Redox Biology 26 (2019) 101293

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

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

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ARTICLE INFO

Keywords:
OxyR
Sulfane sulfur
Escherichia coli
Thioredoxin
Glutaredoxin

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 H_2O_2, 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 (H_2S) 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 in plant [5]. The mechanism of H_2S signaling is often via protein persulfidation. H_2S cannot directly react with protein thiols, but sulfane sulfur, its oxidation product, readily reacts with thiols to generate persulfides [6,7].

Sulfane sulfur species include hydrogen polysulfide (H_2S_n, $n \geq 2$), organic polysulfide (RSS_nH, RSS_nR, $n \geq 2$), and organic persulfide (RSSH), which can be produced from H_2S oxidation or from the metabolism of cysteine and cystine. The endogenous H_2S_n 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 (S^0) to protein thiols to generate protein-SSH, 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 H_2S_n [22], inducing protein persulfidation as a possible toxic mechanism [23]. Fungi may use glutathione to reduce polysulfide to H_2S as a detoxification mechanism [22,24]. Organosulfur compounds can be used to treat antibiotic-resistant bacteria, and they are converted to H_2S_n 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

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https://doi.org/10.1016/j.redox.2019.101293
Received 17 May 2019; Received in revised form 24 July 2019; Accepted 7 August 2019
Available online 08 August 2019

discovered. 3-Mercaptopyruvate sulfurtransferase and cysteinyl-tRNA synthetase produce sulfane sulfur from cysteine [8,26,27]. Cystathionine β-synthase and cystathionine γ-lyase can produce H_2S from cysteine and sulfane sulfur from cystine [13]. Since cellular cystine concentration is very low, these enzymes are likely to generate H_2S instead of sulfane sulfur [10]. Sulfide:quinone oxidoreductase and superoxide dismutase produce sulfane sulfur from H_2S [28,29]. Most microorganisms possess several of these pathways. *Escherichia coli* contains 3-mercaptopyruvate sulfurtransferase, cysteinyl-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 H_2S [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 H_2S [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 regulated by guanosine 3′,5′-tetraphosphate, and the expression of GrxC is regulated by cAMP, both of which are nutrient-dependent messengers [38,39]. These four enzymes are highly abundant in *E. coli*; together they account for more than 1% of total protein [40–42]. The expression of GrxA, TrxC, and KatG (catalase) is regulated by OxyR upon exposure to H_2O_2. 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 Cys^{199} and Cys^{208} or oxidizes Cys^{199} to C199-SOH, but the exact mechanism is still in debate [46–48]. Herein, we showed that sulfane sulfur modified OxyR at Cys^{199} 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 H_2S.

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* strains were grown in Lysogeny broth (LB) medium. Antibiotics (50 μg/ml) were added when required. SSP4 (3′,6′-Di(*O*-thiosalicyl) fluorecein) was purchased from DOJINDO MOLECULAR TECHNOLOGIES. H_2S_n was prepared by following Kamyshny & 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 H_2S_n, where n varies from 2 to 8 [51], but at low concentration and neutral pH, H_2S_2 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 OD_{600nm} = 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 CstR-based reporting system was used for real-time analysis. *cstR* gene was chemically synthesized by Genewiz (Shanghai) company and expressed with P_{lacI} promoter in pTrcHis2A plasmid, where the *trc* promoter was replaced by the CstR cognate promoter, and a *mkate* gene (with a C-terminus degradation tag *ssrA*) 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 (OD_{600nm} = 10) in 1 M NaOH, 0.1% SDS and 0.3 M ascorbic acid to lysis 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 $FeCl_3$; 20 mM N,*N*-dimethyl-*p*-phenylenediamine; 7.2 M HCl) to detect S^{2-} 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 OD_{600nm} of one was converted to one μL of cellular volume (https://bionumbers.hms.harvard.edu/search.aspx).

2.3. H_2S production analysis

Production of H_2S was determined by using a previously reported method [53]. Briefly, H_2S 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 20% from 35 to 37 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. H_2S_n inhibition and induction tests

For growth inhibition test, middle-log phased *E. coli* cells (OD_{600nm} = 0.8) were diluted and dripped in freshly prepared LB agar medium containing 0 or 100 μM H_2S_n 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 *trxC*, *grxA*, or *katG* native promoter in pTrchis2A plasmid (Table S2, entries 17–19). The *oxyR* or its mutant gene was expressed under the P_{lacI} 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 (OD_{600nm} = 0.5, in liquid LB) were incubated with 600 μM H_2S_n 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 OD_{600nm} reached 0.4, and then 200 μM H_2S_n were added into anaerobic bottle. After 60 min, cells were collected by centrifugation 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 H_2S_n

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 OD_{600nm} reacted 0.6–0.8, 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and cells were cultured for additional 16 h at 16 °C. Cells were then harvested and disrupted through a high pressure cracker SOCH-18 (STANSTED); 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 NaH_2PO_4, 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 H_2S_n or H_2O_2 reaction, the mole ratio of reduced OxyR to H_2S_n or H_2O_2 was 1:10. After incubating the mixture for 30 min at RT, unreacted H_2S_n or H_2O_2 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 H_2S_n-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 98% acetonitrile) at 300 nl/min was used; solvent A was 0.1% formic acid in 2% acetonitrile. The eluent was ionized and electrosprayed via LTQ-Orbitrap Velos Pro CID mass spectrometer (Thermo Scientific), which run in data-dependent acquisition mode with Xcalibur 2.2.0 software (Thermo Scientific). 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 Purexpress *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, H_2S_n-treated or H_2O_2-treated protein, 200 ng DNA fragment containing P_{trxC}-*mKate*, 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 OD_{600nm} reached 0.5, and 500 μM H_2S_n or 500 μM H_2O_2 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 Bioanalyzer 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 Bioanalyzer 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 Srandalone 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 started to accumulate at mid-log phase and reached the maximum at early stationary phase (Fig. 1A). With the ascorbate reduction method [52], we determined cellular sulfane sulfur in cells harvested at 10 h as 381 ± 69 μM, similar to a previous report that the endogenous GSSH of mice brain tissue is around 150 μM [13]. When the stationary-phased cells were transferred into fresh medium (OD_{600nm} = 1), their intracellular sulfane sulfur decreased quickly with concomitant release of H_2S (Fig. 1B). This phenomenon suggests that sulfane sulfur may be reduced to H_2S 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

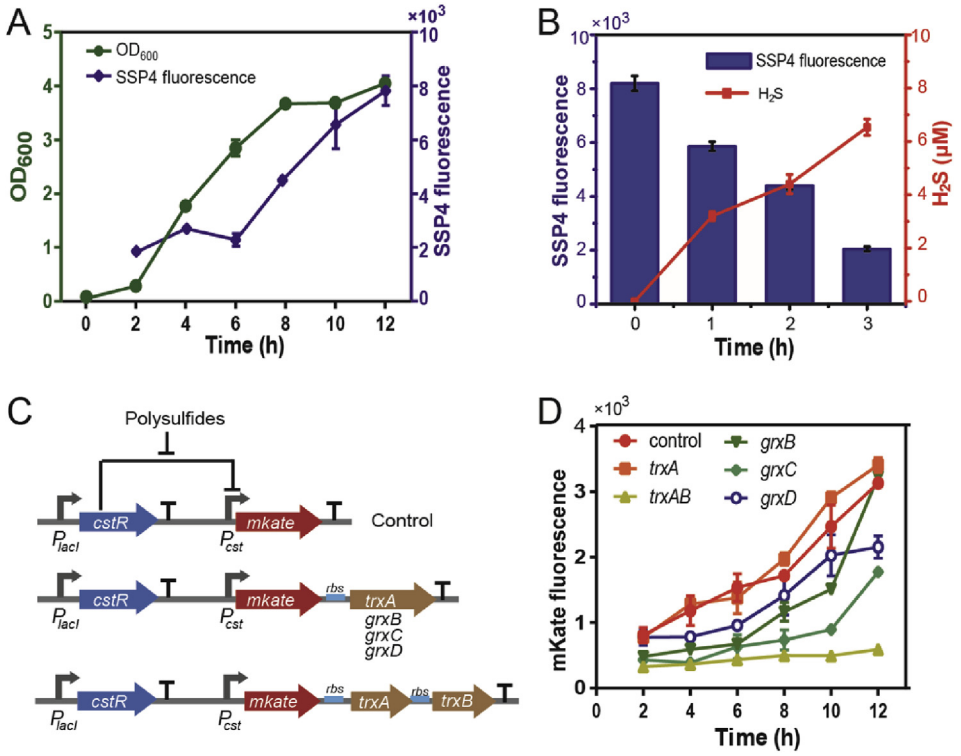

Fig. 1. Endogenous sulfane sulfur production and reduction in *E. coli*.
A *E. coli* accumulated sulfane sulfur during late-log and stationary phases. (n ≥ 3 for each group).
B *E. coli* cells at stationary phase reduced intracellular sulfane sulfur to H_2S after being transferred to fresh LB. (n ≥ 3 for each group).
C CstR-based reporters for real-time monitoring intracellular sulfane sulfur.
D Overexpression of thioredoxins and glutaredoxins decreased intracellular sulfane sulfur, as indicated by mKate fluorescence. (n ≥ 3 for each group).
Data information: In (A, B and D), data are presented as mean ± SEM.

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 (Fig. 1D). When GrxB, GrxC, or GrxD was co-transcribed with mKate under the control of CstR, their expression could partially decrease the 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 TrxB (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 *Pcst* 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_2S_n than wt*

We deleted *oxyR* gene in *E. coli* and observed that the mutant became more sensitive to exogenously added H_2S_n under both aerobic and anaerobic conditions (Fig. 2A and B). In LB medium without added H_2S_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_2S_n (Fig. 2A and B). The results indicated that OxyR plays an important role in dealing with the exogenous H_2S_n stress and the effects are more dramatic under anaerobic conditions. In addition, we noticed that *E. coli* showed higher H_2S_n 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_{600nm} of 1, the decrease of intracellular sulfane sulfur and the release of H_2S 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_2S.

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_2S_n, but resulted in obviously higher expression when H_2S_n was added (Fig. 3A). In *E. coli ΔoxyR*, the three promoters led to constantly low expressions of *mKate* with or without added H_2S_n (Fig. 3B). Complementation of *oxyR* restored the mutant's response to H_2S_n (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_2S_n 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

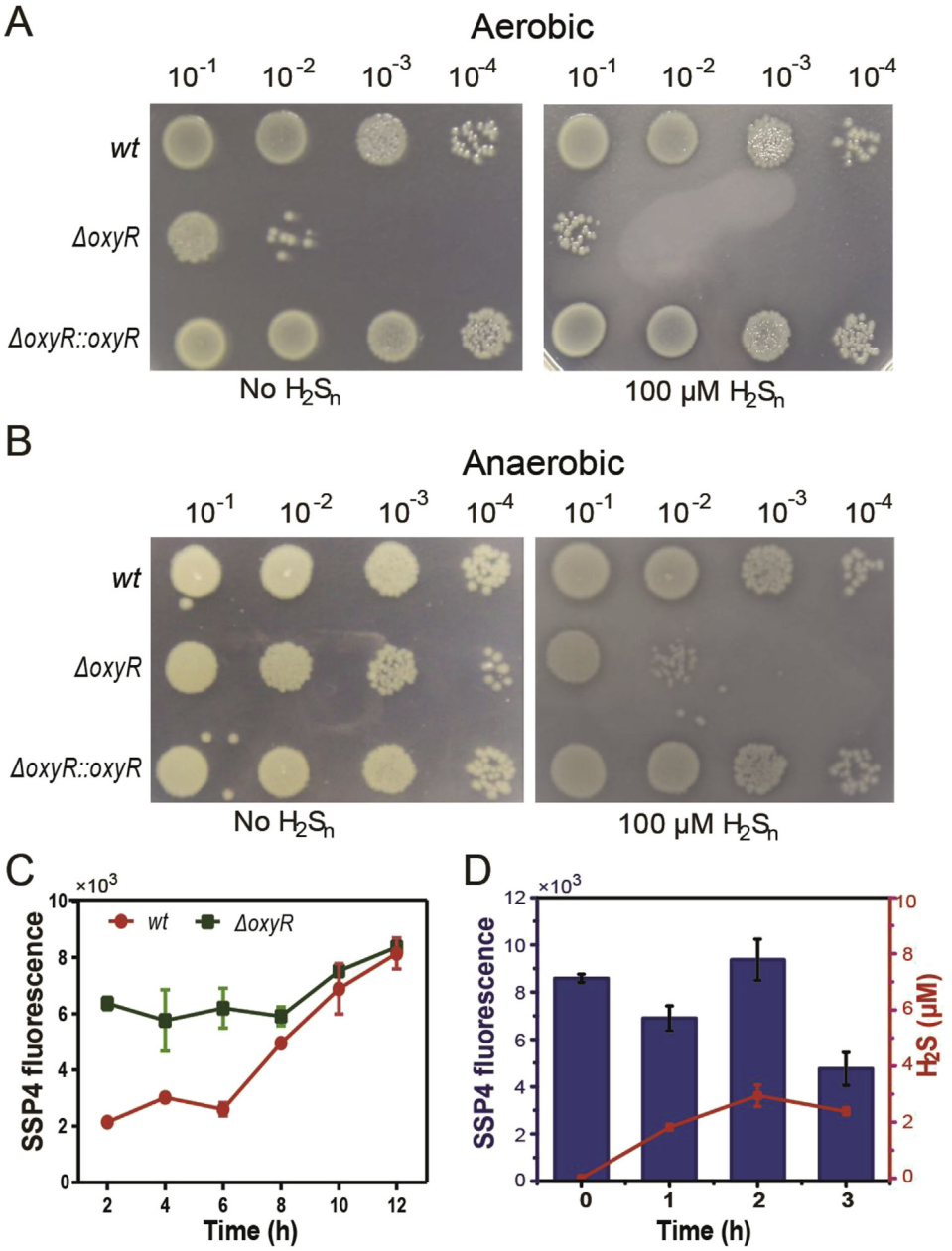

Fig. 2. OxyR affects RSS reduction in *E. coli*.

A, B The *oxyR* deletion made *E. coli* more sensitive to exogenous H_2S_n stress under both aerobic and anaerobic conditions; the *oxyR* complementation restored the muntant's resistance to H_2S_n stress.

C *E. coli ΔoxyR* accumulated more endogenous sulfane sulfur than *E. coli wt* during growth in LB. ($n \geq 3$ for each group).

D After *E. coli* cells at stationary phase were transferred into fresh LB medium, *E. coli ΔoxyR* cells reduced endogenous sulfane sulfur to H_2S more slowly than *E. coli wt* as shown in Fig. 1B. ($n \geq 3$ for each group).

Data information: In (C, D), data are presented as mean ± SEM.

sulfide. When we used *E. coli* cells harboring a sulfide:quinone oxidoreductase of *C. pinatubonensis* JMP134, the added sulfide was oxidized to H_2S_n [29], which induced the expression of *trxC*, *grxA* and *katG* (Fig. S2 B).

We also tested the H_2S_n 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_2S_n were added (Fig. 4A), but not in *E. coli ΔoxyR* (Fig. 4B). Less H_2S_n 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_2S_n (Fig. 4C).

3.5. H_2S_n-treated OxyR activates the transcription of TrxC

The activation of OxyR by H_2S_n 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_2S_n to activate OxyR. The DTT-treated or H_2S_n-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_2S_n-treated OxyR led to high expression of *mKate* (Fig. 5). These results indicated that H_2S_n modifies OxyR, which enhances the expression from the *trxC* promoter.

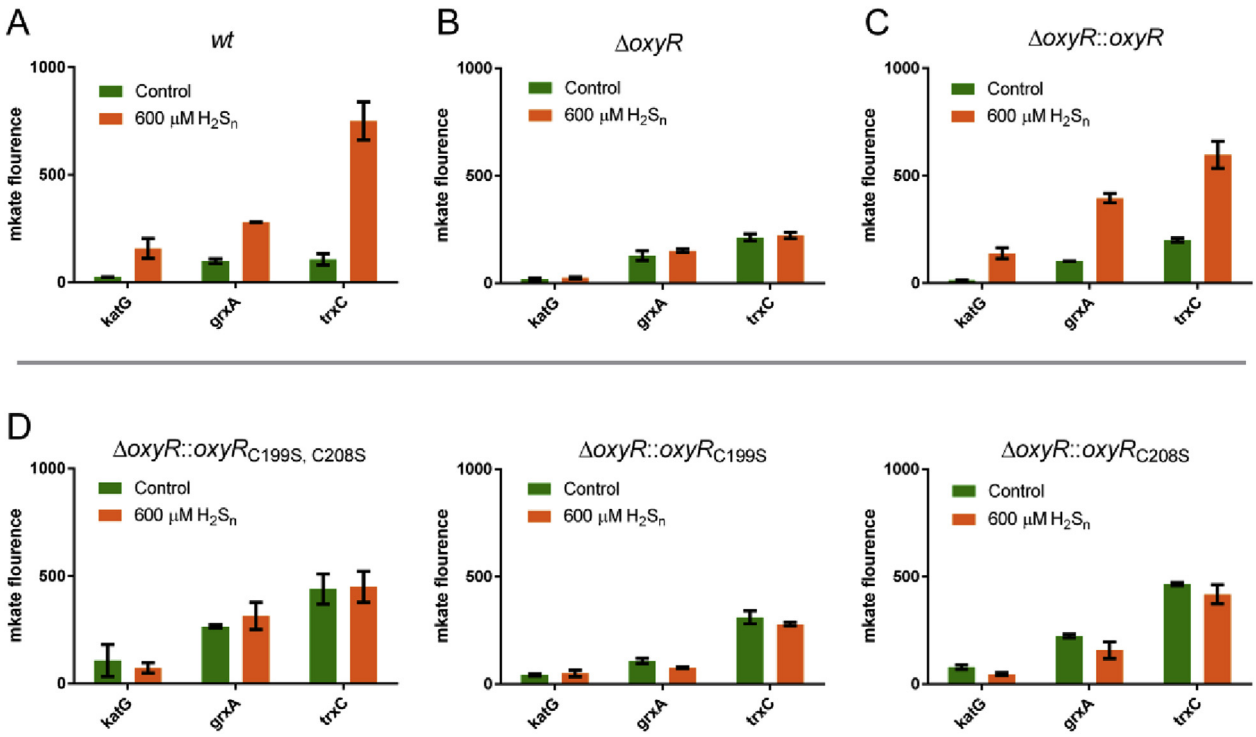

Fig. 3. H_2S_n upregulated expression of *katG*, *grxA*, and *trxC* via OxyR under aerobic conditions.
A H_2S_n induced the expression of *katG*, *grxA*, and *trxC* in *E. coli wt*. (n ≥ 3 for each group).
B The induction effect was lost in *E. coli ΔoxyR*. (n ≥ 3 for each group).
C OxyR complementation recovered the induction effect. (n ≥ 3 for each group).
D Cys199 and Cys208 single or double mutants lost the induction effect. (n ≥ 3 for each group).
Data information: In (A–D), data are presented as mean ± SEM.

3.6. Compare the induction effect of H_2S_n with that of H_2O_2

We used 100–600 μM H_2S_n or H_2O_2 to treat *E. coli* wt strains containing the reporting plasmids as mentioned in 3.4. At 100–200 μM level, H_2S_n and H_2O_2 showed similar activation effects on *trxC*, *grxA* and *katG* promoters. However, at the dosage > 400 μM level, H_2S_n had obviously higher activation effects than H_2O_2 (Fig. 6A–C). For confirmation, we also compared their activation effects using *in vitro* transcription-translation with DTT-reduced, H_2S_n-treated, or H_2O_2-treated OxyR (500 ng). H_2S_n also showed higher activation effect than H_2O_2 in *in vitro* transcription-translation of *mKate* (Fig. 6D).

In addition, we analyzed the expression induced by adding H_2S_n or H_2O_2 to *E. coli* cells. The RT-qPCR results showed that once H_2S_n (600 μM) was added, expression of *trxC* was rapidly increased in 2 min, indicating H_2S_n quickly reacts with OxyR. However, the expression was significantly decreased at 10 min and finally dropped to untreated level at 20 min H_2O_2 (600 μM) addition showed the same trend, but with less expression of *trxC* (Fig. 6E).

3.7. H_2S_n-treatment causes the persulfidation of OxyR Cys^{199} in vitro

OxyR contains six cysteine residues. Previous studies indicated that two of them (Cys^{199} and Cys^{208}) are involved in ROS sensing [48]. We constructed an $OxyR_{4C\rightarrow A}$ 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_2S_n. Whereas, $OxyR_{C199S}$, $OxyR_{C208S}$, and $OxyR_{C199S;\ C208S}$ all lost the regulation

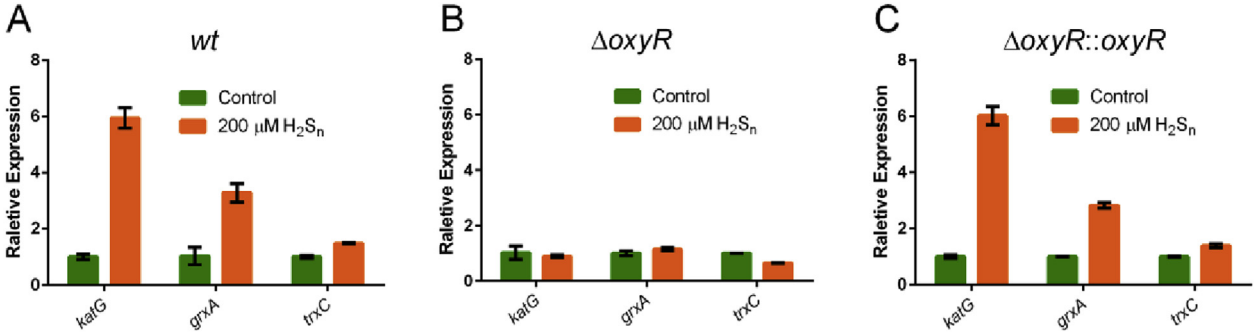

Fig. 4. H_2S_n upregulated expression of *katG*, *grxA*, and *trxC* via OxyR under anaerobic conditions.
A H_2S_n induced the expression of *katG*, *grxA*, and *trxC* in *E. coli wt*. (n ≥ 3 for each group).
B The induction effect was lost in *E. coli ΔoxyR*. (n ≥ 3 for each group).
C OxyR complementation recovered the induction effect. (n ≥ 3 for each group).
Data information: Data are presented as mean ± SEM.

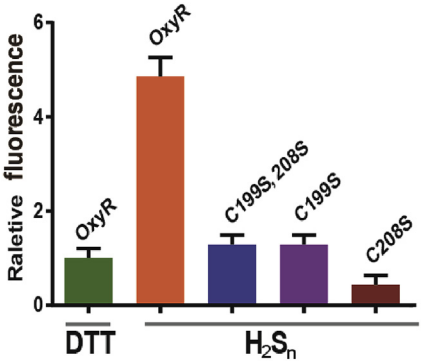

Fig. 5. *In vitro* transcription-translation analysis of H_2S_n activation of OxyR and its mutants. Purified OxyR and its mutants were treated with DTT to ensure their thiols were in the reduce form; The proteins were then treated with H_2S_n to generate H_2S_n modified protein. The *in vitro* transcription-translation system contained P_{trxC}-*mKate* DNA fragment (200 ng) and DTT-reduced or H_2S_n-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.

function (Figs. 3D and 5). Together, these results indicated that the same as in ROS sensing, Cys^{199} and Cys^{208} are involved in H_2S_n sensing.

To find out the molecular mechanism on how OxyR senses H_2S_n, mass spectrometry analysis was performed to analyze the H_2S_n-treated OxyR. A short peptide (MW: 1356.67) containing Cys^{199} but not Cys^{208} was identified (peptide 1, Fig. 7 and Fig. S3) and about 20% of it contained Cys^{199}-SSH (MW: 1388.64) (peptide 2, Fig. 7 and Fig. S4), according to the peak area in MS^1 spectrogram. A peptide containing Cys^{208} was also found, but the Cys^{208} was not modified by iodoacetamide (IAM) (MW: 2144.87) (peptide 3, Fig. 6 and Fig. S5), which is consistent with a previous report that Cys^{208} is buried in the protein and is not accessible to IAM [47]. No peptide containing both Cys^{199} and Cys^{208} was detected. The *in vitro* experiments indicated that H_2S_n reacts with Cys^{199} of OxyR, generating Cys^{199} persulfidation with no detectable disulfide or $–S_n–$ (n ≥ 3) bond between Cys^{199} and Cys^{208}.

3.8. Global transcriptome analysis of H_2S_n-stressed and H_2O_2-stressed *E. coli*

The effects of H_2S_n stress and H_2O_2 stress on gene expression in *E. coli* were tested. H_2O_2 had more upregulated genes (Fig. 8A), while H_2S_n 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, H_2S_n 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 H_2O_2 stress upregulated more genes pertaining to ribonucleotide binding (GO:0032553) and

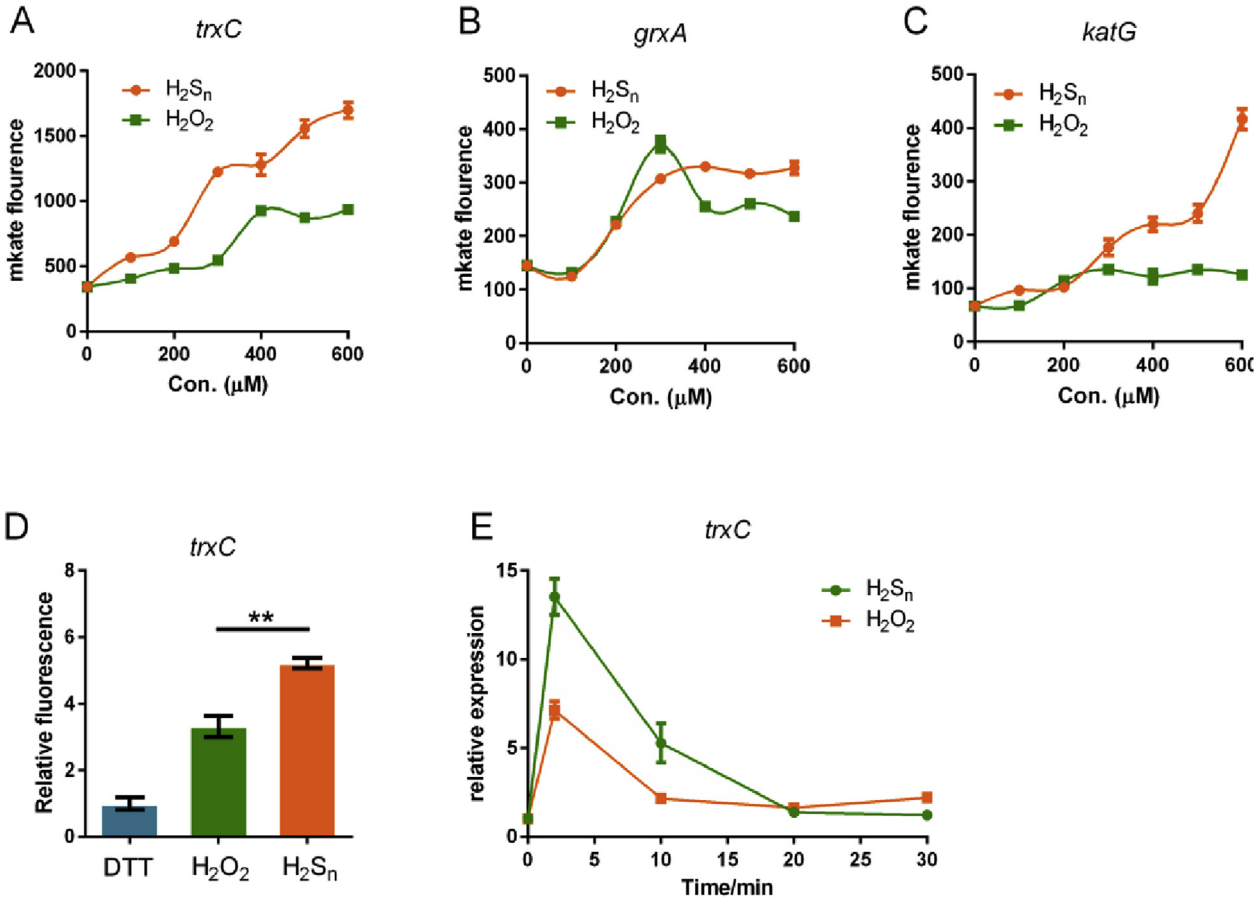

Fig. 6. Comparison of the activation effect of H_2S_n and H_2O_2.
A-C H_2S_n or H_2O_2 (100–600 μM) was used to treat *E. coli* wt strains containing reporter plasmids. (n ≥ 3 for each group)
D Purified OxyR and its mutants were treated with DTT to ensure their thiols were in the reduce form; The proteins were then treated with H_2S_n or H_2O_2 to generate H_2S_n- or H_2O_2-modified OxyR. The *in vitro* transcription-translation system contained P_{trxC}-*mKate* DNA fragment (200 ng) and DTT-reduced, H_2S_n- or H_2O_2-treated OxyR (500 ng) and the expressed mKate was analyzed with the fluorescence photometer Synergy H1. (n ≥ 3 for each group)
E H_2S_n or H_2O_2 (400 μM) was used to treat *E. coli* wt. RT-qPCR was used to quantify the expression of *trxC*. (n ≥ 3 for each group).

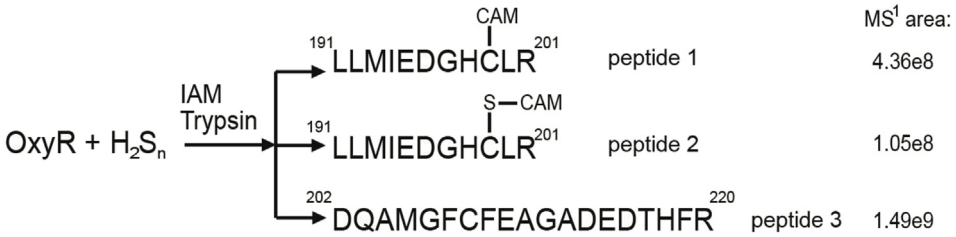

Fig. 7. LTQ-Orbitrap tandem mass analysis of H_2S_n-reacted OxyR.
Data information: MS data of the peptides are provided in Fig. S3 ~ 5.

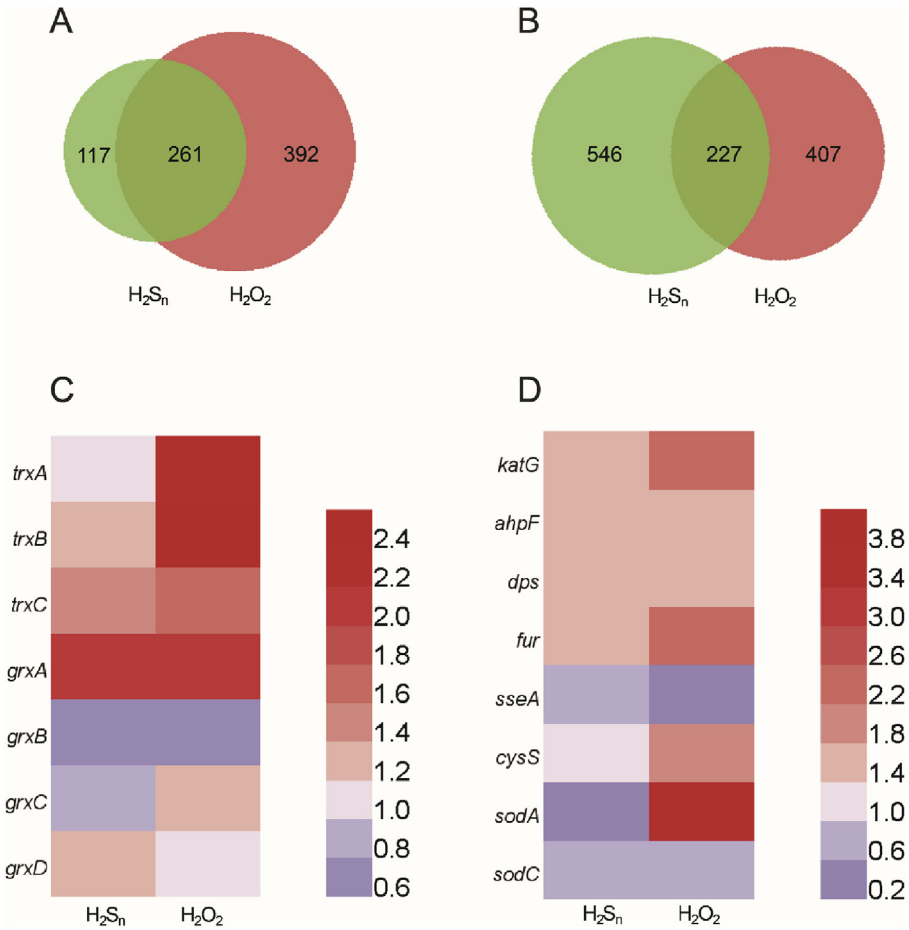

Fig. 8. Transcriptome analysis of H_2S_n- or H_2O_2-stressed *E. coli*.
A Numbers of transcriptionally upregulated genes under H_2S_n and H_2O_2 stresses.
B Numbers of transcriptionally downregulated genes under H_2S_n and H_2O_2 stresses.
C, D Transcriptional changes of genes related to sulfane sulfur and oxidative stress.

carbohydrate derivative binding (GO:0097367), and downregulated no gene pertaining to cellular components (Fig. S6 and Fig. S7). The TCA cycle is upregulated by H_2S_n stress but downregulated by H_2O_2 stress; biosynthesis of secondary metabolites (i.e. serine hydroxymethyltransferase, beta-gulcosidase, 3-deoxy-7-phosphoheptulonate synthase, etc.) is downregulated by H_2S_n stress but not affected by H_2O_2 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 *trxB*, *trxC*, and *katG*, the degrees of upregulation were different (Fig. 8C&D). The expression of *sseA* encoding 3-mercaptopyruvate sulfurtransferase was down regulated by H_2S_n and H_2O_2. The expression of *cysS* encoding cysteinyl-tRNA synthetase was not affected by H_2S_n, but upregulated by H_2O_2. For *grxC* and *sodA*, the effects of H_2S_n and H_2O_2 were opposite (Fig. 8D). For *fur*, its expression was upregulated in both H_2S_n-and H_2O_2-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 H_2O_2 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 Bataproteobacteria, 478 Alphaproteobacteria, 287 Corynebacteriales,

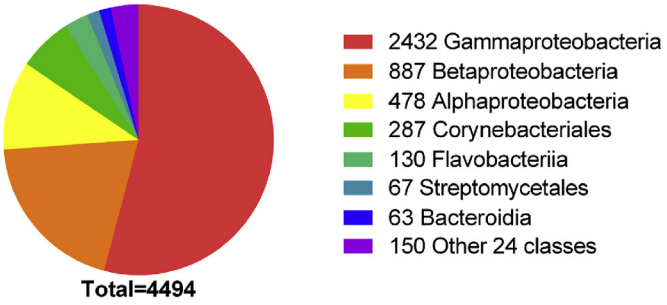

Fig. 9. The distribution of OxyRs in sequenced bacterial genomes.

130 Flavobacteiriia, 67 Streptomycetales, and 63 Bacterioidia; 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 *Porphromonas* spp. For anaerobic bacteria, OxyR is likely used to deal with H_2S_n 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 using 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 H_2S 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 H_2S_n.

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 H_2S_n stress (Fig. 2A&B) than *E. coli wt*. Second, the constructed reporter systems containing the promoters of *trxC, grxA and katG* display OxyR-dependent induction by H_2S_n (Figs. 3 and 4); the two Cys residues C199 and C208 are required for the induction. Third, *in vitro* transcription and translation results show that H_2S_n-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 C199 persulfide (Cys^{199}-SSH) upon H_2S_n treatment. The same modification may happen *in vivo* when *E. coli* is confronting H_2S_n stress. C208 is also indispensable in H_2S_n 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 H_2S_n-treated OxyR, which is consistent with a previous study indicating that no disulfide bond-linked peptide (Cys199-Cys208) can be identified in H_2O_2-treated OxyR [47]. Therefore, OxyR C199 persulfidation is likely the mechanism of sensing H_2S_n.

Thus, the H_2S_n-treated OxyR activates the expression of glutaredoxin and thioredoxin that reduce H_2S_n to H_2S in *E. coli* (Graphical abstract). H_2S_n-stress also activates the expression of *katG*, and catalase is known to oxidize H_2S_n to sulfur oxides [51] (Graphical abstract).

There are overlaps between the regulated genes under H_2O_2 stress or H_2S_n stress (Fig. 8). The difference could result from the modification variations of OxyR by H_2O_2 or H_2S_n. 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-SSH 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 H_2O_2 stress, and other gene regulators can also be affected by H_2O_2, including the global gene regulator McbR in *E. coli* [65]. H_2S_n 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 H_2S_n 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 "housekeeping" role; whereas, the OxyR activated GrxA, TrxC and KatG are involved in dealing with sulfane sulfur stress. Glutaredoxins and thioredoxins reduce sulfane sulfur to H_2S, which is released [22,30,37]. For bacteria and animals with sulfide:quinone oxidoreductase, the released H_2S is captured and oxidized back to sulfane sulfur under aerobic conditions [66]. For *E. coli* and bacteria without sulfide:quinone oxidoreductase, H_2S will be released and evaporated into the gas phase [19,30]. Under anaerobic conditions, H_2S is usually released due to the requirement of O_2 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* H_2O_2) [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 O_2 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 H_2O_2 only under aerobic conditions; whereas, it responses to H_2S_n 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 Cys^{199} 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.

Acknowledgements

The work was financially supported by grants from the National Natural Science Foundation of China (91751207, 31770093), the National Key Research and Development Program of China

(2016YFA0601103), and the Natural Science Foundation of Shandong Province, China (ZR2016CM03, ZR2017ZB0210).

Appendix A. Supplementary data

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

References

[1] C. Hine, E. Harputlugil, Y. Zhang, C. Ruckenstuhl, B.C. Lee, L. Brace, A. Longchamp, J. Treviño-Villarreal, P. Mejia, C.K. Ozaki, Endogenous hydrogen sulfide production is essential for dietary restriction benefits, Cell 160 (2015) 132–144.

[2] K. Abe, H. Kimura, The possible role of hydrogen sulfide as an endogenous neuromodulator, J. Neurosci. 16 (1996) 1066–1071.

[3] W.J. Cai, M.J. Wang, L.H. Ju, C. Wang, Y.C. Zhu, Hydrogen sulfide induces human colon cancer cell proliferation: role of Akt, ERK and p21, Cell Biol. Int. 34 (2010) 565–572.

[4] X.H. Gao, D. Krokowski, B.J. Guan, I. Bederman, M. Majumder, M. Parisien, L. Diatchenko, O. Kabil, B. Willard, R. Banerjee, B. Wang, G. Bebek, C.R. Evans, P.L. Fox, S.L. Gerson, C.L. Hoppel, M. Liu, P. Arvan, M. Hatzoglou, Quantitative H_2S-mediated protein sulfhydration reveals metabolic reprogramming during the integrated stress response, Elife 4 (2015) e10067.

[5] M. Lisjak, N. Srivastava, T. Teklic, L. Civale, K. Lewandowski, I. Wilson, M.E. Wood, M. Whiteman, J.T. Hancock, A novel hydrogen sulfide donor causes stomatal opening and reduces nitric oxide accumulation, Plant Physiol. Biochem. 48 (2010) 931–935.

[6] T.V. Mishanina, M. Libiad, R. Banerjee, Biogenesis of reactive sulfur species for signaling by hydrogen sulfide oxidation pathways, Nat. Chem. Biol. 11 (2015) 457–464.

[7] J.I. Toohey, Sulfur signaling: is the agent sulfide or sulfane? Anal. Biochem. 413 (2011) 1–7.

[8] Y. Kimura, Y. Mikami, K. Osumi, M. Tsugane, J-i Oka, H. Kimura, Polysulfides are possible H_2S-derived signaling molecules in rat brain, FASEB J. 27 (2013) 2451–2457.

[9] T. Sawa, K. Ono, H. Tsutsuki, T. Zhang, T. Ida, M. Nishida, T. Akaike, Chapter One. Reactive cysteine persulphides: occurrence, biosynthesis, antioxidant activity, methodologies, and bacterial persulphide signalling, in: R.K. Poole (Ed.), Advances in Microbial Physiology, vol. 72, Academic Press, 2018, pp. 1–28.

[10] P.K. Yadav, M. Martinov, V. Vitvitsky, J. Seravalli, R. Wedmann, M.R. Filipovic, R. Banerjee, Biosynthesis and reactivity of cysteine persulfides in signaling, J. Am. Chem. Soc. 138 (2015) 289–299.

[11] K. Ono, T. Akaike, T. Sawa, Y. Kumagai, D.A. Wink, D.J. Tantillo, A.J. Hobbs, P. Nagy, M. Xian, J. Lin, The redox chemistry and chemical biology of H_2S, hydropersulfides and derived species: implications to their possible biological activity and utility, Free Radical Biol. Med. 77 (2014) 82–94.

[12] C.M. Park, L. Weerasinghe, J.J. Day, J.M. Fukuto, X. Ming, Persulfides: current knowledge and challenges in chemistry and chemical biology, Mol. Biosyst. 11 (2015) 1775–1785.

[13] T. Ida, T. Sawa, H. Ihara, Y. Tsuchiya, Y. Watanabe, Y. Kumagai, M. Suematsu, H. Motohashi, S. Fujii, T. Matsunaga, M. Yamamoto, K. Ono, N.O. Devarie-Baez, M. Xian, J.M. Fukuto, T. Akaike, Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling, Proc. Natl. Acad. Sci. 111 (2014) 7606–7611.

[14] A.K. Mustafa, M.M. Gadalla, N. Sen, S. Kim, W. Mu, S.K. Gazi, R.K. Barrow, G. Yang, R. Wang, S.H. Snyder, H_2S signals through protein S-sulfhydration, Sci. Signal. 2 (2009) ra72.

[15] B.D. Paul, S.H. Snyder, Chapter five. Protein sulfhydration, in: E. Cadenas, L. Packer (Eds.), Methods in Enzymology, vol. 555, Academic Press, 2015, pp. 79–90.

[16] S. Fujii, T. Sawa, H. Motohashi, T. Akaike, Persulfide synthases that are functionally coupled with translation mediate sulfur respiration in mammalian cells, Br. J. Pharmacol. 176 (2019) 607–615.

[17] H. Peng, Y. Zhang, L.D. Palmer, T.E. Kehl-Fie, E.P. Skaar, J.C. Trinidad, D.P. Giedroc, Hydrogen sulfide and reactive sulfur species impact proteome S-sulfhydration and global virulence regulation in *Staphylococcus aureus*, ACS Infect. Dis. 3 (2017) 744–755.

[18] J.M. Fukuto, L.J. Ignarro, P. Nagy, D.A. Wink, C.G. Kevil, M. Feelisch, M.M. Cortese-Krott, C.L. Bianco, Y. Kumagai, A.J. Hobbs, J. Lin, T. Ida, T. Akaike, Biological hydropersulfides and related polysulfides – a new concept and perspective in redox biology, FEBS (Fed. Eur. Biochem. Soc.) Lett. 592 (2018) 2140–2152.

[19] K. Li, Y. Xin, G. Xuan, R. Zhao, H. Liu, Y. Xia, L. Xun, *Escherichia coli* uses different enzymes to produce H_2S and reactive sulfane sulfur from *L*-cysteine, Front. Microbiol. 10 (2019) 298.

[20] J. Williams, R. Cooper, The oldest fungicide and newest phytoalexin–a reappraisal of the fungitoxicity of elemental sulphur, Plant Pathol. 53 (2004) 263–279.

[21] M. Rai, A.P. Ingle, P. Paralikar, Sulfur and sulfur nanoparticles as potential antimicrobials: from traditional medicine to nanomedicine, Expert Rev. Anti-infect. Ther. 14 (2016) 969–978.

[22] I. Sato, K. Shimatani, K. Fujita, T. Abe, M. Shimizu, T. Fujii, T. Hoshino, N. Takaya, Glutathione reductase/glutathione is responsible for cytotoxic elemental sulfur tolerance via polysulfide shuttle in fungi, J. Biol. Chem. 286 (2011) 20283–20291.

[23] R.A. Islamov, I. Bishimova, A.N. Sabitov, A.I. Ilin, M.M. Burkitbaev, Lack of mutagenic activity of sulfur nanoparticles in micronucleus test on L5178Y Cell Culture, Cell & Tissue Biology 12 (2018) 27–32.

[24] S.R. Choudhury, A. Mandal, M. Ghosh, S. Basu, D. Chakravorty, A. Goswami, Investigation of antimicrobial physiology of orthorhombic and monoclinic nanoallotropes of sulfur at the interface of transcriptome and metabolome, Appl. Microbiol. Biotechnol. 97 (2013) 5965–5978.

[25] Z. Xu, Z. Qiu, Q. Liu, Y. Huang, D. Li, X. Shen, K. Fan, J. Xi, Y. Gu, Y. Tang, J. Jiang, J. Xu, J. He, X. Gao, Y. Liu, H. Koo, X. Yan, L. Gao, Converting organosulfur compounds to inorganic polysulfides against resistant bacterial infections, Nat. Commun. 9 (2018) 3713.

[26] T. Akaike, T. Ida, F.-Y. Wei, M. Nishida, Y. Kumagai, M.M. Alam, H. Ihara, T. Sawa, T. Matsunaga, S. Kasamatsu, A. Nishimura, M. Morita, K. Tomizawa, A. Nishimura, S. Watanabe, K. Inaba, H. Shima, N. Tanuma, M. Jung, S. Fujii, Y. Watanabe, M. Ohmuraya, P. Nagy, M. Feelisch, J.M. Fukuto, H. Motohashi, Cysteinyl-tRNA synthetase governs cysteine polysulfidation and mitochondrial bioenergetics, Nat. Commun. 8 (2017) 1177.

[27] N. Nagahara, S. Koike, T. Nirasawa, H. Kimura, Y. Ogasawara, Alternative pathway of H_2S and polysulfides production from sulfurated catalytic-cysteine of reaction intermediates of 3-mercaptopyruvate sulfurtransferase, Biochem. Biophys. Res. Commun. 496 (2018) 648–653.

[28] K.R. Olson, Y. Gao, F. Arif, K. Arora, S. Patel, E.R. Deleon, T.R. Sutton, M. Feelisch, M.M. Cortesekrott, K.D. Straub, Metabolism of hydrogen sulfide (H_2S) and production of reactive sulfur species (RSS) by superoxide dismutase, Redox Biol. 15 (2018) 74–85.

[29] Y. Xin, H. Liu, F. Cui, H. Liu, L. Xun, Recombinant *Escherichia coli* with sulfide: quinone oxidoreductase and persulfide dioxygenase rapidly oxidises sulfide to sulfite and thiosulfate via a new pathway, Environ. Microbiol. 18 (2016) 5123–5136.

[30] Y. Xia, C. Lü, N. Hou, Y. Xin, J. Liu, H. Liu, L. Xun, Sulfide production and oxidation by heterotrophic bacteria under aerobic conditions, ISME J. 11 (2017) 2754–2766.

[31] N.P.V. de Lira, B.A. Pauletti, A.C. Marques, C.A. Perez, R. Caserta, A.A. de Souza, A.E. Vercesi, A.F. Paes Leme, C.E. Benedetti, BigR is a sulfide sensor that regulates a sulfur transferase/dioxygenase required for aerobic respiration of plant bacteria under sulfide stress, Sci. Rep. 8 (2018) 3508.

[32] H. Li, J. Li, C. Lü, Y. Xia, Y. Xin, H. Liu, L. Xun, H. Liu, FisR activates σ^{54}-dependent transcription of sulfide-oxidizing genes in *Cupriavidus pinatubonensis* JMP 134, Mol. Microbiol. 105 (2017) 373–384.

[33] J.L. Luebke, J. Shen, K.E. Bruce, T.E. Kehl-Fie, H. Peng, E.P. Skaar, D.P. Giedroc, The CsoR-like sulfurtransferase repressor (CstR) is a persulfide sensor in *Staphylococcus aureus*, Mol. Microbiol. 94 (2014) 1343–1360.

[34] É. Dóka, I. Pader, A. Bíró, K. Johansson, Q. Cheng, K. Ballagó, J.R. Prigge, D. Pastor-Flores, T.P. Dick, E.E. Schmidt, E.S.J. Arnér, P. Nagy, A novel persulfide detection method reveals protein persulfide- and polysulfide-reducing functions of thioredoxin and glutathione systems, Sci. Adv. 2 (2016) e1500968.

[35] R. Wedmann, C. Onderka, S. Wei, I.A. Szijártó, J.L. Miljkovic, A. Mitrovic, M. Lange, S. Savitsky, P.K. Yadav, R. Torregrossa, E.G. Harrer, T. Harrer, I. Ishii, M. Gollasch, M.E. Wood, E. Galardon, M. Xian, M. Whiteman, R. Banerjee, M.R. Filipovic, Improved tag-switch method reveals that thioredoxin acts as depersulfidase and controls the intracellular levels of protein persulfidation, Chem. Sci. 7 (2016) 3414–3426.

[36] F. Carbonero, A.C. Benefiel, A.H. Alizadeh-Ghamsari, H.R. Gaskins, Microbial pathways in colonic sulfur metabolism and links with health and disease, Front. Physiol. 3 (2012) 448.

[37] A. Tsuyoshi, H. Takayuki, N. Akira, T. Naoki, Anaerobic elemental sulfur reduction by fungus *Fusarium oxysporum*, Biosci. Biotechnol. Biochem. 71 (2007) 2402–2407.

[38] C.J. Lim, T. Daws, M. Gerami-Nejad, J.A. Fuchs, Growth-phase regulation of the *Escherichia coli* thioredoxin gene, Biochim. Biophys. Acta 1491 (2000) 1–6.

[39] A. Srivatsan, J.D. Wang, Control of bacterial transcription, translation and replication by (p)ppGpp, Curr. Opin. Microbiol. 11 (2008) 100–105.

[40] A.P. Fernandes, M. Fladvad, C. Berndt, C. Andrésen, C.H. Lillig, P. Neubauer, M. Sunnerhagen, A. Holmgren, A. Vlamis-Gardikas, A novel monothiol glutaredoxin (Grx4) from *Escherichia coli* can serve as a substrate for thioredoxin reductase, J. Biol. Chem. 280 (2005) 24544–24552.

[41] A.P. Fernandes, A. Holmgren, Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system, Antioxidants Redox Signal. 6 (2004) 63–74.

[42] D. Ritz, H. Patel, B. Doan, M. Zheng, F. Åslund, G. Storz, J. Beckwith, Thioredoxin 2 is involved in the oxidative stress response in *Escherichia coli*, J. Biol. Chem. 275 (2000) 2505–2512.

[43] R.M. Gutierrez-Rios, J.A. Freyre-Gonzalez, O. Resendis, J. Collado-Vides, M. Saier, G. Gosset, Identification of regulatory network topological units coordinating the genome-wide transcriptional response to glucose in *Escherichia coli*, BMC Microbiol. 7 (2007) 53.

[44] M.F. Christman, R.W. Morgan, F.S. Jacobson, B.N. Ames, Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*, Cell 41 (1985) 753–762.

[45] G. Storz, L.A. Tartaglia, B.N. Ames, Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation, Science 248 (1990) 189–194.

[46] H. Choi, S. Kim, P. Mukhopadhyay, S. Cho, J. Woo, G. Storz, S.E. Ryu, Structural basis of the redox switch in the OxyR transcription factor, Cell 105 (2001) 103–113.

[47] S.O. Kim, K. Merchant, R. Nudelman, W.F.B. Jr, T. Keng, J. Deangelo, A. Hausladen, J.S. Stamler, OxyR: a molecular code for redox-related signaling, Cell 109 (2002) 383–396.

[48] M. Zheng, F. Åslund, G. Storz, Activation of the OxyR transcription factor by reversible disulfide bond formation, Science 279 (1998) 1718–1722.

[49] K.A. Datsenko, B.L. Wanner, One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products, Proc. Natl. Acad. Sci. 97 (2000)

6640–6645.
[50] A. Kamyshny, C.G. Borkenstein, T.G. Ferdelman, Protocol for quantitative detection of elemental sulfur and polysulfide zero-valent sulfur distribution in natural aquatic samples, Geostand. Geoanal. Res. 33 (2009) 415–435.
[51] K.R. Olson, Y. Gao, E.R. DeLeon, M. Arif, F. Arif, N. Arora, K.D. Straub, Catalase as a sulfide-sulfur oxido-reductase: an ancient (and modern?) regulator of reactive sulfur species (RSS), Redox Biol. 12 (2017) 325–339.
[52] M. Ikeda, Y. Ishima, A. Shibata, V.T.G. Chuang, T. Sawa, H. Ihara, H. Watanabe, M. Xian, Y. Ouchi, T. Shimizu, H. Ando, M. Ukawa, T. Ishida, T. Akaike, M. Otagiri, T. Maruyama, Quantitative determination of polysulfide in albumins, plasma proteins and biological fluid samples using a novel combined assays approach, Anal. Chim. Acta 969 (2017) 18–25.
[53] Y. Kimura, Y. Toyofuku, S. Koike, N. Shibuya, N. Nagahara, D. Lefer, Y. Ogasawara, H. Kimura, Identification of H_2S_3 and H_2S produced by 3-mercaptopyruvate sulfurtransferase in the brain, Sci. Rep. 5 (2015) 14774.
[54] Y. Xia, W. Chu, Q. Qi, L. Xun, New insights into the QuikChangeTM process guide the use of Phusion DNA polymerase for site-directed mutagenesis, Nucleic Acids Res. 43 (2014) e12.
[55] I. Jo, I.-Y. Chung, H.-W. Bae, J.-S. Kim, S. Song, Y.-H. Cho, N.-C. Ha, Structural details of the OxyR peroxide-sensing mechanism, Proc. Natl. Acad. Sci. 112 (2015) 6443–6448.
[56] K. Nakjarung, S. Mongkolsuk, P. Vattanaviboon, The OxyR from *Agrobacterium tumefaciens*: evaluation of its role in the regulation of catalase and peroxide responses, Biochem. Biophys. Res. Commun. 304 (2003) 41–47.
[57] S. Varghese, A. Wu, S. Park, K.R. Imlay, J.A. Imlay, Submicromolar hydrogen peroxide disrupts the ability of Fur protein to control free-iron levels in *Escherichia coli*, Mol. Microbiol. 64 (2007) 822–830.
[58] S. Park, X. You, J.A. Imlay, Substantial DNA damage from submicromolar intracellular hydrogen peroxide detected in Hpx-mutants of *Escherichia coli*, Proc. Natl. Acad. Sci. 102 (2005) 9317–9322.
[59] X. Hu, H. Li, X. Zhang, Z. Chen, R. Zhao, N. Hou, J. Liu, L. Xun, H. Liu, Developing polysulfide-sensitive GFPs for real-time analysis of polysulfides in live cells and subcellular organelles, Anal. Chem. 91 (2019) 3893–3901.
[60] H. Li, H. Liu, Z. Chen, R. Zhao, Q. Wang, M. Ran, Y. Xia, X. Hu, J. Liu, M. Xian, L. Xun, Using resonance synchronous spectroscopy to characterize the reactivity and electrophilicity of biologically relevant sulfane sulfur, Redox Biol. 24 (2019) 101179.
[61] Y. Mikami, N. Shibuya, Y. Kimura, N. Nagahara, Y. Ogasawara, H. Kimura, Thioredoxin and dihydrolipoic acid are required for 3-mercaptopyruvate sulfurtransferase to produce hydrogen sulfide, Biochem. J. 439 (2011) 479–485.
[62] P.K. Yadav, K. Yamada, T. Chiku, M. Koutmos, R. Banerjee, Structure and kinetic analysis of H_2S production by human mercaptopyruvate sulfurtransferase, J. Biol. Chem. 288 (2013) 20002–20013.
[63] V. Haridas, S.O. Kim, G. Nishimura, A. Hausladen, J.S. Stamler, Gutterman JU, Avicinylation (thioesterification): a protein modification that can regulate the response to oxidative and nitrosative stress, Proc. Natl. Acad. Sci. U. S. A 102 (2005) 10088–10093.
[64] D. Seth, A. Hausladen, Y.-J. Wang, J.S. Stamler, Endogenous protein S-nitrosylation in *E. coli*: regulation by OxyR, Science 336 (2012) 470–473.
[65] L. Yu, W. Li, K. Qi, S. Wang, X. Chen, J. Ni, R. Deng, F. Shang, T. Xue, McbR is involved in biofilm formation and H_2O_2 stress response in avian pathogenic *Escherichia coli* X40, Poult. Sci. (2019), https://doi.org/10.3382/ps/pez205 [Epub ahead of print].
[66] E. Lagoutte, S. Mimoun, M. Andriamihaja, C. Chaumontet, F. Blachier, F. Bouillaud, Oxidation of hydrogen sulfide remains a priority in mammalian cells and causes reverse electron transfer in colonocytes, Biochim. Biophys. Acta Bioenerg. 1797 (2010) 1500–1511.
[67] M.G. Mehta-Kolte, D. Loutey, O. Wang, M.D. Youngblut, C.G. Hubbard, K.M. Wetmore, M.E. Conrad, J.D. Coates, Mechanism of H_2S oxidation by the dissimilatory perchlorate-reducing microorganism *Azospira suillum* PS, mBio 8 (2017) e02023-02016.
[68] E.R. DeLeon, Y. Gao, E. Huang, M. Arif, N. Arora, A. Divietro, S. Patel, K.R. Olson, A case of mistaken identity: are reactive oxygen species actually reactive sulfide species? Am. J. Physiol. Regul. Integr. Comp. Physiol. 310 (2016) R549–R560.
[69] S. Koike, Y. Ogasawara, N. Shibuya, H. Kimura, K. Ishii, Polysulfide exerts a protective effect against cytotoxicity caused by t-buthylhydroperoxide through Nrf2 signaling in neuroblastoma cells, FEBS (Fed. Eur. Biochem. Soc.) Lett. 587 (2013) 3548–3555.