Transcriptional regulation of the sulfate-starvation-induced gene *sfnA* by a σ^{54} -dependent activator of *Pseudomonas putida*

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The σ^{54} -dependent transcriptional regulator SfnR is essential for the use of dimethyl sulfone (DMSO₂) as a sulfur source by *Pseudomonas putida* DS1. SfnR binds three SfnR-binding sites (sites 1, 2 and 3) within an intergenic region of the divergently transcribed *sfnAB* and *sfnFG* gene clusters. The site 1 region, proximal to the *sfnF* gene, is indispensable for the expression of the *sfnFG* operon, which encodes components of DMSO₂ monooxygenase. We investigated the transcriptional regulation of the *sfnAB* operon and possible functions of the *sfnA* gene. RT-PCR analysis revealed that the *sfnAB* gene cluster, which is similar to homologues of the acyl-CoA dehydrogenase family, was transcribed as an operon, and its expression was regulated by SfnR under conditions of sulfate starvation. Deletion analyses using *lacZ* as a reporter demonstrated that the region up to at least –138 bp from the transcription start point of *sfnA* (containing sites 2 and 3) was necessary for the expression of the *sfnAB* operon. A growth test of the *sfnA*-disrupted mutant revealed the possibility that *sfnA* may be involved in the use of methanethiol as a sulfur source.

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INTRODUCTION

Sulfur is an essential element for bacterial growth and is normally acquired through the assimilation of inorganic sulfate. However, in soil environments sulfate may not be freely available because inorganic sulfate constitutes less than 5% of the total sulfur in these environments (Autry & Fitzgerald, 1990). In addition, most of the residual sulfur is present in the soil as peptides/amino acids, sulfate esters and/or sulfonates (Autry & Fitzgerald, 1990). Thus, several soil bacterial species have evolved the ability to use a wide variety of organosulfur compounds, and both the genes and enzymes involved in the use of such compounds (especially the alkanesulfonates, sulfate esters and taurine) have been studied in detail (see reviews by Kertesz, 2000, 2004). When the preferred inorganic sulfur compounds are not available, these bacteria produce a set of proteins to adapt to sulfur limitation (Kertesz *et al.*, 1993; Coppée *et al.*, 2001). These proteins are

known as sulfate-starvation-induced proteins and include metabolic enzymes, regulatory proteins, and transport systems for scavenging sulfur from organosulfur compounds. This sulfate-starvation response plays an important role in the survival of bacteria in soil environments (Kahnert *et al.*, 2002). Although the emission of dimethylsulfide (DMS) from soils has been reported (Minami, 1982), very little is known about the mechanism of the sulfate-starvation response in the use of organosulfides.

We isolated *Pseudomonas putida* strain DS1, which uses DMS as a sulfur source and desulfurizes it via DMSO, dimethyl sulfone (DMSO₂), and methanesulfonate (MSA; Fig. 1a; Endoh *et al.*, 2003a). As reported for other *Pseudomonas* and *Escherichia coli* strains (Eichhorn *et al.*, 1999; Kahnert *et al.*, 2000; van der Ploeg *et al.*, 1999), desulfonation of MSA to provide inorganic sulfur for growth is catalysed by SsuD (FMNH₂-dependent monooxygenase) with an NAD(P)H-dependent FMN reductase (Endoh *et al.*, 2003a). The *ssuD* gene of DS1 is located within the *ssu* operon (*ssuEADCBF*; Endoh *et al.*, 2003a). An FMNH₂-dependent monooxygenase, SfnG, which is

Abbreviations: DMS, dimethylsulfide; DMSO₂, dimethyl sulfone; IIRS, imperfect inverted repeat sequence; MSA, methanesulfonate; UAS, upstream activating sequence.

involved in the conversion of DMSO₂ to MSA, has also been identified, and the *sfnG* gene is located within the *sfnFG* operon (Endoh *et al.*, 2005). Interestingly, the expression of the *sfnFG* operon is controlled by a novel σ^{54} -dependent transcriptional regulator, SfnR, that is similar to NtrC-type regulators, but lacks the N-terminal phospho-receiver domain (Endoh *et al.*, 2003b, 2005).

Transcriptional regulation of the sulfate-starvation-induced genes has been extensively investigated in *Pseudomonas aeruginosa*, as well as in *E. coli*, and the LysR-family regulator CysB plays a key role in the expression of genes involved in both the cysteine biosynthetic pathway and organosulfur assimilation (e.g. the *ssu* operon; Delic-Attree *et al.*, 1997; Hummerjohann *et al.*, 2000). Four other LysR-family regulators of organosulfur metabolism, SdsB (Davison *et al.*, 1992), AsfR (Vermeij *et al.*, 1999), SftR (Kahnert *et al.*, 2002) and SsuR (Iwanicka-Nowicka *et al.*, 2007), have also been discovered in *Pseudomonas* (or *Burkholderia*) strains. However, before our report on SfnR (Endoh *et al.*, 2003b), there had been no reports that this σ^{54} -dependent transcriptional regulator was involved in sulfur assimilation (Cases *et al.*, 2003). Thus, this was apparently the first study to suggest that at least two different sulfate-starvation response mechanisms function in *P. putida* for organosulfide or organosulfone assimilation; i.e. (1) conversion of DMSO₂ to MSA, controlled by a σ^{54} -dependent transcriptional

regulator, and (2) conversion of MSA to sulfite, controlled by a σ^{70} -dependent LysR-family regulator.

SfnR binds three DNA regions (sites 1, 2 and 3) upstream of the *sfnFG* operon (Fig. 1b; Endoh *et al.*, 2005). Only the site 1 region, which is proximal to the *sfnF* gene and contains the two overlapping imperfect inverted repeat sequences (IIRSs), is necessary for the SfnR-dependent expression of the *sfnFG* operon (Fig. 1b; Endoh *et al.*, 2005). However, the functions of the other SfnR-binding sites (sites 2 and 3) remain unknown. Because these SfnR-binding sites are located in the *sfnF*–*sfnA* intergenic region (Fig. 1b), it is possible that sites 2 and 3 may be involved in the expression of the *sfnAB* operon. If the *sfnA* and *sfnB* genes, both of which exhibit sequence identity with the acyl-CoA dehydrogenase family protein, are indeed targets of a σ^{54} -dependent transcriptional regulator, the function of these genes in the assimilation of organosulfur compounds warrants further research. Therefore, we conducted a transcriptional analysis of the *sfnAB* operon to identify other σ^{54} -dependent organosulfur-assimilating genes in addition to the *sfnFG* operon.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used are listed in Table 1. *Pseudomonas putida*

Fig. 1. (a) Proposed dimethyl sulfide (DMS) desulfurization pathway in *P. putida* strain DS1. DMS is thought to be oxidized in two steps by unidentified monooxygenases via the conversion of dimethyl sulfoxide (DMSO) to dimethyl sulfone (DMSO₂). DMSO₂ is then converted to methanesulfonate (MSA) by the FMN-dependent monooxygenase, SfnG. Sulfite is finally released from MSA by the FMN-dependent monooxygenase, SsuD. The reduced FMN required for the activity of SfnG and SsuD could be provided by the FMN reductase, SfnE. (b) Organization of the *sfnAB*–*sfnFG* gene cluster of *P. putida* strain DS1. Pentagonal boxes indicate the length and direction of the *sfn* genes. Dots with numbers (1, 2 and 3) represent the location of the three SfnR-binding sites (sites 1, 2 and 3, respectively), which were previously identified by DNase I footprinting (Endoh *et al.*, 2005). Double-headed arrows indicate the locations of the DNA fragments amplified by RT-PCR (see text and Fig. 2).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Bacterial strains		
<i>Escherichia coli</i>		
JM109	e14 [−] (<i>mcrA</i>) <i>recA1</i> Δ (<i>lac-proAB</i>) <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>hsdR17</i> (r _K [−] m _K ⁺) <i>relA1</i> <i>supE44</i> [F' <i>traD36 proAB lacI^q</i> Δ M15]	Sambrook et al. (2001)
DH5 α	F [−] Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> (r _K [−] m _K ⁺) <i>recA1</i> <i>endA1</i> <i>deoR</i> <i>thi-1</i> <i>supE44</i> <i>gyrA96</i> <i>relA1</i>	Sambrook et al. (2001)
BL21(DE3)	F [−] <i>ompT</i> <i>hsdR17</i> (r _B [−] m _B ⁺) <i>gal</i> <i>dcm</i> (DE3)	Novagen
<i>Pseudomonas putida</i>		
DS1	Wild-type, DMS-using strain	Endoh et al. (2003a)
Dfi74 J	Derivatives of DS1 containing <i>sfnR</i> ::Tn5, DMSO ₂ -use deficiency	Endoh et al. (2003a)
UK1	Derivatives of DS1 containing <i>ssuD</i> :: <i>kan</i> , MSA-use deficiency	Endoh et al. (2003a)
SAK1	Derivatives of DS1 containing Δ <i>sfnA</i> :: <i>kan</i>	Endoh et al. (2005)
SBK1	Derivatives of DS1 containing Δ <i>sfnB</i> :: <i>kan</i>	Endoh et al. (2005)
Plasmids		
pBluescriptII	Cloning vector, Ap ^r	Stratagene
pT7Blue (R)	Ap ^r , T-vector	Novagen
pET26b(+))	Expression vector, T7 promoter	Novagen
pBBad22T	Cloning vector, P _{araBAD} promoter, Tet ^r	Sukchawalit et al. (1999)
pMElacZ	pME4510 derivative, Gm ^r , <i>lacZ</i>	Endoh et al. (2003b)
pMEsfnA-PE	pMElacZ containing the <i>sfnA</i> region from −328 to +188	This study
pMEPsfnA-58	pMElacZ containing the <i>sfnA</i> region from −58 to +56	This study
pMEPsfnA-108	pMElacZ containing the <i>sfnA</i> region from −108 to +56	This study
pMEPsfnA-138	pMElacZ containing the <i>sfnA</i> region from −138 to +56	This study
pMEPsfnA-188	pMElacZ containing the <i>sfnA</i> region from −188 to +56	This study
pMEPsfnA-328	pMElacZ containing the <i>sfnA</i> region from −328 to +56	This study
pMEPsfnA-138SM1	pMElacZ containing the <i>sfnA</i> region from −138 to +56 with one mutation in IIRS of site 2	This study
pMEPsfnA-138SM2	pMElacZ containing the <i>sfnA</i> region from −138 to +56 with one mutation in IIRS of site 2	This study
pMEPsfnA-138DM3	pMElacZ containing the <i>sfnA</i> region from −138 to +56 with two mutations in IIRS of site 2	This study
pEN18	pBluescriptII containing <i>sfnAB</i> and <i>sfnFG</i>	Endoh et al. (2005)
pEN10	pBBad22T containing <i>sfnB</i>	Endoh et al. (2005)
pETsfnE	pET26b(+) containing <i>N-ht-sfnE</i>	Endoh et al. (2005)

DS1 was cultivated with various sulfur sources using established methods (Endoh et al., 2005). *E. coli* strains for cloning were cultivated at 37 °C using LB broth, 2 × YT medium, or Terrific broth, as described by Sambrook & Russell (2001). Ampicillin (Ap), tetracycline (Tet), gentamicin (Gm), kanamycin (Km), chloramphenicol (Cm), and IPTG were added to the media (when necessary) at final concentrations of 50, 50, 15, 50 and 30 µg ml^{−1}, and 0.1 mM, respectively. In some cases, Km and Gm chloride were prepared from sulfate salts using ion exchange (van der Ploeg et al., 1996). Briefly, the sulfate ion of commercially available Km and Gm was replaced by chloride by passage over the Cl[−] form of Amberlite IRA400 J Cl resin, elution with water and lyophilization.

DNA manipulation. Total DNA preparation, plasmid isolation, restriction enzyme digestion and transformation of *E. coli* were performed as described elsewhere (Sambrook & Russell, 2001). The DNA ligation kit version 2 (Takara) and EZNA gel extraction kit (Omega Bio-tek) were used according to the manufacturer's instructions. Other commercially available enzymes and kits were used as indicated by the respective manufacturers. *P. putida* was transformed by electroporation in a 0.1 cm cuvette (25 µF, 200 Ω,

17 kV cm^{−1}) using a GenePulserII apparatus (Bio-Rad). DNA sequencing was conducted using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Reverse transcription (RT) PCR analysis. Strain DS1 and Dfi74 J (*sfnR* disruptant with Tn5) were grown to early exponential phase in sulfur-free mineral medium (SFMM) (Omori et al., 1995) supplemented with either sulfate or MSA (to final substrate concentrations of 1 mM) as a sulfur source. A 2 ml volume of the respective cultures was then centrifuged, and total RNA from the harvested cells was extracted using an RNeasy Mini kit (Qiagen) or NucleoSpin RNA II (Macherey-Nagel), combined with RQ1 RNase-Free DNase (Promega) according to the manufacturer's instructions. RT-PCR was performed with a TaKaRa One Step RNA PCR kit (AMV) (Takara Shuzo). The gene-specific reverse primers used to synthesize cDNA were *ssuE*-RV for *ssuE*, *sfnA*-RV for *sfnA*, and *sfnB*-RT-r for the *sfnA-sfnB* intergenic region (Table 2). The reaction mixture (50 µl) contained 5 µl 10 × One Step RNA PCR buffer, 5 µl MgCl₂, 5 µl dNTP, 40 U RNase inhibitor, 5 U AMV RTase XL, 5 U AMV-optimized Taq, 1 µl each forward and reverse primer, and 1 µg total RNA prepared as described

Table 2. Primer sequences used

Designation	Experiment	Sequence (5'–3')
sfnA-RT-f	RT-PCR	CAATATTGCCGCCGAAGCTG
sfnB-RT-r	RT-PCR	GTGATTCTGCGGTAAGCTGG
ssuE-FW	RT-PCR	GGAAGCGCTCATATGCTGGTCTCTCAATCG
ssuE-RV	RT-PCR	GCGGGTTGAGCCGGCTCGAGGCTCCAGCGAG
sfnA-FW	RT-PCR	GGTGCAGGAATTCACCCATCGAGGAGAACTGC
sfnA-RV	RT-PCR	AATGGAAGCTTCAGCGCTGCTTGGCACCACC
sfnA-RV-PE	Primer extension	<u>AAGCTT</u> GAGCCAGCGGATGGGCT
sfnA-PE1	Primer extension	CGATGCGCGCAAAGATTGG
sfnA-RVrep	<i>lacZ</i> reporter assay	<u>AAGCTT</u> AGGCAGGTGGGCTTGAGCGGTCAT
sfnA-FW-1	<i>lacZ</i> reporter assay, primer extension	<u>GAATTC</u> CCTGATGACGCTGTGTTCATGCA
sfnA-FW-2	<i>lacZ</i> reporter assay	<u>GAATTC</u> GGGCACAAGCAGGCTGTTGAATC
sfnA-FW-3	<i>lacZ</i> reporter assay	<u>GAATTC</u> AGGCGCCGAATGACCGCCTGG
sfnA-FW-4	<i>lacZ</i> reporter assay	<u>GAATTC</u> TGCGACAGGCTGTTGCCAGCG
sfnA-FW-5	<i>lacZ</i> reporter assay	<u>GAATTC</u> CAAGCATTTGATTTAAAGCAC
sfnA-FW-3M1	<i>lacZ</i> reporter assay	<u>GAATTC</u> AGGCGCCGAACCCCGCCTGG
sfnA-FW-3M2	<i>lacZ</i> reporter assay	<u>GAATTC</u> AGGCGCCGAATGACCGCTGGCAGGGGCC
sfnA-FW-3M12	<i>lacZ</i> reporter assay	<u>GAATTC</u> CAGGCGCCGAACCCCGCCTGGCAGGGGCC

*The underlined and double-underlined sequences represent a *Hind*III site and an *Eco*RI site, respectively.

above. PCR was performed using a PCR Thermal Cycler Dice (Takara Shuzo) as follows: 50 °C for 30 min and 94 °C for 2 min, followed by 25 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1.5 min.

Primer extension analysis. For the construction of pMEsfnA-PE, the DNA region containing the 5'-terminal end of *sfnA* and upstream sequences was amplified with a primer set (sfnA-FW-1 and sfnA-RV-PE; Table 2), using pEN18 (Endoh *et al.*, 2005) as a template. Each amplified fragment was cloned into a pT7Blue (R) vector (Novagene), and the nucleotide sequences of each reporter plasmid were confirmed. Clones were digested with both *Hind*III and *Eco*RI (sites incorporated in the primers; Table 2), and the fragments were then cloned between the *Hind*III and *Eco*RI sites of pMElacZ (Endoh *et al.*, 2003b) to produce pMEsfnA-PE. Total RNA (5 µg) from DS1 (pMEsfnA-PE) grown on MSA as a sulfur source was subjected to an RT reaction using SuperScript III reverse transcriptase (Invitrogen) and a primer, sfnA-PE1 (Table 2), whose 5' end was labelled with IRD₈₀₀ (Aloka). The primer extension products were purified using phenol/chloroform extraction and ethanol precipitation. The products were subjected to electrophoresis together with a sequencing reaction using the same primer and a Li-Cor model 4200I-2 Auto DNA sequencer running Base ImagLR data collection software 4.0 (LI-COR), according to the manufacturer's instructions.

β-Galactosidase reporter assay. For the construction of a series of *sfnA-lacZ* transcriptional fusion plasmids (pMEPsfnA-328, pMEPsfnA-188, pMEPsfnA-138, pMEPsfnA-108, pMEPsfnA-58, pMEPsfnA-138SM1, pMEPsfnA-138SM2, and pMEPsfnA-138DM3), the regions upstream of *sfnA* were amplified using a reverse primer (sfnA-RVrep) and a series of forward primers (sfnA-FW-1 to sfnA-FW-5; sfnA-FW-3M1 to sfnA-FW-3M12; Table 2), using pEN18 (Endoh *et al.*, 2005) as a template. Each amplified fragment was cloned into a pT7Blue (R) vector (Novagene), and the nucleotide sequences of each reporter plasmid were confirmed. Clones were digested with both *Hind*III and *Eco*RI (sites incorporated in the primers; Table 2), and the fragments were then cloned between the *Hind*III and *Eco*RI sites of pMElacZ (Endoh *et al.*, 2003b). The strains of *P. putida* transformed using reporter plasmids were grown to

exponential phase on SFMM containing 1 mM sulfate or 1 mM organosulfur compound at 30 °C. A *lacZ* assay was conducted on the cultures according to Miller (1972), with some modifications.

Measurement of growth characteristics. *P. putida* strains were grown in 1 ml SFMM with 1 mM DMSO₂ as a sulfur source at 30 °C. Cells were harvested by centrifugation and washed three times by suspension in 1 ml SFMM. The washed cells were resuspended into 1 ml SFMM containing 1 mM of the appropriate sulfur source. The sulfur sources used were benzylmethyl sulfide, benzylmethyl sulfone, methanethiol, ethanesulfonate, propanesulfonate, butanesulfonate, pentanesulfonate, taurine and hexylsulfate. After appropriate intervals, OD₅₅₀ was measured using a spectrophotometer (model DU-7400, Beckman).

Chemicals. All chemicals used were of the highest purity commercially available (i.e. 98–100%; Merck, Sigma-Aldrich, Kanto Chemical, Wako Pure Chemical, Nacalai Tesque).

RESULTS

The *sfnAB* operon is regulated by SfnR under sulfate starvation

To investigate the co-transcription of the *sfnA* and *sfnB* genes, RT-PCR was performed with total RNA from sulfate- or MSA-grown DS1 cells and a set of primers (sfnA-RT-f and sfnB-RT-r) based on the 3'-terminus of *sfnA* and the 5'-terminus of *sfnB* (Table 2). The expected 760 bp amplified product was only observed in the reaction mixture containing total RNA from MSA-grown cells (data not shown), indicating that the *sfnAB* gene cluster was organized as a sulfate-starvation-induced operon. Subsequently, RT-PCR analysis was performed to investigate whether the expression of the *sfnA* gene was controlled by a novel σ^{54} -dependent regulator (SfnR)

under sulfate starvation. Total RNA prepared from both MSA-grown DS1 and Dfi74 J (*sfnR*::Tn5; DMSO₂-use deficiency) was subjected to RT-PCR with the primer sets *ssuE*-FW/RV for *ssuE* and *sfnA*-FW/RV for *sfnA* (Table 2). The amplified product of the *ssuE* gene, which is expressed under sulfate starvation, but is not regulated by SfnR (Endoh *et al.*, 2003a, b), was observed in both cells. In contrast, the amplified product of the *sfnA* gene was not found in the reaction mixture containing total RNA from the MSA-grown Dfi74 J cells (Fig. 2). These results indicate that SfnR is involved in the upregulation of the expression of the *sfnAB* operon under sulfate limitation.

Determination of the transcription start point of the *sfnA* gene

The results of the RT-PCR analysis pointed to the existence of an σ^{54} -dependent promoter activated by SfnR in the region upstream of the *sfnA* gene. To determine the transcriptional start point of *sfnA*, total RNA was extracted from DS1(pMEsfnA-PE) cells grown on sulfate or MSA, and a primer extension analysis was then performed. These analyses revealed one transcriptional start point (a single G base) 32 bp upstream from the translation start point of *sfnA*, but only in MSA-grown cells (Fig. 3, lane 2). Through comparison with the σ^{54} -dependent consensus promoter (5'-TGGCACN₅TTGCW-3') of *E. coli* (Reitzer & Schneider, 2001), the promoter of the *sfnAB* operon was determined to be 5'-GGGCACGGAGTTTGCG-3' (underlined sequences are highly conserved; Fig. 3).

Identification of the DNA region required for expression of the *sfnAB* operon

To identify the DNA region required for the *sfnR*-dependent expression of the *sfnAB* operon, deletion analyses of the region upstream of the *sfnA* gene were performed using *lacZ* as a reporter. Strain DS1 was transformed by each *sfnA'*-*lacZ* reporter plasmid (Fig. 4),

Fig. 2. RT-PCR analysis with primer sets *ssuE*-FW/RV for *ssuE* and *sfnA*-FW/RV for *sfnA* (Table 2). Total RNA was prepared from cultures of strains DS1 and Dfi74 J (*sfnR* disruptant) on sulfate or methanesulfonate (MSA); 1 µg total RNA was subjected to RT-PCR. In controls total DNA was used as template.

Fig. 3. Determination of the transcriptional start point of *sfnA* by primer extension. The arrow indicates the primer extension product generated using the primer *sfnA*-PE1 (Table 2) annealed to a specific site within the *sfnA* gene. Lanes G, A, T and C correspond to the sequence ladder generated using the same primer, and the sequence pattern is shown on the right. In the sequence of the *sfnF*-*sfnA* intergenic region, the position of the transcriptional start point is shown. The promoter of the *sfnAB* operon containing a consensus σ^{54} -binding sequence is shown in bold. The boxed sequences (sites 1, 2 and 3) indicate the SfnR-binding regions (Endoh *et al.*, 2005).

and the β -galactosidase activities of the resultant transformants were measured in the presence of sulfate or DMSO₂. In the reaction mixtures with DMSO₂, the deletion of the region containing only site 1 (located upstream of position -138 from the *sfnA* transcription start point) had no effect on the induction of β -galactosidase activity. However, deletion of the region containing site 2 (located upstream of position -108 from the *sfnA* transcription start point) exhibited very low β -galactosidase activity. In contrast, the β -galactosidase activities of the transformants were very low in reaction mixtures with sulfate (Fig. 4), and no activity was observed in DS1 harbouring pMElacZ (control vector). These results indicate that the DNA region downstream of position -138 with respect to the *sfnA* transcription start point was necessary for SfnR-dependent expression of the *sfnAB* operon.

Fig. 4. Deletion analysis of the *sfnA* upstream region. The *sfnA* regions fused with *lacZ* are represented by the lines on the left. β -Galactosidase activities of the DS1 transformants grown on sulfate (white bars) or DMSO_2 (black bars) are shown on the right; results are presented as the mean $\pm$ SD of three independent experiments.

Mutational analysis of SfnR-binding sites

We investigated whether the 18 bp imperfect inverted repeat sequence (IIRS; 5'-CTGTN₁₀ACAG-3') in site 2 (previously expected to be a candidate for the consensus sequence bound by SfnR; Endoh *et al.*, 2005) plays an important role in the expression of the *sfnAB* operon. Three *sfnA'*-*lacZ* reporter plasmids with mutations in one or two of the IIRSs were constructed (Fig. 5a), and β -galactosidase activities were compared with those of the *sfnA'*-*lacZ* reporter plasmids without mutations. All mutations within the IIRS of site 2 led to the loss of the induction of β -galactosidase activity under sulfate

starvation (Fig. 5b), suggesting that this IIRS in site 2 is a potential upstream activating sequence (UAS) for SfnR binding.

Growth characteristics of SAK1 and SBK1

According to the results of the transcriptional analyses, the expression of the *sfnA* and *sfnB* genes was apparently sulfate-starvation-induced and regulated by SfnR. However, strains SAK1 (*sfnA* disruptant) and SBK1 (*sfnB* disruptant) are able to use DMS, DMSO , DMSO_2 , MSA, diethyl sulfone, hexanesulfonate, methionine, methionine sulfoxide and methionine sulfone as sulfur sources (Endoh

Fig. 5. Mutational analysis of SfnR-binding sites. (a) The mutated sequences (bold italics) in the reporter plasmids. (b) β -Galactosidase activities of the DS1 transformants grown on sulfate (white bars) or DMSO_2 (black bars). Results are presented as the mean $\pm$ SD of three independent experiments.

et al., 2005). Therefore, to determine the function of these genes, the growth characteristics of SAK1 and SBK1 were investigated further using other organosulfur compounds as sulfur sources. Among the organosulfur compounds tested, differences in growth were only reliably observed when methanethiol was used as a sulfur source. Whereas SBK1 had nearly identical growth capabilities to wild-type DS1, SAK1 exhibited a reduced growth rate compared to DS1 (Fig. 6). Similar results were obtained when SAK1 (pEN10) carrying the *sfnB* expression plasmid was used to avoid a polar effect (data not shown). This observation suggests that SfnA functions in the assimilation of methanethiol. Because UK1 (*ssuD*, encoding MSA-sulfoxatase, disruptant with *kan*) lost the ability to grow on methanethiol (Fig. 6), we hypothesize that SfnA may catalyse an enzymic reaction (oxygenation) involved in the conversion of methanethiol to MSA.

DISCUSSION

The σ^{54} -dependent transcriptional regulator SfnR binds the *sfnA*–*sfnF* intergenic region and upregulates the expression of the *sfnFG* operon, which is essential for the DMS and DMSO₂ metabolism of strain DS1 (Endoh *et al.*, 2005). Of the three SfnR-binding sites within the intergenic region, the site 1 region is indispensable for the expression of these catabolic genes (Endoh *et al.*, 2005). To find other target genes controlled by this novel transcriptional regulator, we investigated whether SfnR-binding sites other than site 1 are involved in the expression of the *sfnAB* operon. In addition to the DNase I footprinting previously conducted (Endoh *et al.*, 2005), the RT-PCR analysis, primer extension and *lacZ* reporter assays demonstrated that the expression of the *sfnAB* operon was activated by the binding of SfnR to sites 2 and 3.

Fig. 6. Growth of strain DS1 and its derivatives with methanethiol as a sulfur source. Symbols: ◆, DS1 (wild-type); ▲, SAK1 (*sfnA* disruptant); ●, SBK1 (*sfnB* disruptant); ■, UK1 (*ssuD* disruptant). Results are presented as the mean of three independent experiments; error bars (SD) are smaller than the symbols.

We demonstrated that two divergently oriented transcriptional units (*sfnFG* and *sfnAB*) were regulated by SfnR, both of which were strictly regulated in response to sulfate limitation. However, the features of their UASs bound by SfnR were quite different. Normally, two adjacent UASs for a σ^{54} -dependent activator are placed in the correct configuration, and their sequences are formed by two IIRSs (approx. 16 bp) with a spacing of 29–42 bp between the centres of the palindromes (Pérez-Martín & de Lorenzo, 1996; Tropel & van der Meer, 2002). For transcriptional activation of the *sfnAB* operon, two adjacent UASs (i.e. IIRS in site 2 and IIRS in site 3) were placed in the usual position with a space of 30 bp between the centres of the palindromes. In contrast, the two UASs (IIRSs in site 1) for the expression of *sfnFG* operon were partially overlapping (Endoh *et al.*, 2005). Therefore, the relationship between the promoter geometry bound by large protein complexes of SfnR and the transcriptional activation levels of the respective operons warrants further investigation.

Both *sfnA* and *sfnB* are not required for DMS and DMSO₂ metabolism (Endoh *et al.*, 2005). Thus, we investigated the function of the SfnR-dependent sulfate-starvation-induced gene, *sfnA*, in organosulfur assimilation. SAK1 (*sfnA* : *kan*) repeatedly exhibited reduced growth compared to the wild-type strain on methanethiol. This suggests that the *sfnA* gene may function in methanethiol metabolism; however, the detailed oxidative pathway for degradation of methanethiol as a sulfur source has not yet been identified. Vermeij & Kertesz (1999) reported that *P. putida* converted methanethiol to MSA through an unknown pathway and subsequently desulfonated MSA to yield sulfite. Because UK1 (*ssuD* : *kan*) failed to grow on methanethiol (Fig. 6), DS1 also metabolized methanethiol to sulfite via MSA. Considering that SAK1 can grow on MSA as a sulfur source, the *sfnA* gene may be involved in the metabolic step from methanethiol to MSA. To elucidate the methanethiol metabolic pathway at the molecular level, strain DS1 must be transposon-mutagenized to obtain methanethiol-deficient mutants. Such an investigation may help explain why SAK1 growth was restored to nearly the same level as in DS1 or SBK1 (Fig. 6) because possible methanethiol metabolite candidates (e.g. methanesulfinate) are non-enzymically oxidized to MSA in the liquid phase by hydroxyl radicals (Arsene *et al.*, 2002; Bardouki *et al.*, 2002). SfnA may be involved in the oxidation of such metabolic intermediates of methanethiol.

Currently, genome sequences are available for several *Pseudomonas* species (*Pseudomonas* Genome Project; www.pseudomonas.com). According to a homology search, their genomes contain many FMNH₂-dependent monooxygenase homologues (acyl-CoA dehydrogenase-family homologues) such as the *sfnA* and *sfnB* genes. However, many of their functions remain unknown. In strain DS1, we also found at least five sulfate-starvation-induced genes encoding FMNH₂-dependent monooxygenase homologues [i.e. *ssuD* (Endoh *et al.*, 2003a), *sfnC*

(Endoh *et al.*, 2003b), *sfnG*, *sfnA* and *sfnB* (Endoh *et al.*, 2005)], but the enzymic functions of SfnC, SfnA and SfnB are not known. Further study of SfnA may provide insights into the functions of such unknown sulfate-starvation-induced genes in organosulfur assimilation.

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