SfnR2 Regulates Dimethyl Sulfide-Related Utilization in *Pseudomonas aeruginosa* PAO1

Benjamin R. Lundgren,^a Zaara Sarwar,^b Kyle S. Feldman,^a Joseph M. Shoytush,^a Christopher T. Nomura^{a,c,d}

^aDepartment of Chemistry, State University of New York, College of Environmental Science and Forestry, Syracuse, New York, USA

^bDepartment of Biology, The College of New Jersey, Ewing, New Jersey, USA

^cCenter for Applied Microbiology, State University of New York, College of Environmental Science and Forestry, Syracuse, New York, USA

^dHubei Collaborative Innovation Center for Green Transformation of Bio-Resources, College of Life Sciences, Hubei University, People's Republic of China

ABSTRACT Dimethyl sulfide (DMS) is a volatile sulfur compound produced mainly from the degradation of dimethylsulfoniopropionate (DMSP) in marine environments. DMS undergoes oxidation to form dimethyl sulfoxide (DMSO), dimethyl sulfone (DMSO₂), and methanesulfonate (MSA), all of which occur in terrestrial environments and are accessible for consumption by various microorganisms. The purpose of the present study was to determine how the enhancer-binding proteins SfnR1 and SfnR2 contribute to the utilization of DMS and its derivatives in *Pseudomonas aeruginosa* PAO1. First, results from cell growth experiments showed that deletion of either *sfnR2* or *sfnG*, a gene encoding a DMSO₂-monooxygenase, significantly inhibits the ability of *P. aeruginosa* PAO1 to use DMSP, DMS, DMSO, and DMSO₂ as sulfur sources. Deletion of the *sfnR1* or *msuEDC* genes, which encode a MSA desulfurization pathway, did not abolish the growth of *P. aeruginosa* PAO1 on any sulfur compound tested. Second, data collected from β -galactosidase assays revealed that the *msuEDC-sfnR1* operon and the *sfnG* gene are induced in response to sulfur limitation or nonpreferred sulfur sources, such as DMSP, DMS, and DMSO, etc. Importantly, SfnR2 (and not SfnR1) is essential for this induction. Expression of *sfnR2* is induced under sulfur limitation but independently of SfnR1 or SfnR2. Finally, the results of this study suggest that the main function of SfnR2 is to direct the initial activation of the *msuEDC-sfnR1* operon in response to sulfur limitation or nonpreferred sulfur sources. Once expressed, SfnR1 contributes to the expression of *msuEDC-sfnR1*, *sfnG*, and other target genes involved in DMS-related metabolism in *P. aeruginosa* PAO1.

IMPORTANCE Dimethyl sulfide (DMS) is an important environmental source of sulfur, carbon, and/or energy for microorganisms. For various bacteria, including *Pseudomonas*, *Xanthomonas*, and *Azotobacter*, DMS utilization is thought to be controlled by the transcriptional regulator SfnR. Adding more complexity, some bacteria, such as *Acinetobacter baumannii*, *Enterobacter cloacae*, and *Pseudomonas aeruginosa*, possess two, nonidentical SfnR proteins. In this study, we demonstrate that SfnR2 and not SfnR1 is the principal regulator of DMS metabolism in *P. aeruginosa* PAO1. Results suggest that SfnR1 has a supportive but nonessential role in the positive regulation of genes required for DMS utilization. This study not only enhances our understanding of SfnR regulation but, importantly, also provides a framework for addressing gene regulation through dual SfnR proteins in other bacteria.

KEYWORDS *Pseudomonas*, RpoN, SfnR, dimethyl sulfide, dimethyl sulfone, dimethyl sulfoxide, enhancer-binding protein, methanesulfonate, sulfur limitation

Dimethyl sulfide (DMS) is a volatile sulfur compound that is widely recognized for its central role in the global sulfur cycle and impact on climate regulation (1). DMS is generated through various anthropogenic, geochemical, and biological processes and

Citation Lundgren BR, Sarwar Z, Feldman KS, Shoytush JM, Nomura CT. 2019. SfnR2 regulates dimethyl sulfide-related utilization in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 201:e00606-18. <https://doi.org/10.1128/JB.00606-18>.

Editor George O'Toole, Geisel School of Medicine at Dartmouth

Copyright © 2019 American Society for Microbiology. All Rights Reserved.

Address correspondence to Christopher T. Nomura, ctnomura@esf.edu.

Received 3 October 2018

Accepted 20 November 2018

Accepted manuscript posted online 26 November 2018

Published 28 January 2019

annually accounts for ~50% of the total sulfur flux in the atmosphere (2, 3). The main source of atmospheric DMS is the microbial degradation of dimethylsulfoniopropionate (DMSP), an osmolyte occurring in phytoplankton, algae, and halophilic plant species (4, 5). The DMS formed from DMSP degradation is either metabolized by marine microorganisms (5, 6) or released into the atmosphere, where it undergoes oxidation to yield products such as dimethyl sulfoxide (DMSO), dimethyl sulfone (DMSO₂), and methanesulfonate (MSA) (7, 8). These oxidized products are later deposited into terrestrial environments through precipitation.

Many microorganisms can use DMS and its derivatives as a source of sulfur, carbon, and/or energy (2, 7). In bacteria, this utilization is often part of the sulfur limitation response, a condition that occurs when cells no longer have access to a preferred sulfur source, e.g., inorganic sulfate, thiosulfate, or cysteine. Proteins required for the uptake and metabolism of different types of nonpreferred sulfur sources become expressed under sulfur limitation (9, 10). Interestingly, in addition to being a part of the sulfur limitation response, DMS utilization in *Pseudomonas putida* DS1 is specifically regulated by an enhancer-binding protein (EBP) known as SfnR, for dimethyl sulfone regulator (11). In *P. putida* DS1, the utilization of DMS proceeds through a series of oxidation reactions: DMS → DMSO → DMSO₂ → methanesulfinate → MSA → sulfite (11, 12). Of all the reactions in this pathway, it is the formation of MSA from DMSO₂ that is completely dependent on SfnR regulation in *P. putida* DS1 (11). Specifically, SfnR positively regulates the expression of the *sfnG* gene (13), which encodes a Reduced flavin mononucleotide (FMN_{H₂})-dependent monooxygenase that catalyzes the oxidation of DMSO₂ to methanesulfinate (14). Both SfnR and SfnG are required for DMS, DMSO, and DMSO₂ utilization in *P. putida* DS1 (11–13).

SfnR EBPs are encoded in the genomes of many other *Pseudomonas* spp. (15, 16), suggesting that DMS utilization and its regulation by SfnR are similar among them. More intriguingly, while other species of *Pseudomonas* possess only a single *sfnR* gene, *Pseudomonas aeruginosa* actually has two genes encoding putative SfnR EBPs. In *P. aeruginosa* PAO1, these SfnR EBPs are encoded by the PA2354 (*sfnR1*) and PA2359 (*sfnR2*) genes. SfnR1 and SfnR2 are not identical proteins; they share 65% similarity with one another. Furthermore, the *sfnR2* gene is monocistronic, whereas *sfnR1* is in an operon with the *msuEDC* genes (16). The *msuEDC* genes were previously shown to be involved in the desulfurization of MSA to sulfite in *P. aeruginosa* PAO1 (17). Apart from this limited information, nothing has been reported regarding DMS utilization or its regulation by SfnR1 and/or SfnR2 in *P. aeruginosa*.

The presence of dual SfnR EBPs raises thought-provoking questions regarding the regulation of DMS utilization in *P. aeruginosa*. Does each SfnR protein regulate the utilization of a specific compound (DMS versus MSA), or is DMS-related utilization mutually coregulated by SfnR1 and SfnR2? The purpose of this study was to define some of the fundamental factors and control points surrounding SfnR regulation in *P. aeruginosa* PAO1. This includes determining the requirements for the *sfnR1* and *sfnR2* genes in the utilization of DMS and DMS-related compounds. The necessities for the *msuEDC* and *sfnG* genes in DMS-related utilization were also addressed, since they are believed to be key targets of SfnR regulation. Finally, how SfnR1, SfnR2, and various sulfur sources affect or contribute to the expression of genes involved in DMS-related utilization was investigated.

RESULTS

The *sfnR2* gene is required for the utilization of DMS, DMSO, and DMSO₂ as sulfur sources in *P. aeruginosa* PAO1. SfnR was shown to have an essential role in the utilization of DMS, DMSO, and DMSO₂ in *P. putida* DS1 (11, 12). Our initial focus was to confirm that SfnR1 and/or SfnR2 has a similar essential role in *P. aeruginosa* PAO1. In addition, because they are proposed targets of SfnR regulation and encode proteins central to DMS-related utilization, the *msuEDC* and *sfnG* genes were included in this analysis. To this end, the following gene deletion mutants of *P. aeruginosa* PAO1 were constructed: $\Delta sfnR1$, $\Delta sfnR2$, $\Delta sfnR1 \Delta sfnR2::tet$, $\Delta msuEDC$, and $\Delta sfnG$. Attempts were

FIG 1 Utilization of DMS, DMSO, and DMSO₂ as sulfur sources requires the *sfnR2* gene in *P. aeruginosa* PAO1. The Δ sfnR1, Δ sfnR2, Δ sfnR1 Δ sfnR2::tet, Δ msuEDC, and Δ sfnG gene deletion mutants, as well as wild-type *P. aeruginosa* PAO1, were grown in acetate-minimal medium supplemented with 5.0 mM DMS (A), 0.5 mM DMSO (B), 0.5 mM DMSO₂ (C), or 0.5 mM MSA (D) as the sole sulfur source. Data points represent mean values ($n = 3$) with standard deviations (SDs) of <5.0%. Error bars were omitted to improve clarity.

made to construct an entirely markerless Δ sfnR1 Δ sfnR2 double mutant. We were successful in constructing both Δ sfnR1 *sfnR2*::FRT-*aacC1*-FRT and *sfnR1*::FRT-*aacC1*-FRT Δ sfnR2 mutants, but the subsequent FLP-FRT recombination step did not yield the expected Δ sfnR2 and Δ sfnR1 mutations, respectively. Instead, PCR products with larger sizes than those for the expected Δ sfnR1 and Δ sfnR2 mutations were observed for each mutant, suggesting that the second round of FLP-FRT recombination resulted in genomic rearrangement surrounding these loci. This is possibly due to the close proximity of multiple FRT sites. This was not investigated further.

The growth of the Δ sfnR1, Δ sfnR2, Δ sfnR1 Δ sfnR2::tet, Δ msuEDC, and Δ sfnG mutants, as well as wild-type *P. aeruginosa* PAO1, was measured in acetate-minimal medium supplemented with DMS, DMSO, DMSO₂, or MSA as the sole sulfur source (Fig. 1). When DMS served as the sole sulfur source (Fig. 1A), no growth was observed for the Δ sfnR2 and Δ sfnR1 Δ sfnR2::tet mutants, while the Δ sfnR1 mutant displayed wild-type growth. Both the Δ msuEDC and Δ sfnG mutants yielded significantly lower cell densities than that of wild-type *P. aeruginosa* PAO1 throughout the growth period on DMS. In the

presence of DMSO as the sole sulfur source (Fig. 1B), both the $\Delta sfnR2$ and $\Delta sfnR1 \Delta sfnR2::tet$ mutants exhibited no growth. The $\Delta sfnR1$, $\Delta msuEDC$, and $\Delta sfnG$ mutants all had significantly lower cell densities than that of wild-type *P. aeruginosa* PAO1 on DMSO. Only at 14 h postinoculation did the cell densities of the $\Delta sfnR1$ and $\Delta msuEDC$ mutants reach levels similar to that of wild-type *P. aeruginosa* PAO1 on DMSO. In contrast, the $\Delta sfnG$ mutant never achieved cell densities comparable to that of wild-type *P. aeruginosa* PAO1 on DMSO. In the presence of $DMSO_2$ as the sole sulfur source (Fig. 1C), the $\Delta sfnR1 \Delta sfnR2::tet$ mutant showed no growth, whereas the $\Delta sfnR2$ mutant grew poorly, reaching a maximum optical density at 600 nm (OD_{600}) of 0.150, compared to 0.430 for wild-type *P. aeruginosa* PAO1. The cell densities of the $\Delta sfnR1$, $\Delta msuEDC$, and $\Delta sfnG$ mutants were significantly lower than that of wild-type *P. aeruginosa* PAO1 on $DMSO_2$. However, at 14 h postinoculation, the cell densities of the $\Delta sfnR1$ and $\Delta msuEDC$ mutants were similar to that of wild-type *P. aeruginosa* PAO1 on $DMSO_2$. The $\Delta sfnG$ mutant never reached cell densities comparable to that of wild-type *P. aeruginosa* PAO1 on $DMSO_2$. Finally, wild-type growth was observed for all five mutants on MSA as the sole sulfur source (Fig. 1D). The results of the cell growth experiments demonstrate that the *sfnR2* and *sfnG* genes are crucial for the growth of *P. aeruginosa* PAO1 on DMS, DMSO, and $DMSO_2$ as sulfur sources.

Plasmid-based expression of *sfnR1* or *sfnR2* restores the growth of the $\Delta sfnR2$ mutants on DMS, DMSO, and $DMSO_2$. The results from the cell growth experiments indicate that the *sfnR1* and *sfnR2* genes are not identical regarding the utilization of DMS, DMSO, and $DMSO_2$ in *P. aeruginosa* PAO1. Nonetheless, because the SfnR1 and SfnR2 proteins share 65% similarity to one another, it was believed that the *sfnR1* gene is an adequate substitute for *sfnR2* if expressed from a nonregulated, constitutive promoter. This hypothesis was tested by first cloning the *sfnR1* and *sfnR2* genes under the control of the *lac* promoter of the broad-host-range plasmid pBBR1MCS-5 to generate pBRL754 and pBRL755, respectively. Next, the plasmids pBRL754 and pBRL755 were each introduced into the $\Delta sfnR2$ and $\Delta sfnR1 \Delta sfnR2::tet$ mutants, and the transformed cells were grown in acetate-minimal medium supplemented with DMS, DMSO, or $DMSO_2$ as the sole sulfur source (Fig. 2). As anticipated, the $\Delta sfnR2$ and $\Delta sfnR1 \Delta sfnR2::tet$ mutants carrying pBRL754 (*sfnR1*⁺) displayed wild-type growth on DMS (Fig. 2A), DMSO (Fig. 2B), and $DMSO_2$ (Fig. 2C). Identical results were observed for the $\Delta sfnR2$ and $\Delta sfnR1 \Delta sfnR2::tet$ mutants harboring pBRL755 (*sfnR2*⁺) (Fig. 2). These findings argue that the utilization of DMS, DMSO, and $DMSO_2$ in *P. aeruginosa* PAO1 does not require both SfnR1 and SfnR2. Only one of the SfnR proteins needs to be present to support the growth of *P. aeruginosa* PAO1 on these sulfur compounds.

Genetic complementation experiments for the $\Delta sfnG$ mutant were also performed. Expression of *sfnG* from the *lac* promoter of pBBR1MCS-5 resulted in a >3-fold increase in the cell growth or densities of the $\Delta sfnG$ mutant on DMS, DMSO, or $DMSO_2$ (Fig. 3). Although plasmid-based expression of *sfnG* increased the overall growth of the $\Delta sfnG$ mutant, its cell densities never reached wild-type levels. This partial growth restoration of the $\Delta sfnG$ mutant is potentially a result of suboptimal expression of *sfnG* from the *lac* promoter of pBBR1MCS-5.

The *sfnR2* gene is required for induction of *msuE-lacZ*, *sfnR1-lacZ*, and *sfnG-lacZ* reporters in response to DMS, DMSO, $DMSO_2$, and MSA in *P. aeruginosa* PAO1. The findings from the genetic complementation experiments suggest that the SfnR1 and SfnR2 proteins are interchangeable. This means that the differences observed in the requirements for the *sfnR1* and *sfnR2* genes in DMS-related utilization might be more of a direct result of their expression levels and not the biochemical characteristics of their encoded proteins. To investigate their expression, the 5' regulatory regions upstream of the *sfnR1* and *sfnR2* open reading frames (ORFs) were individually fused to the *lacZ* ORF of *Escherichia coli*, and the *lacZ* fusions were each cloned into a promoterless plasmid to generate the *sfnR1-lacZ* and *sfnR2-lacZ* reporters (Fig. 4). Reporters for *msuE* (*msuE-lacZ*) and *sfnG* (*sfnG-lacZ*) were also constructed. The reporters were introduced into wild-type *P. aeruginosa* PAO1 and the $\Delta sfnR1$, $\Delta sfnR2$, and $\Delta sfnR1$

FIG 2 Plasmid-based expression of either *sfnR1* or *sfnR2* rescues the growth of the $\Delta sfnR2$ mutants on DMS, DMSO, and DMSO₂. The *sfnR1* and *sfnR2* genes were individually cloned under the control of the *lac* promoter of the broad-host-range plasmid pBBR1MCS-5 to generate pBRL754 and pBRL755, respectively. The plasmids pBRL754 and pBRL755 and the empty vector pBBR1MCS-5 were each electroporated into the $\Delta sfnR2$ and $\Delta sfnR1 \Delta sfnR2::tet$ mutants, and the recombinant strains were grown in acetate-minimal medium supplemented with 5.0 mM DMS (A), 0.5 mM DMSO (B), or 0.5 mM DMSO₂ (C) as the sole sulfur source. Growth was also measured for wild-type *P. aeruginosa* PAO1 carrying the empty vector pBBR1MCS-5. Data points represent mean values ($n = 3$) with SDs of <5.0%. Error bars were omitted to improve clarity.

$\Delta sfnR2::tet$ mutants, and the expression levels of all four reporters were determined in response to sulfate, DMS, DMSO, DMSO₂, and MSA.

As shown in Fig. 5A for *P. aeruginosa* PAO1, the expression level of *msuE-lacZ* increased >8-fold in response to DMS, DMSO, or DMSO₂ and increased >2-fold in the presence of MSA. The expression level of *sfnR1-lacZ* increased >4-fold in response to DMS, DMSO, or DMSO₂, while MSA caused a >2-fold increase. The expression level of *sfnR2-lacZ* increased >2-fold in response to DMS, DMSO, or DMSO₂ but did not significantly change in the presence of MSA. Finally, the expression level of *sfnG-lacZ* increased >4-fold in response to DMS, DMSO, or DMSO₂ and increased >3-fold in response to MSA.

In the $\Delta sfnR1$ mutant (Fig. 5B), the expression levels or changes observed for *msuE-lacZ*, *sfnR1-lacZ*, *sfnR2-lacZ*, and *sfnG-lacZ* were similar in magnitude to those measured for wild-type *P. aeruginosa* PAO1. In contrast, the expression of *msuE-lacZ*, *sfnR1-lacZ*, and *sfnG-lacZ* did not change in response to DMS, DMSO, DMSO₂, or MSA in either the $\Delta sfnR2$ mutant (Fig. 5C) or the $\Delta sfnR1 \Delta sfnR2::tet$ mutant (Fig. 5D). The

FIG 3 Plasmid-based expression of *sfnG* restores the growth of the $\Delta sfnG$ mutant on DMS, DMSO, and DMSO₂. The *sfnG* gene was cloned under the control of the *lac* promoter of the broad-host-range plasmid pBBR1MCS-5 to generate pBRL753. The $\Delta sfnG$ mutant harboring either pBRL753 (*sfnG*⁺) or the empty vector pBBR1MCS-5 was grown in acetate-minimal medium supplemented with 5.0 mM DMS (A), 0.5 mM DMSO (B), or 0.5 mM DMSO₂ (C) as the sole sulfur source. Growth was also measured for wild-type *P. aeruginosa* PAO1 carrying the empty vector pBBR1MCS-5. Data points represent mean values ($n = 3$) with SDs of <5.0%. Error bars were omitted to improve clarity.

expression level of *sfnR2-lacZ*, however, increased by >2-fold in response to DMS, DMSO, or DMSO₂ in the $\Delta sfnR2$ and $\Delta sfnR1 \Delta sfnR2::tet$ mutants. These results indicate that in response to DMS, DMSO, DMSO₂, and MSA, the expression of the *msuEDC-sfnR1* and *sfnG* genes is dependent upon SfnR2 (and not SfnR1) in *P. aeruginosa* PAO1, whereas the expression of *sfnR2* is independent of SfnR1 and SfnR2.

SfnR1 or SfnR2 activates expression of *msuE-lacZ* and *sfnG-lacZ* in *E. coli*. The *sfnR2* gene was essential for the expression of the *msuE-lacZ* and *sfnG-lacZ* reporters in *P. aeruginosa* PAO1. This result is consistent with SfnR2 being a direct regulator or activator of the *msuEDC-sfnR1* and *sfnG* genes. Moreover, because plasmid-based expression of either *sfnR1* or *sfnR2* rectified the growth deficiencies of the $\Delta sfnR2$ mutants (Fig. 2), it would suggest that SfnR1 and SfnR2 are functionally similar, and therefore, SfnR1 also serves as an activator of the *msuEDC-sfnR1* and *sfnG* genes. We initially tested this hypothesis by measuring the expression levels of the SfnR target gene reporters, *msuE-lacZ* and *sfnG-lacZ*, in *E. coli* DH5 α cells that simultaneously expressed either *sfnR1* or *sfnR2* from the *trc* promoter of the plasmid pTrc99a. As shown in Fig. 6A, expression levels of *msuE-lacZ* were >3-fold higher in *E. coli* DH5 α cells that

A. *msuEDC-sfnR1* and *sfnR2* genes **B. *msuE-lacZ*** **C. *sfnR1-lacZ*** **D. *sfnR2-lacZ*** **E. *sfnG-lacZ***

FIG 4 LacZ reporters used to measure the expression of the *msuEDC-sfnR1*, *sfnR2*, and *sfnG* genes of *P. aeruginosa* PAO1. (A) The *msuEDC-sfnR1* operon and the *sfnR2* gene, which is monocistronic, are clustered near one another on the chromosome of *P. aeruginosa* PAO1. The intervening PA2358 gene encodes a hypothetical protein with no predicted function. (B to D) The *msuE-lacZ* (B), *sfnR1-lacZ* (C), and *sfnR2-lacZ* (D) reporters were constructed by fusing the 5' regulatory region upstream of each ORF to the *lacZ* gene of *E. coli* and then cloning the *lacZ* fusion into the promoterless plasmid Δ Plac-pBBR1MCS-5. (E) The *sfnG-lacZ* reporter was constructed in an identical manner.

expressed either *sfnR1* or *sfnR2* than in cells that harbored the empty vector pTrc99a. Interestingly, the addition of DMS, DMSO, DMSO₂, or MSA had no effect on the expression on *msuE-lacZ*. For *sfnG-lacZ* (Fig. 6B), its expression levels increased by >200-fold in *E. coli* DH5 α cells that expressed *sfnR1*. In comparison, expression levels of *sfnG-lacZ* increased >10-fold when *sfnR2* was expressed. Similar to *msuE-lacZ*, the expression levels of *sfnG-lacZ* in *E. coli* DH5 α were not affected by DMS, DMSO, DMSO₂, or MSA.

His₆-SfnR1 and His₆-SfnR2 bind to the 5' regulatory regions of the *msuE* and *sfnG* genes. The 18-bp consensus sequence 5'-CTGT-N₁₀-ACAC-3' was identified as the DNA-binding site for SfnR of *P. putida* DS1 (13). In the 5' regulatory regions of *msuE* and *sfnG*, located ~150 bp upstream of each respective ORF, are two putative SfnR-binding sites separated by a 13-bp spacer. To confirm that SfnR1 and SfnR2 bind to the *msuE* and *sfnG* loci, electrophoretic mobility shift assays (EMSAs) were conducted using a Cy5-labeled DNA probe resembling the ~250-bp 5' regulatory region of *msuE* or *sfnG* and N-terminal, polyhistidine-tagged (His₆) SfnR1 and SfnR2.

As shown in Fig. 7, shifts in migration were observed for both the *msuE* and *sfnG* probes in the presence of His₆-SfnR1 or His₆-SfnR2. Neither His₆-SfnR1 nor His₆-SfnR2

A. PAO1 **B. $\Delta sfnR1$** **C. $\Delta sfnR2$** **D. $\Delta sfnR1 \Delta sfnR2::tet$**

FIG 5 Induction of *msuE-lacZ*, *sfnR1-lacZ*, and *sfnG-lacZ* in response to DMS, DMSO, DMSO₂, or MSA requires the *sfnR2* gene in *P. aeruginosa* PAO1. Expression of *msuE-lacZ*, *sfnR1-lacZ*, *sfnG-lacZ*, and *sfnR2-lacZ* was investigated in wild-type *P. aeruginosa* PAO1 (A) and the $\Delta sfnR1$ (B), $\Delta sfnR2$ (C), and $\Delta sfnR1 \Delta sfnR2::tet$ (D) mutants. LacZ activity was measured from recombinant cells grown for 4.0 h in acetate-minimal medium supplemented with 5.0 mM sulfate, DMS, DMSO, DMSO₂, or MSA as the sole sulfur source. Data points represent mean values ($n = 3$) $\pm$ SDs. ANOVA with Dunnett's *post hoc* test (α value, 0.05) was done to identify significant differences ($P < 0.0001$), which are indicated with asterisks.

exhibited binding toward a nonspecific DNA probe. The affinity of His₆-SfnR1 and His₆-SfnR2 for the *msuE* and *sfnG* probes was investigated by changing the concentration of each protein in the presence of a fixed amount of the probe. These experiments showed that migratory shifts of the *msuE* probe required a minimum concentration of 62.5 nM His₆-SfnR1 or 125 nM His₆-SfnR2 (Fig. 8A). In comparison, shifts of the *sfnG* probe required a minimum concentration of 125 nM His₆-SfnR1 or His₆-SfnR2 (Fig. 8B). For all experiments, His₆-SfnR1 displayed tighter binding to the *msuE* and *sfnG* probes than did His₆-SfnR2. This suggests that SfnR1 has a stronger binding affinity for the *msuE* and *sfnG* loci than that of SfnR2. As discussed below, these differences in binding affinity are attributed to dissimilarities or variations in the DNA-binding domains of SfnR1 and SfnR2.

FIG 6 Expression of *msuE-lacZ* and *sfnG-lacZ* in *E. coli* is not influenced by DMS, DMSO, DMSO₂, or MSA but requires the heterologous expression of either the *sfnR1* or *sfnR2* gene. The *sfnR1* and *sfnR2* genes were individually cloned under the control of the *trc* promoter of the plasmid pTrc99a to generate pBRL756 and pBRL757, respectively. The plasmid pBRL756 (+ *sfnR1*) or pBRL757 (+ *sfnR2*) or the empty vector pTrc99a was cotransformed with either the *msuE-lacZ* or *sfnG-lacZ* reporter into *E. coli* DH5 α . LacZ activity was measured from recombinant cells grown for 4.0 h in LB supplemented with no sulfur source (no addition) or 5.0 mM DMS, DMSO, DMSO₂, or MSA. Data points represent mean values ($n = 3$) $\pm$ SDs. ANOVA with Dunnett's *post hoc* test (α value, 0.05) was done to identify significant differences ($P < 0.0001$), which are indicated with asterisks.

The *sfnR2* gene is required for the utilization of methionine sulfone and DMSP as sulfur sources in *P. aeruginosa* PAO1. Up to this point, this study focused on SfnR regulation as it pertains to the utilization of DMS, DMSO, DMSO₂, and MSA in *P. aeruginosa* PAO1. It was not yet known whether SfnR regulation has a more global role or general impact in the utilization of other sulfoxides, sulfones, and sulfonates. This was addressed by measuring the growth of *P. aeruginosa* PAO1 and the Δ *sfnR1*, Δ *sfnR2*, Δ *sfnR1* Δ *sfnR2::tet*, Δ *msuEDC*, and Δ *sfnG* mutants in acetate-minimal medium supplemented with sulfoxides, sulfones, and sulfonates that varied in structure and size. Tested sulfur sources included methionine sulfoxide, dibutyl sulfoxide, methionine sulfone, dibutyl sulfone, propanesulfonate, pentanesulfonate, or DMSP.

Wild-type growth was observed for all mutants on methionine sulfoxide as the sole sulfur source (Fig. 9A). In contrast, no growth was detected for the Δ *sfnR2* and Δ *sfnR1* Δ *sfnR2::tet* mutants on methionine sulfone (Fig. 9B), whereas the Δ *sfnR1*, Δ *msuEDC*, and Δ *sfnG* mutants displayed wild-type growth on this sulfur source. All mutants grew similarly to wild-type cells on propanesulfonate (Fig. 9C) and pentanesulfonate (Fig. 9D). No growth was observed for any cell type on dibutyl sulfoxide or dibutyl sulfone. Finally, although wild-type growth was observed for the Δ *sfnR1* and Δ *msuEDC* mutants on DMSP, this compound was not a viable sulfur source for the Δ *sfnR2* or Δ *sfnR1* Δ *sfnR2::tet* mutant (Fig. 10). The Δ *sfnG* mutant had a growth reduction of $>50\%$ compared to wild-type *P. aeruginosa* PAO1 on DMSP (Fig. 10).

The effects of these various sulfoxides, sulfones, and sulfonates on the expression of *msuE-lacZ*, *sfnR1-lacZ*, *sfnG-lacZ*, and *sfnR2-lacZ* were also determined. For *P. aeruginosa* PAO1 (Fig. 11A), the presence of propanesulfonate or pentanesulfonate as the sole sulfur source resulted in a >2 -fold increase in the expression levels of *msuE-lacZ*, *sfnR1-lacZ*, and *sfnG-lacZ*. Neither propanesulfonate nor pentanesulfonate significantly induced the expression of *sfnR2-lacZ* in *P. aeruginosa* PAO1. The effects observed for

FIG 7 His₆-SfnR1 and His₆-SfnR2 bind to the 5' regulatory regions of *msuE* and *sfnG*. (A) A total of 2.0 nM the Cy5-labeled *msuE* or nonspecific *gcvH2* probe was incubated in the absence or presence of 250 nM His₆-SfnR1. (B) A total of 2.0 nM the Cy5-labeled *sfnG* or nonspecific *gcvH2* probe was incubated in the absence or presence of 250 nM His₆-SfnR2. (C) A total of 2.0 nM the Cy5-labeled *sfnG* or nonspecific *gcvH2* probe was incubated in the absence or presence of 250 nM His₆-SfnR1 (lanes 1 to 4) or His₆-SfnR2 (lanes 5 to 8).

propanesulfonate and pentanesulfonate on the expression of *msuE-lacZ*, *sfnR1-lacZ*, *sfnG-lacZ*, and *sfnR2-lacZ* were similar to those of MSA. The presence of methionine sulfoxide as the sole sulfur source induced the expression of *msuE-lacZ* by 2-fold but did not significantly change the expression of *sfnR1-lacZ*, *sfnG-lacZ*, or *sfnR2-lacZ* (Fig. 11A). Dibutyl sulfoxide and methionine sulfone had similar effects; either one induced the expression of *msuE-lacZ*, *sfnR1-lacZ*, and *sfnG-lacZ* by 2-fold, whereas the expression of *sfnR2-lacZ* did not change significantly. The presence of dibutyl sulfone as the sole sulfur source led to a 2-fold increase in the expression levels of *sfnR1-lacZ* and *sfnG-lacZ*, but it had no effect on the expression of *msuE-lacZ* or *sfnR2-lacZ*. It should be noted that in the absence of any sulfur source, the expression levels of *msuE-lacZ*, *sfnR1-lacZ*, *sfnG-lacZ*, and *sfnR2-lacZ* all increased by >2-fold.

In contrast to those in *P. aeruginosa* PAO1, the expression levels of *msuE-lacZ*, *sfnR1-lacZ*, and *sfnG-lacZ* did not change or increase (no induction) in the presence of any sulfoxide, sulfone, or sulfonate in the $\Delta sfnR1 \Delta sfnR2::tet$ mutant (Fig. 11B). Furthermore, the absence of any sulfur source did not induce the expression of *msuE-lacZ*, *sfnR1-lacZ*, or *sfnG-lacZ* in the $\Delta sfnR1 \Delta sfnR2::tet$ mutant. The expression level of *sfnR2-lacZ*, however, increased >2-fold in the absence of any sulfur source or in the presence of dibutyl sulfone or DMSP.

Inorganic sulfate represses expression of *msuE-lacZ*, *sfnR1-lacZ*, *sfnG-lacZ*, and *sfnR2-lacZ* in *P. aeruginosa* PAO1. Expressions of *msuE-lacZ*, *sfnR1-lacZ*, *sfnG-lacZ*, and *sfnR2-lacZ* are induced in *P. aeruginosa* PAO1 when cells are grown in the absence of any sulfur source. This suggests that sulfur limitation might be an additional control point in the expression of the *msuEDC-sfnR1*, *sfnR2*, and *sfnG* genes. To confirm that sulfate has an overriding, negative effect on the expression of these genes, *P. aeruginosa* PAO1 harboring *msuE-lacZ*, *sfnR1-lacZ*, *sfnG-lacZ*, or *sfnR2-lacZ* was grown in

FIG 8 His₆-SfnR1 and His₆-SfnR2 display different binding affinities. (A) The Cy5-labeled *msuE* or *sfnG* probe (2.0 nM) was titrated against increasing concentrations of His₆-SfnR1 (2-fold serial increase from 0 to 500 nM). (B) The Cy5-labeled *msuE* or *sfnG* probe (2.0 nM) was titrated against increasing concentrations of His₆-SfnR2 (2-fold serial increase from 0 to 500 nM).

acetate-minimal medium supplemented with (i) DMS, DMSO, DMSO₂, or MSA or (ii) a combination of inorganic sulfate plus DMS, DMSO, DMSO₂, or MSA. The addition of inorganic sulfate to medium supplemented with DMS, DMSO, or DMSO₂ caused a >5-fold decrease in the expression levels of *msuE-lacZ*, *sfnR1-lacZ*, and *sfnG-lacZ* (Fig. 12). A similar trend was observed for *sfnR2-lacZ*, whose expression level decreased by >2-fold with the addition of inorganic sulfate. Regarding MSA, the addition of inorganic sulfate resulted in a >2-fold decrease in the expression levels of *msuE-lacZ* and *sfnG-lacZ*, but it did not have a significant effect on the expression of *sfnR1-lacZ* and *sfnR2-lacZ*.

DISCUSSION

SfnR2 is essential for DMS-related utilization in *P. aeruginosa* PAO1. The results of this study provide a foundational or working model for SfnR regulation and its biological functions in *P. aeruginosa* PAO1 (Fig. 13). As noted above, *P. aeruginosa* is unique among *Pseudomonas* spp. in that it possesses two genes encoding putative SfnR EBPs. The *sfnR1* gene of *P. aeruginosa* is in an operon with the *msuEDC* genes, which encode enzymes involved in the desulfurization of MSA (17). Similar to *sfnR1* of *P. aeruginosa*, the *sfnR* genes of other *Pseudomonas* spp. are localized within operons: *msuC-sfnR* in *P. putida* KT2440, *sfnEC-sfnR* in *P. putida* DS1, *ssuD-sfnR* in *P. syringae* pv. tomato DC3000, and *msuC-sfnR* in *P. fluorescens* SBW25 (15, 16). In regard to amino acid sequence, SfnR1 of *P. aeruginosa* PAO1 is highly similar (~80% identity) to the SfnR EBPs of other *Pseudomonas* spp. In comparison, SfnR2 of *P. aeruginosa* PAO1 shares ~60% identity to these other SfnR EBPs, including SfnR1. The *sfnR2* gene of *P. aeruginosa* PAO1 is monocistronic and separated from the *msuEDC-sfnR1* operon by the PA2358 gene, encoding a hypothetical protein.

Based on its high sequence similarity to SfnR of *P. putida* DS1, one might expect that SfnR1 would be the principal regulator of DMS-related utilization in *P. aeruginosa* PAO1. However, the results of this study clearly show that SfnR2 fulfills this role. Like SfnR of *P. putida* DS1, SfnR2 was required for the utilization of DMS, DMSO, and DMSO₂ in *P. aeruginosa* PAO1 (Fig. 1). The monooxygenase SfnG (PA3954) was also required for maximum growth on these compounds, and the expression of the *sfnG* gene was shown to be positively regulated by SfnR2 (Fig. 5 and 6). In addition, our findings

FIG 9 Utilization of methionine sulfone as a sulfur source requires the *sfnR2* gene in *P. aeruginosa* PAO1. Wild-type *P. aeruginosa* PAO1 and the $\Delta sfnR1$, $\Delta sfnR2$, $\Delta sfnR1 \Delta sfnR2::tet$, $\Delta msuEDC$, and $\Delta sfnG$ gene deletion mutants were grown in acetate-minimal medium supplemented with 0.5 mM methionine sulfoxide (MetSO) (A), methionine sulfone (MetSO₂) (B), propanesulfonate (PrSA) (C), or pentanesulfonate (PeSA) (D) as the sole sulfur source. Data points represent mean values ($n = 3$) with SDs of <5.0%. Error bars were omitted to improve clarity.

suggest that *SfnR2* positively regulates the expression of the *msuEDC-sfnR1* operon in *P. aeruginosa* PAO1 (Fig. 5 and 6). Although not essential, the *msuEDC* and *sfnR1* genes were necessary for optimal growth on DMSO and DMSO₂. The *msuEDC* genes (but not *sfnR1*) were also needed for maximum growth on DMS. These findings indicate that *SfnR1* and *MsuEDC* are contributing factors in the utilization of DMS-related compounds in *P. aeruginosa* PAO1.

The utilization of alkanesulfonates, including MSA, propanesulfonate, and pentanesulfonate, did not require the *msuEDC*, *sfnG*, *sfnR1*, or *sfnR2* gene in *P. aeruginosa* PAO1 (Fig. 9). The original study that characterized the *msuEDC* genes of *P. aeruginosa* PAO1 reported that disruption of this locus did not affect the growth of this bacterium on alkanesulfonates (17). Our findings support this previous observation. The nonessential nature of the *msuEDC* genes in alkanesulfonate utilization is attributed to genetic redundancy. The *ssu* operon of *Pseudomonas* spp. encodes proteins involved in the desulfurization of various organosulfur compounds, including alkanesulfonates

FIG 10 Utilization of DMSP as a sulfur source requires the *sfnR2* gene in *P. aeruginosa* PAO1. Wild-type *P. aeruginosa* PAO1 and the $\Delta sfnR1$, $\Delta sfnR2$, $\Delta sfnR1 \Delta sfnR2::tet$, $\Delta msuEDC$, and $\Delta sfnG$ gene deletion mutants were grown in acetate-minimal medium supplemented with 0.5 mM DMSP as the sole sulfur source. Data points represent mean values ($n = 3$) with SDs of <5.0%. Error bars were omitted to improve clarity.

(18). Such redundancy would explain why the *msuEDC* genes are not necessary for the utilization of DMS, DMSO, DMSO₂, and alkanesulfonates in *P. aeruginosa* PAO1. It should be noted that growth on MSA in *P. putida* DS1 did not require SfnR (11).

Results of this study revealed that SfnR regulation in *P. aeruginosa* is not limited to the utilization of DMS, DMSO, and DMSO₂. Namely, SfnR2 was necessary for the utilization of DMSP and methionine sulfone in *P. aeruginosa* PAO1 (Fig. 9 and 10). DMSP is metabolized in *P. aeruginosa* PAO1 through the demethylation pathway (19), which generates acetaldehyde, carbon dioxide, and methanethiol as products. Methanethiol can be converted into hydrogen sulfide via methanethiol oxidase, or it can be methylated by a thiol methyltransferase to yield DMS (2, 20, 21). There is no experimental or bioinformatic evidence supporting the existence of a methanethiol oxidase in *P. aeruginosa* PAO1, and although a methanethiol-specific methyltransferase has been characterized (20), there are no proteins similar to this enzyme encoded in the genome of *P. aeruginosa* PAO1. The *sfnG* gene was required for growth on DMSP (Fig. 10), arguing that DMSO₂ is a metabolic intermediate of DMSP utilization in *P. aeruginosa*. This suggests that the methylation of methanethiol to form DMS is occurring in *P. aeruginosa* PAO1 grown on DMSP, but the thiol methyltransferase(s) catalyzing this reaction remains a mystery.

Free and bound methionine is susceptible to oxidation (22, 23). Consequently, oxidized derivatives such as methionine sulfoxide and methionine sulfone are common constituents of biologically derived soil matter and important sources of sulfur (24). The *sfnR2* gene was found to be essential for growth on methionine sulfone but not methionine sulfoxide (Fig. 9). In regard to the latter, *P. aeruginosa* PAO1 harbors two reductases, MsrA (PA5018) and MsrB (PA2827), that convert methionine sulfoxide into methionine (25). MsrA is constitutively expressed, and the presence of these enzymes bypasses any need for SfnR2 in the utilization of methionine sulfoxide. For methionine sulfone, it is not understood how SfnR2 affects the utilization of this compound. Unlike the $\Delta sfnR2$ and $\Delta sfnR1 \Delta sfnR2::tet$ mutants, the $\Delta sfnG$ mutant exhibited wild-type growth on methionine sulfone. Our current hypothesis is that SfnR2 upregulates the expression of a monooxygenase or reductase that has a broad substrate tolerance, enabling it to convert methionine sulfone into methionine sulfoxide. It is possible that

A. PAO1 **B. Δ sfnR1 Δ sfnR2::tet**

FIG 11 Sulfur limitation induces expression of *msuE-lacZ*, *sfnR1-lacZ*, *sfnG-lacZ*, and *sfnR2-lacZ* in *P. aeruginosa* PAO1. The effects of various sulfoxides, sulfones, sulfonates, and sulfur limitation on the expression of *msuE-lacZ*, *sfnR1-lacZ*, *sfnG-lacZ*, and *sfnR2-lacZ* were determined for wild-type *P. aeruginosa* PAO1 (A) and the Δ sfnR1 Δ sfnR2::tet mutant (B). LacZ activity was measured from recombinant cells grown for 4.0 h in acetate-minimal medium supplemented with no sulfur source or 5.0 mM sulfate, DMSP, DMSO, methionine sulfoxide (MetSO), dibutyl sulfoxide (DBSO), DMSO₂, methionine sulfone (MetSO₂), dibutyl sulfone (DBSO₂), MSA, propanesulfonate (PrSA), or pentanesulfonate (PeSA). Data points represent mean values ($n = 3$) $\pm$ SDs. ANOVA with Dunnett's *post hoc* test (α value, 0.05) was done to identify significant differences ($P < 0.0001$), which are indicated with asterisks.

this monooxygenase is the same one that converts DMS into DMSO and/or DMSO into DMSO₂. Attempts are being made to identify and characterize the monooxygenase(s) catalyzing these reactions.

SfnR2 is the principal regulator of DMS-related utilization in *P. aeruginosa* PAO1. Several control points surrounding SfnR regulation in *P. aeruginosa* PAO1 were identified. Importantly, results from the LacZ assays demonstrate that the *sfnR2* gene is essential for the activation of the *msuEDC-sfnR1* and *sfnG* genes in *P. aeruginosa* PAO1 in response to DMS, DMSO, and/or DMSO₂ as the sole sulfur source (Fig. 5). The findings from the *E. coli* LacZ assays (Fig. 6) and EMSAs (Fig. 7 and 8) further suggest that SfnR2 is a direct regulator or activator of the *msuEDC-sfnR1* and *sfnG* genes. In sharp contrast, deletion of the *sfnR1* gene had no significant effect on the expression of the *msuEDC-sfnR1* and *sfnG* genes in *P. aeruginosa* PAO1. This result, combined with the data from

FIG 12 Sulfate represses expression of *msuE-lacZ*, *sfnR1-lacZ*, *sfnG-lacZ*, and *sfnR2-lacZ* in *P. aeruginosa* PAO1. Wild-type *P. aeruginosa* PAO1 harboring *msuE-lacZ* (A), *sfnR1-lacZ* (B), *sfnG-lacZ* (C), or *sfnR2-lacZ* (D) was grown in acetate-minimal medium supplemented with (i) 5.0 mM DMS, DMSO, DMSO₂, or MSA (– sulfate) or (ii) 0.5 mM sulfate plus 5.0 mM DMS, DMSO, DMSO₂, or MSA (+ sulfate). LacZ activity was measured from recombinant cells grown for 4.0 h in each respective medium. Data points represent mean values ($n = 3$) $\pm$ SDs. ANOVA with Dunnett's *post hoc* test (α value, 0.05) was done to identify significant differences ($P < 0.0001$), which are indicated with asterisks.

the cell growth experiments (Fig. 1), strongly argues that SfnR1 has no pivotal role in the regulation and utilization of DMSP, DMS, DMSO, DMSO₂, and/or methionine sulfone.

Results from the genetic complementation experiments, *E. coli* LacZ assays, and EMSAs suggest that SfnR1 and SfnR2 share some functional similarities, and therefore, SfnR1 is believed to contribute (in a nonessential manner) to the expression of the *msuEDC-sfnR1* and *sfnG* genes in *P. aeruginosa* PAO1. Notably, data from the *E. coli* LacZ assays and EMSAs indicate that SfnR1 interacts more strongly with the 5' regulatory regions of *msuEDC-sfnR1* and *sfnG* than does SfnR2. Predicted DNA-binding sites for SfnR of *P. putida* DS1 are located within the 5' regulatory regions of the *msuEDC-sfnR1* and *sfnG* genes. It is extremely probable that these DNA-binding sites are recognized and bound by SfnR1 given that the putative DNA-binding domains (the last 76 amino acid residues of the C terminus) of SfnR1 and SfnR of *P. putida* DS1 are highly similar (71% identity and 84% similarity). SfnR2 is also believed to recognize these same DNA-binding sites but to a lesser extent than that of SfnR1; i.e., the DNA-binding domains of SfnR2 and SfnR of *P. putida* DS1 are less similar (60% identity and 74% similarity). We are currently conducting a more in-depth biochemical analysis of SfnR1 and SfnR2 in order to determine their exact DNA-binding sites and specificities. Knowledge of their DNA-binding specificities coupled to a transcriptomic study of the *sfnR* mutants are expected to clarify the molecular targets of regulation by SfnR1 and SfnR2 in *P. aeruginosa* PAO1.

Sulfur limitation controls SfnR regulation in *P. aeruginosa* PAO1. The master regulator CysB was shown to upregulate the expression of the *sfnEC-sfnR* operon of *P. putida* DS1 in response to sulfur limitation (11, 26). Under sulfur limitation, CysB positively regulates the expression of numerous genes encoding proteins involved in the uptake and assimilation of various, nonpreferred sulfur sources, including alkane-sulfonates, sulfamates, and sulfate esters (9). Surprisingly, little is known about the scope and magnitude of CysB regulation in *P. aeruginosa*, and as a result, we cannot readily predict control points for CysB in SfnR regulation. However, the expression of *sfnR2-lacZ* was repressed in the presence of sulfate and induced under sulfur limitation (Fig. 11 and 12), which are characteristics associated with CysB regulation. The conse-

FIG 13 DMS-related utilization and its regulation by SfnR2 in *P. aeruginosa* PAO1. Utilization of DMS, DMSO, and DMSO₂ is controlled by a gene-regulatory hierarchy consisting of sulfur limitation and the EBPs SfnR1 and SfnR2. In the absence of a preferred sulfur source or sulfur limitation, the expression of the *sfnR2* gene is induced. Although the exact mechanism for this induction is unclear, it is hypothesized that the master regulator CysB is involved in the activation of the *sfnR2* gene. Once expressed, SfnR2 activates the transcription of the *msuEDC-sfnR1* and *sfnG* genes from RpoN-recognized promoters (P_{RpoN}) that are positioned upstream of the target genes. SfnR1 also participates in the activation of the *msuEDC-sfnR1* and *sfnG* genes. Because EBPs are oligomeric proteins that form hexamers or heptamers (39), SfnR1 and SfnR2 are depicted as hypothetical hexamers (homomeric and/or heteromeric). SfnR2 and SfnR1 are hypothesized to regulate the expression of additional, unidentified genes involved in DMS utilization, including genes encoding monooxygenases catalyzing the oxidation of DMS to DMSO and of DMSO to DMSO₂.

quences of this control point would generate a gene-regulatory hierarchy in which the first key step is CysB upregulating the expression of *sfnR2* in response to sulfur limitation in *P. aeruginosa* PAO1 (Fig. 13). This is followed by SfnR2 upregulating the expression of the *msuEDC-sfnR1* operon, the *sfnG* gene, and other yet-to-be-identified genes involved in DMS-related utilization. Once expressed, SfnR1 then participates in the activation of SfnR target genes.

The proposed gene-regulatory hierarchy implies that DMS-related utilization occurs only in the absence of a preferred sulfur source and is part of the global response to sulfur limitation in *P. aeruginosa* PAO1. In other words, genes associated with DMS-related utilization are upregulated primarily in response to sulfur limitation and not the presence of DMS or DMS-related compounds. Support for this hypothesis includes the LacZ findings of this study, which showed that the expression of *sfnR2-lacZ*, *msuE-lacZ*, *sfnR1-lacZ*, and *sfnG-lacZ* was upregulated when cells were grown in the absence of a sulfur source or in the sole presence of nonpreferred sulfur sources such as alkanesulfonates, DMS, DMSO, and DMSO₂, etc. (Fig. 11). Inorganic sulfate had a dominant, repressive effect on the expression of these reporters (Fig. 12). Additionally, in a previously reported study (27), several genes associated with sulfur metabolism, including *msuEDC-sfnR1*, *sfnR2*, and *sfnG*, displayed elevated transcript levels when *P. aeruginosa* PAO1 was subjected to sulfur limitation.

Transcriptional activities of SfnR1 and SfnR2 are constitutive. Results from the *E. coli* and *P. aeruginosa* LacZ assays (Fig. 6 and 11) demonstrate that DMS, DMSO, and/or DMSO₂ is not essential for SfnR1 and SfnR2 transcriptional activity. The expres-

sion of *msuE-lacZ*, *sfnR1-lacZ*, and *sfnG-lacZ* was upregulated by SfnR1 and/or SfnR2 in the absence of exogenous DMS, DMSO, and DMSO₂. These findings suggest that the activities of SfnR1 and SfnR2 are constitutive and not dependent on interactions with DMS-related compounds. Such uncontrollable or constitutive activity might account for why the expression of the *sfnR* genes is tightly regulated by sulfur limitation in *P. aeruginosa* PAO1 and *P. putida* DS1.

RpoN has a putative role in SfnR regulation in *P. aeruginosa* PAO1. It is pertinent to briefly discuss the significance of the alternative sigma factor σ^{54} or RpoN in terms of SfnR regulation and DMS-related utilization in *P. aeruginosa* PAO1, although this was not investigated in the present study. SfnR1 and SfnR2 are predicted EBPs, which means that their amino acid sequences possess unique compositions or structural motifs that enable them to interact specifically with RpoN to mediate transcription initiation (28, 29). Consequently, RpoN is considered to be involved in the transcriptional activation of SfnR target genes in *P. aeruginosa*. Such a role is supported by both bioinformatics and experimental evidence. First, putative RpoN-controlled promoters are located upstream of the *msuE* and *sfnG* genes in *P. aeruginosa* strains PAO1 and PA14 (<http://www.sigma54.ca>) (30). SfnR-binding sites are located within ~100 bp upstream of the RpoN-controlled promoters for both *msuE* and *sfnG* in *P. aeruginosa* PAO1. Second, results from chromatin immunoprecipitation sequencing have identified the upstream regions of *msuE* and *sfnG* as binding sites for RpoN in *P. aeruginosa* PA14 (30). Finally, the *rpoN* gene was required for DMS, DMSO, and DMSO₂ utilization in *P. putida* DS1 (11). We are currently exploring the mechanisms surrounding the transcriptional activation of *msuE* and *sfnG* by SfnR1/SfnR2 *in vitro*. Results of this future study will clarify the role of RpoN in the transcriptional activation of SfnR target genes.

Regulation by dual SfnR EBPs. SfnR regulation in *P. aeruginosa* is both similar to and different from that of *P. putida* DS1. The key similarity is that the expressions of both *sfnR2* in *P. aeruginosa* PAO1 and *sfnR* in *P. putida* DS1 are upregulated in response to sulfur limitation. In both bacteria, the utilization of DMS-related compounds is ultimately controlled by sulfur limitation. However, the expression of *sfnR* in *P. putida* DS1 is repressed by SfnR (26), whereas the expression of *sfnR1* in *P. aeruginosa* PAO1 is activated by SfnR2 and SfnR1. There are no sequences resembling SfnR-binding sites in the 5' regulatory region of the *sfnR2* gene, which suggests that neither SfnR1 nor SfnR2 directly regulates *sfnR2* expression. Additionally, the expression of *sfnR2-lacZ* in the $\Delta sfnR1$, $\Delta sfnR2$, and $\Delta sfnR1 \Delta sfnR2::tet$ mutants was not statistically different from that in wild-type *P. aeruginosa* PAO1 (Fig. 5). Nonetheless, a more detailed investigation into the transcriptional mechanisms surrounding the *sfnR2* gene is required to conclusively rule out direct regulation by SfnR1 and/or SfnR2.

Another similarity between SfnR of *P. putida* DS1 and SfnR1/SfnR2 of *P. aeruginosa* PAO1 is the target gene *sfnG*. In *P. putida* DS1, SfnR positively regulates the expression of the *sfnFG* and *sfnAB* operons (13, 31). Our findings indicate that SfnR1 and SfnR2 positively regulate the expression of the *msuEDC-sfnR1* and *sfnG* genes in *P. aeruginosa* PAO1. The *sfnG* gene, however, is predicted to form an operon with the downstream *PA3955* and *PA3956* genes (16). The *PA3955* gene encodes a putative SfnG-like protein, and *PA3956* encodes a hypothetical protein. The expression of the entire *sfnG-PA3955-PA3956* operon is potentially upregulated by SfnR1 and SfnR2 in *P. aeruginosa* PAO1. Additional targets of SfnR regulation in *P. aeruginosa* PAO1 can be proposed. SfnR-binding sites and RpoN-controlled promoters are found in the 5' regulatory regions of the *PA2326* gene and the *PA2346-PA2351* operon in *P. aeruginosa* PAO1 (<http://www.sigma54.ca>). The *PA2326* and *PA2348* genes encode monooxygenases with unknown functions. Both *PA2346* and *PA2347* encode SfnB-like proteins, while the *PA2349-PA2351* operon encodes transport proteins. Similar to *msuEDC-sfnR1* and *sfnG*, the *PA2326* and *PA2346-PA2351* genes were upregulated under sulfur limitation and shown to be regulated by RpoN in *P. aeruginosa* PA14 (27, 30). These enzymes or proteins might play a role in the oxidation of DMS to DMSO and/or of DMSO to DMSO₂. We are currently

TABLE 1 Bacteria used in this study

Strain	Relevant characteristic(s)	Reference or source
<i>Pseudomonas aeruginosa</i>		
PAO1	Wild type (parental strain)	40
$\Delta sfnR1$	$\Delta sfnR1$ ($\Delta PA2354$)	This study
$\Delta msuEDC$	$\Delta msuEDC$ ($\Delta PA2357$ - $\Delta PA2355$)	This study
$\Delta sfnR2$	$\Delta sfnR2$ ($\Delta PA2359$)	This study
$\Delta sfnR1 \Delta sfnR2::tet$	$\Delta sfnR1 \Delta sfnR2::tet$ ($\Delta PA2354 \Delta PA2359::tet$)	This study
$\Delta sfnG$	$\Delta sfnG$ ($\Delta PA3954$)	This study
<i>Escherichia coli</i>		
DH5 α	F ⁻ $\phi 80lacZ\Delta M15 \Delta(lacZYA-argF)U169 recA1 endA1 hsdR17(r_K^- m_K^+)$ <i>phoA supE44 λ^- thi-1 gyrA96 relA1</i>	Invitrogen
BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ(DE3 [<i>lacI lacUV5-T7p07 ind1 sam7 nin5</i>])</i> <i>[malB⁺]_{K-12}(λ^S)</i>	EMD Millipore

investigating these genes and using transcriptomics to confirm and identify other genes regulated by SfnR1 and SfnR2 in *P. aeruginosa* PAO1.

Intriguingly, similar to *P. aeruginosa*, the bacteria *Acinetobacter baumannii* and *Enterobacter cloacae* each possess an SfnR1- and SfnR2-type EBP. In fact, the entire *sfnR2*-*PA2358*-*msuEDC*-*sfnR1* gene cluster is conserved among the three bacteria. Such conservation leads one to speculate that the lifestyles or biological niches of these bacteria may benefit from regulation by dual SfnR EBPs. This subject warrants exploration.

MATERIALS AND METHODS

Bacteria, media, and antibiotic selection. Bacteria used in the study are given in Table 1. Bacteria were grown in Lennox broth (LB) or acetate-minimal medium consisting of 30 mM sodium acetate, 22 mM KH₂PO₄, 42 mM Na₂HPO₄, 18.7 mM NH₄Cl, 8.6 mM NaCl, 1.0 mM MgCl₂, and 5.0 μ M FeCl₃ (pH 7.0) supplemented with either 0.5 or 5.0 mM sulfur source. Sulfur sources were prepared as 100 mM stocks, filter sterilized, and used as indicated per experiment. Solid bacteriological medium was prepared with the addition of BD Difco agar at 15 g liter⁻¹. For plasmid selection in *E. coli*, media were supplemented with carbenicillin (100 μ g ml⁻¹), gentamicin (30 μ g ml⁻¹), kanamycin (50 μ g ml⁻¹), and/or tetracycline (15 μ g ml⁻¹). For plasmid and marker selection in *P. aeruginosa* PAO1, media were supplemented with carbenicillin (200 μ g ml⁻¹), gentamicin (30 μ g ml⁻¹), or tetracycline (50 μ g ml⁻¹).

General molecular biology methods. Plasmids and oligonucleotides used in the study are given in Tables 2 and 3, respectively. Materials used for cloning were nucleic acid purification kits (Promega) and molecular biology enzymes (New England Biolabs), including Phusion DNA polymerase, restriction endonucleases, and T4 DNA ligase. PCRs were conducted in accordance with the recommended thermocycling conditions given for the Phusion DNA polymerase. All cloned genes and DNA fragments were sequenced (Genewiz) to confirm their identities.

Cloning of the *sfnR1*, *sfnR2*, and *sfnG* genes. The *sfnR1*, *sfnR2*, and *sfnG* ORFs were PCR amplified from genomic DNA of *P. aeruginosa* PAO1 with the primer pairs ZS471.f/ZS471.r, ZS470.f/ZS470.r, and BL666.f/BL666.r, respectively. The *sfnR1*, *sfnR2*, and *sfnG* PCR products were gel purified and cloned into pCR-Blunt (Invitrogen) to generate the plasmids pBRL745, pBRL746, and pBRL748, respectively. The *sfnR1* and *sfnR2* ORFs were individually subcloned into (i) the EcoRI site of pBBR1MCS-5, (ii) the EcoRI site of pTrc99a, and (iii) the NdeI/BamHI sites of pET15b. The *sfnG* ORF from pBRL748 was subcloned into the XbaI/SacI sites of pBBR1MCS-5 to yield pBRL753.

Cloning of *msuE-lacZ*, *sfnR1-lacZ*, *sfnR2-lacZ*, and *sfnG-lacZ* reporters. The 5' regulatory regions upstream of the *msuE*, *sfnR1*, *sfnR2*, and *sfnG* ORFs were PCR amplified from genomic DNA of *P. aeruginosa* PAO1 using the primer pairs BL631.f/BL631.r1, BL631.f/BL631.r2, BL632.f/BL632.r, and BL665.f/BL665.r, respectively. In parallel, the β -galactosidase ORF (*lacZ*) of *E. coli* was PCR amplified with the primer pair BL342.f/BL342.r and subsequently gel purified. The gel-purified *lacZ* fragment was then individually fused to the PCR-amplified 5' regulatory regions of *sfnR1*, *sfnR2*, *msuE*, and *sfnG* through fusion PCR (32). The resulting *msuE-lacZ*, *sfnR1-lacZ*, *sfnR2-lacZ*, and *sfnG-lacZ* fusions were gel purified and cloned into pCR-Blunt (Invitrogen) to generate the plasmids pBRL696, pBRL697, pBRL698, and pBRL742, respectively. These plasmids were double digested with HindIII/XbaI, and the liberated *msuE-lacZ*, *sfnR1-lacZ*, *sfnR2-lacZ*, and *sfnG-lacZ* fragments were each subcloned into the corresponding HindIII/XbaI sites of $\Delta Plac$ -pBBR1MCS-5 (32) to yield pBRL721, pBRL722, pBRL723, and pBRL747, respectively.

Construction of mutants. The *msuEDC*, *sfnR1*, *sfnR2*, and *sfnG* genes were deleted from the genome of *P. aeruginosa* PAO1 using the method of Choi and Schweizer (33). Cloning of gene deletion plasmids, mutant selection, and marker removal, etc., were done using established procedures (33, 34). Briefly, the chromosomal mutations *msuEDC::FRT-aacC1-FRT*, *sfnR1::FRT-aacC1-FRT*, *sfnR2::FRT-aacC1-FRT*, and *sfnG::FRT-aacC1-FRT* were generated by electroporating *P. aeruginosa* PAO1 with the gene deletion plasmids

TABLE 2 Plasmids used in this study^a

Plasmid	Relevant characteristic(s)	Source or reference
pCR-Blunt	Cloning plasmid; Km ^r	Invitrogen
pDONR221	Gateway cloning plasmid; Km ^r	Invitrogen
pJET1.2	Cloning plasmid; Cb ^r	Thermo Fisher Scientific
pET15b	Expression plasmid; Cb ^r	EMD Millipore
pTrc99a	Expression plasmid; Cb ^r	Pharmacia
pBBR1MCS-5	Broad-host-range plasmid; Gm ^r	41
pEX18ApGW	Gene deletion plasmid of <i>P. aeruginosa</i> ; Cb ^r	33
pFLP2	Plasmid carrying FLP recombinase for removal of <i>FRT-aacC1-FRT</i> chromosomal markers; Cb ^r	33
pPS856	PCR template for <i>FRT-aacC-FRT</i> marker	33
pBR322	PCR template for <i>tet</i> marker	New England Biolabs
$\Delta Plac$ -pBBR1MCS-5	pBBR1MCS-5 minus <i>lac</i> promoter; Gm ^r	32
pBRL693	<i>sfnR1::FRT-aacC1-FRT</i> in pDONR221; Km ^r Gm ^r	This study
pBRL694	<i>sfnR2::FRT-aacC1-FRT</i> in pDONR221; Km ^r Gm ^r	This study
pBRL695	<i>msuEDC::FRT-aacC1-FRT</i> in pDONR221; Km ^r Gm ^r	This study
pBRL696	<i>msuE-lacZ</i> in pCR-Blunt; Km ^r	This study
pBRL697	<i>sfnR1-lacZ</i> in pCR-Blunt; Km ^r	This study
pBRL698	<i>sfnR2-lacZ</i> in pCR-Blunt; Km ^r	This study
pBRL710	<i>sfnR1::FRT-aacC1-FRT</i> in pEX18ApGW; Cb ^r Gm ^r	This study
pBRL711	<i>sfnR2::FRT-aacC1-FRT</i> in pEX18ApGW; Cb ^r Gm ^r	This study
pBRL712	<i>msuEDC::FRT-aacC1-FRT</i> in pEX18ApGW; Cb ^r Gm ^r	This study
pBRL721	<i>msuE-lacZ</i> in pCR-Blunt; Km ^r	This study
pBRL722	<i>sfnR1-lacZ</i> in $\Delta Plac$ -pBBR1MCS-5; Gm ^r	This study
pBRL723	<i>sfnR2-lacZ</i> in $\Delta Plac$ -pBBR1MCS-5; Gm ^r	This study
pBRL730	<i>sfnR2::tet</i> in pDONR221; Km ^r Tc ^r	This study
pBRL731	<i>sfnR2::tet</i> in pEX18ApGW; Cb ^r Tc ^r	This study
pBRL741	<i>sfnG::FRT-aacC1-FRT</i> in pDONR221; Km ^r Gm ^r	This study
pBRL742	<i>sfnG-lacZ</i> in pCR-Blunt; Km ^r	This study
pBRL744	<i>sfnG::FRT-aacC1-FRT</i> in pEX18ApGW; Cb ^r Gm ^r	This study
pBRL745	<i>sfnR1</i> in pCR-Blunt; Km ^r	This study
pBRL746	<i>sfnR2</i> in pCR-Blunt; Km ^r	This study
pBRL747	<i>sfnG-lacZ</i> in $\Delta Plac$ -pBBR1MCS-5; Gm ^r	This study
pBRL748	<i>sfnG</i> in pCR-Blunt; Km ^r	This study
pBRL753	<i>sfnG</i> in pBBR1MCS-5; Gm ^r	This study
pBRL754	<i>sfnR1</i> in pBBR1MCS-5; Gm ^r	This study
pBRL755	<i>sfnR2</i> in pBBR1MCS-5; Gm ^r	This study
pBRL756	<i>sfnR1</i> in pTrc99a; Cb ^r	This study
pBRL757	<i>sfnR2</i> in pTrc99a; Cb ^r	This study
pZS455	<i>sfnR1</i> in pET15b; Cb ^r	This study
pZS458	<i>msuE</i> EMSA probe in pJET1.2; Cb ^r	This study
pZS459	<i>sfnR2</i> in pET15b; Cb ^r	This study
pKSF01	<i>sfnG</i> EMSA probe in pJET1.2; Cb ^r	This study

^aPlasmids carry the *bla*, *aacC1*, *aph*, and/or *tet* gene, encoding resistance to carbenicillin (Cb^r), gentamicin (Gm^r), kanamycin (Km^r), and tetracycline (Tc^r), respectively.

pBRL712, pBRL710, pBRL711, and pBRL744, respectively. Following selection and verification of double-crossover mutants, the gentamicin selection markers (*aacC1*) were removed via FLP-mediated excision to give the markerless mutants $\Delta msuEDC$, $\Delta sfnR1$, $\Delta sfnR2$, and $\Delta sfnG$. For constructing the $\Delta sfnR1 \Delta sfnR2::tet$ mutant, the $\Delta sfnR1$ mutant was electroporated with pBRL731 (*sfnR2::tet* in pEX18ApGW), and putative mutants were selected on LB agar supplemented with 50 $\mu\text{g ml}^{-1}$ of tetracycline. Double-crossover mutants were identified and verified by PCR.

Cell growth experiments. A microplate assay was used to measure sulfur utilization of *P. aeruginosa* PAO1 and the gene deletion mutants $\Delta msuEDC$, $\Delta sfnG$, $\Delta sfnR1$, $\Delta sfnR2$, and $\Delta sfnR1 \Delta sfnR2::tet$. First, bacteria were grown on LB agar plates for 18 h at 37°C to yield single colonies. Next, in triplicate, a single colony from each plate was used to inoculate 2.0 ml of LB in a 16- by 100-mm culture tube, and the inoculated cultures were grown for 18 h at 37°C at 200 rpm. Cells from the LB-grown cultures were harvested by centrifugation and then washed with an equal volume of 0.95% NaCl to remove any residual sulfur-containing compounds. The washed cells were suspended in an equal volume of 0.95% NaCl, and the cell suspensions were used at 1.0% (vol/vol) (2 μl) to inoculate 0.2 ml of acetate-minimal medium in a 96-well flat-bottom polystyrene plate (Falcon). Acetate-minimal medium was supplemented with 0.5 mM sulfur source: DMSP, DMSO, methionine sulfoxide, dibutylsulfoxide, DMSO₂, methionine sulfone, dibutylsulfone, MSA, propanesulfonate, or pentanesulfonate. DMS was used at a final concentration of 5.0 mM to account for its high volatility. The inoculated microplate was incubated at 37°C with

TABLE 3 Oligonucleotides used in this study^a

Oligonucleotide	Sequence	Purpose(s)
BL342.f	ATGACCATGATTACGGATTCAGT	<i>E. coli lacZ</i>
BL342.r	GCAGTTATTTTACACACAGACCACTGGTA	<i>E. coli lacZ</i>
BL625.f	TACAAAAAGCAGGCTGAACGCTTCTACCCATACTC	<i>sfnR1</i> UpF-GWL
BL625.r	TCAGAGCGCTTTTGAAGCTAATTCGGCTGAGGTTGTGGATATGC	<i>sfnR1</i> UpR-Gm
BL626.f	AGGAACCTCAAGATCCCCAATTCGGAGCATTGTTGCAGCAGG	<i>sfnR1</i> DnF-Gm
BL626.r	TACAAGAAAGCTGGGTTTCAGATCGACAACCGCACC	<i>sfnR1</i> DnR-GWR
BL627.f	TACAAAAAGCAGGCTGAGCTGACCAATCGGTGC	<i>sfnR2</i> UpF-GWL
BL627.r2	CGGATTCACCACTCCAAGAATTGGCGAAAGCACCGCAGTTGAC	<i>sfnR2</i> UpR-Tc
BL628.f2	ATCGATGATAAGCTGTCAAACATGAGACCTGCACCTCATCGACAT	<i>sfnR2</i> DnF-Tc
BL628.r	TACAAGAAAGCTGGGTCCAGCGAGACCACTGTTGTG	<i>sfnR2</i> DnR-GWR
BL629.f	TACAAAAAGCAGGCTCTTCAAAGTCGTCGCCGTCT	<i>msuEDC</i> UpF-GWL
BL629.r	TCAGAGCGCTTTTGAAGCTAATTCGCCTGGAAGAAGCTGAACAGC	<i>msuEDC</i> UpR-Gm
BL630.f	AGGAACCTCAAGATCCCCAATTCGGAACAGGTGGACGTCTACC	<i>msuEDC</i> DnF-Gm
BL630.r	TACAAGAAAGCTGGGTGGTTCGGCTGGATCTCCAG	<i>msuEDC</i> DnR-GWR
BL631.f	CAGTCACAGCAACCTTGGTTTC	<i>msu-lacZ</i> , <i>sfnR1-lacZ</i>
BL631.r1	GCCAGTGAATCCGTAATCATGGTCATGGGATTTCCCTGTGGAC	<i>msu-lacZ</i>
BL631.r2	GCCAGTGAATCCGTAATCATGGTCATGAGTAGAAGGTCGGTTG	<i>sfnR1-lacZ</i>
BL632.f2	CTTGCTCAAGGAGTTTCATCG	<i>sfnR2-lacZ</i>
BL632.r	GCCAGTGAATCCGTAATCATGGTCATGCTGGGTTCCGAGCC	<i>sfnR2-lacZ</i>
BL663.f	TACAAAAAGCAGGCTGATGGCAGCGGTTCTTCATA	<i>sfnG</i> UpF-GWL
BL663.r	TCAGAGCGCTTTTGAAGCTAATTCGCGATCACCTTGAGCTTCTCG	<i>sfnG</i> UpR-Gm
BL664.f	AGGAACCTCAAGATCCCCAATTCGGCTTCAAGACCAACCTGATC	<i>sfnG</i> DnF-Gm
BL664.r	TACAAGAAAGCTGGGTGGCAGATCGGATACGAGC	<i>sfnG</i> DnR-GWR
BL665.f	GTGTTTCGAGAAGAACGTTCC	<i>sfnG-lacZ</i>
BL665.r	CCAGTGAATCCGTAATCATGGTCATCGGGGTATCTCCGG	<i>sfnG-lacZ</i>
BL666.f	GCTCTAGAGATGGCAGGTTCTTCATAG	<i>sfnG</i> ORF
BL666.r	GCGAGCTCGCAGATCGGATACGAGC	<i>sfnG</i> ORF
ZS470.f	GCGGGGCATATGTCCGTCATCACCCATC	<i>sfnR2</i> ORF
ZS470.r	GAGCGAGAATTCTCACTTGCCCAACAGCGAG	<i>sfnR2</i> ORF
ZS471.f	GCGGGGCATATGAACGCTTCTACCCATAC	<i>sfnR1</i> ORF
ZS471.r	GAGCGAGAATTCTCAGATCGACAACCGCAC	<i>sfnR1</i> ORF
ZS488.f	CATTGCGCAATGGCCAG	<i>msuE</i> probe
ZS488.r	CGGGGAAATGTGCAATTTTC	<i>msuE</i> probe
ZS521.f	GATTTCCACGGCACCCCG	<i>sfnG</i> probe
ZS521.r	CCCGATTATGTTTTCAATC	<i>sfnG</i> probe
JRH05.f	GGCTCGAGTTTTTCAGCAAGAT	Cy5-labeled primer for pJET1.2
JRH05.r	GAATATTGTAGGAGATCTTCTAGAAAG	Cy5-labeled primer for pJET1.2

^aThe sequence and purpose of each oligonucleotide are given. Note that the oligonucleotides used in the construction of gene deletion plasmids are designated UpF (up forward), UpR (up reverse), DnF (down forward), DnR (down reverse), GWR (gateway right), and/or GWL (gateway left) in accordance with the nomenclature established by Choi and Schweizer (33). Abbreviations: Gm, gentamicin; Tc, tetracycline. *lacZ*, β -galactosidase gene.

medium shaking in a BioTek Synergy H4 Multi-Mode microplate reader. The optical density at 600 nm (OD_{600}) was measured hourly. Analysis of variance (ANOVA) with Dunnett's *post hoc* test (α value, 0.05) was done to identify significant differences ($P < 0.0001$) in the measured OD_{600} values between samples.

For the genetic complementation experiments, plasmids carrying *sfnR1* (pBRL754), *sfnR2* (pBRL755), or an empty equivalent (pBBR1MCS-5) were electroporated into the Δ *sfnR2* and Δ *sfnR1* Δ *sfnR2::tet* mutants. The Δ *sfnG* mutant was electroporated with either pBBR1MCS-5 or pBRL753. *P. aeruginosa* PAO1 harboring pBBR1MCS-5 was used as the positive control. The growth of these recombinant *P. aeruginosa* strains on DMS, DMSO, or DMSO₂ was performed exactly as described above except that the LB agar plates and LB liquid media were supplemented with 30 μ g ml⁻¹ of gentamicin for plasmid selection. In contrast, the acetate-minimal media used for these specific experiments were not supplemented with gentamicin due to the presence of sulfate in the purchased antibiotic preparation (gentamicin sulfate). Previous studies by our group have shown that the addition of an antibiotic for plasmid selection is not necessary for genetic complementation experiments involving *P. aeruginosa* (32, 35).

LacZ assays. Each strain and/or condition was tested in triplicate ($n = 3$). The first set of LacZ assays aimed to determine how the *sfnR1* and *sfnR2* genes affect the expression of *msuE-lacZ*, *sfnR1-lacZ*, *sfnR2-lacZ*, and *sfnG-lacZ*. The *msuE-lacZ* (pBRL721), *sfnR1-lacZ* (pBRL722), *sfnR2-lacZ* (pBRL723), and *sfnG-lacZ* (pBRL747) reporters were introduced into *P. aeruginosa* PAO1 and its mutants (Δ *sfnR1*, Δ *sfnR2*, and Δ *sfnR1* Δ *sfnR2::tet*) by electroporation. Following initial selection, recombinant strains were grown in 2.0 ml of LB supplemented with 30 μ g ml⁻¹ of gentamicin in a 16- by 100-mm culture tube for 18 h at 37°C at 200 rpm. Acetate-minimal medium supplemented with 0.5 mM sodium sulfate and 30 μ g ml⁻¹ of gentamicin was inoculated with 1.0% (vol/vol) of an LB-grown culture. The inoculated cultures were then grown for 24 h at 37°C at 200 rpm. Cells from these cultures were harvested via centrifugation and subsequently washed with an equal volume of 0.95% NaCl to remove any residual sulfur-containing compounds. The washed cells were resuspended in an equal volume of acetate-minimal medium supplemented with 5.0 mM sulfur source: sodium sulfate, DMS, DMSO, DMSO₂, or MSA. Gentamicin was

not added to the acetate-minimal medium due to the presence of sulfate in its preparation. Each cell suspension (0.2-ml aliquot) was added to a 96-well flat-bottom polystyrene plate (Falcon), and the inoculated plate was grown statically for 4.0 h at 37°C. LacZ activity was then measured from the cells using the Miller assay as described previously (36, 37) and is reported in Miller units. ANOVA with Dunnett's *post hoc* test (α value, 0.05) was performed to identify significant differences ($P < 0.0001$) in the LacZ activities between samples.

The second set of LacZ assays focused on determining how various sulfoxides, sulfones, and sulfonates affect the expression of *msuE-lacZ*, *sfnR1-lacZ*, *sfnR2-lacZ*, and *sfnG-lacZ* in *P. aeruginosa* PAO1 and the $\Delta sfnR1 \Delta sfnR2::tet$ mutant. These assays were conducted exactly as described above for the first set except that additional sulfur sources were tested, including no sulfur source, methionine sulfoxide, dibutylsulfoxide, methionine sulfone, dibutylsulfone, propanesulfonate, pentanesulfonate, and DMSP. Each sulfur source was used at a final concentration of 5.0 mM. For the third and final set, the effect of sodium sulfate on the expression of *msuE-lacZ*, *sfnR1-lacZ*, *sfnR2-lacZ*, and *sfnG-lacZ* in *P. aeruginosa* PAO1 was measured. These assays were conducted exactly as described above for the other sets except that the sulfur source used was a combination of sodium sulfate (final concentration of 0.5 mM) plus DMS, DMSO, DMSO₂, or MSA (final concentration of 5.0 mM).

For the LacZ assays in *E. coli*, *msuE-lacZ* (pBRL721) or *sfnG-lacZ* (pBRL747) was cotransformed with a plasmid carrying either *sfnR1* (pBRL756), *sfnR2* (pBRL757), or an empty equivalent (pTrc99a) into the *lacZ*-null strain *E. coli* DH5 α . The recombinant *E. coli* strains were grown, washed, and assayed under conditions identical to those described above for *Pseudomonas aeruginosa* except for antibiotic selection, which consisted of 100 $\mu\text{g ml}^{-1}$ carbenicillin and 30 $\mu\text{g ml}^{-1}$ gentamicin for the *E. coli* studies.

Electrophoretic mobility shift assays. The *sfnR1* and *sfnR2* genes were expressed and purified as N-terminal polyhistidine-tagged (His₆) proteins, His₆-SfnR1 and His₆-SfnR2. The *sfnR1*-pET15b (pZS455) and *sfnR2*-pET15b (pZS459) plasmids were each transformed into *E. coli* BL21(DE3), and the recombinant cells were grown in 2XYT medium (16 g liter⁻¹ tryptone, 10 g liter⁻¹ yeast extract, 5 g liter⁻¹ NaCl) at 37°C at 200 rpm until an OD₆₀₀ of 0.6 was reached. Isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the cultures at a final concentration of 0.1 mM, and the induced cultures were incubated for 16 h at 16°C at 200 rpm. Cells were harvested by centrifugation, and the recombinant His₆-SfnR1 and His₆-SfnR2 proteins were purified from the collected cells through nickel affinity chromatography as previously described (38).

The 5' regulatory regions (~250 bp) of the *msuE* and *sfnG* genes were PCR amplified from genomic DNA of *P. aeruginosa* PAO1 with the primer pairs ZS488.f/ZS488.r and ZS521.f/ZS521.r, respectively. The *msuE* and *sfnG* PCR products were gel purified and subsequently cloned into pJET1.2 (Thermo Fisher Scientific), yielding the plasmids pZS458 and pKSF01, respectively. The Cy5-labeled *msuE* and *sfnG* probes were generated by PCR comprised of the Cy5-labeled primers JRH05.f and JRH05.r, which anneal specifically to pJET1.2 (35), and either pZS458 or pKSF01 as the template. The resulting Cy5-labeled *msuE* and *sfnG* PCR products were gel purified and quantified using a NanoDrop spectrophotometer. EMSAs were carried out as previously described (35, 38). Briefly, 250 nM His₆-SfnR1 or His₆-SfnR2 was incubated with 2.0 nM Cy5-labeled *msuE*, *sfnG*, or *gcvH2* probe in EMSA buffer (25 mM Tris-acetate [pH 8.0], 8.0 mM magnesium acetate, 10 mM KCl, 1.0 mM dithiothreitol) for 30 min at 30°C. The *gcvH2* probe, which matches the 5' regulatory region of an operon encoding a glycine cleavage system, served as a nonspecific DNA probe or negative control (38). In the second set of EMSAs, 2.0 nM Cy5-labeled *msuE* or *sfnG* probe was incubated with 0, 7.8, 15.6, 31.25, 62.5, 125, 250, or 500 nM His₆-SfnR1 or His₆-SfnR2 in EMSA buffer for 30 min at 30°C.

ACKNOWLEDGMENTS

This study was funded by the NIH (2R15GM104880-03 to B.R.L. and C.T.N.).

The funding agency had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

We also gratefully acknowledge support from the Undergraduate Honors Program of SUNY-ESF, the Center for Applied Microbiology at SUNY-ESF, and CSTEP at SUNY-ESF.

REFERENCES

1. Sievert S, Kiene RP, Schulz-Vogt H. 2007. The sulfur cycle. *Oceanography* 20:117–123. <https://doi.org/10.5670/oceanog.2007.55>.
2. Lomans BP, van der Drift C, Pol A, Op den Camp HJ. 2002. Microbial cycling of volatile organic sulfur compounds. *Cell Mol Life Sci* 59: 575–588. <https://doi.org/10.1007/s00018-002-8450-6>.
3. Kettle AJ, Andreae MO. 2000. Flux of dimethylsulfide from the oceans: a comparison of updated data sets and flux models. *J Geophys Res Atmos* 105:26793–26808. <https://doi.org/10.1029/2000JD900252>.
4. Kiene RP. 1990. Dimethyl sulfide production from dimethylsulfoniopropionate in coastal seawater samples and bacterial cultures. *Appl Environ Microbiol* 56:3292–3297.
5. Yoch DC. 2002. Dimethylsulfoniopropionate: its sources, role in the marine food web, and biological degradation to dimethylsulfide. *Appl Environ Microbiol* 68:5804–5815. <https://doi.org/10.1128/AEM.68.12.5804-5815.2002>.
6. Kiene RP, Bates TS. 1990. Biological removal of dimethyl sulphide from sea water. *Nature* 345:702–705. <https://doi.org/10.1038/345702a0>.
7. Bentley R, Chasteen TG. 2004. Environmental VOSCs—formation and degradation of dimethyl sulfide, methanethiol and related materials. *Chemosphere* 55:291–317. <https://doi.org/10.1016/j.chemosphere.2003.12.017>.
8. Barnes I, Hjorth J, Mihalopoulos N. 2006. Dimethyl sulfide and dimethyl sulfoxide and their oxidation in the atmosphere. *Chem Rev* 106: 940–975. <https://doi.org/10.1021/cr020529+>.
9. Kertesz MA. 2000. Riding the sulfur cycle—metabolism of sulfonates and sulfate esters in gram-negative bacteria. *FEMS Microbiol Rev* 24:135–175.

10. Cook AM, Laue H, Junker F. 1998. Microbial desulfonation. *FEMS Microbiol Rev* 22:399–419. <https://doi.org/10.1111/j.1574-6976.1998.tb00378.x>.
11. Endoh T, Habe H, Yoshida T, Nojiri H, Omori T. 2003. A CysB-regulated and sigma54-dependent regulator, SfnR, is essential for dimethyl sulfone metabolism of *Pseudomonas putida* strain DS1. *Microbiology* 149:991–1000. <https://doi.org/10.1099/mic.0.26031-0>.
12. Endoh T, Kasuga K, Horinouchi M, Yoshida T, Habe H, Nojiri H, Omori T. 2003. Characterization and identification of genes essential for dimethyl sulfide utilization in *Pseudomonas putida* strain DS1. *Appl Microbiol Biotechnol* 62:83–91. <https://doi.org/10.1007/s00253-003-1233-7>.
13. Endoh T, Habe H, Nojiri H, Yamane H, Omori T. 2005. The sigma54-dependent transcriptional activator SfnR regulates the expression of the *Pseudomonas putida* sfnFG operon responsible for dimethyl sulphone utilization. *Mol Microbiol* 55:897–911. <https://doi.org/10.1111/j.1365-2958.2004.04431.x>.
14. Wicht DK. 2016. The reduced flavin-dependent monooxygenase SfnG converts dimethylsulfone to methanesulfinate. *Arch Biochem Biophys* 604:159–166. <https://doi.org/10.1016/j.abb.2016.07.001>.
15. Kertesz MA. 2004. Metabolism of sulphur-containing organic compounds, p 323–357. In Ramos J-L (ed), *Pseudomonas: biosynthesis of macromolecules and molecular metabolism*, vol 3. Springer, New York, NY.
16. Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FS. 2016. Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome database. *Nucleic Acids Res* 44:D646–D653. <https://doi.org/10.1093/nar/gkv1227>.
17. Kertesz MA, Schmidt-Larbig K, Wüest T. 1999. A novel reduced flavin mononucleotide-dependent methanesulfonate sulfonase encoded by the sulfur-regulated *msu* operon of *Pseudomonas aeruginosa*. *J Bacteriol* 181:1464–1473.
18. Kahnert A, Vermeij P, Wietek C, James P, Leisinger T, Kertesz MA. 2000. The *ssu* locus plays a key role in organosulfur metabolism in *Pseudomonas putida* S-313. *J Bacteriol* 182:2869–2878. <https://doi.org/10.1128/JB.182.10.2869-2878.2000>.
19. Bullock HA, Reisch CR, Burns AS, Moran MA, Whitman WB. 2014. Regulatory and functional diversity of methylmercaptopyruvate coenzyme A ligases from the dimethylsulfoniopropionate demethylation pathway in *Ruegeria pomeroyi* DSS-3 and other proteobacteria. *J Bacteriol* 196:1275–1285. <https://doi.org/10.1128/JB.00026-14>.
20. Carrión O, Curson AR, Kumaresan D, Fu Y, Lang AS, Mercadé E, Todd JD. 2015. A novel pathway producing dimethylsulphide in bacteria is widespread in soil environments. *Nat Commun* 6:6579. <https://doi.org/10.1038/ncomms7579>.
21. Drotar A, Burton GA, Jr, Tavernier JE, Fall R. 1987. Widespread occurrence of bacterial thiol methyltransferases and the biogenic emission of methylated sulfur gases. *Appl Environ Microbiol* 53:1626–1631.
22. Vogt W. 1995. Oxidation of methionyl residues in proteins: tools, targets, and reversal. *Free Radic Biol Med* 18:93–105. [https://doi.org/10.1016/0891-5849\(94\)00158-G](https://doi.org/10.1016/0891-5849(94)00158-G).
23. Grimaud R, Ezraty B, Mitchell JK, Lafitte D, Briand C, Derrick PJ, Barras F. 2001. Repair of oxidized proteins. Identification of a new methionine sulfoxide reductase. *J Biol Chem* 276:48915–48920. <https://doi.org/10.1074/jbc.M105509200>.
24. Scherer HW. 2009. Sulfur in soils. *J Plant Nutr Soil Sci* 172:326–335. <https://doi.org/10.1002/jpln.200900037>.
25. Romsang A, Atichartpongkul S, Trinachartvanit W, Vattanaviboon P, Mongkolsuk S. 2013. Gene expression and physiological role of *Pseudomonas aeruginosa* methionine sulfoxide reductases during oxidative stress. *J Bacteriol* 195:3299–3308. <https://doi.org/10.1128/JB.00167-13>.
26. Kouzuma A, Endoh T, Omori T, Nojiri H, Yamane H, Habe H. 2008. Transcription factors CysB and SfnR constitute the hierarchical regulatory system for the sulfate starvation response in *Pseudomonas putida*. *J Bacteriol* 190:4521–4531. <https://doi.org/10.1128/JB.00217-08>.
27. Tralau T, Vuilleumier S, Thibault C, Campbell BJ, Hart CA, Kertesz MA. 2007. Transcriptomic analysis of the sulfate starvation response of *Pseudomonas aeruginosa*. *J Bacteriol* 189:6743–6750. <https://doi.org/10.1128/JB.00889-07>.
28. Studholme DJ, Dixon R. 2003. Domain architectures of sigma54-dependent transcriptional activators. *J Bacteriol* 185:1757–1767. <https://doi.org/10.1128/JB.185.6.1757-1767.2003>.
29. Bush M, Dixon R. 2012. The role of bacterial enhancer binding proteins as specialized activators of sigma54-dependent transcription. *Microbiol Mol Biol Rev* 76:497–529. <https://doi.org/10.1128/MMBR.00006-12>.
30. Schulz S, Eckweiler D, Bielecka A, Nicolai T, Franke R, Dötsch A, Hornischer K, Bruchmann S, Duvel J, Häussler S. 2015. Elucidation of sigma factor-associated networks in *Pseudomonas aeruginosa* reveals a modular architecture with limited and function-specific crosstalk. *PLoS Pathog* 11:e1004744. <https://doi.org/10.1371/journal.ppat.1004744>.
31. Habe H, Kouzuma A, Endoh T, Omori T, Yamane H, Nojiri H. 2007. Transcriptional regulation of the sulfate-starvation-induced gene *sfnA* by a sigma54-dependent activator of *Pseudomonas putida*. *Microbiology* 153:3091–3098. <https://doi.org/10.1099/mic.0.2007/008151-0>.
32. Lundgren BR, Villegas-Peñaranda LR, Harris JR, Mottern AM, Dunn DM, Boddy CN, Nomura CT. 2014. Genetic analysis of the assimilation of C₅-dicarboxylic acids in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 196:2543–2551. <https://doi.org/10.1128/JB.01615-14>.
33. Choi KH, Schweizer HP. 2005. An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. *BMC Microbiol* 5:30. <https://doi.org/10.1186/1471-2180-5-30>.
34. Lundgren BR, Connolly MP, Choudhary P, Brookins-Little TS, Chatterjee S, Raina R, Nomura CT. 2015. Defining the metabolic functions and roles in virulence of the *rpoN1* and *rpoN2* genes in *Ralstonia solanacearum* GM1000. *PLoS One* 10:e0144852. <https://doi.org/10.1371/journal.pone.0144852>.
35. Lundgren BR, Harris JR, Sarwar Z, Scheel RA, Nomura CT. 2015. The metabolism of (*R*)-3-hydroxybutyrate is regulated by the enhancer-binding protein PA2005 and the alternative sigma factor RpoN in *Pseudomonas aeruginosa* PAO1. *Microbiology* 161:2232–2242. <https://doi.org/10.1099/mic.0.000163>.
36. Lundgren BR, Thornton W, Dornan MH, Villegas-Peñaranda LR, Boddy CN, Nomura CT. 2013. Gene PA2449 is essential for glycine metabolism and pyocyanin biosynthesis in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 195:2087–2100. <https://doi.org/10.1128/JB.02205-12>.
37. Zhang X, Bremer H. 1995. Control of the *Escherichia coli* *rrnB* P1 promoter strength by ppGpp. *J Biol Chem* 270:11181–11189. <https://doi.org/10.1074/jbc.270.19.11181>.
38. Sarwar Z, Lundgren BR, Grassa MT, Wang MX, Gribble M, Moffat JF, Nomura CT. 2016. GcsR, a TyrR-like enhancer-binding protein, regulates expression of the glycine cleavage system in *Pseudomonas aeruginosa* PAO1. *mSphere* 1:e00020-16. <https://doi.org/10.1128/mSphere.00020-16>.
39. Beck LL, Smith TG, Hoover TR. 2007. Look, no hands! Unconventional transcriptional activators in bacteria. *Trends Microbiol* 15:530–537. <https://doi.org/10.1016/j.tim.2007.09.008>.
40. Heurlier K, Dénervaud V, Pessi G, Reimann C, Haas D. 2003. Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 185:2227–2235. <https://doi.org/10.1128/JB.185.7.2227-2235.2003>.
41. Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, II, Peterson KM. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166:175–176. [https://doi.org/10.1016/0378-1119\(95\)00584-1](https://doi.org/10.1016/0378-1119(95)00584-1).