Mechanism of H$_2$S-mediated protection against oxidative stress in Escherichia coli

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Endogenous hydrogen sulfide (H$_2$S) renders bacteria highly resistant to oxidative stress, but its mechanism remains poorly understood. Here, we report that 3-mercaptopyruvate sulfurtransferase (3MST) is the major source of endogenous H$_2$S in *Escherichia coli*. Cellular resistance to H$_2$O$_2$ strongly depends on the activity of *mstA*, a gene that encodes 3MST. Deletion of the ferric uptake regulator (Fur) renders Δ*mstA* cells hypersensitive to H$_2$O$_2$. Conversely, induction of chromosomal *mstA* from a strong pLtetO-1 promoter (*P_{ltetO-1}mstA*) renders Δ*fur* cells fully resistant to H$_2$O$_2$. Furthermore, the endogenous level of H$_2$S is reduced in Δ*fur* or Δ*soda* Δ*sodB* cells but restored after the addition of an iron chelator dipiridyl. Using a highly sensitive reporter of the global response to DNA damage (SOS) and the TUNEL assay, we show that 3MST-derived H$_2$S protects chromosomal DNA from oxidative damage. We also show that the induction of the CysB regulon in response to oxidative stress depends on 3MST, whereas the CysB-regulated l-cystine transporter, TcyP, plays the principle role in the 3MST-mediated generation of H$_2$S. These findings led us to propose a model to explain the interplay between l-cystine metabolism, H$_2$S production, and oxidative stress, in which 3MST protects *E. coli* against oxidative stress via l-cysteine utilization and H$_2$S-mediated sequestration of free iron necessary for the genotoxic Fenton reaction.

hydrogen sulfide | oxidative stress | cysteine | sulfur metabolism | antibiotics

**H**ydrogen sulfide (H$_2$S) is well-recognized as a second messenger implicated in many physiological processes in mammals, including synaptic transmission, vascular tone, inflammation, angiogenesis, and protection from oxidative stress (1). The latter function of H$_2$S seems to be universal, because it has been implicated in bacterial defense against reactive oxygen species (ROS) and antibiotics-induced oxidative damage (2). H$_2$S can also kill microorganisms by inhibiting antioxidant enzymes during induced oxidative stress (3, 4). These seemingly contradictory attributes of H$_2$S highlight its concentration-dependent dual nature: at high concentration, it is a toxic gas, and at lower physiological concentrations, it is a signaling and/or protective molecule.

In *Escherichia coli* grown in Luria–Bertani broth, 3-mercaptopyruvate sulfurtransferase (3MST) is responsible for the bulk of endogenous H$_2$S generated from l-cysteine (2). Although *E. coli* has several known l-cysteine desulfhydrases (CDs), including O-acetylserine sulfhydrylases A and B (CysK and CysM), cystathionine β-lyases A and B (MetC and MatY), and tryptophanase (TnaA), that can, in principle, generate H$_2$S as a by-product of l-cysteine degradation, their contribution to H$_2$S production under normal growth conditions has not been established (2, 5). Because l-cysteine can be toxic to bacteria (6, 7), its intracellular level is tightly controlled. Excess l-cysteine inhibits the activity of l-serine O-acetyltransferase, a key enzyme in the l-cysteine biosynthetic pathway (8). The LysR-type transcriptional regulator, CysB, controls expression of genes involved in cysteine biosynthesis and sulfur assimilation. CysB binds the inducer, N-acetyl-l-serine (NAS), the product of a nonenzymatic rearrangement of O-acetyl-l-serine (OAS) that activates its binding to promoter DNA sequences (9). It has been shown that a high level of intracellular l-cysteine promotes the Fenton reaction (10):

\[
\text{L-cysteine} + \text{Fe}^{3+} \rightarrow \text{L-cystine} + \text{Fe}^{2+}
\]

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH} + \text{OH}^{-}
\]

This process is potentially toxic to the cell, because the resulting hydroxyl radicals damage nucleic acids, carbonylate proteins, and peroxidate lipids (11–13).

In our previous experiments, we showed in various bacterial species that an exogenous H$_2$S donor could suppress H$_2$O$_2$-mediated DNA damage (2). Here, we extend these findings to show that 3MST-mediated endogenous production of H$_2$S suppresses oxidative stress in *E. coli* by sequestering free iron required to drive the genotoxic Fenton reaction. Furthermore, we elucidate the complex interplay between 3MST and the CysB regulon that controls intracellular l-cysteine as a rate-limiting factor in H$_2$O$_2$-driven cytotoxicity.

**Results**

3MST-Derived H$_2$S Protects *E. coli* from Sequestration of Free Iron. To study the biochemistry of endogenous H$_2$S in *E. coli* and determine whether it is cytoprotective against ROS, we

**Significance**

Hydrogen sulfide (H$_2$S) is a highly toxic gas that interferes with cellular respiration; however, at low physiological amounts, it plays an important role in cell signaling. Remarkably, in bacteria, endogenously produced H$_2$S has been recently recognized as a general protective molecule, which renders multiple bacterial species highly resistant to oxidative stress and various classes of antibiotics. The mechanism of this phenomenon remains poorly understood. In this paper, we use *Escherichia coli* as a model system to elucidate its major enzymatic source of H$_2$S and establish the principle biochemical pathways that account for H$_2$S-mediated protection against reactive oxygen species. Understanding those mechanisms has far-reaching implications in preventing bacterial resistance and designing effective antimicrobial therapies.


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generated *E. coli* strains either lacking a chromosomal copy of the 3MST-encoding gene *mstA* (also known as *sseA*) or carrying it under a strong pLtet-O1 promoter (*P* _tet*-mstA*). After induced, *P* _tet*-derived 3MST should remain at a constantly high level. We used two complementary methods to quantify the level of H$_2$S production by *E. coli* cells (Fig. 1). The first method is based on the specific reactivity of lead acetate [Pb(Ac)$_2$] with H$_2$S, resulting in a brown lead sulfide stain. The rate of staining on a Pb(Ac)$_2$-soaked paper strip is directly proportional to the concentration of H$_2$S (14). The second method uses the twister internal charge transfer (TICT)-based fluorescent probe for H$_2$S (15). The TICT probe is cell-permeable and allows for monitoring exogenous and endogenous H$_2$S in living cells. Both methods consistently show that 3MST-deficient *E. coli* exhibit reduced level of H$_2$S production, whereas *P* _tet*-mstA* cells produce much more H$_2$S compared with the WT (Fig. 1).

Next, we examined the sensitivity of those cells to peroxide. H$_2$O$_2$ was added to midlog phase cultures (OD$_{600}$ ~ 0.2) at time 0, and the percentages of viable cells in the population were measured at intervals of 10, 20, and 30 min (Fig. S2A). After 20 min of treatment with 2 mM H$_2$O$_2$, the viabilities of WT and Δ*mstA* cells were reduced by ~10 and 25%, respectively. *P* _tet*-mstA* displayed no loss of viability (Fig. S2A). Notably, the exposure of WT cells to peroxide stimulated H$_2$S production (Fig. 1B), indicating that cells respond to oxidative stress by stimulating the activity of 3MST.

H$_2$O$_2$ is only mildly genotoxic to WT K-12 *E. coli*, which contains little free iron (16). We, therefore, sought to promote Fenton chemistry by elevating intracellular free iron in all three strains. Ferric uptake regulator (Fur) is the master transcriptional regulator of iron uptake and homeostasis in *E. coli* (17, 18). For example, Fur represses a small RNA RyhB, which negatively regulates a number of iron-containing proteins in *E. coli* (19). Fur deletion results in a constitutive import of iron (20, 21) and hypersensitivity to oxidative DNA damage (22). Accordingly, inactivation of *fur*, with or without *ryhB*, resulted in a 40-fold increase in cell death from H$_2$O$_2$ (Fig. 2B and C). The survivability of Δ*fur*, or Δ*fur* Δ*ryhB*, cells deficient in H$_2$S production (Δ*mstA*) decreased much more drastically (~360-fold). In contrast, Δ*fur*, or Δ*fur* Δ*ryhB*, cells that overproduced H$_2$S (*P* _tet*-mstA*) displayed almost complete loss of susceptibility to H$_2$O$_2$ (Fig. 2B and C), suggesting that H$_2$S counteracts the toxicity of H$_2$O$_2$ by sequestering the excess of free iron in Fur-deficient cells.

In support of this conclusion, we showed that the addition of FeCl$_3$ reduces the amount of H$_2$S in *P* _tet*-mstA* cells (Fig. 3A). We also observed a significant H$_2$S reduction in *P* _tet*-mstA* cells deleted of *fur* or *sodA/sodB* (Fig. 3A). The levels of free chelatable iron in Δ*fur*, Δ*sodA/sodB* mutants and the triple Δ*fur* Δ*sodA* Δ*sodB* mutant are ~8- and 17-fold higher, respectively, compared with WT cells (21). Accordingly, we observed the largest decrease in detectable H$_2$S in *P* _tet*-mstA* cells in the Δ*fur* Δ*sodA* Δ*sodB* mutant (Fig. 3A). Moreover, addition of an iron chelator, 2,2’-dipyridyl, fully restored the high level of H$_2$S in all *P* _tet*-mstA* strains (Fig. 3A). Because the inactivation of *fur* or *sodA/sodB* did not affect the level of *mstA* gene expression (Fig. S1), we conclude that the level of H$_2$S generated by 3MST is inversely proportional to the level of intracellular free iron. Taken together, these results argue that endogenous H$_2$S protects against H$_2$O$_2$-mediated toxicity by directly sequestering Fe$^{2+}$.

**3MST Is the Major CD That Protects Genomic DNA from Oxidative Damage.** Formation of double-strand breaks (DSBs) in DNA is the primary cause of bacterial cell death resulting from exposure to peroxide (23). These DSBs are the result of the toxic effects of the hydroxyl radical generated by the Fenton reaction (24). To examine whether endogenous H$_2$S protects bacteria from DNA damage caused by the Fenton reaction, we first examined its effect on the global response to DNA damage (SOS). We used a pColD’::lux reporter plasmid to directly monitor SOS activation in response to DNA damage (25). Fig. 3B shows the bioluminescence induction curves as a function of H$_2$O$_2$ concentrations in Δ*fur*, Δ*fur* Δ*mstA*, and Δ*fur* *P* _tet*-mstA* cells carrying pColD’::lux. In Δ*fur* cells, SOS induction begins at a concentration of 5 μM H$_2$O$_2$ and reaches a maximum at 80 μM followed by the decrease of bioluminescence caused by cell death. The Δ*fur* Δ*mstA* mutant exhibits a maximal SOS response at the lower concentration of H$_2$O$_2$.
The high level of resistance to oxidative stress observed in P. tetrazyma ΔcysB τau cells is directly attributable to the efficient sequestration of free iron but also, may be because of a higher rate of utilization via the sequential action of aspartate aminotransferase (AspC) and 3MST. Indeed, 3MST is not only responsible for the bulk of H₂S generated in P. tetrazyma cells in response to the addition of fur or sodA sodB genes. Such deletions cause a drastic increase in the intracellular free iron content (21). Addition of 200 μM 2,2'-dipyridyl (dp), an iron chelator, restores H₂S to its original level in each case. The values (percentages) are means from three independent experiments with a margin of error less than 10%. (B) 3MST-derived H₂S renders cells less susceptible to DNA damage as evidenced by the higher H₂O₂ concentration necessary to induce the SOS response in P. tetrazyma cells (Fig. S3). SOS response was monitored by bioluminescence of the lux biosensor (pColD::lux) in Δfur, Δfur ΔmstA, Δfur P. tetrazyma, and WT cells in the presence of different concentrations of H₂O₂. Iₐ/Iₒ indicates the induction factor in percentage compared with the maximal intensity of bioluminescence of samples in the presence of H₂O₂. Values are means ± SD from three experiments. (C) 3MST-derived H₂S renders cells less susceptible to H₂O₂-induced DNA breaks as detected by TUNEL. The graph shows the percentage of gated propidium iodide cells that are TUNEL-positive as detected by fluorescence intensity greater than that of untreated cells. Statistical evaluation (one-way ANOVA and Tukey’s post hoc test) was performed to evaluate differences in the cell population.

In contrast, Δfur P. tetrazyma ΔcysB τau cells reach the peak of bioluminescence intensity at a much higher H₂O₂ concentration (~1 mM), which is similar to that of the WT (Fig. 3B). These data indicate that endogenous H₂S significantly augments cellular tolerance to the Fenton reaction.

To further assess DNA damage after H₂O₂ treatment, we used an assay in which 3'-OH DNA ends were labeled with TUNEL followed by analysis by flow cytometry (Fig. 3C and Fig. S2). The percentage of TUNEL-positive cells, after gating for propidium iodide-stained cells, was significantly higher in Δfur ΔmstA than WT or Δfur P. tetrazyma cells. However, there was no significant difference in the percentages of the TUNEL-positive cells between treated WT and Δfur P. tetrazyma ΔcysB τau cells. Moreover, at the 5 mM concentration of H₂O₂, the threshold of detection for TUNEL-positive cells is minimal for WT and Δfur P. tetrazyma ΔcysB τau-treated cells. These results show directly that endogenous H₂S effectively protects chromosomal DNA from H₂O₂-induced DSBs.

The high level of resistance to oxidative stress observed in P. tetrazyma ΔcysB τau cells may not be only caused by the efficient sequestration of free iron but also, may be because of a higher rate of L-cysteine utilization via the sequential action of aspartate aminotransferase (AspC) and 3MST. L-cysteine promotes the Fenton reaction by effectively reducing Fe³⁺ to Fe⁺⁺ (10). Therefore, the intensive L-cysteine degradation in P. tetrazyma cells can also contribute to the suppression of the Fenton reaction.

E. coli has five known CDs in addition to 3MST, which are capable of degrading L-cysteine to pyruvate, ammonia, and sulfide. However, a quintuple mutant of ΔmstA ΔmetC ΔcysK ΔcysM ΔmstA ΔmstB ΔmstY retains significant CD activity, which is increased in the presence of ΔcysB (5), suggesting that the major enzyme responsible for converting L-cysteine to H₂S is 3MST. Indeed, 3MST is not only responsible for the bulk of H₂S during normal growth in rich media but also, generates more H₂S under exposure to peroxide (Fig. 1C). In contrast, TnaA, which is considered to be the predominant CD (5), contributes little to the overall level of endogenous H₂S (Fig. S5A) and does not influence bacterial susceptibility to H₂O₂, irrespective of Fur (Fig. S5B).

**Functional Interaction Between 3MST and CysB.** CysB is a master transcriptional regulator of sulfur metabolism that senses the level of endogenous L-cysteine (8). To further evaluate the impact of 3MST on endogenous L-cysteine catabolism, we used quantitative RT-PCR (qRT-PCR) to measure the expression of the CysB-dependent genes, cysK, cysP, and tau, in ΔmstA and P. tetrazyma cells. Transcription of all three genes was mildly decreased in ΔmstA cells compared with WT cells (Fig. 4A). In P. tetrazyma cells, however, cysK, cysP, and tau were induced ~11-, 8-, and 5-fold, respectively. The induction of these genes is strictly dependent on CysB, because cysB inactivation reduced their expression to the background level (Fig. 4A). We infer that the induction of CysB-dependent genes was caused by the induction of cysB itself (Fig. 4A), which is likely to occur because of the increased L-cysteine degradation in P. tetrazyma cells. Indeed, L-cysteine is involved in feedback inhibition of serine acetyltransferase, CysE, which generates OAS, a precursor of an autoinducer for CysB, NAS (Fig. S4) (9). Accordingly, the addition of exogenous L-cysteine to the Ptet-ΔmstA strain reduced the expression of all CysB-regulated genes to the basal level (Fig. 4A).

We next examined the effect of 3MST on the CysB regulon during oxidative stress. Treatment of WT cells with 2 mM H₂O₂ for 20 min resulted in ~5-, 23-, 10-, and 14-fold inductions of cysB, cysK, cysP, and tau, respectively (Fig. 4B). In contrast, the induction of CysB-regulated genes in response to H₂O₂ was completely abolished in ΔmstA cells (Fig. 4B). Moreover, inactivation of cysB almost completely abolished H₂S generation by P. tetrazyma cells (Fig. 5A). We suggest that, without CysB, the transport of L-cysteine into
the cell is abrogated, hence the inability of 3MST to generate H$_2$S and protect against oxidative stress.

To test this hypothesis, we placed the chromosomal copy of the major t-cysteine importer, TcyP (26, 27), under the strong P$tet$ promoter. TcyP is normally under the positive control of CysB (28). P$tet$-TcyP fully restored 3MST-dependent H$_2$S production in P$tet$-mstA cells (Fig. 5A). Moreover, P$tet$-tcyP increased H$_2$S production in cysB$^-$ cells carrying mstA under its native promoter (Fig. 5B). Because the deletion of mstA in P$tet$-tcyP cells abolishes H$_2$S production, we conclude that 3MST is the sole source of H$_2$S in E. coli grown in Luria–Bertani broth. These results argue that, under conditions of cysteine overflow, the AspC-3MST system generates a sufficient amount of H$_2$S to render cells resistant to oxidative stress. To maintain such a protective level of H$_2$S under oxidative stress, the enhanced influx of t-cysteine must occur. Accordingly, the expression of tcyP is strongly induced in response to H$_2$O$_2$ treatment (Fig. S7). Moreover, this induction is strictly dependent of 3MST activity: deletion of mstA abolishes tcyP induction, whereas P$tet$-mstA increases it (Fig. S7).

Discussion

The purpose of this work is to explain the mechanism of H$_2$S-mediated protection against oxidative stress and establish the biochemical pathway of H$_2$S production in response to stress in E. coli. The results determine that the AspC-3MST pathway is the principle source of H$_2$S in E. coli grown in rich medium containing cysteine (Fig. S4). It has been assumed that TnaA could be the major CD and potential generator of H$_2$S in E. coli (5). However, our previous work showed that inactivation of TnaA ($\Delta$tnaA) or other known desulphhydrases ($\Delta$mstA, $\Delta$mstC, $\Delta$cysK, $\Delta$cysM, and $\Delta$mstY) does not significantly alter the level of endogenous H$_2$S (2). Here, we provide independent support for this conclusion and show that the inactivation or overexpression of TnaA does not function at all in H$_2$S-mediated protection against oxidative stress (Fig. S3). Rather, 3MST is central.

The protective function of 3MST becomes most apparent in Fur-deficient cells, in which the level of intracellular iron (Fe$^{2+}$) substantially increased (22). The $\Delta$mstA $\Delta$fur double mutant exhibited a 360-fold increase in sensitivity to H$_2$O$_2$ compared with its $\Delta$mstA fur$^+$ counterpart (Fig. 2), which showed an ~10-fold increase in peroxide sensitivity compared with the $\Delta$fur mutant. This sensitivity correlates well with the dramatic increase in genomic DNA DSBs (Fig. 3C and Fig. S2). Remarkably, endogenous overproduction of H$_2$S from the chromosomal P$tet$-mstA completely protects Fur-deficient cells from H$_2$O$_2$ toxicity and DNA damage. Furthermore, we found that the level of H$_2$S in P$tet$-mstA cells is reduced in $\Delta$fur or $\Delta$mstA $\Delta$mstB cells but can be restored after addition of the Fe$^{2+}$ chelator, 2,2’-dipyridyl (Fig. 3A). These data imply that 3MST renders E. coli resistant to oxidative stress via H$_2$S-mediated sequestration of Fe$^{2+}$, thereby diminishing the genotoxic Fenton reaction (Fig. 5C).

Because the amino acids in Luria–Bertani broth are the main carbon source (29), we postulate that Luria–Bertani broth-derived t-cysteine/cysteine is the principle substrate for H$_2$S production by AspC-3MST. Indeed, the deletion of cysB abolishes the generation of H$_2$S in P$tet$-mstA cells. CysB positively regulates not only the genes responsible for t-cysteine bio-synthesis but also, tcyP and tcyJ, which encode the two t-cysteine transporters, the symporter TcyP and the ATP binding cassette importer TcyJ, respectively (27). Therefore, the inability of the P$tet$-mstA $\Delta$cysB mutant to generate H$_2$S can be caused by reduced production of endogenous t-cysteine, disruption of t-cysteine import from the Luria–Bertani broth medium, or both. We found that the introduction of the constitutively active form of tcyP (P$tet$-tcyP) (Fig. 5A), but not tcyJ (P$tet$-tcyJ) (Fig. S8), fully restores the generation of H$_2$S in CysB-deficient P$tet$-mstA cells. Remarkably, we found that the constitutive expression of tcyP also leads to overproduction of H$_2$S in cells with native expression of mstA (Fig. 5B). Thus, the main source of H$_2$S generated by 3MST is t-cysteine/cysteine imported from the Luria–Bertani broth medium by the TcyP transporter (Fig. 5C). This conclusion is consistent with the observation that, unlike TcyJ, TcyP functions predominantly as a nutrient importer under normal growth conditions (26).

Our results also reveal the reciprocal interaction between 3MST and the CysB regulon under normal growth conditions and during oxidative stress. The high level of 3MST expression in P$tet$-mstA cells resulted in cysB induction and its target genes (cysK, cysP, and tau), whereas in the absence of 3MST, the expression of all CysB-regulated genes was diminished (Fig. 4A). Remarkably, 3MST deficiency also abolished H$_2$O$_2$-mediated induction of CysB-dependent genes (Fig. 4B). It has been reported that at least three such genes (cysK, cysP, and tcyJ) are highly up-regulated in response to H$_2$O$_2$ in an OxyR-independent manner (26, 30). The mechanism of such an induction remains unknown. Our results suggest the following model, which explains the interplay between oxidative stress, activation of the CysB regulon, and 3MST-dependent generation of H$_2$S (Fig. 5C). The sulfhydryl group of t-cysteine reacts with H$_2$O$_2$ in the periplasm to yield t-cystine (26). This reaction lowers the intracellular level of t-cysteine leading to the induction of the CysB regulon, including the TcyP transporter, thereby boosting the t-cysteine/cysteine influx into the cytoplasm. The increased flow of t-cysteine stimulates H$_2$S production by the AspC-3MST pathway, leading to sequestration of Fe$^{2+}$ and suppression of the Fenton reaction (Fig. 5C). Inactivation of 3MST halts the conversion of l-cysteine to H$_2$S, leading to accumulation of intracellular l-cysteine, thereby preventing H$_2$O$_2$-dependent induction of CysB-regulated genes and fueling the genotoxic Fenton reaction.

Understanding the mechanism of H$_2$S-mediated protection against ROS has important implications for bacterial resistance to antibiotics (31, 32). Pharmacological inhibition of bacterial H$_2$S production may facilitate rapid bacterial killing, which would not only widen the therapeutic window for many classes of
bactericidal antibiotics but also, diminish the rate at which bacterial acquire resistance to such antibiotics (33).

Materials and Methods

Strains and Growth Conditions. All E. coli strains used in this work are listed in Table S1. BW25113 and its derivatives (single-gene deletion mutants) were obtained from the E. coli Keio Knockout Collection (Thermo Scientific) (34). Details of strain constructions are described in SI Materials and Methods. P1 transduction was used to introduce mutations into new strains (35). When necessary, Cam or Kan drug resistance markers were excised from strains using the flippase activity of pCP20 followed by loss of the plasmid at non-permissive temperature (36). All mutations were verified by PCR and gel analysis. DNA manipulations and the transformation of E. coli strains were performed according to standard methods (37). Luria–Bertani broth complete medium was used for the general cultivation of E. coli. When appropriate, antibiotics were added at 40 μg/mL (for kanamycin), 30 μg/mL (for chloramphenicol), and 100 μg/mL (for ampicillin). For solid medium, 1.5% agar was added.

Generation of Growth Curves. Growth curves were obtained on a Bioscreen C automated growth analysis system. Subcultures of specified strains were grown overnight at 37 °C, diluted in fresh medium at 1:100, inoculated into honeycomb wells in triplicate, and grown at 37 °C with maximum shaking on the platform of the Bioscreen C instrument. When the cultures reached an OD600 of 0.2, cells were treated with H2O2 (2 mM) and incubated at 37 °C for 10 h. OD600 values were recorded automatically at specified times, and the mean value of the triplicate cultures was plotted.

Generation of Survival Curves. Overnight cultures were inoculated into Luria–Bertani broth and grown at 37 °C to -~× 106 cells per 1 mL. Cells were then treated with H2O2 (2 mM) and after 10 or 20 min of incubation, samples were diluted, plated on Luria–Bertani broth agar, and incubated at 37 °C for 16–18 h. Cell survival was determined by counting cfu and is shown as the mean value ± SD from three independent experiments.

H2S Detection. To monitor H2S production, we used a Pb(Ac)2 detection method (14) and the TICT-based fluorescent H2S probe (BH- HS) (15). Overnight cultures were diluted 1:500 in Luria–Bertani broth and incubated at 37 °C with aeration (250 rpm) for 18–20 or 3–4 h for Pb(Ac)2 or BH-HS, respectively. Before incubation, the paper strips saturated with 2% Pb(Ac)2 were affixed to the inner wall of a cultural tube above the level of the liquid culture of WT or mutant bacteria. Stained paper strips were scanned and quantified with an Alpha Imager (Imgen Technologies). BH-HS (5 μM) was added to liquid bacterial culture, and after 40 min, the aliquots were taken for fluorescent microscopy (API DeltaVision PersonalDV system with Olympus IX-71 inverted microscope base). Images were taken with an Olympus Plan-Neofluar 40× oil immersion. A Cy3 (Molecular Probes Inc.) was used to quantitate fluorescence. The results were normalized according to the ODs.

RNA Extraction and qRT-PCR. E. coli K-12 MG1655 cells were grown until OD600 of 0.6, and total RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to the manufacturer’s protocol. All RNA samples were treated with DNaseI (Fermentas); 500 ng total RNA was reverse-transcribed with 100 U SuperScript III enzyme from the First-Strand Synthesis Kit for RT-PCR (Invitrogen) according to the manufacturer’s protocol in the presence of appropriate gene-specific primers (Table S2). One microlitre reverse transcription reaction was used as the template for real-time PCR. The gene def encoding peptide deformylase was used for normalization. Each real-time PCR reaction included 2 μL cDNA, 10 μL SYBR Green PCR Master Mix (Applied), 12 μL nuclease-free H2O, 1 μL 10 μM forward primer, 1 μL 10 μM reverse primer, and 1 μL cDNA template. Amplifications were carried out using the DTite 51 Cyclersystem (DNA Technology). Reaction products were analyzed using 2% agarose electrophoresis to confirm that the detected signals originated from products of expected lengths. Each qRT-PCR was performed at least in triplicate, and average data are reported. Error bars correspond to the SD.

TUNEL Assay. Cells were grown until OD600 of 0.4, and 1 mL aliquots were treated with 5 mM H2O2 for 30 min. Cells were fixed and labeled using a 2% paraformaldehyde, and then, they were incubated on ice. After 1 h, cells were centrifuged, washed, and resuspended in 10% ethanol overnight at -20 °C. The next day, cells were centrifuged, washed, and resuspended in 50 μL TUNEL reaction mix for 2 h at 37 °C. After the labeling reaction was stopped, the cells were counterstained with propidium iodide/RNAscope A and analyzed by flow cytometry on the FACSCalibur.

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