Full Paper

Generation of hydrogen sulfide from sulfur assimilation in *Escherichia coli*

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Many organisms produce endogenous hydrogen sulfide (H₂S) as a by-product of protein, peptide, or L-cysteine degradation. Recent reports concerning mammalian cells have demonstrated that H₂S acts as a signaling molecule playing important roles in various biological processes. In contrast to mammals, bacterial H₂S signaling remains unclear. In this work, we demonstrate that *Escherichia coli* generates H₂S through the assimilation of inorganic sulfur, without L-cysteine degradation. Comparison of phenotypes and genomes between laboratory *E. coli* K-12 strains revealed a major contribution of CRP (a protein that controls the expression of numerous genes involved in glycolysis) to H₂S generation. We found that H₂S was produced by cells growing in a synthetic minimal medium containing thiosulfate as a sole inorganic sulfur source, but not in a medium only containing sulfate. Furthermore, *E. coli* generated H₂S in a CRP-dependent manner as a response to glucose starvation. These results indicate that CRP plays a key role in the generation of H₂S coupled to thiosulfate assimilation, whose molecular mechanisms remains to be elucidated. Here, we propose a potential biological role of the H₂S as a signaling mediator for a cross-talk between carbon and sulfur metabolism in *E. coli*.

Key Words: CRP; *Escherichia coli*; hydrogen sulfide; sulfur assimilation; thiosulfate

Abbreviations: H₂S, hydrogen sulfide; Cys, L-

cysteine; Kmr, kanamycin resistance; Cmr, chloramphenicol resistance; ORFs, open reading frames

Introduction

Hydrogen sulfide (H₂S) has been classically known as a toxic gas, but it is also produced by different organisms via enzymatic degradation of L-cysteine (Cys). In mammalian cells, it acts as an important signaling molecule involved in a wide variety of biological functions from vasorelaxation to neurotransmission (Gadalla and Snyder, 2010; Ingenbleek and Kimura, 2013; Kimura, 2016). Recently, Shatalin et al. (2011) reported that bacteria generates H₂S from Cys upon antibiotic's exposure to an active scavenging system for a reactive oxygen species. Therefore, nowadays, H₂S is recognized as a signaling molecule in prokaryotes as well, although little is known about the physiological role of H₂S apart from the antibiotic's response in bacteria.

It is accepted that some *Enterobacteriaceae*, including *Escherichia coli*, do not actively produce H₂S (Neidhardt and Curtiss, 1996). Indeed, unlike *Salmonella*, *E. coli* does not have both a Cys-specific degradation enzyme and a H₂S-specific export system for the detoxification of H₂S. However, in the presence of a high concentration of extracellular or intracellular Cys (e.g. an extra supply of Cys in a growth medium or Cys overproduction in fermentation conditions), the bacterial cells produce H₂S to avoid Cys accumulation. *E. coli* has several Cys-degrading enzymes that convert Cys into pyruvate, ammonia, and H₂S (Awano et al., 2005; Delwiche, 1951). Degradation of ex-

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Table 1. *E. coli* strains used in this study.

Strain	Genotype	Reference or source
MG1655	F ⁻ λ <i>ilvG-rfb-50 rph-1</i>	Laboratory strain
JM101	F ⁺ <i>traD36 proA⁺B⁺ lacI^q Δ(lacZ)M15/Δ(lac-proAB) glnV thi</i>	Laboratory strain
JM109	F ⁺ <i>traD36 proA⁺B⁺ lacI^q Δ(lacZ)M15/Δ(lac-proAB) glnV44 e14⁻ gyrA96 recA1 relA1 endA1 thi hsdR17</i>	Laboratory strain
W3110	F ⁻ λ <i>rph-1INV(rrnD rrnE)</i>	Laboratory strain
BW25113	<i>rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1</i>	Laboratory strain (Baba et al., 2006)
JW5702	BW25113 Δ <i>crp</i> ::Km ^r	(Baba et al., 2006)
J-GLT	<i>E. coli</i> strain (JM101 Δ <i>ggt ΔybiK ΔyliABCD</i>) harboring glutathione-producing plasmid pGH183	kindly provided by Kyowa Hakko Bio Co., Ltd. (Tokyo, Japan)
J-GLT Δ <i>crp</i>	J-GLT Δ <i>crp</i> ::Cm ^r	This study

Table 2. Primers used in this study.

Oligonucleotide	Sequence
CRP-KN	GGCGTTATCTGGCTCTGGAGAAAGCTTATAACAGAGGATAACCGCGCATGATTCCGGGGATCC
CRP-KC	CTACCAGGTAACGCGCCACTCCGACGGGATTAACGAGTGCCGTAACGACTGTAGGCTGGAGCTGCTTCG

cess Cys might be beneficial for the cells to minimize the effect of cytotoxicity of Cys, but to still reuse pyruvate and NH₃ as carbon and nitrogen sources. Thus, we have so far observed H₂S generation under artificial conditions containing abundant Cys and antibiotic's exposure.

Unlike higher eukaryotes, *E. coli* has two well-known pathways for *de novo* biosynthesis of Cys which generates sulfide as an intermediate product (Supplementary Fig. S1) (Aguilar-Barajas et al., 2011; Nakatani et al., 2012). The process of sulfate and thiosulfate reduction produces sulfite, which is then converted to sulfide by enzymes of the sulfate pathway. The generated sulfide is converted into Cys by the enzyme CysK (Boronat et al., 1984). Although the sulfide can be H₂S and released into extracellular environments, it hardly occurs under physiological conditions since the sulfide rapidly assimilated into Cys. The contribution of the sulfur assimilation to H₂S generation remains unclear.

Since Cys and its derivatives have crucial functions in cellular metabolism, their production by fermentation is important in terms of its applications (Breuer et al., 2004). In the processes of *E. coli* fermentative overproduction, H₂S gas is generated from the cells. This is often considered as a serious risk to human life in the manufacturing environment due to the H₂S toxicity. Therefore, it is important to understand the potential mechanisms of H₂S production in *E. coli*. In order to investigate novel H₂S generation, we focused on sulfur assimilation pathways, and explored their contribution to H₂S generation in the presence of inorganic sulfur compounds, sulfate, or thiosulfate as a sole sulfur source. Our findings show a hitherto unknown mechanism of H₂S generation through thiosulfate assimilation without Cys degradation and provide important insights about the cross-talk between sulfur, carbon, and energy metabolisms in *E. coli*.

Materials and Methods

Bacterial strains and cell growth. The *E. coli* strains used in this study are listed in Table 1. A fermentative strain, *E.*

coli J-GLT, was kindly provided by Kyowa Hakko Bio Co., Ltd. (Tokyo, Japan) in which a glutathione biosynthetic system was modified to produce high-level glutathione, and then used as an experimental strain. A *crp* deletion mutation was introduced to J-GLT by the method of Baba et al. (2006) using the pKD334 carrying chloramphenicol resistance (Cm^r) gene and the primers listed in Table 2. To cultivate J-GLT strains, we used the synthetic medium as follows below: 6 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 5 g/L Difco Pepton, 10 g/L Difco Yeast Extract, 10 mg/L thiamine-HCl, 10 mg/L MnSO₄·7H₂O, 5 g/L (NH₄)₂SO₄, 10 mM Na₂S₂O₃, and 1 g/L glucose, which is termed FM_{J-GLT} in this report. For the other *E. coli* strains, we used LB medium or the synthetic minimal medium M9_S (6 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 0.5 g/L NaCl, 1 g/L NH₄Cl, 1 mM MgCl₂, 0.5 mg/L thiamine-HCl, 0.01 g/L MnCl₂, 0.2 mg/L FeCl₃, and 1 g/L glucose) supplemented with all amino acids (1 g/L) except for Cys and L-methionine. For growth as a sole inorganic sulfur source, the medium was supplemented with Na₂SO₄ (1 mM) or Na₂S₂O₃ (1 mM). When required, kanamycin (Km) and chloramphenicol (Cm) were added at concentrations of 50 and 30 μg/ml, respectively. Cultures were incubated aerobically by vigorous shaking at 30°C. Growth was monitored using a U-1100 Spectrophotometer (HITACHI) to measure optical density at 660 nm.

Determination of H₂S and glucose concentrations. Determining the concentration of H₂S was carried out as indicated below. For the main culture, performed in a jar-fermenter (MBC-3; Sanki Seiki, Osaka, Japan), 2 L of the synthetic medium (described above) was prepared. *E. coli* cells were precultured overnight in the synthetic medium supplemented with Na₂SO₄ (1 mM) or Na₂S₂O₃ (1 mM) at 30°C. The overnight cell culture was inoculated into the synthetic medium supplemented with an appropriate sulfur source at OD₆₆₀ = 0.1, and then incubated in a jar-fermenter at 30°C with stirring at 480 rpm. The culture was aerated at 1 L/min. Concentrations of H₂S produced by *E. coli* cells were quantified by using a Personal H₂S

Fig. 1. Comparison of H_2S generation between laboratory strains.

E. coli cells were grown in $FM_{J\text{-}GLT}$ medium at 30°C for 24 h. Lead acetate-soaked paper strips produce lead sulfide (brown coloration) when they react with H_2S . Data are shown by a gray scale and are representative of the results of three independent experiments.

Fig. 2. Impact of single-gene deletion on H_2S generation.

E. coli cells were grown in LB containing 0.4% glucose at 30°C for 24 h. Data are shown by a gray scale and are representative of the results of two independent experiments.

Detector XS-2200 (New Cosmos Electric).

For easy and sensitive detection of H_2S , we employed a lead acetate detection method (Shatalin et al., 2011). A piece of lead (II) acetate paper (GE Healthcare) was affixed to the inner wall of a cultural cube. The overnight cell culture was inoculated into a medium (appropriately supplemented with a sulfur source depending on the purpose of the experiment) at $OD_{660} = 0.1$, and then incubated at 30°C. Residual glucose in the supernatant was determined enzymatically using a commercial glucose kit (Glucose CII-test kit, Wako). The absorbance at 505 nm was measured using a U-1100 Spectrophotometer (HITACHI).

Results

CRP is involved in H_2S generation of *E. coli*.

E. coli J-GLT strain from JM101 produces high glutathione levels, but does not produce Cys when grown in the fermentation medium $FM_{J\text{-}GLT}$ (see Materials and Methods). We noticed that H_2S was generated from the cultured cells (Supplementary Fig. S2). Notably, H_2S production began upon glucose starvation from the medium. To test the generality of the H_2S generation in *E. coli* laboratory strains, we cultivated five *E. coli* K-12 derivatives (JM101, JM109, BW25113, W3110, and MG1655) in the $FM_{J\text{-}GLT}$ medium. To detect H_2S , we used lead (II) acetate paper, which reacts with H_2S forming a brown-colored

precipitate of lead sulfide. Among the tested strains, BW25113 and MG1655 produced a relatively higher amount of H_2S , and JM101 produced lower amounts of H_2S (Fig. 1). By contrast, JM109 and W3110 did not produce any detectable amounts of H_2S . These results suggest that genetic differences between the *E. coli* K-12 strains affects H_2S generation.

A detailed genomic comparison between MG1655 and W3110 was conducted which revealed genetic differences in fourteen ORFs, genomic insertions in six genes (*dcuC*, *gatA*, *rscC*, *tdcD*, *tnaB*, *alsK*), and sequence differences in eight genes (*ycdT*, *acnA*, *intQ*, *yedJ*, *rpoS*, *crp*, *rrlE*, *ducA*) (Hayashi et al., 2006). Thus, we examined the effect of single-gene deletion on H_2S production. Based on the previous report, we prepared the corresponding ten mutant strains from the Keio collection (Baba et al., 2006), excluding non-providing mutants due to their essentiality for cell viability. Wild-type BW25113 (WT), the parent of the Keio strains, was used as a positive control. Whereas eight disruptants ($\Delta dcuC$, $\Delta rscC$, $\Delta tnaB$, $\Delta ycdT$, $\Delta acnA$, $\Delta intQ$, $\Delta yedJ$, $\Delta ducA$) produced H_2S at the same level with the WT strain, Δcrp and $\Delta rpoS$ cells hardly produced any H_2S (Fig. 2). These results indicated that CRP and RpoS are required for H_2S generation. Δcrp cells grew normally under standard laboratory culture conditions, while $\Delta rpoS$ cells exhibited growth retardation compared with the WT cells (data not shown). Therefore, we focused on CRP in our further investigations.

Fig. 3. H₂S generation in M9_s medium supplemented with sulfate or thiosulfate.

E. coli cells (open symbols: WT; filled symbols: Δ*crp*) were grown in liquid M9_s medium supplemented with Na₂SO₄ (1 mM) or Na₂S₂O₃ (1 mM) as a sole sulfur source at 30°C. Measurements of glucose and H₂S were started after 2 h. A. Na₂SO₄, B. Na₂S₂O₃, and C. *E. coli* cells were grown in liquid M9_s medium supplemented with Na₂SO₄ (1 mM) or Na₂S₂O₃ (1 mM) as the sole sulfur source at 30°C for 12 h.

Endogenous *E. coli* H₂S is generated by thiosulfate assimilation via CRP function.

To clarify whether degradation of organic sulfur compounds contributes to H₂S generation, we cultivated WT cells in M9_s with sulfate, or thiosulfate, as a sole sulfur source. As shown in Fig. 3, the WT strain only generates H₂S in the presence of thiosulfate. Interestingly, H₂S generation started right after glucose deprivation from the culture media. These results demonstrate that H₂S generation induced by glucose starvation depends on the process of thiosulfate assimilation. To explore the involvement of CRP in H₂S generation from thiosulfate, we cultured the Δ*crp* mutant strain in M9_s medium supplemented with thiosulfate and tested its H₂S generation. Compared with the WT cells, H₂S generation in the Δ*crp* cells was dramatically reduced (Figs. 3B and 3C). Likewise, a remarkable decrease of H₂S production was observed in Δ*crp* cells with a J-GLT genetic background (Supplementary Fig. S2). Our results suggest that deletion of the *crp* gene is a novel strategy to produce a sulfur-containing organic compound (e.g. glutathione) whilst minimizing H₂S generation, which

can be a risk for human life and product yield.

We previously reported that thiosulfate, rather than sulfate, is the preferred sulfur source for Cys production in *E. coli* (Nakatani et al., 2012). It has also been reported that Rhodanese-like protein SseA, a 3-mercaptopyruvate sulfurtransferase (Spallarossa et al., 2004), is the major H₂S-producing enzyme via Cys degradation in *E. coli* (Shatalin et al., 2011). Therefore, we tested whether the Δ*sseA* showed reduced H₂S generation comparable with our new target CRP. However, we could not observe any obvious difference between WT and Δ*sseA* cells for H₂S generation (Fig. 3C) in M9_s media containing thiosulfate. This suggests that the SseA does not contribute to H₂S generation upon glucose starvation under this condition.

Discussion

Most studies for bacterial H₂S have been conducted using a nutrient-rich medium, such as LB. Under such conditions, *E. coli* does not require *de novo* synthesis of Cys (and Cys derivatives), and directly uptakes Cys from the

medium. When excess Cys is added, *E. coli* converts Cys into H₂S to maintain a low level concentration of cytotoxic Cys. Production of H₂S as a result of the degradation of organic sulfur compounds takes place in all living organisms; this phenomenon has been well characterized (Kimura, 2016; Li et al., 2011; Shatalin et al., 2011). However, H₂S generation from inorganic sulfur compounds and its physiological role in *E. coli*, remains unclear. Transiently-generated H₂S during fermentation is a serious problem for the production of sulfur-containing amino acids because of their toxicity.

In this study, we identified that *E. coli* K-12 generates H₂S from thiosulfate but not from sulfate (Figs. 3A and 3B). *E. coli* has two enzymes that assimilate inorganic sulfur: CysK, and CysM. While the former enzyme utilizes sulfide as a sulfur donor, the latter enzyme uses thiosulfate. CysK synthesizes Cys from *O*-acetyl-L-serine and sulfide, but CysM can utilize thiosulfate instead of sulfide (Supplementary Fig. 1). CysM produces *S*-sulfocysteine, which is later converted to Cys and sulfite by the enzymes NrdH or Grx1 (Nakatani et al., 2012). Recently, we reported a CysM-independent pathway for Cys synthesis from thiosulfate involving rhodanese-like sulfurtransferases (rhodanese) (Supplementary Fig. 1) (Kawano et al., 2017). In this pathway, the thiol group of the catalytic Cys residue of the rhodanese is persulfurated by the sulfane sulfur of thiosulfate, simultaneously releasing sulfite. The generated sulfite is recruited into the conventional sulfate pathway to be used for Cys synthesis. The role of the sulfur atom bound to the Cys residue as persulfide remains unclear. It may serve a purpose unrelated to Cys synthesis, for instance, such as H₂S signaling.

SseA (a protein with rhodanese activity) did not contribute to H₂S generation (Fig. 3C). It is possible that other rhodanases are involved in H₂S generation. We recently observed that upon deletion of *cysA* (the gene encoding the thiosulfate and sulfate importer) (Hryniewicz et al., 1990), cells could grow in M9s containing thiosulfate as a sole sulfur source, but not sulfate (our unpublished data). These observations suggest the existence of an unknown thiosulfate-specific uptake system. YeeD is a small rhodanese, consisting of a single domain. Interestingly, the *yeeD* gene is located downstream of the *yeeE* gene, which encodes an unknown functional transporter. These genes might be transcribed as a polycistronic *yeeED* operon (Mendoza-Vargas et al., 2009). We found that the Δ *cysA* Δ *yeeED* double mutant strain cannot grow in the minimal medium containing thiosulfate as a sole sulfur source (our unpublished data), suggesting that only CysTWAP and YeeED are responsible for thiosulfate uptake in *E. coli* (Supplementary Fig. S3). We believe that the YeeED system contributes to assimilate thiosulfate and the subsequent generation of H₂S, and are currently analyzing the function of YeeED to clarify the molecular mechanism of H₂S generation coupled with carbon and thiosulfate metabolism.

In this study, we also found that glucose starvation triggered H₂S production from thiosulfate and that the H₂S induced by glucose starvation requires a CRP function (Figs. 3B and 3C). Because the CRP function depends on cyclic-AMP (Shimada et al., 2011), we believe that ade-

nylate cyclase CyaA is also required for H₂S generation. CRP is a global transcriptional regulator that controls the expression of numerous genes of metabolic enzymes that respond to carbon source availability (Shimada et al., 2011; Zheng et al., 2004). Considering previously reported H₂S functions as a signaling molecule, it is plausible that H₂S generation by the assimilation of thiosulfate plays an important role in the interrelationship between sulfur, carbon, and energy metabolism via transcriptional regulation by CRP. The importance of CRP for H₂S generation was also reported in *Shewanella oneidensis*, where *crp* deletion completely abolished H₂S generation (Wu et al., 2015). Genetic studies in *S. oneidensis* showed that CRP directly controls the transcription of the operons encoding *sirACD* and *psrABC*. The SirACD and PsrABC complexes act as sulfite and sulfide reductases, respectively, and are predominantly responsible for H₂S generation via the respiration of sulfur species (Wu et al., 2015). These results show that the importance of CRP for H₂S generation is due to the regulation of these operons. CRP plays a crucial role in *S. oneidensis*, acting as a switch between aerobic and anaerobic respirations. However, *E. coli* does not have homologs for these genes. We searched the CRP-binding motif for the genes encoding rhodanese involved in thiosulfate metabolism (Kawano et al., 2017), and found the predicted sequence in the upstream region of *glpE* gene. Furthermore, global transcriptional analyses of *E. coli* have suggested that Crp regulates the expression of gene for *glpE* (Choi et al., 1991; Shimada et al., 2011). We examined the effect of the deletion of the *glpE*-encoding gene on H₂S generation, but no effect was observed on H₂S generation in Δ *glpE* (Supplementary Fig. S3). We propose that CRP controls some unknown genes encoding enzymes involved in H₂S generation. Characterization of revertants isolated from Δ *crp* cells, whose H₂S generation capacity is restored, may provide some clues.

H₂S accumulation is toxic to mammalian cells because it inhibits cytochrome *c* oxidase (Li et al., 2011; Nicholls et al., 2013). In eukaryotes, sulfide quinone oxidoreductase (SQR), a mitochondrial membrane flavoprotein that oxidizes H₂S to protein-bound persulfide, acts as a detoxification enzyme for H₂S (Kabil and Banerjee, 2010). SQR transports two electrons to the oxidized form of coenzyme Q and to the complex III of the respiratory chain; at the same time, it uses the sulfur species as acceptors, forming thiosulfate and other thiols. In the intestinal environment, thiosulfate produced by mammalian cells is metabolized by the intestinal flora. Intestinal bacteria might use the energetically favored thiosulfate rather than sulfate to synthesize Cys. The endogenously generated H₂S from intestinal bacteria might be utilized as a signal for Cys production. CRP-regulated H₂S might function as a crucial role in the aspect of energy conservation when carbon and energy sources are limited.

Conclusions

We found that a H₂S is produced by cells growing in a synthetic minimal medium containing thiosulfate as a sole inorganic sulfur source, but not sulfate. In addition, *E. coli* generated the H₂S in a CRP-dependent manner as a re-

sponse to glucose starvation. Furthermore, we also suggest that some rhodanese are involved in the generation of H₂S. These results propose that the H₂S in bacteria as well as in eukaryote might be utilized as a signal for environmental response.

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Supplementary Materials

Supplementary figures are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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