent oxidoreductase	17.27	17.42
Hden_0685	LipS2, radical SAM protein	14.96	15.70
Hden_0686	Lpl(AB), lipoate--protein ligase family protein	16.04	18.56
Hden_0687	LipX, GMP synthase - glutamine amidotransferase domain-like protein	15.25	17.49
Hden_0688	DsrE3C, DsrE/DsrF/DrsH-like family protein	10.84	15.72
Hden_0689	sHdrC1	8.19	11.79
Hden_0690	sHdrB1	7.40	10.69
Hden_0691	sHdrA, FAD-dependent oxidoreductase	8.21	11.65
Hden_0692	sHdrH	7.93	12.36
Hden_0693	sHdrC2	6.72	9.96
Hden_0694	sHdrB2	6.37	8.71
Hden_0695	sHdrI	5.24	7.90
Hden_0696	LbpA2	4.17	6.12
Hden_0697	Cytochrome P450	4.77	ns
Hden_0698	TusA family sulfurtransferase	4.88	ns
Hden_0699	SoxT1B, YeeE/YedE family protein	2.69	ns
Hden_0701	SoxS, thioredoxin family protein	13.69	ns
Hden_0702	Sulfur oxidation c-type cytochrome SoxX	12.43	ns
Hden_0703	Sulfur oxidation c-type cytochrome SoxA	17.19	ns
Hden_0704	Thiosulfate oxidation carrier protein SoxY	17.44	ns
Hden_0705	Thiosulfate oxidation carrier complex protein SoxZ	16.72	ns
Hden_0706	Thiosulfohydrolase SoxB	14.50	ns
Hden_0834	YeiH family protein, sulfite export	ns	3.70
Carbon metabolism			
Hden_0802	DUF3734 domain-containing protein	2.42	ns
Hden_2747	Acyl CoA:acetate/3-ketoacid CoA transferase	2.33	2.12
Heme degradation and iron acquisition			
Hden_0540	TonB-dependent heme receptor	4.61	ns
Hden_0541	Heme degrading monooxygenase HmoA	3.45	3.02
Hden_0542	TonB family protein	3.20	2.82
Hden_0874	Hemin uptake protein HemP	3.53	3.58
Hden_0875	Heme degrading monooxygenase HmoA	2.86	2.62
Hden_0876	Heme utilization cystosolic carrier protein ChuX/HutX	2.88	2.69
Hden_0877	Heme transport system substrate-binding protein ChuT	2.97	2.77
Hden_0878	Heme transport system permease protein ChuU	2.82	2.74
Hden_0879	Heme transport system ATP-binding protein, HmuV	2.64	2.68

Hden_1331	TonB-dependent siderophore receptor	2.54	2.24
Hden_1332	PepSY domain-containing protein	2.23	ns
Hden_1333	hypothetical protein	2.25	ns
Hden_3200	hypothetical protein	10.51	8.75
Hden_3201	hypothetical protein	9.99	7.27
Hden_3202	Hemin uptake protein HemP	8.85	6.35
Transport			
Hden_0532	ABC transporter substrate-binding protein, branched chain amino acid transport	2.73	ns
Hden_2931	Potassium-transporting ATPase subunit KdpA	2.92	2.08
Hden_3198	YceI family protein, periplasmic, polyisoprenoid-binding	2.23	2.04
Hden_3199	Cytochrome *b*, YceJ	2.64	2.31
Regulation			
Hden_0594	helix-turn-helix domain-containing protein	2.60	2.39
Hden_0722	response regulator transcription factor, LuxR family	2.58	
Hden_2164	AraC family transcriptional regulator	3.05	ns
Respiration and electron transport			
Hden_2084	pseudoazurin	4.56	4.11
Hden_3539	cupredoxin domain-containing protein	2.40	2.12
Hden_2748	c-type cytochrome	3.50	3.17
Hden_2908	cytochrome c oxidase subunit II	2.32	2.33
Other			
Hden_0136	hypothetical protein	1.00	2.27
Hden_0441	glycosyltransferase	1.00	6.50
Hden_0457	hypothetical protein	2.29	2.30
Hden_0523	zf-HC2 domain-containing protein	ns	2.25
Hden_0525	catalase family peroxidase	ns	3.92
Hden_0738	hypothetical protein	2.18	2.23
Hden_0914	hypothetical protein	ns	5.23
Hden_0990	hypothetical protein	ns	2.01
Hden_1114	hypothetical protein	2.51	2.34
Hden_1235	phage GP46 family protein	ns	2.06
Hden_1416	hypothetical protein	2.01	ns
Hden_1432	DUF3307 domain-containing protein	ns	2.26
Hden_1518	hypothetical protein	ns	2.28
Hden_2458	hypothetical protein	9.73	6.35
Hden_2517	hypothetical protein	2.49	ns
Hden_2518	catalase	2.85	2.73
Hden_2542	class I SAM-dependent methyltransferase	ns	2.15
Hden_2599	hypothetical protein	2.31	2.54
Hden_2684	hypothetical protein	ns	2.30
Hden_2944	DUF3302 domain-containing protein	2.01	ns
Hden_2965	hypothetical protein	ns	2.41
Hden_2982	hypothetical protein	ns	2.35
Hden_3015	DNA cytosine methyltransferase	ns	2.22
Hden_3020	hypothetical protein	ns	2.70
Hden_3022	hypothetical protein	3.27	ns
Hden_3142	FHA domain-containing protein	2.14	2.10
Hden_3444	hypothetical protein	ns	2.19
Hden_3518	hypothetical protein	2.54	2.18
Hden_R0029		2.00	2.21

Hden_R0052	ns	2.47

ns, not significant

[a] Gene names obtained using sequence similarities in Uniprot or NCBI databases

** Significance threshold set at >2-fold change and $p<0.001$;

Supplementary Table 4. mRNAseq analysis of *H. denitrificans* strains *ΔtsdA ΔsoxR* and *ΔtsdA ΔshdrR*, part 2. Genes with lower mRNA abundance in the regulator-deficient mutants than in the reference strain in the absence of thiosulfate. Potential regulatory proteins associated with genes for respiratory proteins are printed in bold and not arranged under the headline "regulation".

Locus tag	Annotation[a]	Δ*tsdA* Δ*soxR* vs. Δ*tsdA* Fold change**	Δ*tsdA* Δ*shdrR* vs. Δ*tsdA* Fold change**
Biosynthesis of metabolites and cofactors			
PQQ			
Hden_0547	FmdE family protein, Flag1 repressor motif	0.175	0.184
Hden_0550	urate hydroxylase PuuD	ns	0.427
Hden_0551	pyrroloquinoline quinone biosynthesis protein PqqE	0.424	0.362
Hden_0552	pyrroloquinoline quinone biosynthesis peptide chaperone PqqD	0.190	0.185
Hden_0553	pyrroloquinoline quinone precursor peptide PqqA	0.232	0.321
Fatty acids			
Hden_0554	beta-ketoacyl-ACP synthase FabF	0.067	0.056
Hden_0555	beta-ketoacyl-ACP synthase FabF	0.057	0.036
Hden_0556	zinc-binding dehydrogenase, putative enoyl-ACP reductase FabI function	0.012	0.008
Hden_0557	beta-ketoacyl-ACP synthase FabF	0.007	0.012
Hden_0558	beta-ketoacyl-ACP synthase FabF	0.010	0.016
Hden_0559	beta-hydroxyacyl-ACP dehydratase FabZ	0.004	0.006
Hden_0560	acyl carrier protein	0.001	0.003
Hden_0561	3-oxoacyl-ACP reductase, FabG	0.002	0.004
Hden_0562	HAD-IIIC family phosphatase, putative involvement in methoxymalonyl-ACP biosynthesis, FkbH-like protein	0.025	0.038
Hden_0563	acyl carrier protein	0.199	0.218
Ubiquinone			
Hden_0564	UbiX family flavin prenyltransferase	0.219	0.049
Hden_0565	UbiD family decarboxylase	0.033	0.068
Hden_0566	UbiT ubiquinone biosynthesis accessory factor UbiT, SCP2 sterol-binding domain-containing protein	0.046	0.071
Hden_0567	O_2-independent ubiquinone biosynthesis protein UbiU	0.028	0.073
Hden_0568	O_2-independent ubiquinone biosynthesis protein UbiV	0.107	0.084
Hden_0569	Cytochrome P450	0.185	0.168
Hden_0570	**Crp/Fnr family transcriptional regulator**	0.138	0.093
Hden_0571	DUF2478 domain-containing protein	0.411	0.421
Respiration and electron transport			
Hden_0508	Cupin domain-containing protein	0.173	0.238
Hden_0509	SPW repeat protein	0.238	0.321
Hden_0510	Ferredoxin-NADP reductase	0.200	0.211
Hden_0572	Hypothetical protein	0.042	0.097
Hden_0573	4Fe-4S binding protein	0.047	0.036
Hden_0574	Periplasmic cupredoxin domain-containing protein	0.032	0.032
Hden_0579	NorE	ns	0.423
Hden_0581	Nitric oxide reductase subunit C, NorC	0.066	0.068
Hden_0582	Nitric oxide reductase subunit B, NorB	0.215	0.177
Hden_0583	Nitric oxide reductase NorQ protein	0.453	0.396

Hden_0584	Nitric oxide reductase NorD protein, VWA domain-containing protein	0.457	0.486
Hden_0585	Cytochrome c, hypothetical protein	0.388	0.461
Hden_0587	DUF2946 domain-containing protein	0.336	0.376
Hden_0589	Hypothetical protein	0.137	0.131
Hden_0590	NnrS family protein, involved in response/tolerance to NO	0.309	0.251
Hden_0591	Copper-containing nitrite reductase apoprotein NirK	0.189	0.213
Hden_0592	Host attachment family protein	0.042	0.042
Hden_0595	**Helix-turn-helix domain-containing protein**	0.037	0.043
Hden_0596	**PAS domain-containing protein**	0.259	0.309
Hden_0597	**Signal transduction histidine kinase, nitrite/nitrate specific NarQ**	0.073	0.074
Hden_0598	**Two-component system response regulator NarL**	0.059	0.062
Hden_0673	NitT/TauT family transport system substrate-binding protein, nitrate/sulfonate transport	0.138	0.154
Hden_0674	NitT/TauT family transport system permease protein	0.243	0.217
Hden_0675	NitT/TauT family transport system ATP-binding protein	0.271	0.297
Hden_0676	NnrS family protein, involved in response/tolerance to NO	0.317	0.349
Hden_0677	NnrS family protein, involved in response/tolerance to NO	0.491	0.412
Hden_0922	VOC family protein	0.201	0.326
Hden_0924	Porin	0.086	0.090
Hden_0925	NarK, nitrate/nitrite antiporter	0.019	0.015
Hden_0926	Nitrate reductase subunit alpha, NarG	0.179	0.152
Hden_1054	Cytochrome c	0.160	0.172
Hden_1055	Cytochrome c_{550} domain protein	0.007	0.010
Hden_1483	Cytochrome c family protein	0.480	1.000
Hden_1879	Permease protein NosY, copper transport	0.408	1.000
Hden_1880	ABC transporter ATP-binding protein NosF, copper transport	0.466	0.460
Hden_1881	Periplasmic nitrous oxide reductase family maturation protein NosD	0.266	0.306
Hden_1882	TAT-dependent nitrous-oxide reductase NosZ	0.126	0.130
Hden_1883	NosR/NirI family protein	0.031	0.037
Hden_1884	Ferritin family protein	0.247	0.253
Hden_1937	NADH-quinone oxidoreductase subunit NuoF	0.481	1.000
Hden_2045	FixH family protein	0.107	0.107
Hden_2046	Cytochrome c oxidase accessory protein CcoG	0.064	0.071
Hden_2047	Cytochrome-*c* oxidase *cbb*$_3$-type subunit III	0.012	0.014
Hden_2048	cbb3-type cytochrome c oxidase subunit 3	0.006	0.010
Hden_2049	Cytochrome-*c* oxidase *cbb*$_3$-type subunit II	0.007	0.011
Hden_2050	Cytochrome-*c* oxidase *cbb*$_3$-type subunit I	0.005	0.008
Carbon metabolism			
Hden_0042	Poly(3-hydroxybutyrate) depolymerase	0.435	0.484
Hden_0607	NAD-dependent formate dehydrogenase	3.915	2.864
Sulfur metabolism			
Hden_0759	SufS family cysteine desulfurase	ns	0.472
Hden_1046	Sulfate adenylyltransferase subunit CysN	0.250	0.388
Hden_1047	Sulfate adenylyltransferase subunit CysD	0.426	ns

Hden_1491	NADPH-dependent assimilatory sulfite reductase hemoprotein subunit, CysI	0.433	ns
Transport			
Hden_2042	Sulfite exporter TauE/SafE family protein	0.438	0.376
Hden_2044	cadmium-translocating P-type ATPase	0.151	0.142
Hden_2136	DHA2 family efflux MFS transporter permease subunit	0.384	0.261
Heme degradation and iron acquisition			
Hden_0575	FtrA, periplasmic iron binding protein	0.014	0.019
Hden_0576	HemN, oxygen-independent coproporphyrinogen III oxidase	0.040	0.043
Hden_0599	Heme anaerobic degradation, anaerobilin synthase ChuW/HutW	0.239	0.185
Regulation			
Hden_0099	PAS domain-containing protein	0.118	0.120
Hden_2177	Crp/Fnr family transcriptional regulator	0.022	0.021
Hden_2274	NnrS family protein, involved in response to NO	0.391	0.360
Hden_3436	response regulator	0.441	1.000
Other			
Hden_0086	Group II truncated hemoglobin	0.092	0.087
Hden_0095	HPF/RaiA family ribosome-associated protein	0.070	0.085
Hden_0096	Zinc-dependent alcohol dehydrogenase family protein	0.036	0.040
Hden_0097	Flavin reductase family protein	0.070	0.074
Hden_0174	Hypothetical protein	0.261	1.000
Hden_0328	Hypothetical protein	0.399	0.361
Hden_0672	TonB-dependent receptor	0.150	0.151
Hden_0959	5-aminolevulinate synthase	0.400	0.430
Hden_1119	Phage tail tape measure protein	ns	0.444
Hden_1171	Hypothetical protein	0.491	ns
Hden_1773	radical SAM protein	0.272	0.313
Hden_1841	Universal stress protein	0.099	0.102
Hden_1876	Hypothetical protein	0.038	0.043
Hden_2272	Membrane protein	0.276	0.180
Hden_2281	HD domain-containing protein	ns	0.464
Hden_2596	Alpha/beta fold hydrolase	0.395	0.367
Hden_2615	Hypothetical protein	0.117	0.092
Hden_2827	Ferric reductase-like transmembrane domain-containing protein	0.024	0.025
Hden_2910	Hypothetical protein	0.397	0.458
Hden_3135	Circularly permuted type 2 ATP-grasp protein	0.487	ns

ns, not significant

[a] Gene names obtained using sequence similarities in Uniprot or NCBI databases

** Significance threshold set at <0.5-fold change and $p<0.001$;

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The sulfane-sulfur responsive transcriptional repressor sHdrR: Properties and binding sites

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ABSTRACT

Organisms have a variety of strategies for sensing and responding to inorganic sulfur compounds such as thiosulfate. In the Alphaproteobacterium *Hyphomicrobium denitrificans*, one such strategy is the use of an ArsR-SmtB transcriptional regulator, sHdrR, as a key regulator that senses sulfane sulfur and represses the expression of sulfur oxidation genes in the absence of an oxidizable sulfur compound. Electrophoretic mobility shift assays (EMSAs) revealed that sHdrR binds DNA at promoter regions overlapping the -35 and -10 sites upstream of the divergently transcribed *soxY-soxA* and *soxT1A-shdrR* gene sets, involving direct and inverted repeat sequences. sHdrR was compared with SoxR, another ArsR-SmtB-type transcriptional regulator. sHdrR contains three cysteine residues, two of which, Cys^{50} and Cys^{116}, are conserved in homologs from other bacteria. *In vitro* assays indicate that upon exposure to polysulfide, sHdrR undergoes persulfidation and forms a sulfur bridge between Cys^{50} and Cys^{116}. This modification reduces its DNA binding affinity, leading to transcriptional derepression of target genes in the presence of external thiosulfate. A sHdrR Cys^{63}Ser variant is unresponsive to oxidation but responsive to polysulfide *in vitro*, indicating an important role of this residue. Our results establish sHdrR as a sulfane sulfur-responsive repressor and reveal the mechanism of thiosulfate-dependent transcriptional derepression of genes involved in oxidative thiosulfate metabolism in *H. denitrificans.*.

Keywords: *Hyphomicrobium denitrificans*; transcriptional regulation; binding site; reactive sulfur species; repressor.

1 INTRODUCTION

Facultative sulfur-oxidizing chemoorganoheterotrophs such as the Alphaproteobacterium *Hyphomicrobium denitrificans* live in environments that not only contain different amounts of molecular oxygen, but where the organisms may also encounter different concentrations of reduced sulfur compounds. To make the best out of these additional oxidizable substrates, the bacteria must have strategies to perceive their presence. Accordingly, complex regulatory patterns have been reported for facultative sulfur oxidizers, with upregulation usually occurring only in the presence of metabolizable sulfur substrates, whereas the corresponding genes are thought to be always highly expressed in chemolithoautotrophs restricted to the oxidation of sulfur compounds. In *H. denitrificans*, and other Alphaproteobacteria that are not restricted to sulfur oxidation, such as *Rhodovulum sulfidophilum*, *Paracoccus pantotrophus* or *Pseudaminobacter salicylatoxidans*, the ability to oxidize thiosulfate and, depending on the organism, other reduced inorganic and organic sulfur compounds such as sulfide or dimethyl sulfide, is not constitutive but can be induced by the presence of oxidizable sulfur compounds (Rother et al., 2005; Mandal et al., 2007).

The transcriptional regulation of sulfur oxidation in *H. denitrificans* is primarily controlled by two transcription factors: sHdrR and SoxR. Both of them, play a crucial role in transcriptional control of genes encoding Sox, sHdr and associated proteins involved in sulfur transfer and metabolism in *H. denitrificans* (Li et al., 2023b; Li et al., 2023a; Li et al., 2024). The genetic and biochemical functions of SoxR have not only been extensively studied in species such as *Paracoccus pantotrophus* and *Pseudaminobacter salicylatoxidans* (Rother et al., 2005; Lahiri et al., 2006; Mandal et al., 2007) but its role has also been characterized at the molecular level in *H. denitrificans* (Li et al., 2023a). In contrast, the role of sHdrR is less well understood. Available evidence suggests that it regulates the sHdr system by modulating sHdrA protein levels, as shown by Western blot analyses, and that it binds to the upstream region of its own gene (Li et al., 2023b). In addition, the role of sHdrR has been analyzed by targeted qRT-PCR and global RNA-Seq-based analysis (Li et al., 2025). It was shown that *in vivo* sHdrR primarily affects the *lip-shdr-lbpA* genes, which encode the cytoplasmic enzymes essential for sulfite formation. However, its detailed molecular sensing mechanism, particularly whether it involves a sulfane sulfur bridge between two conserved cysteine residues in response to thiosulfate, requires further investigation.

To better understand the function of the repressor sHdrR, we aim to establish the molecular details of the sHdrR-based mode of transcriptional regulation. Here, we investigate the DNA-binding properties of sHdrR, map its binding sites, and narrow down the specific binding regions. Additionally, sHdrR variants were constructed by site-directed mutagenesis and key residues involved in the DNA binding and the potential formation of a sulfane sulfur bridge were analysed.

2 EXPERIMENTAL PROCEDURES

2.1 Bacterial strains, plasmids, primers, and growth conditions

Supplementary Table 1 lists the bacterial strains, and plasmids that were used for this study. *Escherichia coli* strains were grown on complex lysogeny broth (LB) medium (Bertani, 2004) under aerobic conditions at 37°C. *Escherichia coli*. BL21 (DE3) was used for recombinant protein production. *E. coli* strains 10-beta and DH5α were used for molecular cloning. Antibiotics for *E. coli* were used at the following concentrations (in μg ml^{-1}): ampicillin, 100; kanamycin, 50.

2.2 Recombinant DNA techniques

Enzymes (restriction enzymes, T4 ligase and Q5 polymerase) were from New England Biolabs (Ipswich, UK) and used according to the manufacturer's instructions. Oligonucleotides were from Eurofins Genomics Germany GmbH (Ebersberg, Germany). Standard cloning methods were followed (Ausubel et al., 1997). Plasmid DNA and gel-purified fragments were obtained using the GeneJET Kits (Thermo Scientific, Waltham, USA).

2.3 Cloning, site-directed mutagenesis, overproduction and purification of recombinant proteins

A truncated *H. denitrificans shdrR* gene, lacking the sequence for the first 25 amino acids, was amplified and cloned into pET22b (+) via NdeI/NotI, resulting in pET22bHdsHdrR-trunc (Li et al., 2025). Cysteine to serine exchanges were performed using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, UK) according to the manufacturer's instructions and with the primers listed in Supplementary Table 1. Plasmids pET-22b-sHdrR-trunc $C^{50}S$, pET-22b-sHdrR-trunc $C^{116}S$, pET-22b-sHdrR-trunc $C^{50}S$ $C^{116}S$, pET-22b-sHdrR-trunc $C^{63}S$ were constructed. Recombinant sHdrR-trunc proteins and variants were overexpressed in *E. coli* BL21(DE3). The cells were grown at 37°C in 400 ml LB medium containing ampicillin up to an OD_{600} of 0.5-0.6. Expression of *shdrR-trunc* was induced by adding 0.5 mM IPTG. IPTG-induced *E. coli* cells were grown over night at 20°C. Cells were harvested at 14,000 × g for 30 min.The carboxy-terminally His-tagged protein was purified by affinity chromatography on Ni^{2+}-NTA using the same conditions as described for the full length protein (Li et al., 2023b). The Pierce™ BCA protein assay kit (Thermo Scientific, Rockford, USA) was used for protein determination. The SoxR protein was produced with a Strep tag and purified as described earlier (Li et al., 2023b).

2.4 Electrophoretic mobility shift assays (EMSA)

Binding reactions (15 µl) containing truncated sHdrR (WT or variants, up to 700 nM), DNA probe (17 nM), binding buffer, and glycerol were set up and incubated as described before (Li et al., 2025). Reactions were run on 6% native PAGE at 4 °C in 0.25 × TBE with 0.5% glycerol, followed by SYBR Green I staining following previously described procedures (Li et al., 2025). Bound and free DNA were visualized using a ChemiDoc Imaging System (BioRad). The DNA probes consisted of a 362-bp fragment covering the entire intergenic region between the *shdrR* (Hden_0682) and the *soxT1A* (Hden_0681) genes, a 180-bp fragment representing the central part of the first product (created with primers EMSA-Fr2-Fr and EMSA_Fr3-Rev), and a 151-bp fragment situated between the *soxA* (Hden_0703) and *soxY* (Hden_0704) genes. In addition, subfragments covering the intergenic region between *shdrR* and *soxT1A* (fragments A–K) and between *soxA* and *soxY* (fragments L–Q) were generated. All primers used in these experiments are listed in Supplementary Table 1.

2.5 Redox treatments and persulfidation reactions

5 µg of protein was treated with 5 mM DTT, for reduction, 5 mM $CuCl_2$ for oxidation, or 0.5 mM polysulfide for persulfidation in a final volume of 15 µl containing 100 mM Tris-HCl, pH 8.0, 150 mM NaCl. When polysulfide and DTT were applied consecutively, the concentrations were 0.5 mM and 10 mM, respectively for samples analyzed by EMSA. Protein samples used in EMSA experiments were reacted with the reagents for 20 min at 25°C. A polysulfide stock solution (Ikeda et al., 1972) was prepared as described earlier (Li et al., 2023a).

3. RESULTS AND DISCUSSION

3.1 sHdrR-trunc: a stable alternative to the unstable full-length sHdrR

The *H. denitrificans* sHdrR (*Hd*sHdrR) protein is 125 amino acids in length. Originally, full-length sHdrR was expressed as a His-tagged recombinant protein in *E. coli*. Electrophoretic mobility shift assays (EMSA) confirmed its specific binding to a 362 bp DNA fragment located in the *soxT1A-shdrR* intergenic region (Li et al. 2023a). However, the protein turned out to be unstable after purification, leaving only a very short time window for further experiments, such as EMSA. Mass spectrometry revealed a reduction in mass due to the loss N-terminal amino acids (Supplementary Fig. 1). The structures for sHdrR and SoxR were predicted with AlphaFold (Jumper et al., 2021) (Supplementary Fig. 2), showing that the sHdrR N-terminal region forms a long unstructured loop. This could be the reason for the observed instability of this region.To address this issue, we produced a truncated version of sHdrR, sHdrR-trunc (Li et al. 2025). It starts with MTDASIEQ, i.e. the first 25 amino acids are removed. The truncated sHdrR variant remained stable and could be used for a period of seven days when kept on ice (Li et al., 2025). With a stable protein variant at hand we assessed the oligomerization state of sHdrR by size exclusion chromatography. Just as observed for SoxR and all other related ArsR-type transcriptional repressors (Li et al., 2023a), the protein eluted at a k_{av} value corresponding to twice the mass of a 14.9-kDa His-tagged sHdrR-trunc monomer (Supplementary Fig. 3).

3.2 *In vitro,* sHdrR-trunc binds to the same DNA fragments as SoxR

Previous results have provided first indication that sHdrR-trunc binds to the same intergenic regions within the hyphomicrobial sulfur oxidation locus that serve as repressor binding sites for SoxR (Li et al., 2023b, 2025). In fact, the binding region for SoxR had already been narrowed down to a 183 bp fragment in the center of the *soxT1A-shdrR* intergenic region. However, it remained unclear whether sHdrR binds to the same part of the *soxT1A–shdrR* intergenic region and, if yes, how this binding differs from that of SoxR.

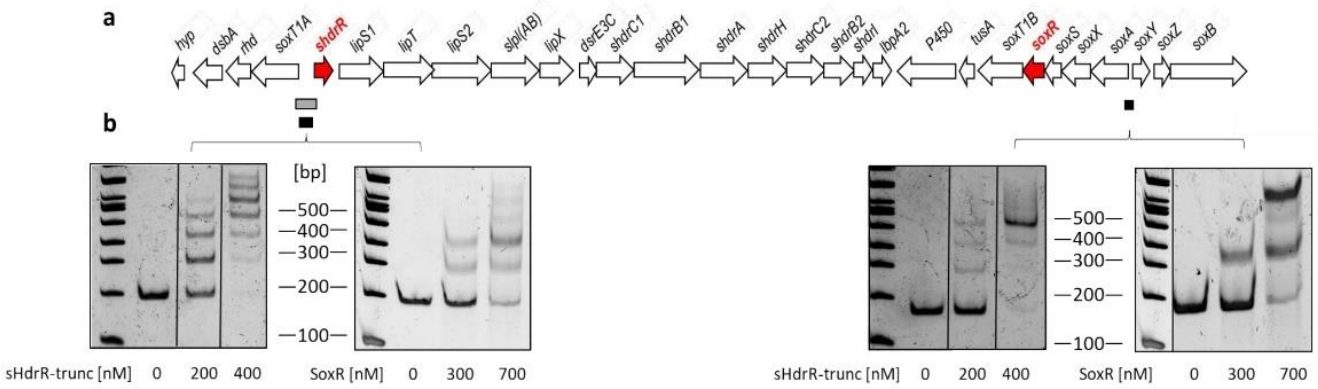


Fig 1. (a) DNA regions tested in EMSA assays for sHdrR binding are indicated as black rectangles below the hyphomicrobial *shdr-sox* genetic island. Fragment size: 362 bp for the *soxT1A-shdrR* intergenic region (gray rectangle), 183 bp for the central part of the *soxT1A-shdrR* intergenic region, 151 bp for the *soxA-soxY* intergenic region**. (b)** EMSA analysis of the 183-bp central part of the *soxT1A-shdrR* intergenic fragment (17 nM) (left) and the 151-bp *soxA-soxY* intergenic fragment (right)with different amounts of sHdrR-trunc (200 and 400 nM), SoxR (300 and 700 nM).Vertical lines separate samples that were run on the same gel but were not directly adjacent.

Here, we show that truncated sHdrR indeed binds to the central part of the *soxT1A–shdrR* intergenic region (Fig. 1). Already at a DNA probe to protein ratio of 12 (200 nM sHdrR-trunc), EMSA revealed multiple shifted bands (Fig. 1b). When the amout of sHdrR-trunc was further increased, super-shifted bands occurred, similar to what has been reported when the entire 362-bp *soxT1A–shdrR* intergenic region was used as a probe (Li et al., 2025). sHdrR also bound effectively to the complete 151-bp *soxA-soxY* intergenic region (Fig. 1b). Taken together, our results indicate that *in vivo* sHdrR-trunc and SoxR can regulate expression of *shdr*-associated genes and *sox*-related genes in a cooperative manner.

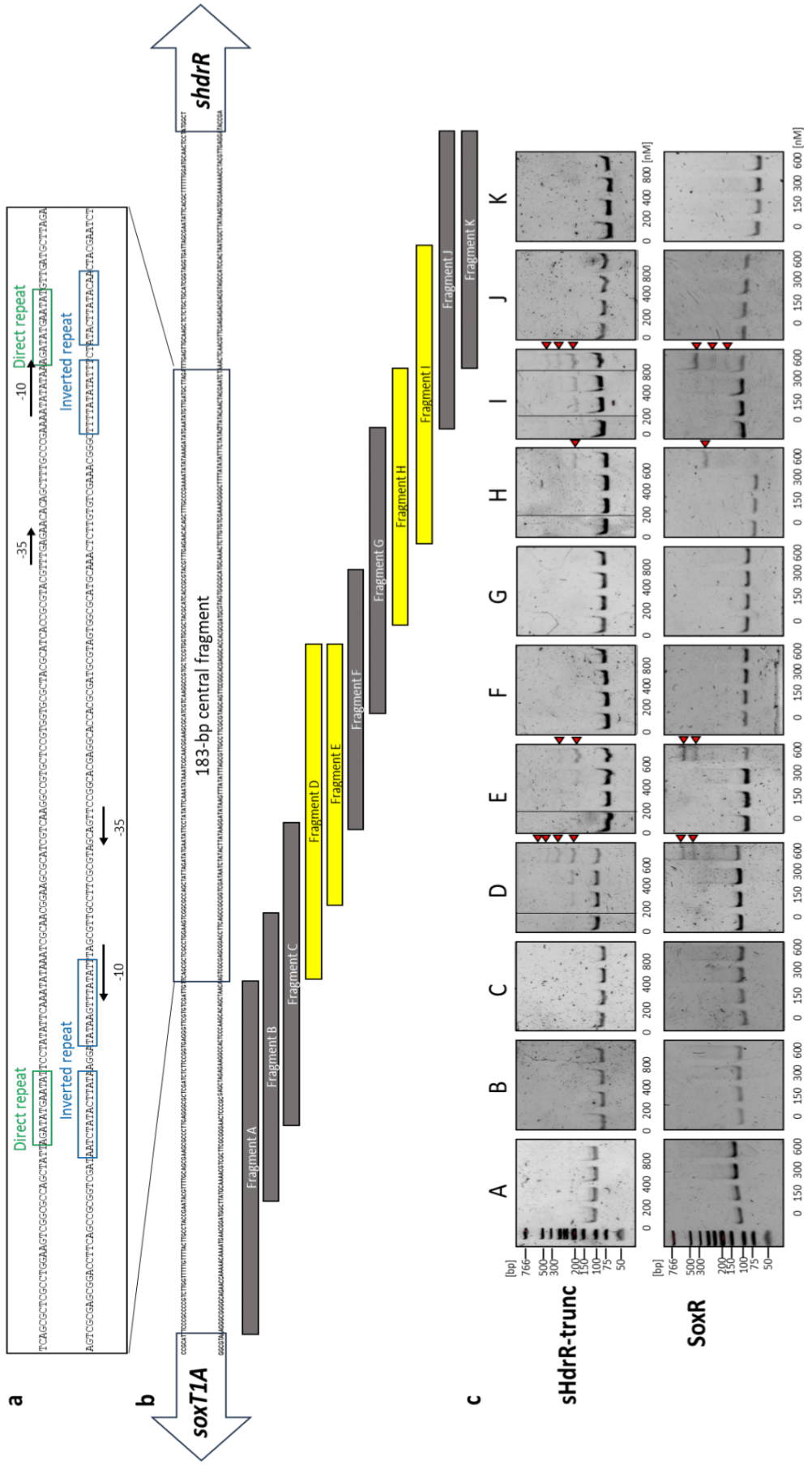
3.3 Mapping of sHdrR and SoxR binding sites

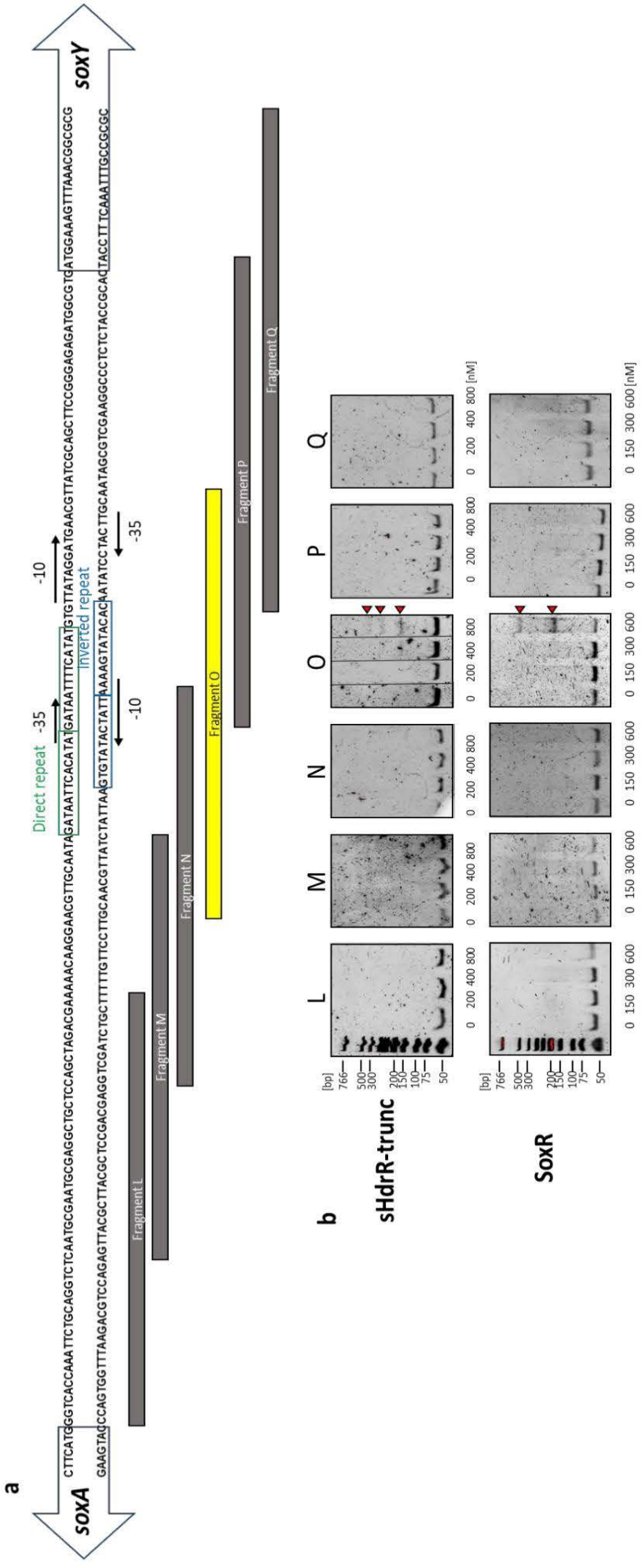
Close analysis of the *soxT1A-shdrR* and *soxY-soxA* intergenic regions, revealed the presence of prominent AT-rich direct and indirect repeats, as well as two divergently oriented -35 and -10 RNA polymerase binding sites predicted by BPROM (Solovyev and Salamov, 2010). These features are shown in Figs. 2a and 3a. The repeats overlap the putative -35 and -10 RNA polymerase binding sites, which are located between the divergently transcribed *soxY* and *soxA,* and *soxT1A* and *shdrR* gene sets (Figs. 2a, 3a). For a detailed characterization of the presumptive sHdrR and SoxR binding sites and definition of a consensus binding motif, we took an experimental approach and analyzed the DNA binding of both proteins to subfragments derived from the *soxT1A-shdrR* and *soxA-soxY* intergenic promoter regions.

For the *soxT1A-shdrR* intergenic locus, the DNA-binding sites for both repressor proteins were narrowed down by generating a series of eleven subfragments, A-K, for EMSA experiments (Fig. 2b). Two different, albeit identical, binding sites for each of the transcriptional regulators became apparent. Notably, the positive fragments D and E cover one of the inverted repeat regions, while fragments H and I cover the other inverted repeat region (Fig. 2a, 2b). SoxR and sHdrR thus interacted with DNA probes containing palindromic AT-rich sequence spanning predicted -10 regions. The binding patterns of sHdrR-trunc and SoxR differ, with sHdrR-trunc showing more shifted bands than SoxR (Fig. 2b). Notably, with fragment D, sHdrR-trunc exhibits a band pattern similar to that observed for its reaction with the central part of the *soxT1A-shdrR* region (compare Figs. 1b and 2c), with one band size being twice that of another. This suggests that the protein may not only act as a dimer, but also form higher oligomers when bound to DNA.

In the next step, we analyzed the sHdrR and SoxR binding sites in the the *soxA-soxY* intergenic region. We refined the DNA-binding sites to <60 bp, by using six subfragments (L to Q) (Fig. 3a). Both, SoxR and sHdrR bound exclusively to subfragment O (Fig. 3b). This fragment contains a striking sequence that is both a direct and an indirect repeat.

Fig 2. (a) Blow up of the 183 bp central fragment of the *soxT1A-shdrR* intergenic region. Predicted -35 and -10 RNA polymerase binding sites are indicated. Direct and indirect repeat regions are mark by green and blue open boxes, respectively. **(b)** Overview of the 362 bp *soxT1A-shdrR* intergenic region. Subfragments “A” to “K” are depicted as boxes. Yellow boxes indicate subfragments bound by sHdrR-trunc and SoxR. Fragments indicated in gray are not shifted in the presence of the regulator proteins. **(c)** EMSA performed with increasing concentrations of purified sHdrR-trunc and SoxR with subfragments A to K. Oligonucleotides used to amplify DNA fragments A to K are indicated. DNA fragments that exhibit shifted binding are marked by arrowheads with red filling. Vertical lines separate samples that were run on the same gel but were not directly adjacent.


a
Direct repeat
-10
-35
Direct repeat
Inverted repeat
Inverted repeat
-35
-10
b
sox T1A
shdrR
183-bp central fragment
Fragment A
Fragment B
Fragment C
Fragment D
Fragment E
Fragment F
Fragment G
Fragment H
Fragment I
Fragment J
Fragment K
c
A
B
C
D
E
F
G
H
I
J
K
sHdrR-trunc
SoxR
[bp]
766
500
300
200
150
100
75
50
0 200 400 800
0 150 300 600
[nM]


a
soxA
soxY
CTTCATGGGTCACCAAATTCTGCAGGTCTCAATGCGAATGCGAGGCTGCTCCAGCTAGACGAAAAACAAGGAAACGTTGCAATAGATAATTCACATATGTGTTATAGGATGAACGTTATCGCAGCTTCCGGGAGAGATGGCGTGATGGAAAGTTTAAACGGCGCG
GAAGTAGCCAGTGGTTTAAGACGTCCAGAGTTACGCTTACGCTCCGACGAGGTCGATCTGCTTTTTGTTCCTTGCAACGTTATCTATTAAGTGTATACTATTTAAAGTATATACACAATATCCTACTTGCAATAGCGTCGAAGGCCCTCTCTACCGCACTACCTTTCAAATTTGCCGCGC
Direct repeat
-35
-10
Inverted repeat
-10
-35
Fragment L
Fragment M
Fragment N
Fragment O
Fragment P
Fragment Q
b
L
M
N
O
P
Q
[bp]
766
500
300
200
150
100
75
50
sHdrR-trunc
SoxR
0 200 400 800 [nM]
0 150 300 600 [nM]

DNase I protection assays determined the SoxR-binding sites in the *sv* (*soxS-soxV*) region of *P. salicylatoxidans* KCT001 (Mandal et al. 2007). The protected site has a size of 30 bp and was described as containing a near-exact direct repeat of seven nucleotides. We cannot confirm this upon close inspection of the sequence (Fig. 4, Kct *SV*). Instead, the sequence contains a near exact inverted repeat as indicated in Fig. 4. The same feature is found in the core SoxR-binding region in *Paracoccus pantotrophus* (Rother et al 2005, Fig. 4, PP *SV*). We aligned these region with the *soxT1A-shdrR* and *soxA-soxY* intergenic regions in *H. denitrificans* (Fig. 4). Notably, the *H. denitrificans soxA-soxY* intergenic region also appears symmetrical, with two CATA sequences being positioned at equivalent sites on either side of the centre of symmetry. This symmetrical arrangement is likely important, as the promoter appears to be bidirectional. The *H. denitrificans soxT1A–shdrR* intergenic region contains two separate binding regions that qualify for binding of sHdrR or SoxR. Each of them features an inverted repeat region and they have a 12-nt exact direct repeat in common. The consensus operator sequence for all cases depicted in Fig. 4 is ATA-N_2-A-N_2-AT-N_4-ATA. In all cases, this consensus element overlaps with the -10 region of the RNA polymerase binding site that is a requirement for promoter activity. Other regulators of the ArsR family also bind to palindromic AT-rich elements that overlap with -10 regions (Barbosa and Benedetti, 2007). Thus, the repressor proteins and RNA polymerase compete for the same promoter sites.

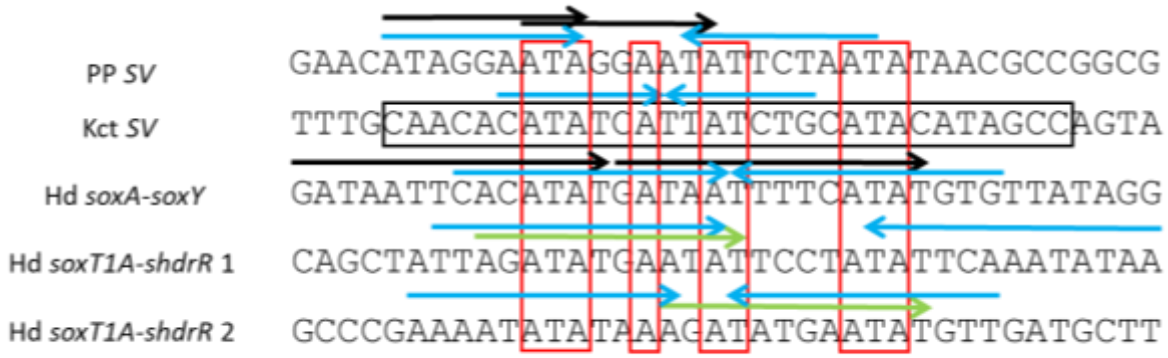


Fig. 4. Alignment of the core SoxR-binding regions of *Paracoccus pantotrophus* (PP *SV*), *P. salicylatoxidans* KCT001 (Kct *SV*) and the core binding regions in the *soxT1A-shdrR* and *soxA-soxY* intergenic regions in *H. denitrificans*. Two binding sites reside in the *soxT1A-shdrR* intergenic region. An open box highlights the region identified by DNase I protection assays in *P. salicylatoxidans* KCT001 (Mandal et al., 2007) . Black arrows above the sequences highlight exact direct repeats. Blue arrows highlight inverted repeats and green arrows indicate the direct repeats located 114 base pair apart in the *soxT1A-shdrR* region (Fig. 2a).

Fig 3. (a) sHdrR-trunc and SoxR specifically bind the 151 bp *soxA-soxY* intergenic subfragments. Predicted -35 and -10 RNA polymerase binding sites are indicated. Direct and indirect repeat regions are mark by green and blue open boxes, respectively. Subfragments “L” to “Q” are depicted as as boxes. Yellow boxes indicate subfragments bound by sHdrR-trunc and SoxR. Fragments indicated in gray are not shifted in the presence of the regulator proteins. **(b)** EMSA were performed using increasing concentrations of purified sHdrR-trunc and SoxR with subfragments L to Q. Oligonucleotides used to amplify DNA fragments L to Q are indicated. DNA fragments that exhibit shifted binding are marked by arrowheads with red filling. Vertical lines separate samples that were run on the same gel but were not directly adjacent.

3.4 sHdrR-trunc binding properties

EMSA assays were performed that allowed more detailed insights into the binding of sHdrR-trunc to the 183-bp central part of the intergenic region between the divergently oriented *soxT1A* and *shdrR* genes (Fig. 5). We had already shown that sHdrR binds to the DNA probe in a concentration-dependent manner, leading to appearance of mutiple shifted bands (Fig. 1b) (Li et al., 2023a), which suggests the formation of higher oligomers just as observed in EMSA experiments with subfragments (Fig. 2c). As sHdrR-related proteins respond to persulfidation (Shimizu and Masuda, 2017; Shimizu et al., 2017), we now assessed the response of sHdrR-trunc to treatment with polysulfide, sulfite, NaHS and thiosulfate in various molar ratios of protein and additive. Whereas sulfite and NaHS had no effect even when present in 40-fold excess compared to the protein (Supplementary Fig. 4), treatment with polysulfide above a molar ratio of 1 caused decreased binding of sHdrR-trunc to the target DNA (Fig. 5a). Compared with polysulfide-treatment, thiosulfate did not cause a significant reduction in DNA affinity (Fig. 5b). This effect is different from that observed with SoxR. Treatment with polysulfide above a molar ratio of 1 compelety prevented binding of SoxR to the target DNA, a shift was no longer observed (Li et al., 2023a). sHdrR requires a higher amount of polysulfide to prevent binding.

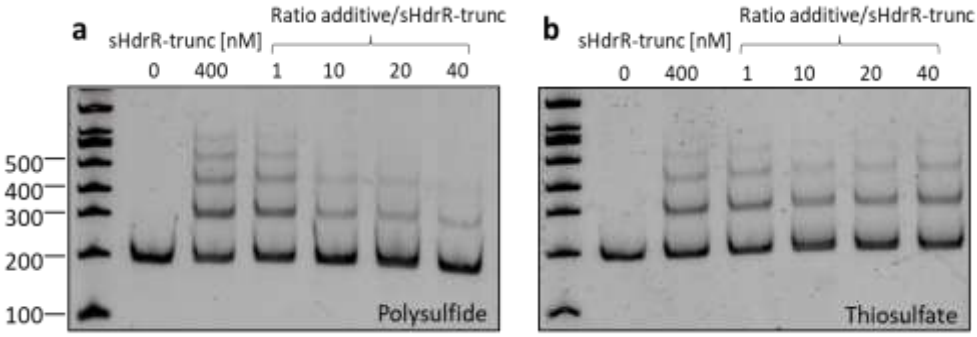


Fig 5. EMSA of the 183-bp central part of the *soxT1A-shdrR* intergenic fragment (17 nM) with sHdrR-trunc pre-induced with increasing amounts of polysulfide **(a)** or thiosulfate **(b)**.

3.5 Relevance of conserved cysteines Cys^{50} and Cys^{116} in sHdrR-trunc

To obtain information about the reactivity of the two conserved cysteines in sHdrR, His-tagged sHdrR-trunc as well as variants with serine in place of either one or both conserved cysteine were constructed, resulting in sHdrR-trunc $Cys^{50}Ser$, sHdrR-trunc $Cys^{116}Ser$, and sHdrR-trunc $Cys^{50}Ser$ $Cys^{116}Ser$. The proteins were overproduced in *E. coli*, purified by affinity chromatography, and used in EMSA experiments with two different DNA probes, the central part of the *soxT1A-shdrR* intergenic region and the *soxA-soxY* intergenic region (Fig. 6).

sHdrR-trunc bound to both DNA probes in a concentration-dependent manner (Fig. 6). Both sHdrR-trunc variants with one cysteine exchanged to serine still bound to their target DNAs, although binding appeared to be less effective, especially with the *soxA-soxY* target. This behaviour is different from that of the *H. denitrificans* SoxR protein, where the variant lacking Cys^{50} is unable to bind to its target DNA (Li et al., 2023a). The sHdrR-trunc variant lacking both conserved cysteines also behaves differently from its SoxR equivalent. While SoxR $Cys^{50}Ser$ $Cys^{116}Ser$ cannot bind DNA under any of the tested conditions (Li et al., 2023a), shifted bands are clearly visible after incubation of the respective double Cys to Ser variant of sHdrR-trunc with its two different target DNAs (Fig. 6).

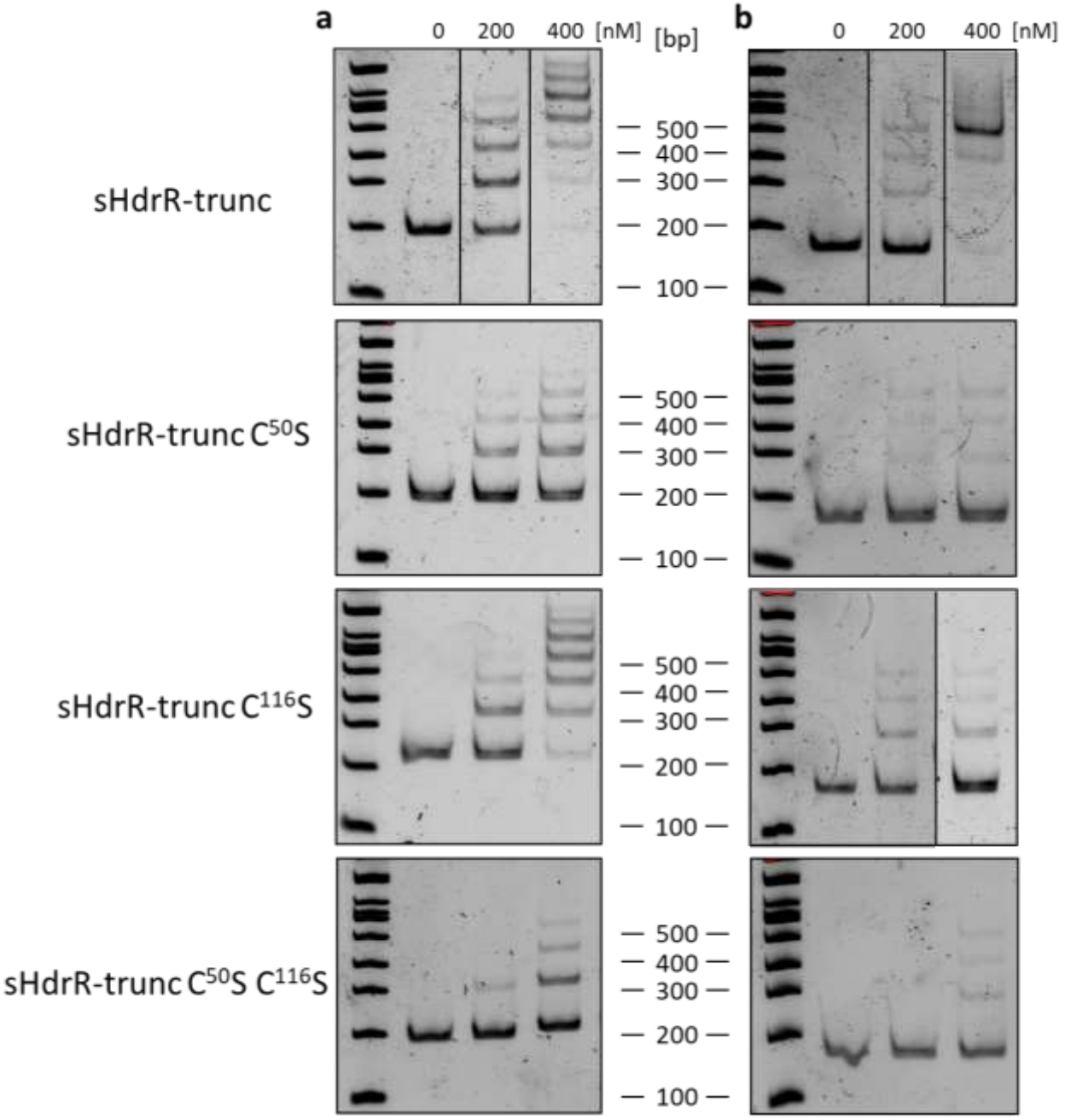


Fig 6. EMSA of the 180-bp central part of the *soxT1A-shdrR* (left)and the 151-bp *soxA-soxY* intergenic fragment (right) (17nM) with 0 nM, 200 nM, and 400 nM sHdrR-trunc and variant proteins as isolated. Vertical lines separate samples that were run on the same gel but were not directly adjacent.

In vivo results point at a reduced DNA binding capacity of the sHdrR $Cys^{116}Ser$ variant and strong binding, that is unresponsive to the presence of reduced sulfur compounds, for the sHdrR variants lacking Cys^{50} or both, Cys^{50} and Cys^{116} (Li et al., 2025). This prompted us to proceed to the next step and to subject all proteins (sHdrR-trunc, sHdrR-trunc $Cys^{50}Ser$, sHdrR-trunc $Cys^{116}Ser$, sHdrR-trunc $Cys^{50}Ser$ $Cys^{116}Ser$) to EMSA analysis with the two different DNA probes in the as-isolated state, after reduction with DTT, after oxidation with $CuCl_2$, and after persulfidation with polysulfide (Fig 7). Reduction of with DTT gave the same results as obtained for the untreated proteins indicating that all proteins are fully reduced upon isolation and remain in this state during storage. Oxidation of sHdrR-trunc prevented binding to both tested DNA probes (Fig 7). However, in line with the results shown in Fig. 5, persulfidation of sHdrR-trunc yielded protein still able to weakly bind to

the central part of the *soxT1A-shdrR* intergenic region. When polysulfide-treated sHdrR-trunc was reduced with DTT in a second step, the protein fully regained its DNA-binding capacity demonstrating that the modification caused by polysulfide was entirely reversible by reduction. This behaviour is compatible with the formation of a sulfur bridge between Cys^{50} and Cys^{116} as has been described for SoxR (Li et al., 2023a). It should be noted, however, that further experiments are required to substantiate this conclusion.

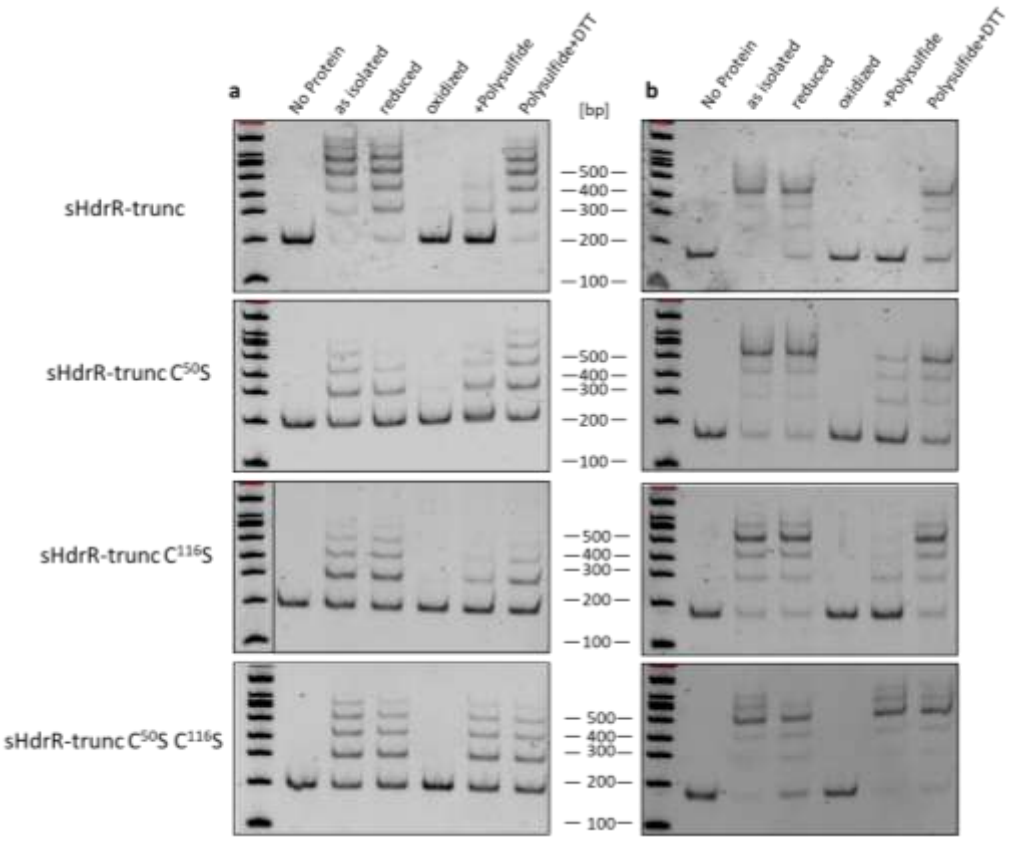


Fig. 7. (a) EMSA of the 183-bp central part of the *soxT1A-shdrR* intergenic fragment (17nM) with 400 nM sHdrR-trunc wild-type and variant proteins as isolated, reduced with DTT, oxidized with $CuCl_2$, treated with polysulfide, and sequentially treated with polysulfide and DTT; **(b)** EMSA of the 151-bp *soxA-soxY* intergenic fragment (17nM) with 400 nM sHdrR-trunc wild-type and variant proteins as isolated, reduced with DTT, oxidized with $CuCl_2$, treated with polysulfide, and sequentially treated with polysulfide and DTT. Vertical lines separate samples that were run on the same gel but were not directly adjacent.

The untreated sHdrR-trunc Cys^{50}Ser, Cys^{116}Ser and Cys^{50}Ser Cys^{116}Ser variants can all bind to both DNA probes (Figs. 6 and 7). The response to oxidation is the same for sHdrR-trunc and all its variants. All proteins loose their DNA binding ability after treatment with $CuCl_2$. Polysulfide treatment appears to have a somewhat stronger effect on sHdrR-trunc Cys^{116}Ser than on sHdrR-trunc Cys^{50}Ser and sHdrR-trunc Cys^{50}Ser Cys^{116}Ser (Fig. 7). Translated into the *in vivo* situation this would mean that sHdrR Cys^{50}Ser and sHdrR lacking both conserved cysteines should constitutively repress the transcription of their target genes, while a strain harboring the sHdrR Cys^{116}Ser should still be responsive to externally available sulfane sulfur. In fact, this is exactly what was observed for the respective mutant strains (Li et al., 2025). In this respect, sHdrR differs significantly from the related SqrR from *Rhodobacter capsulatus*. Both SqrR variants lacking a conserved cysteine can no longer respond to persulfidation *in vitro*, and constitutively repress target gene expression *in vivo* (Shimizu et al., 2017). sHdrR differs from *H. denitrificans* SoxR in that the the SoxR Cys^{50}Ser and SoxR Cys^{50}Ser Cys^{116}Ser variants are unable to bind their target DNA *in vitro*. They are also unresponsive to polysulfide treatment or changes in oxidation state (Li et al., 2023a). Taken together our observations

reveal substantial differences between the *H. denitrificans* transcriptional repressors SoxR and sHdrR. At the amino acid sequence level, the most striking difference between SoxR and sHdrR is the presence of a third cysteine, Cys^{63}, in sHdrR, which may well be responsible for the differences between these closely related proteins.

3.6 First insights into the relevance of Cys^{63}

To learn more about the function the third cysteine in sHdrR, Cys^{63} was changed to serine and the sHdrR-trunc $Cys^{63}Ser$ variant was subjected to EMSA using the central part of the *soxT1A-shdrR* region as a probe (Fig. 8). In the as-isolated state, the variant bound effectively to the DNA. When sHdrR-trunc $Cys^{63}Ser$ was incubated with different sulfur compounds, there were no significant effect observed, except in the case of polysulfide treatment (Figure 8 and Supplementary Fig. 5). Thiosulfate, sulfite, and NaHS did not affect binding, even when applied in 20-fold access over the protein (Fig. 8 and Supplementary Fig 5). The response of sHdrR-trunc $Cys^{63}Ser$ to incubation with polysulfide, sulfite, thiosulfate and NaHS is not significantly different from that of the original sHdrR-trunc protein (compare Figs. 5 and 8).

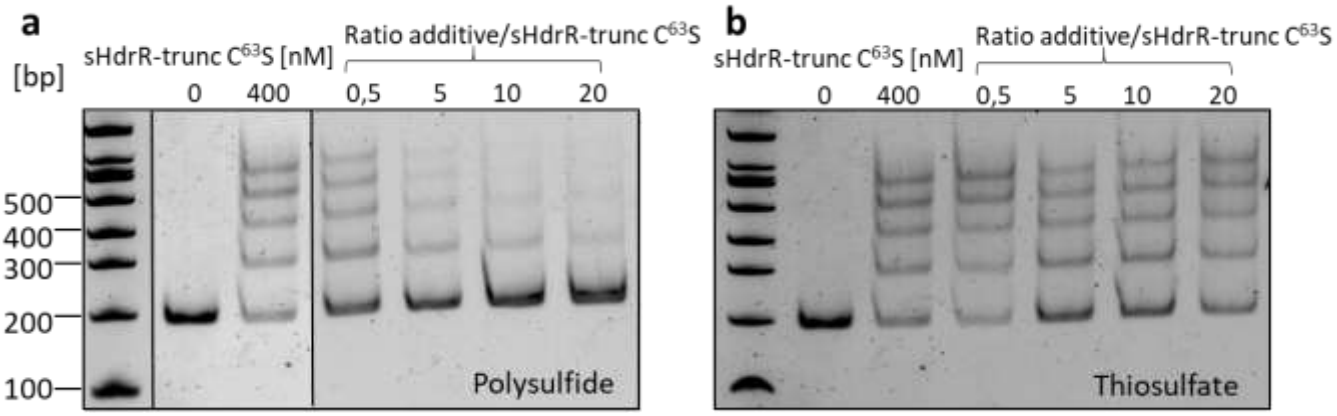


Fig 8. EMSA of the 183-bp central part of the *soxT1A-shdrR* intergenic fragment (17nM) with sHdrR-trunc $Cys^{63}Ser$ pre-induced with increasing amounts polysulfide (**a**) or thiosulfate (**b**). Vertical lines separate samples that were not run on the same gel.

In the next step, EMSA experiments were performed with the as-isolated, reduced, oxidized and polysulfide-treated sHdrR-trunc $Cys^{63}Ser$ variant and two different DNA probes (Fig. 9). In agreement with the results presented in Fig. 8, pre-incubation of the protein lacking Cys^{63} with 0.5 mM polysulfide turned it unable to bind to its DNA targets (Fig. 9), indicating that the remaining cysteine residues (Cys^{50} and Cys^{116}) are responsive to polysulfide treatment. Notably, sHdrR-trunc $Cys^{63}Ser$ showed a weaker response to oxidation by $CuCl_2$ than sHdrR-trunc (Figs. 7 and 9), suggesting that the remaining cysteine residues (Cys^{50} and Cys^{116}) in sHdrR are less susceptible to oxidative modification, i.e. formation of a disulfide bond between them, in the absence than in the presence of Cys^{63}, and that Cys^{63} may play a role in sensing redox signals.

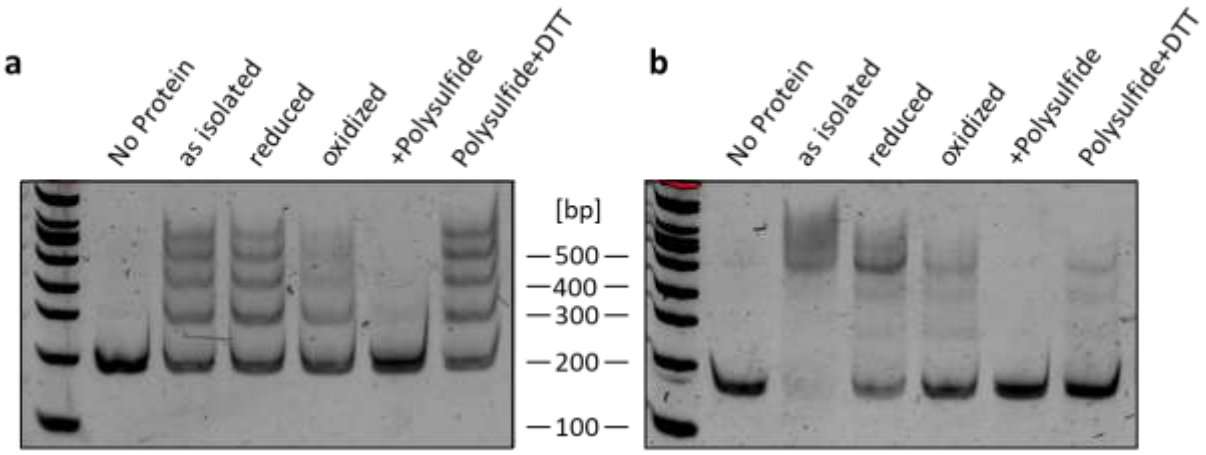


Fig. 9. (a) EMSA of the 183-bp central part of the *soxT1A-shdrR* intergenic fragment (17nM) with 400 nM sHdrR-trunc $Cys^{63}Ser$ proteins as isolated, reduced with DTT, oxidized with $CuCl_2$, treated with polysulfide, and sequentially treated with polysulfide and DTT; **(b)** EMSA of the 151-bp *soxA-soxY* intergenic fragment (17nM) with 400 nM sHdrR-trunc $Cys^{63}Ser$ proteins as isolated, reduced with DTT, oxidized with $CuCl_2$, treated with polysulfide, and sequentially treated with polysulfide and DTT.

4 CONCLUSIONS

Here, we provide first insights into the DNA-binding properties of sHdrR, an ArsR-type regulator that functions as a transcriptional repressor of genes encoding enzymes involved in the oxidation of thiosulfate as a supplemental electron donor in *H. denitrificans*. sHdrR and SoxR co-regulate the sHdr-LbpA-Sox pathway and both sense thiosulfate probably via intracellular polysulfide produced as an intermediate. A stable truncated version of sHdrR displayed strong binding to the *soxT1A-shdrR* intergenic region and to the *soxA-soxY* intergenic region. The sequences of the *soxT1A-shdrR* and the *soxA-soxY* intergenic regions were analyzed and found to contain two and one palindromic AT-rich consensus operator sequences, ATA-N_2-A-N_2-AT-N_4-ATA, respectively, which overlap -10 sites required for attachment of RNA polymerase. sHdrR-trunc and its variants carrying exchanges of relevant cysteine residues exhibited differential responses to polysulfide. In sHdrR, two conserved cysteine residues (Cys^{50} and Cys^{116}) are sensitive to polysulfide treatment, while the third cysteine (Cys^{63}) may play a role in sensing redox signals . Our work expands the understanding of sHdrR as a sulfane sulfur-responsive regulator. However, whether and how the truncated sHdrR differs from the full-length sHdrR *in vitro* remains unknown.

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The sulfane-sulfur responsive transcriptional repressor sHdrR: Properties and binding sites

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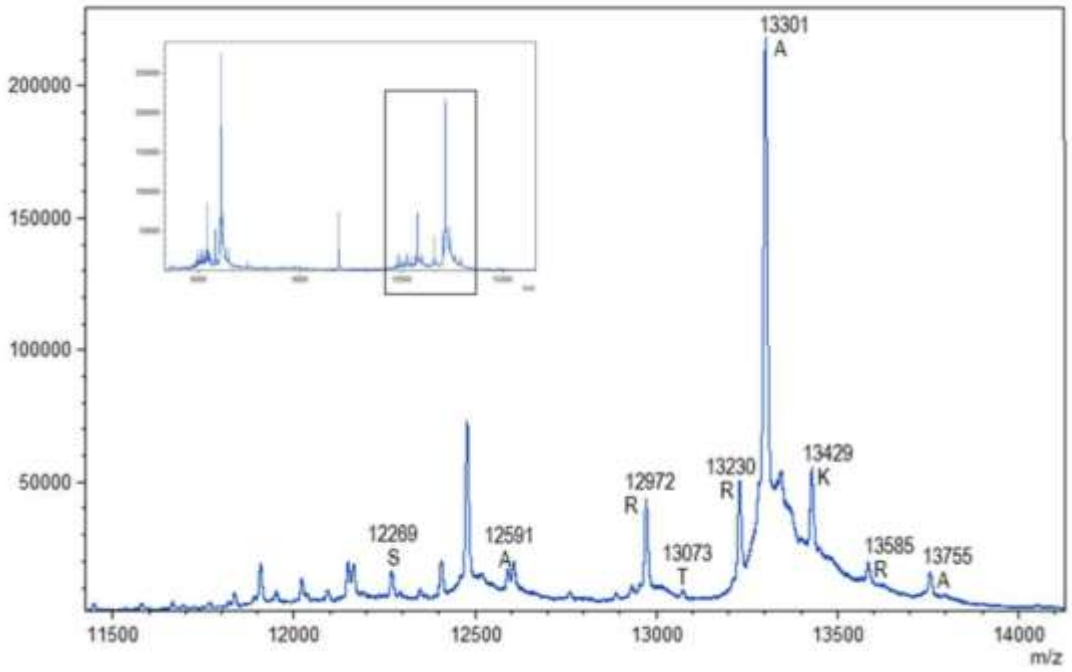
Supplementary Figures and Tables:



Supplementary Fig. 1. Mass spectrometry analysis of full-length sHdrR. A mass matching the full length His-tagged sHdrR protein was not detected. Instead masses matching those for several different N-terminally truncated polypeptides were observed, showing that the sHdrR-N-terminus is highly prone to degradation.

Amino acid sequence of His-tagged sHdrR without initiator methionine (14875 Da):
AVVKPRTNRPAVRKARTRQPALHSTDASIEQATALLRALGSPHRLAILCLLLEGERTVSEICDKIGARQSLVSQHLTR
LRLDGLVKSDRNGYFVSYSLTSAPAQEIIATLHKYYCATSAGKRSNAAALEHHHHHH

13755 Da:
AVRKARTRQPALHSTDASIEQATALLRALGSPHRLAILCLLLEGERTVSEICDKIGARQSLVSQHLTRLRLDGLVKSD
RNGYFVSYSLTSAPAQEIIATLHKYYCATSAGKRSNAAALEHHHHHH

13585 Da:
RKARTRQPALHSTDASIEQATALLRALGSPHRLAILCLLLEGERTVSEICDKIGARQSLVSQHLTRLRLDGLVKSDRN
GYFVSYSLTSAPAQEIIATLHKYYCATSAGKRSNAAALEHHHHHH

13429 Da:
KARTRQPALHSTDASIEQATALLRALGSPHRLAILCLLLEGERTVSEICDKIGARQSLVSQHLTRLRLDGLVKSDRNG
YFVSYSLTSAPAQEIIATLHKYYCATSAGKRSNAAALEHHHHHH

13301 Da:
ARTRQPALHSTDASIEQATALLRALGSPHRLAILCLLLEGERTVSEICDKIGARQSLVSQHLTRLRLDGLVKSDRNGY
FVSYSLTSAPAQEIIATLHKYYCATSAGKRSNAAALEHHHHHH

13230 Da:

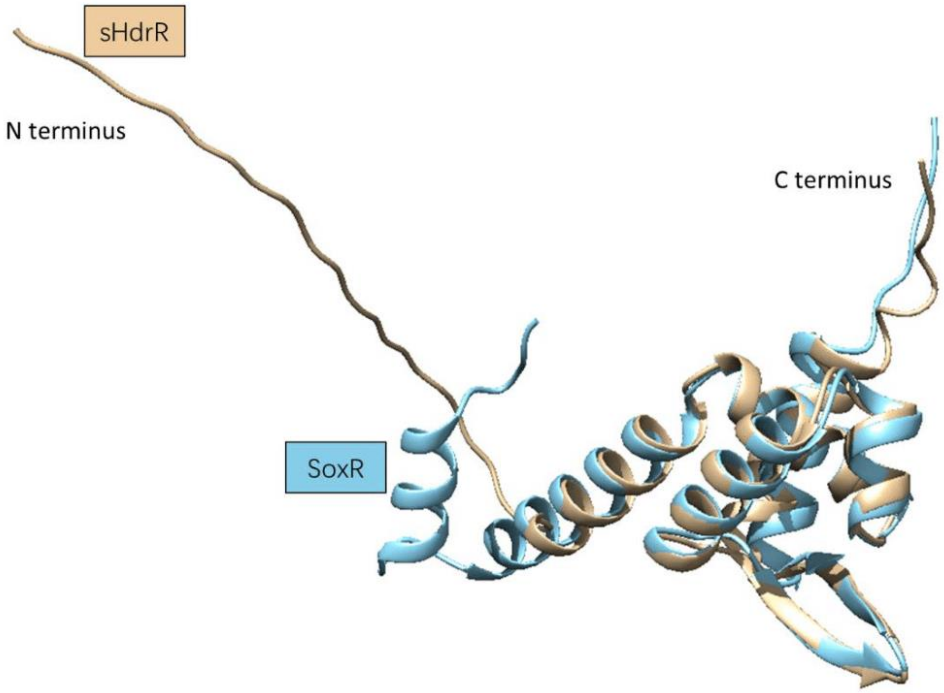
RTRQPALHSTDASIEQATALLRALGSPHRLAILCLLLEGERTVSEICDKIGARQSLVSQHLTRLRLDGLVKSDRNGYF
VSYSLTSAPAQEIIATLHKYYCATSAGKRSNAAALEHHHHHH

13073 Da:
TRQPALHSTDASIEQATALLRALGSPHRLAILCLLLEGERTVSEICDKIGARQSLVSQHLTRLRLDGLVKSDRNGYFV
SYSLTSAPAQEIIATLHKYYCATSAGKRSNAAALEHHHHHH

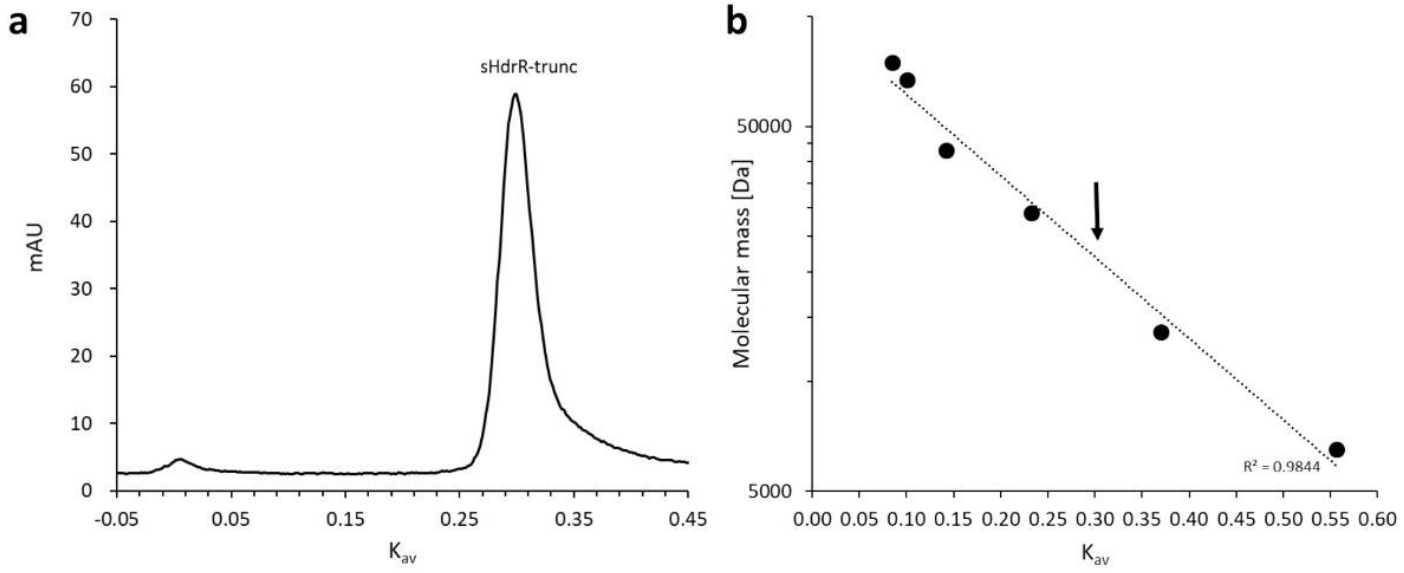
12972 Da:
RQPALHSTDASIEQATALLRALGSPHRLAILCLLLEGERTVSEICDKIGARQSLVSQHLTRLRLDGLVKSDRNGYFVS
YSLTSAPAQEIIATLHKYYCATSAGKRSNAAALEHHHHHH

12591 Da:
ALHSTDASIEQATALLRALGSPHRLAILCLLLEGERTVSEICDKIGARQSLVSQHLTRLRLDGLVKSDRNGYFVSYSLT
SAPAQEIIATLHKYYCATSAGKRSNAAALEHHHHHH

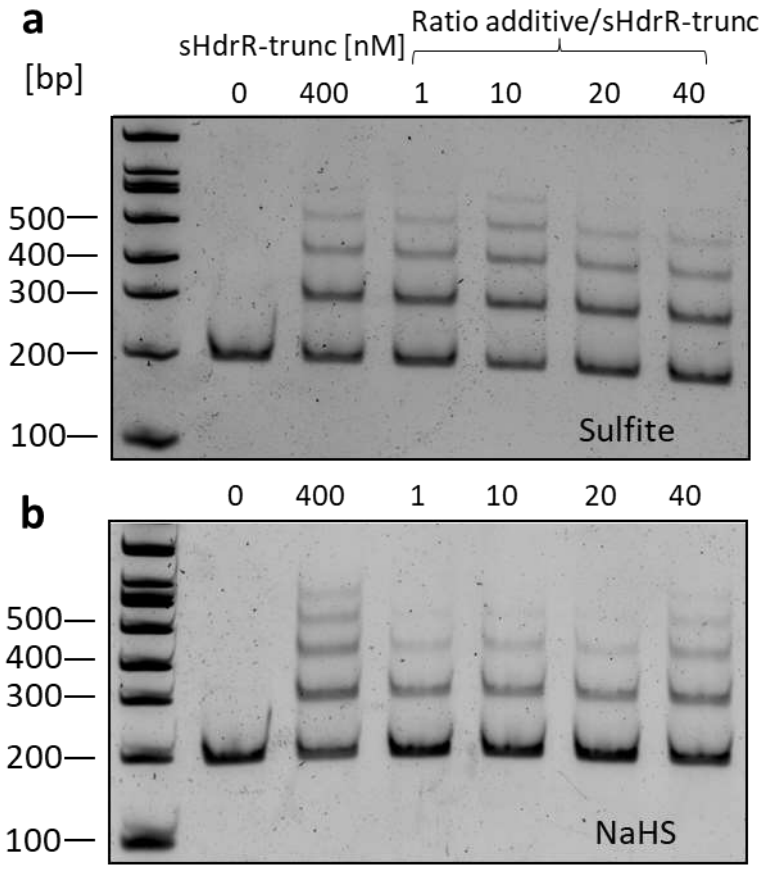
12269 Da:
STDASIEQATALLRALGSPHRLAILCLLLEGERTVSEICDKIGARQSLVSQHLTRLRLDGLVKSDRNGYFVSYSLTSAP
AQEIIATLHKYYCATSAGKRSNAAALEHHHHHH



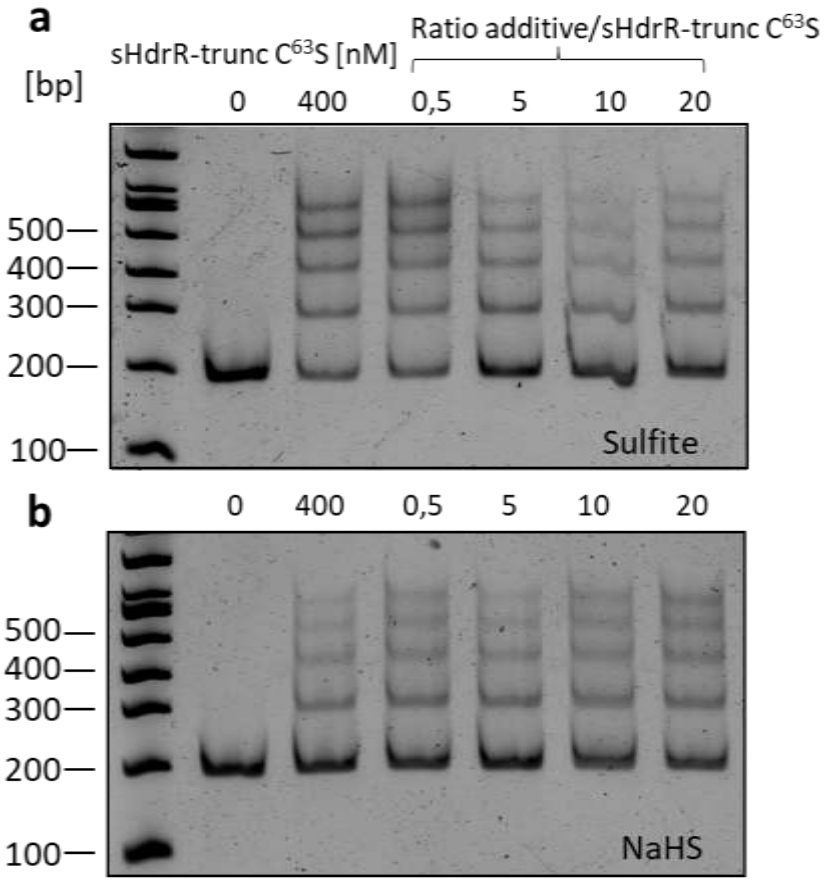
Supplementary Fig. 2. Structures for sHdrR and SoxR predicted by Alphafold (Jumper et al., 2021)**.** sHdrR is shown in khaki, SoxR is shown in light blue.



Supplementary Fig. 3. Conformation of sHdrR-trunc as analyzed by gel permeation chromatography. (a) The elution profile of sHdrR-trunc upon gel filtration on Superdex 75 Increase 10/300 GL (Cytiva, Freiburg, Germany) is depicted. The column was run in 50 mM Tris-HCl, pH 8.0 and 150 mM NaCl at a flow rate of 0.8 mL min^{-1} using an Äkta FPLC system. sHdrR-trunc elutes at a K_{av} of 0.298, which corresponds to a molecular mass of 22.1 kDa and indicates formation of a dimer. **(b)** Calibration curve. The column was calibrated using Blue dextran (2000 kDa), conalbumin (75 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), lactoglobulin (35 kDa), carbonic anhydrase (29 kDa), chymotrypsin (23 kDa), and ribonuclease (13.7 kDa). The calibration curve was plotted using the gel-phase distribution coefficient (K_{av}) versus the logarithm of molecular weight. $K_{av} = (V_e - V_0/V_c - V_0)$, where V_e = elution volume, V_0 = column void volume (7.94 ml based on Blue dextran elution volume) and V_c geometric column volume (24 ml). The black arrow indicates the K_{av} for sHdrR-trunc.



Supplementary Fig 4. EMSA of the 183-bp central part of *soxT1A–shdrR* intergenic fragment with 400 nM sHdrR-trunc reincubated with increasing amounts of sulifte **(a)** or NaHS **(b).**



Supplementary Fig 5. EMSA of the 183-bp central part of *soxT1A–shdrR* intergenic fragment with 400 nM sHdrR-trunc Cys^{63}Ser preincubated with increasing amounts of sulifte **(a)** or NaHS **(b).**

Supplementary Table 1. Strains, plasmids and primers

Strains primers or plasmids	Relevant genotype, description or sequence	Reference or source
Strains		
E. coli 10-beta	Δ*(ara-leu) 7697 araD139 fhuA* Δ*lacX74 galK16 galE15 e14-* φ*80dlacZ*Δ*M15 recA1 relA1 endA1 nupG rpsL* (Str^R) *rph spoT1* Δ*(mrr-hsdRMS-mcrBC)*	New England Biolabs
E. coli DH5α	F$^-$ φ80*lacZ*ΔM15 Δ(*lacZ*YA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_{K^-}, m_{K^+}) *pho*A *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1	New England Biolabs
E. coli BL21 (DE3)	F$^-$*omp*T *hsd*S_B (r_{B^-}, m_{B^-}) *gal dcm* (DE3)	Novagen
Primers		(Li et al., 2023a)
EMSA-Fr	TTCCCGCCCCGTCTTGGTTT	(Li et al., 2023b)
EMSA_Fr2_Fr	TCAGCGCTCGCCTGGAAGTC	(Li et al., 2023a)
EMSA_Fr3_Rev	TCTAAGCATCAACATATTCATATCTTTATATATTTTCG	(Li et al., 2023a)
EMSA-Rev	AGGAGTTGCATCCAAAAAAGCGTG	(Li et al., 2023b)
EMSA-Hden_0703/04-fw	GGGTCACCAAATTCTGCAGGTCTC	(Li et al., 2023a)
EMSA-Hden_0703/04-rev	ATCACGCCATCTCTCCCGGAA	(Li et al., 2023a)
Fr-pET22b-sHdrR-trunc	GGCA**CATATG**ACCGACGCGTCGATCGAACAG (NdeI)	(Li et al., 2025)
Rev-pET22b-sHdrR	TTTT**GCGGCCGC**ATTCGAGCGTTTTCCCGCAC (NotI)	(Li et al., 2023a)
sHdrR_C50S_Down_fwd	TCTCGCGATTTTGTCCCTCCTGCTCGAGGGAGAAAGAACC	(Li et al., 2025)
sHdrR_C50S_UP_rev	GGTTCTTTCTCCCTCGAGCAGGAGGGACAAAATCGCGAGA	(Li et al., 2025)
sHdrR_C116 S_Down_fwd	ACGCTGCATAAGTATTATAGTGCAACGAGTGCGGGAAAAC	(Li et al., 2025)
sHdrR_C116 S_Down_rev	GTTTTCCCGCACTCGTTGCACTATAATACTTATGCAGCGT	(Li et al., 2025)
Fw-pET22b-sHdrR-Cys63	TCCGAAATCTCCGACAAGATC	This work
Rev-pET22b-sHdrR-Cys63	AACGGTTCTTTCTCCCTC	This work
Fragment A		
EMSA-Fr	TTCCCGCCCCGTCTTGGTTT	(Li et al., 2023b)
shdrR_A_Rev	ACAATCGACACGAACCCTCAC	This work
Fragment B		
shdrR_B_Fr	TACGTTTTGCAGCGAAGCGC	This work
EMSA_F1_rev	GACTTCCAGGCGAGCGCTGA	This work
Fragment C		
shdrR_C_Fr	TGAGGGCGCTCGATCTCTT	This work
shdrR_C_Rev	GAATATTCATATCTAATAGCTGGCGCC	This work
Fragment D		
EMSA_Fr2_Fr	TCAGCGCTCGCCTGGAAGTC	(Li et al., 2023a)
EMSA_F2_Rev	ACGGAGCACGGCCTTGACGA	This work
Fragment E		
Fwd_IR_part1_Fr2_sHdrR	CGCCAGCTATTAGATATGAATATTCCT	This work
EMSA_F2_Rev	ACGGAGCACGGCCTTGACGA	This work
Fragment F		
shdrR_F_Fr	CCTATATTCAAATATAAATCGCAACGGAAGC	This work
Rev_SF_sHdrR	ACGTACGCGGTGATGCGTAG	This work

Fragment G		
EMSA_F3_Fr	TCGTCAAGGCCGTGCTCCGT	This work
shdrR_G_Rev	CATATCTTTATATATTTTCGGGCAAAGCTGTG	This work
Fragment H		
Fwd_sHdrR_F7	CTACGCATCACCGCGTACGT	This work
EMSA_Fr3_Rev	TCTAAGCATCAACATATTCATATCTTTATATATTTTCG	(Li et al., 2023a)
Fragment I		
Fwd_sHdrR_F9	GAACACAGCTTTGCCCGAAAATA	This work
shdrR_I_Rev	ATCACCTACCGATGCAGC	This work
Fragment J		
shdrR_J_Fr	AATATGTTGATGCTTAGATTTGAGTTGCA	This work
EMSA-Rev	AGGAGTTGCATCCAAAAAAGCGTG	(Li et al., 2023b)
Fragment K		
shdrR_K_Fr	TTTGAGTTGCAAGCTCTCTGC	This work
EMSA-Rev	AGGAGTTGCATCCAAAAAAGCGTG	(Li et al., 2023b)
Fragment L		
EMSA-Hden_0703/04-fw	GGGTCACCAAATTCTGCAGGTCTC	(Li et al., 2023a)
Rev_SoxR_L	TTCGTCTAGCTGGAGCAGCCTC	This work
Fragment M		
Fwd_soxR_F2	CAATGCGAATGCGAGGCT	This work
Rev_SoxR_F1	TATTGCAACGTTCCTTGTTTTTCGTCT	This work
Fragment N		
Fwd_SoxR_N	CAGCTAGACGAAAAACAAGGAACG	This work
Rev_SoxR_F2	TTATCATATGTGAATTATCTATTGCAACGT	This work
Fragment O		
Fwd_SoxR_F3	ACGTTGCAATAGATAATTCACATATGATAAT	This work
Rev_SoxR_O	ACGTTCATCCTATAACACATATGAAAATTATC	This work
Fragment P		
Fwd_soxR_F4	GATAATTTTCATATGTGTTATAGGATGAACGT	This work
EMSA-Hden_0703/04-rev	ATCACGCCATCTCTCCCGGAA	(Li et al., 2023a)
Fragment Q		
Fwd_soxR_F5	GTTATAGGATGAACGTTATCGCAG	This work
Rev_SoxR_Q	CGCGCCGTTTAAACTTTCCA	This work
Fragment R		
Fwd_soxR_F6	TTATCGCAGCTTCCGGGAGAG	This work
Rev_SoxR_F6	TGAAGGCACGGCGCGA	This work

Plasmids

pET-22b(+)	Ap^r	Novagen
pET-22bHdsHdrR	Ap^r, NdeI-NotI fragment of PCR amplified *shdrR* in NdeI-NotI of pET-22b(+)	(Li et al., 2023b)
pET-22b-SoxR-Strep	Ap^r, NdeI-HindIII fragment of PCR amplified *soxR* in NdeI-HindIII of pET-22b(+)	(Li et al., 2023b)
pET-22b-sHdrR-trunc-C-His	Ap^r, NdeI-NotI fragment of PCR amplified truncated *shdrR* in NdeI-NotI of pET-22b(+)	This work
pET-22b-sHdrR-trunc $C^{50}S$	Apr, pET-22b-sHdrR-trunc-C-His with a $Cys^{50}Ser$ exchange	This work
pET-22b-sHdrR-trunc $C^{116}S$	Ap^r, pET-22b-sHdrR-trunc-C-His with a $Cys^{116}Ser$ exchange	This work

pET-22b-sHdrR-trunc $C^{50}S$ $C^{116}S$	Ap^r, pET-22b-sHdrR-trunc-C-His with $Cys^{50}Ser$ and $Cys^{116}Ser$ exchanges	This work
pET-22b-sHdrR-trunc $C^{63}S$	Apr, pET-22b-sHdrR-trunc-C-His with a $Cys^{63}Ser$ exchange	This work

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