A CysB-regulated and $\sigma^{54}$-dependent regulator, SfnR, is essential for dimethyl sulfone metabolism of *Pseudomonas putida* strain DS1

Takayuki Endoh, Hiroshi Habe, Takako Yoshida, Hideaki Nojiri and Toshio Omori

Biotechnology Research Center, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan

*Pseudomonas putida* strain DS1 utilizes dimethyl sulfide (DMS) as a sulfur source, and desulfurizes it via dimethyl sulfoxide (DMSO), dimethyl sulfone (DMSO$_2$) and methanesulfonate (MSA). Its Tn5 mutant, Dfi74J, no longer utilized DMS, DMSO and DMSO$_2$, but could oxidize DMS to DMSO$_2$, suggesting that the conversion of DMSO$_2$ to MSA was interrupted in the mutant. Sequencing of the Tn5 flanking region of Dfi74J demonstrated that a gene, *sfnR* (designated for dimethyl sulfone utilization), encoding a transcriptional regulator containing an ATP-dependent $\sigma^{54}$-association domain and a DNA-binding domain, was disrupted. *sfnR* is part of an operon with two other genes, *sfnE* and *sfnC*, located immediately upstream of *sfnR* and in the same orientation. The genes encode NADH-dependent FMN reductase (*SfnE*) and FMNH$_2$-dependent monoxygenase (*SfnC*). Complementation of Dfi74J with an *sfnR*-expressing plasmid led to restoration of its growth on DMS, DMSO and DMSO$_2$. An *rpoN*-defective mutant of strain DS1, which lacks the $\sigma^{54}$ factor, grew on MSA, but not on DMS, DMSO and DMSO$_2$, indicating that SfnR controls expression of gene(s) involved in DMSO$_2$ metabolism by interaction with $\sigma^{54}$-RNA polymerase. Northern hybridization and a reporter gene assay with an *sfnR–lacZ* transcriptional fusion elucidated that expression of the *sfnECR* operon was induced under sulfate limitation and was dependent on a LysR-type transcriptional regulator, CysB. This is believed to be the first report that a $\sigma^{54}$-dependent transcriptional regulator induced under sulfate limitation is involved in sulfur assimilation.

INTRODUCTION

Dimethyl sulfide (DMS) is a major biogenic sulfur compound generated by degradation of dimethylsulfiniopropionate by marine microalgae and marine bacteria (Charlson et al., 1987); the total annual flux from the ocean to the atmosphere is estimated to range from $0.5 \times 10^{12}$ to $1.2 \times 10^{12}$ mol S per year, which is 50–60 % of the total natural sulfur flux (Welsh, 2000). DMS in the atmosphere is oxidized to dimethyl sulfoxide (DMSO), methanesulfonate (MSA) and sulfate by hydroxyl radicals, and these compounds form precursors of tropospheric aerosols and cloud condensation nuclei in the atmosphere (Charlson et al., 1987; Welsh, 2000). Accordingly, it has been suggested that DMS plays central roles in the Earth’s radiative balance, its climate, and the global sulfur cycle (Charlson et al., 1987; Lovelock et al., 1972).

A variety of bacteria in aquatic sediments and soil have been reported to utilize DMS and/or its related compounds including DMSO and methanethiol as a carbon, energy or sulfur source (de Bont et al., 1981; Smith & Kelly, 1988; Omori et al., 1995; Horinouchi et al., 1997; Fuse et al., 2000; Lomans et al., 2002). Thus, it has been proposed that bacterial conversion of such organosulfur compounds is involved in the global sulfur cycle.

Several enteric and soil bacteria are able to utilize a variety of organosulfur compounds including taurine, MSA, alkane-sulfonates and/or alkanesulfate esters as sulfur sources (Roberts et al., 1955; Uria-Nickelsen et al., 1993; van der Ploeg et al., 1996, 1998, 1999; Hummerjohann et al., 1998; Vermeij et al., 1999; Kertesz, 1999). Recently, the genes involved in utilization of such organosulfur compounds in *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas putida* and *P. aeruginosa* have been reported. The *tauABCD* and *ssuEADCB* operons of *E. coli* are required for taurine and alkanesulfonate utilization, respectively (van der Ploeg et al., 1996, 1999). The expression of *tau* and *ssu* operons is repressed in the presence of preferred sulfur sources such as...
inorganic sulfate, thiosulfate or cysteine, but induced in their absence (van der Ploeg et al., 1997, 1999). This phenomenon is called the sulfate starvation response (SSR; Quadrini et al., 1996), and is controlled by two LysR-type transcriptional regulators, CysB and Cbi, in E. coli (van der Ploeg et al., 1997, 1999; Bykowski et al., 2002). Also, in P. aeruginosa and P. putida S-313, induction of a set of proteins under sulfate starvation was observed (Quadrini et al., 1999; Kahnert et al., 2000). These sulfate-starvation-induced (SSI) proteins enable bacterial cells to adapt to sulfate limitation, e.g. they include enzymes for scavenging sulfur from suboptimal concentrations of organosulfur compounds. Although CysB plays an essential role in organosulfur utilization in P. aeruginosa (Kertesz et al., 1999; Hummerjohann et al., 2000), there are few reports concerning transcriptional regulation of SSI genes in Pseudomonas sp.

P. putida strain DS1, which utilized DMS or DMSO as a sulfur source, was recently isolated (Endoh et al., 2003). When strain DS1 grew on DMS as a sulfur source, DMSO and dimethyl sulfone (DMSO2) accumulated in the culture. However, the accumulation of DMSO and DMSO2 was repressed in the presence of sulfate. This suggests that enzymes involved in DMS oxidation are regulated by SSR (Endoh et al., 2003). DMS-utilization-defective mutants of strain DS1 were obtained by Tn5 mutagenesis. One mutant, Dfi175, no longer utilized DMS, DMSO, DMSO2 and MSA as sulfur sources, and had a deficiency in the ssuEADCBF operon, which encodes an ABC-transporter (SsuABC), a two-component sulfonate sulfonatase system (SsuED) and a small protein SsuF (Endoh et al., 2003). The ssuEADCBF operon has also been reported to be essential in P. putida S-313 for utilization of organosulfur compounds, including sulfonates, sulfate esters and methionine (Kahner et al., 2000). Although the DMS metabolic pathway of P. putida strain DS1 was proposed as shown in Fig. 1, enzymes or proteins involved in the conversion of DMS to MSA remained unclear.

To identify the genes involved in DMS metabolism of strain DS1, we characterized another Tn5-inserted mutant, Dfi741, which grows on MSA, but not on DMS, DMSO or DMSO2 as sulfur sources. We show here that a novel σ54-dependent transcriptional regulator, SfnR, plays an essential role in expression of gene(s) of DMSO2 metabolism of strain DS1. In addition, the expression of the sfnECR operon is regulated by a LysR-type transcriptional regulator, CysB, during sulfate starvation.

**METHODS**

**Bacterial strains, plasmids, media, and culture conditions.** Bacterial strains and plasmids used are listed in Table 1. A physical map of some of the plasmids constructed in this study is shown in Fig. 2. Sulfur-free mineral medium (SFMM) (Omori et al., 1995) and sulfur-free M9 glucose medium (Sambrook et al., 1989) were used as minimal media for the sulfur utilization tests for Pseudomonas strains. DMSO, DMSO2, or MSA (1 mM) was added to sulfur-free medium as the sulfur source; DMSO was used at 0.5% (v/v), 2 × YT medium (Sambrook et al., 1989) was used as a complete medium for Pseudomonas strains and E. coli. Pseudomonas strains and E. coli were grown at 30°C and 37°C, respectively, with reciprocal shaking (300 strokes min−1). Swarms plates (Köhler et al., 1989) were used to check the motility of the rpoN mutant of strain DS1. Kanamycin (Km) sulfate (50 μg ml−1), gentamicin (Gm) sulfate (15 μg ml−1) and ampicillin (Ap) (50 μg ml−1) were used with 2 × YT. Sulfur-free medium was prepared with 50 μg Km chloride ml−1 and 15 μg Gm chloride ml−1 (van der Ploeg et al., 1996).

**DNA manipulation.** Plasmid DNA was prepared from E. coli by the alkaline lysis method (Sambrook et al., 1989). Total DNA from Pseudomonas strains was prepared as described previously (Sato et al., 1997). Restriction endonuclease (Takara Shuzo) and DNA Ligation Kit version 2 (Takara Shuzo) were used according to the manufacturer’s instruction. Transformation of E. coli was done by published procedures (Sambrook et al., 1989). DNA fragments were extracted from the agarose gel by using Concert Rapid Gel Extraction Systems (Invitrogen) according to the manufacturer’s instruction. We used a modified cosmid vector, SuperCos1 LANE0 (Ap′ Km′; Endoh et al., 2003), for construction of cosmids library and Tn5 rescue from mutants. Southern, Northern and colony hybridization analyses were carried out by standard methods (Sambrook et al., 1989), using digoxigenin (DIG)-labelled probes synthesized with a DIG DNA labelling and detection system (Roche Diagnostics) or 32P-labelled probes synthesized with a Megaprime DNA labelling system (Amersham Pharmacia Biotech) and [α-32P]dCTP (110 TBq mmol−1; Amersham Pharmacia Biotech). P. putida strain DS1 was transformed by electroporation in a 0.1 cm cuvette (25 μF, 200 Ω, 17 kV cm−1), using a GenePulserII apparatus (Bio-Rad). PCR was carried out in a GeneAmp PCR System 9700 (PE Biosystems). Standard reaction mixtures consisted of 50 mmol deoxynucleotide triphosphates, 0–2 U LA Taq DNA polymerase (Takara Shuzo), and 1–100 ng template in a final volume of 50 μl.

**DNA sequence analysis.** DNA sequences were analysed by the chain-termination method with a Li-Cor model 4200L-2 Auto-DNA Sequencer and Base ImagIR Data Collection Software 4.0 (Li-Cor) according to the manufacturer’s instructions. Homology searching was performed using the SWISS-PROT amino acid sequence database or the DDBJ/EMBL/GenBank DNA databases with the BLAST program (Altschul et al., 1997).
### Table 1. Bacterial strains, plasmids, cosmids and primers used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid or oligonucleotide</th>
<th>Relevant features</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>c14^- (mcrA^-) recA1 endA1 gyrA96 thi-1 hsdRI7 (rK mK^+) supE44 relA1 Δ(lac–proAB) F’[traD36 proAB^- lacI^o lacZΔM15]</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td>P. putida</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS1</td>
<td>Wild-type, prototroph, utilizes dimethyl sulfide</td>
<td>Endoh et al. (2003)</td>
</tr>
<tr>
<td>D674J</td>
<td>D5, DMS-, DMSO- and DMSO_2-utilization deficiency, sfrR::Tn5</td>
<td>Endoh et al. (2003)</td>
</tr>
<tr>
<td>Δrpon</td>
<td>D5, Δrpon, Km’</td>
<td>This study</td>
</tr>
<tr>
<td>ΔcysB</td>
<td>D5, ΔcysB, Km’, cysteine auxotroph</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pBBR1MCS-5</td>
<td>Broad-host-range cloning vector, Gm’, lacZa mob</td>
<td>Kovach et al. (1995)</td>
</tr>
<tr>
<td>pBBRsfnR</td>
<td>pBBR1MCS-5 with HindIII–SacI fragment containing sfnR</td>
<td>This study</td>
</tr>
<tr>
<td>pBluescriptII SK(−)</td>
<td>Ap’, cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBSrpoN</td>
<td>pBluescriptII SK(−) with rpoN</td>
<td>This study</td>
</tr>
<tr>
<td>pBSsfn02</td>
<td>pBluescriptII SK(−) with EcoRV–SacI fragment containing sfnECR</td>
<td>This study</td>
</tr>
<tr>
<td>pBSacycB</td>
<td>pBluescriptII SK(−) with cysB internal fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pBSacycB-Km</td>
<td>pBSacycB (bla::kan), Ap’ Km’</td>
<td>This study</td>
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<td>pME4510</td>
<td>Broad-host-range cloning vector, Gm’, lacZa</td>
<td>Rist &amp; Kertesz (1998)</td>
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<td>pMElacZ</td>
<td>pME4510 derivative, Gm’, lacZ</td>
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<td>pMElacZ with EcoRV– HindIII fragment containing sfnE and its promoter region</td>
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<td>pSCafr</td>
<td>SuperCos1NEO with sfnECR operon</td>
<td>This study</td>
</tr>
<tr>
<td>pTkm</td>
<td>pT7blue(R) with kan, Ap’ Km’</td>
<td>Yoshida et al. (2003)</td>
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<td>pUC19 with EcoRI fragment containing sfnR::Tn5</td>
<td>Endoh et al. (2003)</td>
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<td>pUCArp0N</td>
<td>pUC19 with rpoN internal fragment</td>
<td>This study</td>
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<td>pUCArp0N-Km</td>
<td>pUCarp0N (bla::kan), Ap’ Km’</td>
<td>This study</td>
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<tr>
<td>pUCsfn01</td>
<td>pUC19 with SphI–SacI fragment containing sfnECR</td>
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<td><strong>Oligonucleotides</strong></td>
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<tr>
<td>Pp-rpoN-FW</td>
<td>5‘-CTCGAGGAATTCTGAAAACCATCGCTCTGCTTAAAAATG2-3’ EcoRI</td>
<td>This study</td>
</tr>
<tr>
<td>Pp-rpoN-RV</td>
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<td>Pp-cysB-int-F</td>
<td>5‘-GAATTCCGACGGCACTGGCCATACGAC-3’ EcoRI</td>
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<td>5‘-GAATTCCGACGGCACTGGCCATACGAC-3’ EcoRI</td>
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<tr>
<td>lac-hindIII-F</td>
<td>5‘-ATAAACAGCTTCAAGGAAAGAAAACATGACTGACTGATC2-3’ HindIII</td>
<td>This study</td>
</tr>
<tr>
<td>lac-sflI-R</td>
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<td>This study</td>
</tr>
<tr>
<td>rt-sfnC-FW</td>
<td>5‘-TGGCGTCGAACGGCACTGGCGCAACCTGG-3’ Sfl</td>
<td>This study</td>
</tr>
<tr>
<td>rt-sfnR-RV</td>
<td>5‘-GCCTCGACCAGGGACCTGGAGAAGCGAC-3’ Sfl</td>
<td>This study</td>
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</tbody>
</table>

**Plasmid construction.** The rpoN gene was amplified from total DNA of strain DS1 by PCR (98 °C for 5 min; followed by 25 cycles of 98 °C for 1 min, 55 °C for 5 s, 74 °C for 2-5 min; then by 74 °C for 5 min) with a set of primers, Pp-rpoN-FW and Pp-rpoN-RV (Table 1) designed based on the 5’- and 3’-terminal ends, respectively, of rpoN from *P. putida* strains. A 1·5 kb amplified product was digested with EcoRI and ligated into pBluescript II SK(−), to produce pBSrpoN (Table 1), and the insert was then sequenced to confirm that rpoN was encoded. Finally, a Sall–SphI internal region of the insert was subcloned into pUC19 to give pUCArp0N (Table 1), and the Km’ gene cassette (kan) from pTkm (Yoshida et al., 2003) was then inserted into the Scal site in the Ap’ gene of pUCArp0N. The resultant plasmid, pUCarp0N-Km, was used for disruption of rpoN in *P. putida* strain DS1.

The internal region of cysB was amplified from total DNA of strain DS1 by PCR (98 °C for 5 min; followed by 25 cycles of 98 °C for 1 min, 55 °C for 5 s, 74 °C for 1-5 min; then by 74 °C for 3 min) with a set of primers, Pp-cysB-int-F and Pp-cysB-int-R (Table 1) designed based on the internal sequence of cysB from *P. aeruginosa* strain PAO1. The resultant 865 bp product was digested with EcoRI and ligated into pBluescript II SK(−) to give pBSacycB. The insert was then sequenced to confirm that it was the internal region of cysB. Finally, kan was ligated into the Scal site in the Ap’ gene of pBSacycB. The resultant plasmid, pBSacycB-Km, was used for disruption of cysB of strain DS1.

For reporter gene assay, a broad-host-range reporter plasmid, pMElacZ, was constructed. The lacZ gene was amplified from pRS551 (Simons et al., 1987) by PCR (98 °C for 5 min; followed by 25 cycles of 98 °C for 1 min, 55 °C for 5 s, 74 °C for 1-5 min; then by 74 °C for 3 min) with a set of primers, Pp-cysB-int-F and Pp-cysB-int-R (Table 1) designed based on the internal sequence of cysB from *P. aeruginosa* strain PAO1. The resultant 865 bp product was digested with EcoRI and ligated into pBluescript II SK(−) to give pBSacycB. The insert was then sequenced to confirm that it was the internal region of cysB. Finally, kan was ligated into the Scal site in the Ap’ gene of pBSacycB. The resultant plasmid, pBSacycB-Km, was used for disruption of cysB of strain DS1.
containing lacZ was replaced by the HindIII-Sfi-digested lacZ gene, to give pMElacZ. A 1.9 kb EcoRV–HindIII fragment of pBSsnf02 containing sfnE and its promoter region was cloned into Smal–HindIII-digested pMElacZ, to produce pMEsnf-lacZ (Fig. 2). pMEsnf-lacZ was used for assay of sfn promoter activity.

**Measurement of growth characteristics.** *Pseudomonas* strains were grown on 1 ml SFMM with 1 mM cysteine (for the cysB mutant), or on 1 ml SF-M9 glucose medium with 1 mM Na$_2$SO$_4$ (for the ropN mutant) at 30°C with reciprocal shaking (300 strokes per minute), and washed three times by suspending into 1 ml of the respective sulfur-free medium. The washed cells were resuspended into 1 ml of the respective sulfur-free medium and washed three times by suspending into 1 ml of the respective sulfur-free medium. The washed cells were resuspended into 1 ml of the respective sulfur-free medium and washed three times by suspending into 1 ml of the respective sulfur-free medium. The washed cells were resuspended into 1 ml of the respective sulfur-free medium and washed three times by suspending into 1 ml of the respective sulfur-free medium.

**RT-PCR.** RT-PCR was carried out by using TakaraOneStep RNA PCR Kit (Takara Shuzo) and TaKaRa One Step RNA PCR System 9700. The reaction mixture (50 μl) contained 5 μl 10-fold One Step RNA PCR Buffer, 50 μM MgCl$_2$, 10 μM dNTP, 40 U RNase inhibitor, 5 U AMV RTase XL, 5 U AMV-Optimized Taq, 20 μM each of rt-sfnC-FW and rt-sfnR-RV (Table 1), and 1 μg total RNA prepared as described above. The reaction mixture was incubated at 50°C for 30 min, then at 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and was finally ramped down to 4°C by a GeneAmp PCR System 9700.

**Measurement of sfn promoter activity.** Strains DS1(pMEsnf-lacZ) and ΔCYSB(pMEsnf-lacZ) were grown at 30°C on a reciprocal shaker in sets of 20 tubes of SF-M9 glucose medium (1 ml) containing 1 mM cysteine. The cells were harvested and washed by suspending in SF-M9 (1 ml), and then resuspended in SF-M9 (1 ml) containing 10 μM cysteine (10 tubes) or 10 μM cysteine plus 1 mM Na$_2$SO$_4$ (10 tubes). The cell suspensions were incubated at 30°C with reciprocal shaking for 1 h (five tubes) and 5 h (five tubes). Samples (100 μl) of each suspension were subjected to a β-galactosidase assay with n-nitrophenol β-D-galactopyranoside as substrate (Miller, 1992).

**Analytical methods.** DMS, DMSO and DMSO$_2$ accumulated in whole-cell reaction mixtures were detected with a gas chromatograph GC-14B (Shimadzu) fitted with a fused-silica chemically bonded capillary column DB-5 (0.25 mm inside diameter by 30 m, 1 μm film thickness; J&W Scientific). The head pressure of the helium carrier gas was 100 kPa. Each sample was injected into the column at 100°C. After 2 min at 100°C, the column temperature was increased to 200°C at 10°C min$^{-1}$. The peaks of DMS, DMSO and DMSO$_2$ were detected at 2:1 min, 4:8 min and 5:4 min, respectively.

**Chemicals.** Chemicals were of the highest quality available and were purchased from Tokyo Kasei Kogyo or Kanto Chemical.

**RESULTS**

A metabolic step from DMSO$_2$ to MSA is interrupted in DMS-utilization-defective mutant Dfi74J

A DMS-utilization-defective mutant Dfi74J of *P. putida* strain DS1 utilized MSA, but not DMSO or DMSO$_2$, as a sulfur source (Endoh et al., 2003). To determine the disrupted DMS metabolic step of Dfi74J, the capability of this strain to oxidize DMS to DMSO$_2$ was examined by two different whole-cell biotransformation experiments. First, Dfi74J was grown in SFMM containing 1 mM MSA and 0.5% DMS, then 1 μl supernatant of the culture was subjected to GC analysis. MSA was chosen as a sulfur source because it does not repress the SSI DMS oxidation of strain DS1. Two intense peaks with retention times of 2:1 min and 4:8 min, which were identical with those of authentic DMS and DMSO, respectively, were observed (data not shown). Dfi74J cells grown on MSA were suspended in 1 ml SFMM containing 1 mM DMSO, and the resultant cell suspension was incubated at 30°C with reciprocal shaking for 8 h. Then, 1 μl of the supernatant of the cell suspension was analysed by GC. Two intense peaks with retention times of 4:8 min and 5:4 min, which corresponded to those of authentic DMS and DMSO, were observed (data not shown). Therefore, it was demonstrated that Dfi74J still had the capability to convert DMS to DMSO$_2$ under sulfate limitation, suggesting that the metabolic step from DMSO$_2$ to MSA is interrupted in Dfi74J.

**Cloning and sequencing analysis of the Tn5-inserted region in Dfi74J**

To identify the locus disrupted by Tn5 in Dfi74J, the region flanking the Tn5 insertion was cloned by transposon rescue techniques and sequenced. Sequencing of a 3.3 kb SphI–SacI fragment (Fig. 2) revealed that there were three ORFs (these were designated as sfnE, sfnC and sfnR, for dimethyl sulfone utilization), and that Tn5 was inserted at 166 bp upstream from the 3′-terminus of sfnR (Fig. 2). The 2.9 kb region
downstream from the SacI site was also cloned and sequenced. An ORF showing identity with glutathione S-transferase was located approximately 1.2 kb downstream of sfnR in the opposite direction (data not shown), but no ORFs were found immediately downstream of sfnR. All sfn genes were preceded by good consensus ribosome-binding sites (data not shown). The overall G+C content of the coding region was 65.2 mol%. The initial codons of sfnC and sfnR were located immediately downstream from the stop codons of the preceding ORFs (data not shown), indicating that the sfn gene cluster is probably transcribed as a transcriptional unit. By comparison with the E. coli (Reiter & Schneider, 2001), a signature of a 3′-CGGACAGCATGCT-3′; underlined sequences are well conserved) was found 42 bp upstream from the initial codon of sfnE. The deduced amino acid sequence of SfnE showed the highest identity (64% amino acid identity) with MsuE from P. aeruginosa, which was reported to encode an NADH-dependent FMN reductase (Kertesz et al., 1999). SfnC is a 395 amino acid protein showing the highest identity (70%) with MsuC (PA2355) from P. aeruginosa PAO1, whose function has not been fully elucidated (Kertesz et al., 1999; Stover et al., 2000; http://www.pseudomonas.com). However, SfnC also showed identity with two bacterial FMNH2-dependent monoxygenases: TdsC (47%) from Paenibacillus sp. strain A11-2 (Ishii et al., 2000) and DsZC (43%) from Rhodococcus sp. strain IGTS8 (Demone et al., 1994), both of which catalyse stepwise monooxygenation of dibenzothiophene to dibenzothiophene sulfone.

SfnR exhibited identity with putative transcriptional regulators, PA2354 (78%) and PA2359 (60%), from P. aeruginosa PAO1 (Stover et al., 2000; http://www.pseudomonas.com). pa2354 is located immediately downstream of msuC (Stover et al., 2000; http://www.pseudomonas.com), although it has not been confirmed that pa2354 is organized in an operon with the msuEDC operon of PAO1 (Kertesz et al., 1999). Moreover, SfnR also showed identity with several bacterial NtrC-type response regulators such as NtrC (37%) from Agrobacterium tumefaciens (accession no. I39719; Wardhan et al., 1989), HydG (36%) from Salmonella typhimurium (accession no. S19606; Chopra et al., 1991), AtoC (36%) from E. coli (accession no. B64992; Canellakis et al., 1993), and TacA (34%) from Caulobacter crescentus (accession no. AAC45640; Marques et al., 1997). The sequence alignment of SfnR with these response regulators demonstrated that SfnR had the central ATP-dependent 54 factor (RpoN) interaction domain and the C-terminal helix–turn–helix DNA-binding domain. However, SfnR was approximately 100 amino acids shorter than these response regulators, and did not have the N-terminal phospho-receiver domain, which is specific to the NtrC-type response regulators (data not shown). Therefore, SfnR appeared to participate in transcriptional regulation of unknown gene(s) by coordinating with 54 RNA polymerase.

**Complementation of Dfi74J with sfnR**

Dfi74J was complemented with a plasmid expressing sfnR to confirm that the DMSO2-utilization deficiency was caused by the disruption of sfnR. The 1966 bp HindIII–SacI fragment containing the 3′-half of sfnC and the entire sfnR gene (Fig. 2) was cloned into pBBR1MCS-5 (Kovach et al., 1995), to produce pBBRsfnR. Since the lac promoter is expressed constitutively in Pseudomonas species (Rist & Kertesz, 1998), pBBRsfnR should express sfnR in Dfi74J. pBBRsfnR was introduced into Dfi74J by electroporation. pBBRsfnR restored the growth of Dfi74J on DMS, DMSO or DMSO2 (Table 2), indicating that SfnR was essential for DMSO2 metabolism of strain DS1.

**RpoN is involved in the DMSO2 metabolism of strain DS1**

As far as we know there have been no reports of the 54 factor being involved in sulfur assimilation in Gram-negative bacteria. However, the 54 factor interaction domain conserved in SfnR implied involvement of 54 RNA polymerase in the DMSO2 assimilation by strain DS1. To obtain direct evidence, a mutant defective in rpoN was constructed and its sulfur utilization phenotype was determined.

pUCArpON-Km containing the 873 bp Sall–Spfl internal fragment of rpoN was constructed (see Methods). This plasmid was introduced into strain DS1 by electroporation, and a mutant ΔRPN (Km’) was obtained (Fig. 3a). Disruption of rpoN of ΔRPN was confirmed by PCR (Fig. 3b). Mutant ΔRPN formed much smaller colonies on 2 × YT plates than strain DS1 did, and the loss of motility was confirmed on swarm plates (data not shown). Also, ΔRPN could utilize glucose or ammonium chloride, but not succinate or nitrate, as carbon or nitrogen sources (data not shown). These phenotypic features of ΔRPN were in

**Table 2. Sulfur utilization phenotype of the mutant strains**

<table>
<thead>
<tr>
<th>Sulfur source</th>
<th>Growth* (OD550)</th>
<th>ΔRPN (ΔrpoN)</th>
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<tbody>
<tr>
<td>DMS</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>DMSO</td>
<td>++</td>
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</tr>
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*−, OD550 <0.5; +, 0.5 ≤OD550 <1.0; ++, 1.0 ≤OD550; NT, Not tested.
accordance with those of a *P. putida* *rpoN* mutant reported previously (Kühlter *et al.*, 1989). The ΔRPON mutant could utilize MSA, but not DMS, DMSO or DMSO2, as a sulfur source (Table 2). The sulfur utilization selectivity of ΔRPON was the same as that of Dfi74J, suggesting that both SfnR and σ54-RNA polymerase are involved in transcription of gene(s) for DMSO2 metabolism.

**Transcription of the sfnECR operon is induced under sulfate-limited conditions**

Kertesz *et al.* (1999) reported that the *msuEDC* operon of *P. aeruginosa* PAO1 is induced during growth with organosulfur sources such as sulfonates and sulfate esters, but repressed in the presence of sulfate. Since sfn genes and their organization are similar to those of *msu* genes, sfnR is probably expressed under sulfate imitation with sfnEC. The transcriptional regulation and the operon structure of sfnECR gene cluster were therefore investigated by Northern hybridization and RT-PCR.

Total RNAs were prepared from DS1 cells growing exponentially (0.3 < OD<sub>550</sub> < 0.7) in SFMM containing DMSO, Na<sub>2</sub>SO<sub>4</sub>, or DMSO plus Na<sub>2</sub>SO<sub>4</sub>. The total RNAs (10 µg) were subjected to Northern hybridization with probes specific to sfnC or sfnR (Fig. 2). An approximately 3.0 kb band hybridizing with an sfnC or sfnR probe was detected only for total RNA from DMSO-grown cells; the size corresponded to the length of the sfnECR-coding region (Fig. 4a). To confirm the co-transcription of sfnR and sfnC genes, RT-PCR was done using total RNAs from sulfate- or MSA-grown DS1 cells and a set of primers, rt-sfnC-FW and rt-sfnR-RV, designed on the 3'-terminus of sfnC and 5'-terminus of sfnR, respectively (Table 1; Fig. 2). A 400 bp amplified product was observed only in the reaction mixture containing total RNA from MSA-grown cells (Fig. 4b). These findings indicated that the sfnECR gene cluster was organized as an SSI operon, and that conversion of DMSO2 to MSA occurs only under sulfate limitation in strain DS1.

Next, the possibility was examined that the disruption of SfnR affects the transcription of its own sfnECR operon, because the signature of the σ<sup>54</sup>-dependent promoter was located upstream of sfnE. Total RNAs (1 µg) prepared from...
mid-exponential-phase MSA-grown and sulfate-grown Dfi74J cells were subjected to RT-PCR with the primer set shown in Fig. 2. A 400 bp amplified product was observed only in the reaction mixture containing total RNA from Dfi74J cells grown on MSA (Fig. 4b), suggesting that SfnR was not involved in the transcriptional regulation of its own sfn operon.

Expression of the sfnECR operon requires the LysR-type regulator, CysB

It has been suggested that a P. aeruginosa cysB mutant does not utilize MSA probably because of the loss of expression of the msuEDC and ssuEADCBF operons (Kertesz et al., 1999). Also, since the sfnECR operon was induced under sulfate limitation, expression of the sfn operon was suspected to be under the control of CysB.

To examine the involvement of CysB in the expression of the sfn operon, a cysB mutant of strain DS1, ΔCYSB, was constructed by integration of pBSΔcysB-Km (see Methods) into the chromosomal cysB by single-crossover recombination (data not shown). The sfn promoter activity of strain DS1(pMEsfn-lacZ) was then compared with that of ΔCYSB (pMEsfn-lacZ). ΔCYSB could grow on cysteine, but not on sulfate, as a sole sulfur source (data not shown), which was in accordance with the characteristics of the P. aeruginosa cysB mutant reported previously (Kertesz et al., 1999). The β-galactosidase activities of ΔCYSB(pMEsfn-lacZ) were 10-fold and 4.1-fold lower than those of strain DS1 (pMEsfn-lacZ) after 1 h and 5 h under the sulfate-starvation condition, while those of DS1 (pMEsfn-lacZ) and ΔCYSB (pMEsfn-lacZ) were at similar levels in the presence of sulfate (Fig. 5). This suggested that CysB played an important role in the SSI expression of the sfnECR operon.

DISCUSSION

P. putida strain DS1 was known to desulfurize DMS via DMSO, DMSO2 and MSA (Fig. 1), but the genes involved in the conversion of DMS to DMSO2 were previously unclear. In this study, the DMS-, DMSO- and DMSO2-utilization-defective Tn5 mutant D674J was investigated. It was demonstrated that the phenotype of D674J was caused by interruption of the σ54-dependent regulator, SfnR, encoded by the SSI sfnECR operon, and that σ54-RNA polymerase also participated in DMSO2 assimilation. In addition, it was shown that the LysR-type regulator, CysB, activated transcription of the sfnECR operon under sulfate limitation.

The sfnECR operon of strain DS1 showed identity with the msuEDC–pa2354 gene cluster of P. aeruginosa PAO1 at the DNA level (more than 80% similarity). pa2354 is also predicted to be part of an operon with msuEDC and to participate in DMSO2 metabolism of PAO1, because of its ability to grow on DMSO as a sulfur source (Hummerjohann et al., 1998). Also, it is suggested that operons sfnECR and msuEDC–pa2354 come from a common ancestor. However, interestingly, a gene corresponding to msuD, whose product catalyses desulfonation of MSA in P. aeruginosa (Kertesz et al., 1999), was lacking in the sfnECR operon of strain DS1 (Fig. 2). Kertesz et al. (1999) have reported that disruption of msuD does not affect the capacity of P. aeruginosa strain PAO1 to grow on MSA, probably due to the presence of ssuD, whose product shows 72% amino acid identity with MsuD (Kahnert et al., 2000). By contrast, disruption of ssuD in P. putida strains S-313 and DS1 led to loss of the capacity to grow on MSA (Kahnert et al., 2000; Endoh et al., 2003), indicating that ssuEADCBF encodes the sole MSA desulfonation system on P. putida genome. Hence, it is speculated that sulfonate desulfonation systems in P. putida were rearranged in the evolutionary process.

Transcriptional regulators involved in organosulfur assimilation in E. coli or Pseudomonas spp.

So far, several transcriptional regulators involved in expression of SSI organosulfur-assimilating genes have been identified and investigated in E. coli and Pseudomonas spp. In E. coli, two LysR-type transcriptional regulators (CysB and Cbl) control the expression of tauABCD and ssuEADCB under sulfate limitation; these operons are required for taurine and alkanesulfonate utilization, respectively (van der Ploeg et al., 1996, 1999; Bykowski et al., 2002). Although cysB plays an essential role in organosulfur assimilation in P. aeruginosa (Kertesz et al., 1999), a gene corresponding to the E. coli cbl was not found in the P. aeruginosa genome (Stover et al., 2000; http://www.pseudomonas.com). This implies that the SSI genes of Pseudomonas sp. are regulated differently from those of E. coli. Alternatively, three genes, sdsB, asfR and sftR, encoding LysR-type transcriptional regulators have been identified in Pseudomonas sp. (Davison et al., 1992; Vermeij et al., 1999; Kahnert et al., 2002). SdsB has been reported to activate expression of sdsA, whose
product catalyses desulfation of SDS in *Pseudomonas* sp. ATCC 19151 (Davison et al., 1992). AsfR modulates transcription of the *asfABC* operon, whose products (AsfAB but not AsfC) are required for arylsulfonate utilization of *P. putida* S-313 (Vermeij et al., 1999). SfrR activates expression of a gene cluster, *atsBC*, *atsRK* and *sftB–astA*, whose products play an essential role in utilization of aryl- or alkylsulfate esters in *P. putida* S-313 (Kahnert et al., 2002). Accordingly, SfnR identified in strain DS1 is the fourth transcriptional regulator involved in organosulfur assimilation in *Pseudomonas* sp. However, unlike these LysR-type regulators, SfnR is related to NtrC-type transcriptional regulators, suggesting that the mechanism of transcriptional activation is quite different from that of LysR-type regulators. LysR-type regulators usually activate the expression of target genes by interacting with $\sigma^{54}$-RNA polymerase, whereas NtrC-type regulators employ $\sigma^{34}$-RNA polymerase (Rombel et al., 1998; Ramos et al., 1997). To our knowledge this is the first report that a $\sigma^{54}$-dependent transcriptional regulator is involved in sulfur assimilation.

**Relationship between $\sigma^{54}$ and sulfur assimilation**

The $\sigma^{54}$ factor has been generally considered as a sigma factor for nitrogen metabolism in *E. coli*, whereas *P. putida* $\sigma^{54}$-RNA polymerase controls a number of disparate functions including nitrogen metabolism, growth on some carbon sources (Köhler et al., 1989), polar flagellar synthesis (Pandza et al., 2000), and degradation of hydrocarbons such as *m*-xylene (Ramos et al., 1997) and phenols (Shingler et al., 1993). However, the relationship between the $\sigma^{54}$ factor and sulfur metabolism in Gram-negative bacteria has not been reported, probably due to its dispensability for sulfur amino acid biosynthesis. Cases & Lorenzo (2001) proposed that the $\sigma^{54}$ factor of *P. putida* plays important roles in surviving environmental stress. Thus, the involvement of *P. putida* $\sigma^{54}$ factor in SSR is not surprising, because sulfate-limited stress is thought to be ubiquitous in aerobic forest and agricultural soils inhabited by pseudomonads due to the abundance of organosulfur compounds such as sulfonates and sulfate esters rather than inorganic sulfate (Fitzgerald, 1976; Autry & Fitzgerald, 1990).

**Transcriptional regulation of the sfnECR operon in *P. putida* strain DS1**

CysB has been suggested to be involved in transcription of organosulfur-assimilating genes in *P. aeruginosa* (Kertesz et al., 1999; Hummerjohann et al., 2000). However, direct evidence that CysB activates expression of SSI genes has not been obtained, because the *P. aeruginosa* cysB mutant can utilize only preferred sulfur compounds such as cysteine and thiosulfate, which do not induce SSR in pseudomonads. We here obtained direct evidence that CysB protein is involved in activation of transcription of the *sfnECR* operon by a reporter gene assay with supplementation of a small amount of cysteine, which does not prevent the SSR (Fig. 5). However, interestingly, a signature of a $\sigma^{54}$-dependent promoter was found upstream of the *sfnE* site. Since *sfnR* deficiency did not affect its own expression under sulfate limitation (Fig. 4b), it was suggested that *sfnECR* is under the control of an unknown $\sigma^{54}$-dependent regulator. To elucidate that, detailed examination of the transcriptional mechanism of *sfn* promoter will be needed.

**The regulatory model of DMSO$_2$ assimilation in *P. putida***

The cascade regulatory model of DMSO$_2$ metabolism of strain DS1 is proposed as follows. Under sulfate limitation, CysB functions as a master regulator for organosulfur-assimilating genes, and activates expression of an unknown $\sigma^{54}$-dependent regulator. The unknown $\sigma^{54}$-dependent regulator enhances the expression of the *sfnECR* operon. Then, the resultant SfnR activates the expression of gene(s) involved in DMSO$_2$ metabolism and interacting with $\sigma^{54}$ RNA polymerase. In this process, unlike NtrC-type transcriptional regulators, SfnR probably functions only as a transcriptional activator rather than a response regulator, because of the lack of the N-terminal phospho-receiver domain. On the other hand, since Dfi74J grows normally on taurine, alkylsulfonates and sulfate esters (Endoh et al., 2003), SfnR appears not to be involved in transcriptional regulation of the *ssu, tau* and *ats* SSI operons. To obtain direct evidence for the above hypothesis, we are attempting to clone the gene(s) involved in DMSO$_2$ conversion.

**Is an SfnEC two-component monooxygenase system involved in DMS catabolism?**

It is possible that an SfnEC two-component monooxygenase system might be involved in DMS catabolism. *sfnEC* was transcribed together with *sfnR*, and SfnC showed identity (43 %) with DszC from *Rhodococcus* sp. IGTS8, catalysing stepwise monooxygenation of the sulfur atom of dibenzothiophene to dibenzothiophene sulfone (Denome et al., 1994), which is quite similar to oxidation of DMS to DMSO$_2$ in strain DS1 (Endoh et al., 2003). To elucidate our hypothesis, we are also attempting to characterize the SfnEC monooxygenase system.

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Involvement of SfnR in dimethyl sulfone metabolism


