Transcription Factors CysB and SfnR Constitute the Hierarchical Regulatory System for the Sulfate Starvation Response in *Pseudomonas putida*\(^\dagger\)

Atsushi Kouzuma,\(^1\) Takayuki Endoh,\(^1\) Toshio Omori,\(^2\) Hideaki Nojiri,\(^1\) Hisakazu Yamane,\(^1\) and Hiroshi Habe\(^3\)*

Biotechnology Research Center, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan\(^1\); Department of Industrial Chemistry, Faculty of Engineering, Shibaura Institute of Technology, 3-9-14 Shibaura, Minato-ku, Tokyo 108-8548, Japan\(^2\); and National Institute of Advanced Industrial Science and Technology (AIST), Central 5-2, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, Japan\(^3\)

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*Pseudomonas putida* DS1 is able to utilize dimethyl sulfone as a sulfur source. Expression of the *sfnFG* operon responsible for dimethyl sulfone oxygenation is directly regulated by a \(\alpha\)-dependent transcriptional activator, SfnR, which is encoded within the *sfnECR* operon. We investigated the transcription mechanism for the sulfate starvation-induced expression of these *sfn* operons. Using an in vivo transcription assay and in vitro DNA-binding experiments, we revealed that SfnR negatively regulates the expression of *sfnECR* by binding to the downstream region of the transcription start point. Additionally, we demonstrated that a LysR-type transcriptional regulator, CysB, directly activates the expression of *sfnECR* by binding to its upstream region. CysB is a master regulator that controls the sulfate starvation response of the *sfn* operons, as is the case for the sulfonate utilization genes of *Escherichia coli*, although CysB\(_{DS1}\) appeared to differ from that of *E. coli* CysB in terms of the effect of O-acetylseryserine on DNA-binding ability. Furthermore, we investigated what effector molecules repress the expression of *sfnFG* and *sfnECR* in vivo by using the disruptants of the sulfate assimilatory genes *cysNC* and *cysI*. The measurements of mRNA levels of the *sfn* operons in these gene disruptants suggested that the expression of *sfnFG* is repressed by sulfate itself while the expression of *sfnECR* is repressed by the downstream metabolites in the sulfate assimilatory pathway, such as sulfide and cysteine. These results indicate that SfnR plays a role independent of CysB in the sulfate starvation-induced expression of the *sfn* operons.

Sulfur, an essential element for bacterial growth, is assimilated from a range of sources. The preferred sulfur source for bacteria is inorganic sulfate, but in soil environments, sulfate may not be freely available because inorganic sulfate constitutes less than 5% of the total sulfur (1). The remainder is largely in the form of sulfate esters and carbon-bound sulfur such as peptides/amino acids and sulfonates (1). Volatile organic sulfur compounds, for example, dimethyl sulfide (DMS) and methanethiol, are also widely distributed in the environment because they are emitted from oceans (2), freshwater sediments (28, 29), and soils (26, 31). Thus, several soil bacterial species have developed a number of systems that allow the use of these organosulfur compounds.

Metabolic use of these organosulfur compounds, especially alkanesulfonates, taurine, and alkanesulfate ester, has been extensively investigated in *Escherichia coli* and *Pseudomonas* spp. (reviewed in reference 19). Bacterial organosulfur assimilation is mediated by the sulfate starvation response, which involves a set of proteins synthesized by several species of bacteria when the supply of their preferred sulfur sources, such as inorganic sulfate, cysteine, and thiosulfate, is limited. These proteins are known as sulfate starvation-induced (SSI) proteins and include enzymes and transport systems for scavenging sulfur from organic compounds (19).

Transcriptional regulation of the SSI genes has been investigated extensively in *E. coli*, but knowledge is limited to the two well-characterized operons involved in sulfonate and taurine utilization, i.e., *ssuEADCB* and *tauABCD*, respectively (reviewed in reference 38). In *E. coli*, a LysR-type transcriptional regulator, CysB, which is highly conserved among gram-negative bacteria, plays a key role not only in the expression of the cys genes responsible for sulfate assimilation but also in that of the SSI genes. Full expression of the CysB-regulated genes requires a signal for sulfur limitation provided by an internal inducer molecule, N-acetylseryserine or O-acetylseryserine (34). Additionally, sulfide and thiosulfate act as anti-inducers by inhibiting the binding of CysB protein to cys promoters (12, 35). A LysR-type transcriptional regulatory gene, *cbl*, one such CysB-regulated gene, is also required for the expression of *ssu* and *tau* operons (15). Under sulfate starvation conditions, Cbl binds to upstream regions of the *ssu* and *tau* operons to activate their transcription. Under sulfate-rich conditions, however, adenosine-5′-phosphosulfate (APS), an intermediate in sulfate assimilation, functions as a core-
pressor of Cbl and inhibits Cbl DNA binding, resulting in a lack of ssu and tau operon expression (4). In contrast, regulation of SSI gene expression in soil bacteria such as pseudomonads is believed to be quite different from that in E. coli because no equivalent to the cbl gene exists in the pseudomonad genome (Pseudomonas Genome Project [www.pseudomonas.com]). Some specific regulators have been reported to be involved in the regulation of the respective organosulfur assimilatory genes (5, 18, 40); however, it remains unknown how pseudomonads perceive sulfate starvation conditions and subsequently control the expression of a variety of organosulfur-assimilating pathways.

In contrast to alkanesulfonates and taurine, little is known about how bacteria scavenge sulfur from volatile organosulfur compounds such as organic sulfides and thiols. Pseudomonas putida DS1 is capable of utilizing DMS as a sulfur source (10), and the DMS metabolic pathway was investigated at the molecular level. DS1 desulfurizes DMS to sulfite via dimethyl sulfoxide, dimethyl sulfone (DMSO₂), methanesulfonate (MSA), and sulfite (Fig. 1A). The desulfonation of MSA requires an FMNH₂-dependent monooxygenase, SsuD, which is conserved among many gram-negative and gram-positive bacteria (10). However, we found that the conversion of DMSO₂ to MSA requires a novel FMNH₂-dependent monooxygenase, SfnG, which is encoded within the sfnFG operon (8). Expression of the sfnFG operon is directly regulated by a novel σ₅₄-dependent transcriptional regulator, SfnR, which is encoded within the sfnECR operon (Fig. 1B) (8, 9). SfnR also activates the expression of the sfnAB operon, and SfnD is suggested to be involved in the use of methanethiol as a sulfur source (11). Expression of the sfnECR, sfnFG, and sfnAB operons is activated under sulfate starvation conditions.

The finding of SfnR implied that sulfone utilization of DS1 is controlled by a novel mechanism for the sulfate starvation response because all of the other known transcriptional regulators of gram-negative bacteria involved in organosulfur metabolism belong to the LysR family, e.g., Cbl and SdsB (5), AsfR (40), SftR (18), and SsuR (16). More interestingly, SfnR lacks an N-terminal regulatory domain, unlike many other σ₅₄-dependent transcriptional regulators (8, 9). Therefore, determining the role that SfnR plays in the sulfate starvation response would be of great interest. However, the transcription mechanism of the sfnR gene itself remains unclear. We previously demonstrated that CysB is necessary for the expression of the sfnECR operon (9), but it remains to be elucidated whether CysB directly activates the expression of the sfnECR operon by binding at its upstream region. Hence, in this study, we first investigated the detailed mechanism of the transcriptional regulation of the sfnECR operon.
TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
| **Bacterial strains** | | |
| Escherichia coli | | |
| DH5α | supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | Laboratory collection |
| SR17-1pir | hsdR thi pro recA1; RP4 integrated into the chromosome (kan::Tn7 tet::Mu) pir | 6 |
| **Pseudomonas putida** | | |
| DS1 | Wild-type, DMS-utilizing strain | 10 |
| SRK1 | Derivative of DS1 containing sfnR::kan, DMSO utilization deficiency | 8 |
| ΔcysNC | Derivative of DS1 containing cysNC::kan, sulfate utilization deficiency | This study |
| ΔcysI | Derivative of DS1 containing cysI::kan, sulfite utilization deficiency | This study |

| Plasmids | | |
| pMElacZ | pME4510 derivative, lacZ Gm’ | 9 |
| pMEsfn-lacZ | pMElacZ with 1.9-kb EcoRV-HindIII fragment containing sfnE and its promoter region | 9 |
| pMEsfnE-500 + 192 | pMElacZ containing the sfnE region from −500 to +192 | This study |
| pMEsfnE-500 + 142 | pMElacZ containing the sfnE region from −500 to +142 | This study |
| pMEsfnE-500 + 100 | pMElacZ containing the sfnE region from −500 to +100 | This study |
| pMEsfnE-500 + 60 | pMElacZ containing the sfnE region from −500 to +60 | This study |
| pMEsfnE-500 + 20 | pMElacZ containing the sfnE region from −500 to +20 | This study |
| pMEsfnE-137 | pMElacZ containing the sfnE region from −137 to +20 | This study |
| pMEsfnE-104 | pMElacZ containing the sfnE region from −104 to +20 | This study |
| pMEsfnE-80 | pMElacZ containing the sfnE region from −80 to +20 | This study |
| pMEsfnE-63 | pMElacZ containing the sfnE region from −63 to +20 | This study |
| pMEsfnE-45 | pMElacZ containing the sfnE region from −45 to +20 | This study |
| pET26b | Expression vector, pT7 promoter | Novagen |
| pETsfnR | pET26b containing N-ht-sfnR | This study |
| pET-C-ht-cysB | pET26b containing C-ht-cysB | This study |
| pBBR1MCS-5 | Broad-host-range vector; Gm’ | 22 |
| pBBR-C-ht-cysB | pBBR1MCS-5 containing C-ht-cysB | This study |
| pG18mobsacB | pk18mobsacB derivative, Km’ | 21 |
| pG18cysNC::Km | pG18mobsacB containing ΔcysNC::kan | This study |
| pG18cysL::Km | pG18mobsacB containing ΔcysNC::kan | This study |

**MATERIALS AND METHODS**

**Chemicals.** The chemicals used in this study were of the highest purity commercially available (Kanto Chemical, Wako Pure Chemical, Tokyo Kasei Kogyo, and Nacalai Tesque). O-Acetyl-L-serine hydrochloride was purchased from Tokyo Kasei Kogyo and used for the electrophoretic mobility shift assay (EMSA).

**Bacterial strains, plasmids, and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. The *P. putida* strains were cultivated at 30°C in Luria-Bertani (LB) medium or sulfur-free mineral medium (SFMM) (33) supplemented with 1 mM of each sulfur source. *Pseudomonas putida* DH5α was grown at 37°C in LB medium. For the overproduction of a protein, *E. coli* BL21(DE3) was grown in 2X yeast extract-triptone medium at 37°C, and isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.3 mM (for C-terminally histidine-tagged CysB production) or 1 mM (for N-terminally histidine-tagged SnfR production) at a turbidity of 0.60 nm of 0.4 to 0.8. The culture was further grown at 25°C for 12 h. Antibiotics were added to the following final concentrations: 100 µg ml⁻¹ ampicillin, 15 µg ml⁻¹ gentamicin, 50 µg ml⁻¹ kanamycin, 10 µg ml⁻¹ tetracycline, and 30 µg ml⁻¹ chloramphenicol. When necessary, kanamycin and gentamicin chloride were prepared from their sulfate salts by ion exchange, as previously described (8).

**DNA manipulation.** Plasmid isolation, restriction enzyme digestion, and transformation of *E. coli* were performed as described elsewhere (36). *Pseudomonas putida* was transformed by electroporation as described previously (8).

**RNA preparation.** Total RNA from the DS1 derivatives was extracted using a NucleoSpin RNA II Kit (Macherey-Nagel) according to the manufacturer’s instructions. The RNA was treated with RNase-free DNase (Promega) and then purified with the NucleoSpin RNA Clean-up Kit (Macherey-Nagel).

**Primer extension analysis.** The total RNA (2.4 µg), extracted from DS1 or its disruptant (8) that harbored pMEsfn-lacZ (Table 1) grown on SFMM with 1 mM sulfate or MSA until the log phase was reached, was subjected to a reverse transcription (RT) reaction with SuperScript III reverse transcriptase (Invitrogen) and an IRD800-labeled primer (Aloka), sfnE-P1 (see Table S1 in the supplemental material). The primer extension products were purified using phenol-chloroform extraction and ethanol precipitation. The products were subjected to electrophoresis together with a sequence reaction using the same primer and a Li-Cor model 4200L-2 Auto-DNA sequencer running Base ImagIR data collection software version 4.0 (Li-Cor) according to the manufacturer’s instructions.

**Construction of plasmids.** To construct lacZ transcriptional fusion plasmids pMEsfnE-500 + 20, pMEsfnE-500 + 60, pMEsfnE-500 + 100, pMEsfnE-500 + 142, and pMEsfnE-500 + 192, the regions upstream of sfnE were amplified from linearized pMEsfnE-lacZ (Table 1) with a forward primer, sfnE-FW-100, and a series of reverse primers (sfnE-RV + 20 through sfnE-RV + 192) (see Table S1 in the supplemental material). To construct plasmids pMEsfnE-157, pMEsfnE-104, pMEsfnE-80, pMEsfnE-63, and pMEsfnE-45, a reverse primer, sfnE-RV + 20, and a series of forward primers (sfnE-FW-137 through sfnE-FW-45) (see Table S1 in the supplemental material) were used. Each amplified fragment was cloned into a pT7Blue (R) vector (Novagen), and the nucleotide sequences of each reporter plasmid were confirmed. Clones were digested with both HindIII and EcoRI (sites incorporated in the primers) (see Table S1 in the supplemental material), and the fragments were then cloned between the HindIII and EcoRI sites of pMElacZ (9).

A plasmid, pET-C-ht-cysB, for the production of the C-terminally histidine-tagged CysB (C-ht-CysB) protein was constructed as follows. We obtained the complete nucleotide sequence of the cysB gene of DS1 from the mini-Tn5 flanking DNA regions of cysH::mini-Tn5 mutants isolated previously (21) because cysB of DS1 lies adjacent to the cysH gene. The DNA fragment containing C-ht-cysB was amplified from total DNA of DS1 with the set of primers cysB-FW and C-ht-cysB (see Table S1 in the supplemental material). The PCR product was cloned into a pT7Blue (R) vector (Novagen), and the nucleotide sequence was confirmed. Clones were digested with both NdeI and EcoRI (sites incorporated in the primers) (see Table S1 in the supplemental material), and the
fragment was then cloned between the corresponding sites of pET26b (Novagen). To construct plasmid pBBR-C-ht-cysB, the XbaI-Sacl fragment containing C-ht-cysB from pET-C-ht-cysB was cloned between the corresponding sites of pBBRIMCS-5 (Table 1).

To construct plasmids pG18cysNC::Km and pG18cysI::Km, a DNA fragment was first generated consisting of the Km cassette flanked by two homology regions with the surrounding sequence of cysNC or the internal sequence of cysI by using a two-step PCR according to a method described previously (21). The nucleotide sequence of the surrounding region of cysNC was obtained from several mini-Tn5-flanking DNA regions of cysNC::mini-Tn5 mutants isolated previously (21). The internal nucleotide sequence of cysI was obtained by sequencing the PCR fragment, which was amplified from total DNA of DS1 with primers KT-ctf-F and KT-ctf-R (see Table S1 in the supplemental material) and designed from the cysI sequence of P. putida KT2440 whose whole genome sequence is available. In the first PCR, about 500 bp of the 5′ and 3′ regions were amplified, which partially overlapped with the Km cassette, using total DNA from DS1 and the primers described below: the 5′ region was amplified using primers cysNC-UpF/cysNC-UpR-Km and cysI-Upf/Cysf-UpR-Km, and the 3′ region was amplified using primers cysNC-DnF-Km/cysNC-DnR or cysI-DnF-Km/cysI-DnR (see Table S1 in the supplemental material). Simultaneously, the Km cassette was amplified as described previously (21). The resultant three PCR products were then assembled during the second PCR. The assembled DNA fragments were ligated into the SmaI site of pG18mobsacB (Table 1) to yield plasmids pG18cysNC::Km and pG18cysI::Km.

β-Galactosidase reporter assay. The P. putida strain harboring a reporter plasmid was grown on SFMM containing 1 mM sulfate or 1 mM MSA until the stationary phase at 30°C. A LacZ assay was performed with the bacterial cultures according to the method of Miller (30).

Purification of proteins. Escherichia coli BL21(DE3) (pETsfnR) or BL21(DE3) (pET-C-ht-cysB) cells producing N-histCysB or C-ht-CysB were collected from 4 ml of culture and suspended into 0.5 ml of IMAC wash buffer (Bio-Nobile). The cell suspension was subjected to ultrasonication with a Branson Sonifier 250D. The cell extract was centrifuged at 10,000 × g for 5 min to remove cell debris. The supernatant containing N-histCysB or C-ht-CysB was subjected to metal chelation chromatography with a QuickPick IMAC (Bio-Nobile) metal affinity kit according to the manufacturer’s instructions. The protein samples eluted by the QuickPick IMAC system were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and stored at 4°C for 48 h. Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad).

EMSA. EMSA was performed with a DIG Gel Shift kit, second generation (Roche Applied Science), according to the manufacturer’s instructions. The EMSA with N-histSnfr was performed as reported previously (8). Probes PhED, PhEDM1, PhEDM2, and PhEDM3 were generated by PCR with the sets of primers listed in Table S1 in the supplemental material and labeled with digoxigenin-11-dUTP at the 3′ termini by terminal transferase. For EMSA with C-ht-CysB, the DNA fragment containing the region from −137 to +20 relative to the transcription start point was generated by PCR with a set of primers sfnECR-F and sfnECR-R (see Table S1 in the supplemental material) and labeled as described above. DNA-binding reactions were performed as described previously (8). The reaction mixtures were incubated for 15 min at 15°C to 25°C. After addition of 5 μl of loading buffer to the mixture, 10-μl aliquots were subjected to electrophoresis (150 V, 45 min, 15 to 25°C) on 6% polyacrylamide gels and Tris-borate-EDTA buffer. Probes in the polyacrylamide gels were visualized with an ImageQuant LAS-4000 (Amersham Biosciences) membranes and then detected with a CSPD system (Roche Applied Science).

DNAase I footprinting. DNAase I footprinting was performed according to a method described previously (8). Briefly, the 245-bp DNA fragments between −189 and +59 relative to the transcriptional start site of sfnE were amplified by PCR with the appropriate pairs of primers (5′-biotin-labeled sfnE-186-FW/1RSDR000-labeled sfnE + 59-RV and 1RSDR000-labeled sfnE-189-RF/5′-biotin-labeled sfnE + 59-RV) (see Table S1 in the supplemental material) and used as probes. The probes binding to C-ht-CysB were digested with 3 units of DNase I by using a two-step PCR according to a method described previously (21). The resultant three PCR products were then assembled during the second PCR. The assembled DNA fragments were ligated into the SmaI site of pG18mobsacB (Table 1) to yield plasmids pG18cysNC::Km and pG18cysI::Km.

RESULTS

Determination of the transcription start point of the sfnECR operon. To characterize the sfnECR promoter, we first investigated the transcription start point of the sfnECR operon. Because we had found a σ54-dependent promoter-like sequence upstream of the sfnE translation start point, we first believed that the expression of sfnECR was autoregulated by SfnR. However, in our previous study, the expression of sfnECR was observed in an sfnR-defective mutant (D6749) (9). Therefore, we postulated that the expression of sfnECR is controlled by another σ54-dependent transcriptional regulator or that additional promoters exist in the upstream region of sfnECR. To test these hypotheses, we carried out primer extension analysis using total RNA from the sfnR disruptant (SRK1) (8) as well as from the wild-type DS1. As a result, one transcription start point was found at 132 bp upstream of this σ54-dependent promoter-like sequence (Fig. 2), indicating that this sequence does not function as a promoter. Instead, a σ70-promoter-like sequence was located upstream of the transcription start point. The −10 region corresponding to this start point is 5′-TATAAA-3′ (position −13 to −8); the −35 region would lie somewhere within the sequence 5′-TTTTTC AT-3′ (position −36 to −29). Interestingly, the amount of the primer extension product from the sfnR disruptant was much larger than that from the wild type, suggesting that SfnR represses the expression of sfnECR containing its own gene. An SfnR-binding motif, which is conserved upstream of sfnFG and sfnAB (5′-CTGTN10ACG-3′; consensus sequence), was also found downstream of the sfnECR transcriptional start point (5′-CTGTN10ACG-3′; position +28 to +45). These results suggest that SfnR represses the expression of sfnECR by binding to this motif.

Involvement of the SfnR-binding motif in repression of sfnECR. To investigate involvement of the SfnR-binding motif located downstream of the transcription start point in the negative regulation of sfnECR, we performed deletion analysis of the DNA region containing this motif using lacZ as a reporter gene. A 3′-deletion series of the DNA region downstream of the sfnECR transcription start point was constructed (Table 1) and was introduced into P. putida DS1 or SRK1, and the LacZ activity of the resultant transformants was measured under sulfate starvation conditions. In the wild type, LacZ activity...
was increased by deletion of the region containing the SfnR-binding motif (+60 to +21 with respect to the sfnE transcription start point) (Fig. 3). However, in SRK1, deletion of the same region did not have any effect on the induction of LacZ activity. These results strongly suggest that SfnR binds to the region from +60 to +21.

To confirm the binding of SfnR to this DNA region, we carried out an EMSA using purified N-terminally histidine-tagged SfnR (N-ht-SfnR) and a 40-bp probe from +60 to +21 relative to the sfnE transcription start point (PbED) (Fig. 4A).

An sfnF promoter probe, Pb10 (8) (Fig. 4A), was used as a positive control. As reported previously (8), Pb10 exhibited two shifted bands (Fig. 4B) because it contains two partially overlapping imperfect inverted repeat sequence (IIRSs; IR1 and IR2, Fig. 4A) for the binding of SfnR. Probe PbED also exhibited two shifted bands (Fig. 4B), suggesting that it contains two SfnR-binding sites in this region. However, the amounts of complex II of PbED were smaller than those of Pb10. In the sequences between Pb10 and PbED, IR1 was highly conserved, but IR2 was not clearly conserved (Fig. 4A). Therefore, we thought that binding of SfnR to IR2 was weaker in PbED than in Pb10. To investigate the involvement of these IIRSs in the binding of SfnR, we performed EMSA with probes mutated in IR1 and/or IR2 (PbEDM1, PbED2, and PbEDM3) (Fig. 4A). PbEDM2 with a mutation in IR2 gave rise to only one shifted band and did not form complex II (Fig. 4B). This result demonstrated that SfnR binds to IR2 in PbED despite the low degree of conservation of IIRS. PbEDM1 with a mutation in IR1 did not produce any shifted bands, even at an N-ht-SfnR concentration of 480 μg/ml. Therefore, we concluded that IR2 alone is insufficient to bind to SfnR in PbED.

Identification of the DNA region required for expression of the sfnE operon. We then carried out a 5′-deletion analysis of the sequence upstream of the sfnE transcription start point, using lacZ as a reporter gene, to determine the minimal region necessary for SSI expression of the sfnE operon. Strain DS1 was transformed by each reporter plasmid (pMEsfnE-137 through pMEsfnE-45) (Fig. 5), and the LacZ activity of the resultant transformant was measured in the presence and absence of sulfate. Deletion from −500 to −138 did not affect the LacZ activities (data not shown). The LacZ activity of pMEsfnE-104, pMEsfnE-80, or pMEsfnE-63 increased in the absence of sulfate, although the LacZ activities appeared to be partially decreased in comparison to that of pMEsfnE-137. However, the LacZ activity of pMEsfnE-45 did not increase in the absence of sulfate and was almost the same as that of pMElacZ (control vector). These results indicate that the DNA region up to 63 bp from the transcription start point of sfnE is necessary for SSI expression of the sfnE operon. In this region, we found an inverted repeat sequence (5′-AAGAATATAGAAATAGAATATAAAA to 3′), at position −57 to −36 (Fig. 2B), which could be involved in transcription activation under sulfate starvation conditions.

Binding of CysB protein to the sfnECR promoter region. We previously demonstrated that CysB of strain DS1 (CysBDS1) is necessary for expression of the sfnE operon by in vivo transcription analysis using the cysB disruptant (9); however, it has remained unknown whether CysBDSS binds directly to the sfnE operon by binding at the region upstream of its promoter. The DNA binding of CysB protein of Salmonella enterica serovar Typhimurium (CysBSTMV) has been well characterized with the cysJIH, cysK, and cysP promoters (12, 32); these studies demonstrated that CysBDSS binds to the AT-rich 40-bp sites just upstream of the −35 regions of the three cys promoters (designated CBS-S, CBS-K, and CBS-P, respectively) in the presence of an inducer, O-acetylserine or N-acetylserine, although these three binding sites showed only a low level of similarity to one another and lacked a strong consensus sequence. As well as these three
binding sites of CysB<sub>ST</sub>, the 40-bp region just upstream of the −35 region of the sfnE promoter is quite AT rich (the A+T content of the region from −74 and −35 is 83%); therefore, we postulated that CysB<sub>DS1</sub> could bind to this promoter region. To test this hypothesis, we performed EMSA with the purified CysB<sub>DS1</sub> protein and the sfnE promoter probe. Because only a partial cysB<sub>DS1</sub> sequence was obtained in our previous study, we first determined the complete cysB<sub>DS1</sub> sequence (GenBank accession no. AB374359) with the method described in Materials and Methods. The cysB<sub>DS1</sub> gene showed 94% nucleotide identity and 100% amino acid identity with cysB of P. putida KT2440 (Pseudomonas Genome Project). For the production of CysB<sub>DS1</sub>, we constructed a plasmid, pET-C-ht-cysB, in which the histidine tag to facilitate the purification was added to the C-terminal region of CysB<sub>DS1</sub> because LysR-type transcriptional regulators contain a DNA-binding domain in their N terminus. C-ht-CysB<sub>DS1</sub> was overproduced with E. coli BL21(DE3)(pET-C-ht-cysB) and was purified in a single step by metal ion affinity chromatography (see Fig. S1 in the supplemental material). The molecular mass of the purified protein was approximately 36 kDa, which is consistent with the deduced molecular mass of C-ht-CysB<sub>DS1</sub>.

We previously reported that the cysB disruptant (ΔCYSB) cannot utilize sulfate as a sulfur source and that the expression level of sfnECR is greatly diminished in ΔCYSB (9). Therefore, we constructed a broad-host-range plasmid that produced C-ht-CysB<sub>DS1</sub>, pBBR-C-ht-cysB (Table 1), and introduced it into ΔCYSB to confirm that C-ht-CysB<sub>DS1</sub> maintains regulatory function in vivo. As a result, ΔCYSB harboring pBBR-C-ht-cysB restored the ability to utilize sulfate (data not shown). We then used quantitative RT-PCR analysis to investigate the expression levels of sfnECR in ΔCYSB (pBBR-C-ht-cysB). As a result, it was shown that ΔCYSB (pBBR-C-ht-cysB) restored the ability to express sfnECR under sulfate starvation conditions (data not shown), indicating that C-ht-CysB<sub>DS1</sub> maintains regulatory function in vivo.

In EMSA with the purified C-ht-CysB<sub>DS1</sub> and the labeled DNA fragment containing the region from −137 to +20 relative to the transcription start point of sfnE, we observed a shifted band, indicating the binding of CysB<sub>DS1</sub> to this region (Fig. 2B). The molecular mass of the complex was approximately 70 kDa, which is consistent with the predicted mass of C-ht-CysB<sub>DS1</sub> (36 kDa) and the DNA fragment (34 kDa). We then performed EMSA with the purified CysB<sub>DS1</sub> and the labeled DNA fragment containing the region from −137 to +20 relative to the transcription start point of sfnE, and we observed a shifted band, indicating the binding of CysB<sub>DS1</sub> to this region (Fig. 2B). The molecular mass of the complex was approximately 70 kDa, which is consistent with the predicted mass of C-ht-CysB<sub>DS1</sub> (36 kDa) and the DNA fragment (34 kDa).
Whether sulfate starvation is sensed only by CysB or whether under sulfate starvation conditions, but it remains unknown, the regulation of the hierarchy in the regulation of sulfate starvation response of the sfn operons indicates that CysB is the master regulator located at the top.

The role of cysteine biosynthetic intermediates for repression of the sfn genes. Expression of the sfn operons is activated under sulfate starvation conditions, but it remains unknown whether sulfate starvation is sensed only by CysB or whether SnfR plays a role independent of CysB in the SSI expression of the sfn operons. To estimate individual functions of CysB and SnfR, we investigated whether the expression of their target genes, sfnECR and sfnFG, respectively, is repressed in mutants that were blocked at different steps in cysteine biosynthesis from sulfate.

In P. putida, cysteine has been reported to be synthesized via the following sequence of reactions (3, 20, 39) (Fig. 1A): conversion of sulfate into APS (catalyzed by the cysD and cysNC gene products), reduction of the sulfate moiety to sulfite (catalyzed by the cysH gene product) and then to sulfide (catalyzed by the cysl and cysJ gene products), and finally, incorporation of the sulfide into O-acetylserine to yield cysteine (catalyzed by the cysM or cysK gene product).

We constructed the cysNC or cysI disruptant (ΔcysNC or ΔcysI, respectively), which was unable to utilize sulfate or sulfite, respectively, as a sulfur source (data not shown). We subsequently measured the expression levels of sfnECR and sfnFG in both disruptants in the presence or absence of sulfate by using quantitative RT-PCR analysis (Fig. 7). As expected, in the wild-type strain DS1, mRNA levels of both sfnECR and sfnFG decreased in the presence of sulfate. In the ΔcysNC strain, the mRNA level of sfnFG decreased even in the absence of sulfate and further decreased by half in the presence of sulfate. This result suggests that the expression of sfnFG is repressed by sulfate itself. The reason for the low level of expression of sfnFG in the absence of sulfate can be explained if it is assumed that a “sulfur-free” mineral medium contains residual amounts of sulfate, as reported by Bykowski et al. (4); in the wild-type strain, residual amounts of sulfate are probably immediately metabolized, while in the ΔcysNC strain, sulfate would accumulate to a level sufficient to prevent the expression of sfnFG.

However, the expression of sfnECR was not repressed in the presence of sulfate in either the ΔcysNC or the ΔcysI strain, suggesting that the expression of sfnECR is repressed by metabolites downstream of sulfite, such as sulfide or cysteine. These results indicate that SnfR plays a role independent of CysB in the SSI expression of the sfn operons. Interestingly, in

![Graph](http://jb.asm.org/Downloaded from April 27, 2019 by guest)
the ΔcysNC strain, both in the presence and in the absence of sulfate, the mRNA level of sfnECR increased three- to fourfold over that of the wild-type strain in the absence of sulfate; this pattern was the opposite of that observed with sfnFG. Moreover, in the ΔcysI strain the mRNA level increased only in the presence of sulfate. These observations suggest that the expression level of sfnECR is increased by the accumulation of sulfate.
expression of *sfnECR* is repressed by metabolites downstream of sulfite, such as sulfide or cysteine. This result will provide an important clue to identify an inducer or corepressor molecule for the repression of *sfnECR* under sulfate-rich conditions. In *S. enterica* serovar Typhimurium, sulfide and thiosulfate were reported to act as anti-inducers for CysB<sub>ST</sub> and inhibit both the transcription initiation and binding of CysB<sub>ST</sub> to the target promoter region (12, 35). In addition, cysteine was reported to have a repressive effect on the expression of *cys* genes because this compound inhibits activity of the serine acetyltransferase enzyme required for synthesis of an inducer, O-acetylserine (25). We postulated that sulfide, thiosulfate, or cysteine acts as a corepressor of CysB<sub>DS1</sub>, and we therefore performed EMSA with C-ht-CysB and the *sfnE* promoter probe in the presence of up to 10 mM of each compound; however, these compounds affected neither the abundance nor the relative mobilities of the protein-DNA complexes (data not shown). Further study is therefore necessary to identify the inducer or corepressor molecules of CysB<sub>DS1</sub>.

Measurement of mRNA levels in the disruptants of sulfate assimilatory genes (Fig. 7) also provided genetic evidence that sulfate itself serves as a signaling molecule for sulfate excess in the transcription regulation of *sfnFG*. The expression of *sfnFG* requires a σ<sup>54</sup>-dependent transcriptional activator, SfnR. Many σ<sup>54</sup>-dependent transcriptional regulators share a common domain architecture that includes three domains: a C-terminal DNA-binding domain, a central module for ATPase activity and interaction with σ<sup>54</sup>-RNA polymerase, and an N-terminal regulatory domain (37). However, as reported previously, SfnR lacks an N-terminal regulatory domain (8, 9); therefore, it is of interest in this context to examine how sulfate is sensed and represses the expression of *sfnFG*. Several studies have reported on σ<sup>54</sup>-dependent transcriptional regulators lacking an N-terminal regulatory domain like SfnR. The best characterized of these is the phage shock protein of *E. coli*, PspF, which controls expression of the *pspABCDE* operon required for survival in stationary phase in an alkaline environment (17). PspF has been reported to activate the *psp* operon without phosphorylation or an activation ligand. Instead, its regulatory function is controlled by formation of a repressive complex with the PspA protein (7). In a similar manner, SfnR may be negatively regulated by its partner protein in the presence of sulfate. As reported in our previous study, the phosphoenolpyruvate-dependent phosphotransferase system (PTS) family protein is also involved in the expression of *sfnFG* (21). Further studies are being considered to examine the relationships among PTS, SfnR, and the recognition of sulfate.

SfnR is also involved in the transcription regulation of *sfnECR* containing its own gene by binding to the region downstream of the *sfnE* transcription start point and interrupting transcription. This type of autoregulation has been described for several genes encoding a prokaryote transcriptional activator. Interestingly, the mRNA level of *sfnECR* appeared to increase following the accumulation of sulfate in ΔcysNC and ΔcysI strains (Fig. 7). Therefore, sulfate likely derepresses the expression of *sfnECR*, probably by inhibiting the DNA binding of SfnR, although the mechanism for the recognition of sulfate remains to be elucidated.

The results obtained in the previous and present studies allow us to propose a mechanism for transcription of the *sfn*
genes as described below. The regulatory system controlled by CysB and SfnR is responsible for the hierarchical utilization of sulfur sources. In the absence of downstream metabolites in the sulfate assimilatory pathway, such as sulfide and cysteine, CysB activates the expression of sfnECR, probably in addition to that of the cys genes responsible for sulfate assimilation. Sulfate itself had no direct effect on the expression, but its conversion to the downstream metabolites resulted in about a 30-fold reduction in the expression levels of sfnECR (see Results). This repressive effect provided by sulfate in strain DS1 appears to be greater than that in E. coli and S. enterica serovar Typhimurium because the expression of cys genes controlled by CysB in these enteric bacteria is only partially repressed in the presence of sulfate (23, 24). When sulfate itself is lacking, in addition to the downstream metabolites, SfnR activates the expression of sfnFG. SfnR also represses sfnECR transcription. This negative autoregulation may contribute to maintaining appropriate levels of sfnR expression. The regulation by SfnR would ensure rapid switching of utilized sulfur sources from DMSO2 to sulfate when sulfate, one of the most preferred sulfur sources, is supplied.

The system for regulation of sfn genes by CysB and SfnR is similar to that for regulation of the ssu genes by CysB and Cbl in E. coli, although in E. coli, APS, which is synthesized in one step from sulfate in the sulfate assimilatory pathway, acts as a signaling molecule for sulfate excess (4). A similar hierarchical regulatory system may also exist for arylsulfatase gene (ataA) expression in Pseudomonas aeruginosa, because at least two independent corepressor molecules are suggested to be involved in the sulfate starvation-inducible expression of ataA, although sulfate itself is not the active corepressor (14). However, as described in the introduction, no equivalent to the cbl gene exists in the pseudomonad genome. Therefore, pseudomonads appear to have developed a different mechanism for a sulfate starvation response from that of E. coli, especially in terms of the recognition of sulfate. In the pseudomonad genome, some orthologs of the sfnR gene are present, for example, PP2771 in P. putida KT2440 (82% amino acid identity), PFL_4158 and PFL_2926 in Pseudomonas fluorescens Pf-5 (79% and 68% amino acid identities, respectively), and PA2354 and PA2359 in P. aeruginosa (79% and 59% amino acid identities, respectively), although all of their functions remain unknown. Therefore, investigating these SfnR homologs form transcriptional networks and are related to the sulfate starvation response in each of these bacteria would be of great interest.

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