OxyR senses sulfane sulfur and activates the genes for its removal in *Escherichia coli*

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**ABSTRACT**

Sulfane sulfur species including hydrogen polysulfide and organic persulfide are newly recognized normal cellular components, and they participate in signaling and protect cells from oxidative stress. Their production has been extensively studied, but their removal is less characterized. Herein, we showed that sulfane sulfur at high levels was toxic to *Escherichia coli* under both anaerobic and aerobic conditions. OxyR, a well-known regulator against H₂O₂, also sensed sulfane sulfur, as revealed via mutational analysis, constructed gene circuits, and in vitro gene expression. Hydrogen polysulfide modified OxyR at Cys199 to form a persulfide OxyR C199-SSH, and the modified OxyR activated the expression of thioredoxin 2 and glutaredoxin 1. The two enzymes are known to reduce sulfane sulfur to hydrogen sulfide. Bioinformatics analysis indicated that OxyR homologs are widely present in bacteria, including obligate anaerobic bacteria. Thus, the OxyR sensing of sulfane sulfur may represent a preserved mechanism for bacteria to deal with sulfane sulfur stress.

1. Introduction

Hydrogen sulfide (H₂S) has been proposed as a gasotransmitter because it is involved in many physiological and pathological processes in animals and plants, such as ageing [1], neuromodulation [2], cancer cell proliferation, metabolic reprogramming [3,4], and stomatal closure [5]. The mechanism of H₂S signaling is often via protein persulfidation. H₂S cannot directly react with protein thiols, but sulfane sulfur, its oxidation product, readily reacts with thiols to generate protein-persulfide (RSSnR, n ≥ 2). Sulfane sulfur species include hydrogen polysulfide (H₂Sn, n ≥ 2), organic polysulfide (RSSₙH, RSSₙ, n ≥ 2), and organic persulfide (RSSₙH), which can be produced from H₂S oxidation or from the metabolism of cysteine and cystine. The endogenous H₂Sn was initially discovered in rat brain [8]. Now, sulfane sulfur are considered as normal cellular components in both prokaryotic and eukaryotic cells [9,10]. Sulfane sulfur possesses both nucleophilic and electrophilic characteristics, while thiol (cysteine, GSH, etc.) is nucleophilic [11,12]. As nucleophiles, sulfane sulfur species are better reductants than thiols [13]; as electrophiles, they can transfer electrophilic sulfane sulfur (SₙH) to protein thiols to generate protein-persulfide, affecting certain protein functions and protecting protein thiols from irreversible oxidation [14,15]. Owing to the dual-reactivities, sulfane sulfur is involved in many cellular processes, such as redox homeostasis, virulence in pathogenic bacteria, and biogenesis of mitochondria [16,17]. Sulfane sulfur also functions as antioxidants inside cells [18,19].

Albeit the good roles, sulfane sulfur may be toxic at high concentrations. Indeed, elemental sulfur has been used as an antimicrobial agent for ages, and its efficiency is likely impaired by its low solubility [20]. Advances in the synthesis of sulfur nanoparticles have significantly increased the antimicrobial efficiency of elemental sulfur [21]. Elemental sulfur is often used as a fungicide. Although its toxicity mechanism is unclear, a recent study suggested that sulfur is transported into the cell in the form of H₂Sn [22], inducing protein persulfidation as a possible toxic mechanism [23]. Fungi may use glutathione to reduce polysulfide to H₂S as a detoxification mechanism [22,24]. Organosulfur compounds can be used to treat antibiotic-resistant bacteria, and they are converted to H₂Sn for the toxicity [25]. Both bacteria and fungi display reduced viability being exposed to sulfane sulfur in excess [22,25]. Therefore, intracellular sulfane sulfur is likely maintained within a range for microorganisms under normal conditions.

Multiple pathways for sulfane sulfur generation have been
discovered. 3-Mercaptopropionate sulfurtransferase and cysteiny1-tRNA synthetase produce sulfane sulfur from cysteine [8,26,27]. Cystathionine β-synthase and cystathionine γ-lyase can produce H2S from cysteine and sulfane sulfur from cystine [13]. Since cellular cystine concentration is very low, these enzymes are likely to generate H2S instead of sulfane sulfur [10]. Sulfidequinone oxidoreductase and superoxide dismutase produce sulfane sulfur from H2S [28,29]. Most microorganisms possess several of these pathways. Escherichia coli contains 3-mercaptopyruvate sulfurtransferase, cysteiny1-tRNA synthetase, and superoxide dismutase that may generate cellular sulfane sulfur.

Microorganisms may use several mechanisms to remove or reduce sulfane sulfur inside cells. Aerobic microorganisms may apply persulfide dioxygenase to remove excessive sulfane sulfur [30], and the persulfide dioxygenase expression can be induced by sulfane sulfur via sulfane sulfur-sensing transcription factors [31–33]. Another possibility is that sulfane sulfur is reduced by glutathione (GSH) or glutaredoxin or thioredoxin to H2S [34,35], which is released out of cells [36], as observed in anaerobically cultured fungi [22,37]. A recent report that two thioredoxin-like proteins catalyze the reduction of protein persulfidation in Staphylococcus aureus also support that thioredoxin participates in the reduction of cellular sulfane sulfur to H2S [17]. However, it is unclear whether sulfane sulfur induces the expression of glutaredoxin and thioredoxin.

E. coli, a common intestinal bacterium, contains three thioredoxins and four glutaredoxins. The expression of TrxA, GrxB, and GrxD is inducible in conditions of stress [41–43]. The expression of GrxA, TrxC, and KatG (catalase) is regulated by OxyR upon exposure to H2O2. These proteins are much less than other thioredoxins and glutaredoxins in E. coli in the absence of oxidative stress [41–43].

OxyR was initially identified as a regulator responding to reactive oxygen species (ROS) [44,45]. ROS triggers the formation of a disulfide bond between Cys199 and Cys208 or oxidizes Cys199 to C199-SOH, but the exact mechanism is still in debate [46–48]. Herein, we showed that sulfane sulfur modified OxyR at Cys199 to form a protein persulfide that in turn activates the expression of thioredoxin, glutaredoxin, and catalase in E. coli, and the induced enzymes reduced cellular sulfane sulfur to H2S.

2. Materials and methods

2.1. Strains, plasmids, and chemicals

All strains and plasmids used in this study are listed in Table S2. Deletion of oxyR was performed following a reported method [49]. E. coli cells strains were grown in Lysogeny broth (LB) medium. Antibiotics (50 μg/ml) were added when required. SSP4 (3’β-Di(0-thiosialicyl) fluorescein) was purchased from DOJINDO MOLECULAR TECHNOLOGIES. H2Sb was prepared by following Kamshny & Alexey’s method [50]. Briefly, 13 mg of sulfur powder and 70 mg of sodium sulfide were added to 5 ml of anoxic distilled water under argon gas. The pH was adjusted to 9.3 with 6 M HCl. The obtained product contained a mixture of H2Sb, where n varies from 2 to 8 [51], but at low concentration and neutral pH, H2S2 is dominant [29].

2.2. Cellular sulfane sulfur analysis

SSP4 probe was used for cellular sulfane sulfur analysis. E. coli cells (1 ml) were taken out from the culture at specific time points and diluted to OD600nm = 1, washed, and resuspended in HEPES buffer (50 mM, pH 7.4); then 10 μM SSP4 and 0.5 mM CTAB were added. After an incubation at 37 °C for 15 min in the dark with gently shaking (125 rpm), reagents were washed off with HEPES buffer (50 mM, pH 7.4). Reacted-cells were subjected to flow cytometry (FACS) analysis by using BD Accuri™ C5. For each sample, > 10,000 cells were analyzed in FL1-A channel. The average fluorescent intensity was used to estimate cellular sulfane sulfur of sampled cells.

The CsoR-based reporting system was used for real-time analysis. csrB gene was chemically synthesized by GeneWiz (Shanghai) company and expressed with Pnad promoter in pTrcHis2A plasmid, where the tsc promoter was replaced by the CsoR cognate promoter, and a mkate gene (with a C-terminus degradation tag scrA) was put after it (Table S2, entry 22). For trxA, trxB, grxB, grxC, or grxD overexpression experiment, the gene was introduced after mkate (Table S2, entries 23–27). E. coli strains containing reporting plasmids were culture in LB medium at 37 °C with shaking (220 rpm). Fluorescence was analyzed by FACS (FL3-A channel, > 10,000 cells).

We used a reported method to quantitate the concentration of cellular sulfane sulfur [52]. Briefly, E. coli cells were harvested and resuspended (OD600nm = 10) in 1 M NaOH, 0.1% SDS and 0.3 M ascorbic acid to lyse cells and reduce cellular sulfane sulfur to sulfide. Then zinc acetate was added to recover the released sulfide as ZnS precipitate. The precipitate was collected by centrifugation and washed with distilled water. The ZnS precipitate were resuspended in 1 ml distilled water and mix with 100 μl methylene blue reagent (30 mM FeCl3; 20 mM N,N-dimethyl-p-phenylenediamine; 7.2 M HCl ) to detect S2− released from ZnS at 670 nm. Sulfane sulfur in the solution was converted to cellular sulfane sulfur by using a reported conversion factor: one ml of cells at OD600nm of one was converted to one μl of cellular volume (https://biionumbers.hms.harvard.edu/search.aspx).

2.3. H2S production analysis

Production of H2S was determined by using a previously reported method [53]. Briefly, H2S was derivatized with mBBr then analyzed by HPLC (LC-20A, Shimadzu) equipped with a fluorescence detector (RF-10AXL, Shimadzu). A C18 reverse phase HPLC column (VP-ODS, 150 × 4 mm, Shimadzu) was pre-equilibrated with 80% Solvent A (10% methanol and 0.25% acetic acid) and 20% Solvent B (90% methanol and 0.25% acetic acid). The column was eluted with the following gradients of Solvent B: 20% from 0 to 10 min; 20–40% from 10 to 25 min; 40%–90% from 25 to 30 min; 90%–100% from 30 to 32 min; 100% from 32 to 35 min; 100 to 35 min; and 20% from 37 to 40 min. The flow rate was 0.75 ml/min. For detection, the excitation wavelength was set to 340 nm and emission wavelength was set to 450 nm.

2.4. H2Sn inhibition and induction tests

For growth inhibition test, middle-log phased E. coli cells (OD600nm = 0.8) were diluted and dripped in freshly prepared LB agar medium containing 0 or 100 μM H2Sn, and incubated in 37 °C under aerobic conditions. For anaerobic conditions, the anaerobic LB agar plates were prepared in an anaerobic glove box and the dilution and drip of E. coli cells also performed in an anaerobic glove box, then incubated in an anaerobic incubator at 37 °C for 24 h. For promoter induction test, a mkate gene was put after trxA, grxB, or katG native promoter in pTrcHis2A plasmid (Table S2, entries 17–19). The oxyR or its mutant gene was expressed under the Pnad promoter in the same plasmid (Table S2, entries 5–16) for complementary experiments. The obtained plasmids were transformed into wt and ΔoxyR strains. Early log-phased E. coli cells (OD600nm = 0.5, in liquid LB) were incubated with 600 μM H2Sn for 2 h. Cells were harvested and washed with HEPES buffer (50 mM, pH 7.4), then subjected to FACS analysis (FL3-A channel, > 10,000 cells).

2.5. Real-time quantitative reverse transcription PCR (RT-qPCR)

RNA sample was prepared by using the TRIzol™ RNA Purification
Kit (12183555, Invitrogen). Total cDNA was synthesized using the All-In-One RT Master Mix (ABM). For RT-qPCR, strains were grown in anaerobic LB medium until OD600nm reached 0.4, and then 200 μM H2S04 were added into anaerobic bottle. After 60 min, cells were centrifuged and RNA was extracted. RT-qPCR was performed by using the Bestar SybrGreen qPCR Mastermix (DBI) and LightCycler 480II (Roche). For calculation the relative expression levels of tested genes, GAPDH gene expression was used as the internal standard.

2.6. Protein purification and reaction with DTT or H2Sn

The oxyR gene with a C-terminal His tag was ligated into pET30. Mutants of oxyR were constructed from this plasmid via site-directed mutagenesis [54]. The obtained plasmids were transformed into E. coli BL21 (DE3). For protein expression, E. coli cells were cultured in LB medium at 25 °C with shaking (150 rpm) until OD600nm reacted 0.6. After incubating the mixture for 1 h at RT, the cells were harvested and disrupted through a high pressure cracker SOCH-18 (STA-NSTED); protein was purified via the Ni-NTA resin (Invitrogen). Buffer exchange of the purified protein was performed by using PD-10 desalting column (GE Healthcare).

Reactions were performed in an anaerobic glove box. 0.6 mg/ml protein was mixed with 200 mM DTT in a pH 8.0 buffer (50 mM NaH2PO4, 300 mM NaCl). After 1-h incubation at RT, the protein was dialyzed against 0.5 M KCl until the dialysis buffer was free of DTNB-titratable SH group. For H2S04 or H2O2 reaction, the mole ratio of reduced OxyR to H2S04 or H2O2 was 1:10. After incubating the mixture for 30 min at RT, unreacted H2S04 or H2O2 was removed via dialysis. The reacted-proteins were sealed and taken out from the glove box to be used in further experiments.

2.7. LC-MS/MS analysis of OxyR

The H2S04-reacted OxyR (0.5 mg/ml) was mixed with iodoacetamide (IAM), and then digested with trypsin by following a previously reported protocol [32]. The Prominence nano-LC system (Shimadzu) equipped with a custom-made silica column (75 μm × 15 cm) packed with 3-μm Reprosil-Pur 120C18-AQ was used for the analysis. For the elution process, a 100 min gradient from 0% to 100% of solvent B (0.1% formic acid in 2% acetonitrile) was applied. The eluent was ionized and elution process, a 100 min gradient from 0% to 100% of solvent B (0.1% formic acid in 2% acetonitrile) was applied. The eluent was ionized and full-scan MS spectra (from 400 to 1800 m/z) were detected in the Orbitrap with a resolution of 60,000 at 400 m/z.

2.8. In vitro transcription-translation analysis

In vitro translation-transcription reactions were performed using the PureExpress In Vitro Protein Synthesis system (NEB #E6800). The reaction solution was prepared in the following order: 10 μl solution A (NEB #E6800), 7.5 μl solution B (NEB #E6800), 2 μl E. coli RNA polymerase (NEB #M0551), 1 μl RNase inhibitor, 500 ng reduced, H2S04-treated or H2O2-treated protein, 200 ng DNA fragment containing PoxycmKate, and RNase free water. The total volume was 25 μl. The solution was incubated at 37 °C for 3 h. After reaction, the translated mKate was diluted four times with distilled water, and assayed by using the Synergy H1 microplate reader. The excitation wavelength was set to 588 nm, and the emission wavelength was set to 633 nm. The fluorescence intensity from reduced OxyR was used as standard; fluorescence intensities from other groups were divided by the standard to calculate the relative expression levels.

2.9. Transcriptomic analysis

E. coli wt strain was cultured in LB medium until OD600nm reached 0.5, and 500 μM H2S04 or 500 μM H2O2 were added. After 20 min of treatment, cells were harvested and total RNA was extracted by using the TRIzol™ RNA Purification Kit (12183555, Invitrogen). RNA quality was assessed with the RNA Nano 6000 Assay Kit of the Agilent Bioanalyser 2100 system (Agilent Technologies). rRNA was removed with the Ribo-Zero rRNA Removal Kit (MRZMB 126, Epicentre Biotechnologies). For cDNA library construction, first-strand cDNA was synthesized by using random hexamer primers from fragmentation of mRNA and second-strand cDNA was synthesized by using a dNTP mixture containing dUTP with DNA polymerase I and RNase H. After adenylation of the ends of blunt-ended DNA fragments, NEBNext index adaptor oligonucleotides were ligated to the cDNA fragments. The second-strand cDNA containing dUTP was digested with the USER enzyme. The first-strand DNA fragments with ligated adaptors on both ends were selectively enriched in a 10-cycle PCR reaction, purified (AMPure XP), and the library was quantified using the Agilent High Sensitivity DNA assay on the Agilent Bioanalyser 2100 system. The library was sequencing on Illumina HiSeq 2500 platform. Sequencing was performed at Beijing Novogene Bioinformatics Technology Co., Ltd. The clean data were obtained from raw data by removing reads containing adapter, poly-N and low-quality reads. The clean reads were aligned with the genome of E. coli BL21 by using Bowtie2-2.2.3. Gene expression was quantified as reads per kilobase of coding sequence per million reads (RPKM) algorithm. Genes with a p-value<0.05 found by DESeq and change fold > 1.5 were considered as significantly differentially expressed. Gene Ontology (GO) and KEGG analyses were performed at NovoMagic platform provided by Beijing Novogene Bioinformatics Technology Co., Ltd.

2.10. Analysis of OxyR distribution in sequenced bacterial genomes

A microbial genomic protein sequence set from NCBI updated until November 11, 2017 was downloaded for OxyR search. The query sequences of OxyR were reported OxyR proteins [46,55,56] were used to search the database by using Standalone BLASTP algorithm with conventional criteria (e-value ≤ 1e−5, coverage ≥ 45%, identity ≥ 30%) to obtain OxyR candidates from 8286 bacterial genomes. A conserved domain PBP2_OxyR and PRK11151 were used as standard features for further filtration of OxyR candidates. The candidates containing PBP2_OxyR or PRK11151 were identified as putative OxyR.

3. Results

3.1. The accumulation and reduction of endogenous sulfane sulfur in E. coli

E. coli cells were cultured in LB medium and harvested at various incubation time. Cellular sulfane sulfur of the sampled cells was determined by using the sulfane sulfur sensitive probe SSP4. The cellular sulfane sulfur may be reduced to H2S by enzymes, such as thioredoxin and glutaredoxin [34,35].

3.2. Thioredoxin and glutaredoxin participate in the reduction of intracellular sulfane sulfur

To confirm the change of intracellular sulfane sulfur, we constructed...
a transcription factor (TF)-based reporting plasmid, which contains a sulfane sulfur-sensing TF (CstR) [33], its cognate promoter ($P_{cst}$), and a red fluorescent protein (mKate, with a C-terminus degradation tag ssrA) (Fig. 1C). Using the reporting plasmid, the increase of intracellular sulfane sulfur in live cells (Fig. 1A) was reported as the mKate fluorescence intensity (Fig. 1D). mKate alone did not affect sulfane sulfur accumulation as reflected by the decreased mKate fluorescence intensity (Fig. 1D). TrxA alone did not affect sulfane sulfur accumulation (Fig. 1D), however, the co-transcription of mKate with TrxA and Trxβ (thioredoxin reductase) prevented the increase of sulfane sulfur during the log phase of growth (Fig. 1D). These results confirmed that thioredoxin and glutaredoxin also reduce sulfane sulfur in vivo.

The artificial operons with thioredoxin or glutaredoxin created negative feedback loops (Fig. 1C&D), maintaining the intracellular sulfane sulfur within a narrow range that was defined by the leaky strength of $P_{cst}$ and the sensitivity of CstR as well as the reductase activity. Since OxyR is known to regulate similar enzymes, we speculated whether OxyR may function in a similar way as CstR in the artificial operons (Fig. 1C).

### 3.3. *E. coli* ΔoxyR is more sensitive to H$_2$S$_n$ than wt

We deleted oxyR gene in *E. coli* and observed that the mutant became more sensitive to exogenously added H$_2$S$_n$ under both aerobic and anaerobic conditions (Fig. 2A and B). In LB medium without added H$_2$S$_n$, *E. coli* ΔoxyR displayed similar growth as wt; however, on LB agar plates the deletion clearly showed dexterous effects on the growth for *E. coli* ΔoxyR under aerobic conditions. After complementing oxyR into *E. coli* ΔoxyR, the strain regained the tolerance to H$_2$S$_n$ (Fig. 2A and B).

The results indicated that OxyR plays an important role in dealing with the exogenous H$_2$Sn stress and the effects are more dramatic under anaerobic conditions. In addition, we noticed that *E. coli* showed higher H$_2$Sn resistance under anaerobic condition compared with that of under aerobic condition, suggesting that oxidative stress may be involved under aerobic conditions.

*E. coli* ΔoxyR had higher intracellular sulfane sulfur than wt did at log-phase (Fig. 2C). When *E. coli* ΔoxyR cells at the stationary phase were transferred into fresh LB medium at OD$_{600}$ of 1, the decrease of intracellular sulfane sulfur and the release of H$_2$S were slower than that of the wt cells (Fig. 2D). The results suggested that OxyR regulates the production of thioredoxin and glutaredoxin that reduce sulfane sulfur to H$_2$S.

### 3.4. OxyR regulates the expression of trxC, grxA and katG under both aerobic and anaerobic conditions

We constructed three reporting plasmids with an mKate gene under the control of the trxC, grxA, or katG promoter. These plasmids were transformed into *E. coli* wt and ΔoxyR, and the recombinant cells were tested under aerobic condition. In wt, all three promoters led to low mKate expression in the absence of H$_2$Sn, but resulted in obviously higher expression when H$_2$Sn was added (Fig. 3A). In *E. coli* ΔoxyR, the three promoters led to constantly low expressions of mKate with or without added H$_2$Sn (Fig. 3B). Complementation of oxyR restored the mutant’s response to H$_2$Sn (Fig. 3C). On the flipside, the introduction of plasmids overexpressing trxC, grxA, or katG in *E. coli* ΔoxyR decreased intracellular sulfane sulfur (Fig. S1).

Since the H$_2$Sn solution contained sulfide, we tested if sulfide alone could induce the gene expression. Sulfide did not induce the expression of related genes in wt (Fig. S2 A), excluding the signal function of
sulfide. When we used E. coli cells harboring a sulfide:quinone oxidoreductase of C. pinatubonensis JMP134, the added sulfide was oxidized to H$_2$Sn [29], which induced the expression of trxC, grxA and katG (Fig. S2 B).

We also tested the H$_2$Sn induction under anaerobic conditions. Since mKate is not fluorescent under anaerobic condition, the gene expression was assayed by using qPCR. Similarly, katG, grxA, and trxC had higher expression in wt when 200 μM H$_2$Sn were added (Fig. 4A), but not in E. coli ΔoxyR (Fig. 4B). Less H$_2$Sn was required under anaerobic conditions than under aerobic conditions, likely due to the increased stability. After complementation, E. coli ΔoxyR::oxyR resumed response to H$_2$Sn (Fig. 4C).

3.5. H$_2$Sn-treated OxyR activates the transcription of TrxC

The activation of OxyR by H$_2$Sn was tested with in vitro transcription-translation assays. The purified OxyR was treated with DTT to ensure that its thiols were in the reduced form, and the reduced OxyR was further treated with H$_2$Sn to activate OxyR. The DTT-treated or H$_2$Sn-treated OxyR was used for in vitro transcription-translation of a DNA fragment containing the trxC promoter and mKate (P$_{trxC}$-mKate). The DTT-treated OxyR resulted in low expression of mKate, while the H$_2$Sn-treated OxyR led to high expression of mKate (Fig. 5). These results indicated that H$_2$Sn modifies OxyR, which enhances the expression from the trxC promoter.
3.6. Compare the induction effect of H$_2$Sn with that of H$_2$O$_2$

We used 100–600 μM H$_2$Sn or H$_2$O$_2$ to treat E. coli wt strains containing the reporting plasmids as mentioned in 3.4. At 100–200 μM level, H$_2$Sn and H$_2$O$_2$ showed similar activation effects on trxC, grxA, and katG promoters. However, at the dosage > 400 μM level, H$_2$Sn had obviously higher activation effects than H$_2$O$_2$ (Fig. 6A–C). For confirmation, we also compared their activation effects using in vitro transcription-translation with DTT-reduced, H$_2$Sn-treated, or H$_2$O$_2$-treated OxyR (500 ng). H$_2$Sn also showed higher activation effect than H$_2$O$_2$ in in vitro transcription-translation of mKate (Fig. 6D).

In addition, we analyzed the expression induced by adding H$_2$Sn or H$_2$O$_2$ to E. coli cells. The RT-qPCR results showed that once H$_2$Sn (600 μM) was added, expression of trxC was rapidly increased in 2 min, indicating H$_2$Sn quickly reacts with OxyR. However, the expression was significantly decreased at 10 min and finally dropped to untreated level at 20 min. H$_2$O$_2$ (600 μM) addition showed the same trend, but with less expression of trxC (Fig. 6E).

3.7. H$_2$Sn-treatment causes the persulfidation of OxyR Cys$^{199}$ in vitro

OxyR contains six cystein residues. Previous studies indicated that two of them (Cys$^{199}$ and Cys$^{208}$) are involved in ROS sensing [48]. We constructed an OxyR$_{C199S}$ mutant (except for Cys$^{199}$ and Cys$^{208}$, the other four cysteines were mutated to alanines) and expressed it in ΔoxyR. The mutant regulated trxC, grxA, and katG promoters essentially the same as the wild-type OxyR in the presence of H$_2$Sn. Whereas, OxyR$_{C199S}$, OxyR$_{C208S}$, and OxyR$_{C199S; C208S}$ all lost the regulation
function (Figs. 3D and 5). Together, these results indicated that the same as in ROS sensing, Cys199 and Cys208 are involved in H2Sn sensing.

To find out the molecular mechanism on how OxyR senses H2Sn, mass spectrometry analysis was performed to analyze the H2Sn-treated OxyR. A short peptide (MW: 1356.67) containing Cys199 but not Cys208 was identified (peptide 1, Fig. 7 and Fig. S3) and about 20% of it contained Cys199-SSH (MW: 1388.64) (peptide 2, Fig. 7 and Fig. S4), according to the peak area in MS1 spectrogram. A peptide containing Cys208 was also found, but the Cys208 was not modified by iodoacetamide (IAM) (MW: 2144.87) (peptide 3, Fig. 6 and Fig. S5), which is consistent with a previous report that Cys208 is buried in the protein and is not accessible to IAM [47]. No peptide containing both Cys199 and Cys208 was detected. The in vitro experiments indicated that H2Sn reacts with Cys199 of OxyR, generating Cys199 persulfidation with no detectable disulfide or −S−S− (n ≥ 3) bond between Cys199 and Cys208.

3.8. Global transcriptome analysis of H2Sn-stressed and H2O2-stressed E. coli

The effects of H2Sn stress and H2O2 stress on gene expression in E. coli were tested. H2O2 had more upregulated genes (Fig. 8A), while H2Sn had more down regulated genes (Fig. 8B). Both had some overlaps. At the global level, there were similarities and differences. Gene ontology (GO) analysis indicated the cellular processes affected by them were different. For instance, H2Sn stress upregulated more genes pertaining to cellular components, e.g., cell part (GO:0044464) and macromolecular complex (GO:0032991), and downregulated more genes pertaining to molecular transducer activity (GO:0060089) and signal transducer activity (GO:0004871); whereas H2O2 stress upregulated more genes pertaining to ribonucleotide binding (GO:0032553) and

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**Fig. 5. In vitro transcription-translation analysis of H2Sn activation of OxyR and its mutants.** Purified OxyR and its mutants were treated with DTT to ensure their thiols were in the reduce form; The proteins were then treated with H2Sn to generate H2Sn-modified protein. The in vitro transcription-translation system containedPtrxC-mKate DNA fragment (200 ng) and DTT-reduced or H2Sn-treated OxyR (500 ng), and the expressed mKate was analyzed with the fluorescence photometer Synergy H1. (n ≥ 3 for each group)

Data information: Data are presented as mean ± SEM.

**Fig. 6. Comparison of the activation effect of H2Sn and H2O2.**

A-C H2Sn or H2O2 (100–600 μM) was used to treat E. coli wt strains containing reporter plasmids. (n ≥ 3 for each group)

D Purified OxyR and its mutants were treated with DTT to ensure their thiols were in the reduce form; The proteins were then treated with H2Sn or H2O2 to generate H2Sn- or H2O2-modified OxyR. The in vitro transcription-translation system containedPtrxC-mKate DNA fragment (200 ng) and DTT-reduced, H2Sn- or H2O2-treated OxyR (500 ng) and the expressed mKate was analyzed with the fluorescence photometer Synergy H1. (n ≥ 3 for each group)

E H2Sn or H2O2 (400 μM) was used to treat E. coli wt. RT-qPCR was used to quantify the expression of trxC. (n ≥ 3 for each group).
carbohydrate derivative binding (GO:0097367), and downregulated no gene pertaining to cellular components (Fig. S6 and Fig. S7). The TCA cycle is upregulated by H₂Sn stress but downregulated by H₂O₂ stress; biosynthesis of secondary metabolites (i.e. serine hydroxymethyltransferase, beta-gulcosidase, 3-deoxy-7-phosphoheptulonate synthase, etc.) is downregulated by H₂Sn stress but not affected by H₂O₂ stress (Fig. S8 and Fig. S9).

For the genes related to oxidative stress or sulfane sulfur, the effects on the expression of grxA, grxB, ahpF, dps, sodC were the same (Fig. 8C&D). For trxA, trxB, and katG, the degrees of upregulation were different (Fig. 8C&D). The expression of sseA encoding 3-mercaptopyruvate sulfurtransferase was down regulated by H₂Sn and H₂O₂. The expression of cysS encoding cysteinyl-tRNA synthetase was not affected by H₂Sn, but upregulated by H₂O₂. For grxC and sodA, the effects of H₂Sn and H₂O₂ were opposite (Fig. 8D). For fur, its expression was upregulated in both H₂Sn- and H₂O₂-treated cells. Fur is the repressor of iron importer and its upregulation can decrease cellular concentration of ferrous iron [57], minimizing hydroxyl radical production via the Fenton reaction when E. coli is under H₂O₂ stress [58]. Whether ferrous iron reacts with H₂Sn to generate further oxidative stress needs further investigation.

3.9. The distribution of OxyR in sequenced bacterial genomes

We invested the distribution of OxyR among 8286 microbial genomic sequences (NCBI updated until November 11, 2017) by using BLAST search, and then confirmed with the conserved domain and phylogenetic tree analysis. 4772 identified OxyR distributed in 4494 bacterial genomes, including 2432 Gammaproteobacteria, 887 Betaproteobacteria, 478 Alphaproteobacteria, 287 Corynebacteriales,
130 Flavobacteriia, 67 Streptomyces, and 63 Bacteriodia; the other 24 classes had a few genomes containing OxyR (Fig. 9 and Table S1). Thus, OxyR is widely distributed in bacteria, including many obligate anaerobic bacteria in the human gut, such as Bacteroides spp., Prevotella spp., and Porphyromonas spp. For anaerobic bacteria, OxyR is likely used to deal with H₂Sn stress.

4. Discussion

The levels of cellular sulfane sulfur vary and reach the highest level in early stationary phase for E. coli in LB medium (Fig. 1). This observation is shown by two approaches: the fluorescent probe SSP4 and a constructed reporting system containing a gene regulator inducible by sulfane sulfur. The results are in agreement with previously reported data by using a sulfane sulfur-sensitive green fluorescent protein [59] or by using resonance synchronous spectroscopy [60]. The accumulated sulfane sulfur is rapidly reduced to H₂S when E. coli cells are transferred into fresh LB medium (Fig. 1B), and the reduction is at least in part catalyzed by glutaredoxin and thioredoxin (Fig. 1D). The participation of these enzymes in reducing sulfane sulfur has been reported. Glutaredoxin and thioredoxin are more effective in reducing sulfane sulfur than GSH in in vitro assays [61,62], and they can also reduce the level of protein persulfidation in vivo [34,35]. Our results support previous reports that these enzymes are involved in reducing sulfane sulfur inside live cells. Further, our results suggest that they play an important role in maintaining cellular sulfane sulfur within a range (Fig. 1D) as well as for the detoxification of added H₂S₈.

Several lines of evidence support that OxyR regulates the expression of glutaredoxin and thioredoxin. First, OxyR is known to regulate certain thioredoxin and glutaredoxin; its deletion mutant, containing more sulfane sulfur on average (Fig. 2C), is more sensitive to H₂Sn stress (Fig. 2A&B) than E. coli wild type. Second, the constructed reporter systems containing the promoters of trxC, grxA and katD display OxyR-dependent induction by H₂Sn₈ (Figs. 3 and 4); the two Cys residues Cys199 and C208 are required for the induction. Third, in vitro transcription and translation results show that H₂Sn₈-treated OxyR activates the transcription of trxC; again, C199 and C208 are required for the induction (Fig. 5). Fourth, MS analysis confirms the formation of OxyR Cys199 persulfide (Cys¹⁹⁹-SH₈) upon H₂Sn₈ treatment. The same modification may happen in vivo when E. coli is confronting H₂Sn₈ stress. C208 is also indispensable in H₂Sn₈ sensing, but its role is unresolved. Although a sulfur bridge between C199 and C208 is possible, our MS data indicated that the sulfur linkage (Cys199-Cys208) is not present in H₂Sn₈-treated OxyR, which is consistent with a previous study indicating that no disulfide bond-linked peptide (Cys199-Cys208) can be identified in H₂O₂-treated OxyR [47]. Therefore, OxyR C199 persulfidation is likely the mechanism of sensing H₂Sn₈.

Thus, the H₂Sn₈-treated OxyR activates the expression of glutaredoxin and thioredoxin that reduce H₂Sn₈ to H₂S in E. coli (Graphical abstract). H₂Sn₈ stress also activates the expression of katG, and catalase is known to oxidize H₂Sn₈ to sulfur oxides [51] (Graphical abstract).

There are overlaps between the regulated genes under H₂O₂ stress or H₂Sn₈ stress (Fig. 8). The difference could result from the modification variations of OxyR by H₂O₂ or H₂Sn₈. Three additional modifications on OxyR Cys199 (C199-SNO, C199-SSG and avicinylation) are also known, resulting in different OxyR configurations, DNA binding affinities, and promoter activities [47,63,64]. Therefore, C199-SSG may lead to an allosteric regulation different from the other modifications [47,63,64], acting as one of multi-level transcriptional responses with the other modifications [47]. Further, OxyR is the major gene regulator responding to H₂O₂ stress, and other gene regulators can also be affected by H₂O₂, including the global gene regulator McbR in E. coli [65]. H₂Sn₈ may also affect other gene regulators, contributing to the variations in gene expression under different stresses.

E. coli is likely to use house-keeping and induced glutaredoxins and thioredoxins to deal with H₂Sn₈ stress. According to the FPKM (expected number of Fragments Per Kilobase of transcript sequence per millions base pairs sequenced) from the transcriptomic sequencing data, we observed that the basic expression levels of TrxA, TrxB, GrxB, GrxC and GrxD are much higher than those of OxyR-regulated GrxA and TrxC. These proteins are regulated by nutrient mediated regulators and are highly abundant in E. coli [38–42], and they may play a “house-keeping” role; whereas, the OxyR activated GrxA, TrxC and KatG are involved in dealing with sulfane sulfur stress. Glutaredoxins and thioredoxins reduce sulfane sulfur to H₂S₈, which is released [22,30,37]. For bacteria and animals with sulfide:quinone oxidoreductase, the released H₂S₈ is captured and oxidized back to sulfane sulfur under aerobic conditions [66]. For E. coli and bacteria without sulfide:quinone oxidoreductase, H₂S will be released and evaporated into the gas phase [19,30]. Under anaerobic conditions, H₂S₈ is usually released due to the requirement of O₂ or an alternative electron acceptor for its oxidation [67].

Both S and O are chalcogens. Sulfane sulfur species are similar chemicals to reactive oxygen species (e.g., HSSH vs H₂O₂) [68], and their modification of proteins is also analogous, i.e., protein-SSH vs protein-SOH [6]. From an evolutionary perspective, the former’s history can be traced back before the Great Oxidation Event (GOE), when O₂ had not been generated by cyanobacteria. As an abundant element on the ancient earth, S should play important roles in ancient microorganisms. Therefore, sulfur metabolism related enzymes should have emerged before the oxygen’s era. It is reasonable to speculate that the anti-ROS proteins are derived from anti-sulfane sulfur ones [51]. Our observation that OxyR responses to both reactive oxygen species and sulfane sulfur supports the hypothesis. OxyR is required to deal with H₂O₂ only under aerobic conditions; whereas, it responds to H₂Sn₈ under both aerobic and anaerobic conditions. Besides OxyR, the signaling pathway of Keap1/Nrf2 responding to antioxidants is also regulated by polysulfides in mouse neuroblastoma cells [69].

In conclusion, we discovered that E. coli uses thioredoxin and glutaredoxin to control homeostasis of intracellular sulfane sulfur. Known bacterial gene regulators sensing sulfane sulfur are specific for activating sulfur-oxidizing genes. OxyR is the first reported global gene factor that functions as a sulfane sulfur sensor via persulfidation of its Cys¹⁹⁹ under both aerobic and anoxic conditions. This is the fifth type of modification for OxyR activation. Since OxyR is widely distributed in both aerobic and anaerobic bacteria, the OxyR-regulated network may represent a conserved mechanism that bacteria can resort to when confronting endogenous and/or exogenous sulfane sulfur stress.

Conflicts of interest

No conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101293.

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