Protective Role of Bacterial Alkanesulfonate Monooxygenase under Oxidative Stress

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ABSTRACT Bacterial alkane metabolism is associated with a number of cellular stresses, including membrane stress and oxidative stress, and the limited uptake of charged ions such as sulfate. In the present study, the genes *ssuD* and *tauD* in *Acinetobacter oleivorans* DR1 cells, which encode an alkanesulfonate monooxygenase and a taurine dioxygenase, respectively, were found to be responsible for hexadecanesulfonate (C₁₆SO₃H) and taurine metabolism, and Cbl was experimentally identified as a potential regulator of *ssuD* and *tauD* expression. The expression of *ssuD* and *tauD* occurred under sulfate-limited conditions generated during *n*-hexadecane degradation. Interestingly, expression analysis and knockout experiments suggested that both genes are required to protect cells against oxidative stress, including that generated by *n*-hexadecane degradation and H₂O₂ exposure. Measurable levels of intracellular hexadecanesulfonate were also produced during *n*-hexadecane degradation. Phylogenetic analysis suggested that *ssuD* and *tauD* are mainly present in soil-dwelling aerobes within the *Betaproteobacteria* and *Gamma*proteobacteria classes, which suggests that they function as controllers of the sulfur cycle and play a protective role against oxidative stress in sulfur-limited conditions.

IMPORTANCE *ssuD* and *tauD*, which play a role in the degradation of organosulfonate, were expressed during *n*-hexadecane metabolism and oxidative stress conditions in *A. oleivorans* DR1. Our study confirmed that hexadecanesulfonate was accidentally generated during bacterial *n*-hexadecane degradation in sulfate-limited conditions. Removal of this by-product by SsuD and TauD must be necessary for bacterial survival under oxidative stress generated during *n*-hexadecane degradation.

KEYWORDS hexadecanesulfonate, SsuD, TauD, *Acinetobacter oleivorans* DR1, oxidative stress, sulfate limitation, soil-dwelling aerobe

Sulfur is critical for the synthesis of amino acids (e.g., cysteine and methionine) and enzyme cofactors (e.g., biotin, coenzyme A, coenzyme M, thiamine, and lipoic acid) in bacteria. Although aquatic environments contain high concentrations of inorganic sulfate (e.g., an average of 28.7 mM in marine environments), in soil environments, inorganic sulfate accounts for only 1 to 5% of the total available sulfur. Most sulfur in soil is held in an organically bound form, such as in sulfate esters or organosulfonates (1, 2). In bacteria, sulfur is mainly acquired through inorganic sulfate (SO₄^{2−}) using the sulfate assimilation pathway (3). SO₄^{2−} is imported into the cell and converted into 3-phosphoadenosine-5-phosphosulfate (PAPS) via adenosine-5-phosphosulfate (APS). Once converted, it is reduced to sulfite (SO₃^{2−}) and sulfide (−HS, H₂S), which can then be incorporated into cysteine synthesis (4).

Because SO₄^{2−} is not abundant in nature, particularly in soil, bacteria regularly face SO₄^{2−}-limited conditions (2). To obtain sulfur from their environment, bacteria utilize organosulfonate or sulfonate-derived compounds (e.g., taurine, aliphatic/aromatic sulfonate, and sulfate ester) with the assistance of enzymes such as alkanesulfonate monooxygenase (SsuD) and taurine dioxygenase (TauD) (2, 5). In response to sulfate

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starvation, the representative global regulator CysB upregulates the expression of the cysteine regulon (i.e., the *cys* operon) and the *ssu* (*ssuEADCB*) and *tau* (*tauABCD*) operons (6). However, the expression of CysB is repressed in the presence of sulfate due to the inhibition of inducer (*N*-acetyl-L-serine) synthesis by L-cysteine or by the production of anti-inducers (e.g., sulfide and thiosulfate), which leads to the lower expression of the genes that respond to sulfate starvation (7). The regulator Cbl is well-known as a nonessential protein for sulfate assimilation, although its knockout strain grew slowly under normal conditions (8). However, Cbl can bind to the *tauA* and *ssu* promoters and control the expression of the *tau* and *ssu* operons (8, 9).

The two-component alkanesulfonate monooxygenase system is composed of SsuD monooxygenase and NADPH-dependent flavin mononucleotide (FMN) reductase (SsuE), which catalyzes the reduction of flavin (FMNH₂) using NADPH. Thus, the oxidation of alkanesulfonate by SsuD occurs after the reduced FMNH₂ has been transferred to SsuD, resulting in the production of aldehyde and sulfite (10). Similarly, TauD requires α -ketoglutarate to mediate the oxidation of taurine (2-aminoethanesulfonate), which produces succinate, aminoacetaldehyde, and sulfite (11).

The sulfur cycle of anaerobic bacteria has been well studied in terms of the alkane degradation process because sulfate is an important electron acceptor for sulfate-reducing bacteria (12). However, the contribution of sulfur metabolism to aerobic alkane degradation remains poorly understood. Generally, aerobic alkane degradation involves the initial oxidation of alkanes by AlkB monooxygenase. AlkB-type alkane monooxygenase, which is composed of rubredoxin (AlkG) and rubredoxin reductase (AlkT), mediates the conversion of alkanes to fatty alcohols, which allows the subsequent oxidation of fatty aldehydes and fatty acids to occur (13). Soil-derived *Acinetobacter oleivorans* DR1 has been studied as an alkane/diesel degrader (14–18). The DR1 strain can utilize C₁₂ to C₃₀ alkanes as a carbon source, with *n*-hexadecane (Hex; C₁₆) reported to be the most favorable substrate for DR1 growth (17). Our previous research demonstrated that the genes involved in sulfate assimilation were highly upregulated in DR1 during Hex metabolism (16). Interestingly, the expression levels of the *ssu* and *tau* operons were also significantly higher in Hex-supplemented media, even though organosulfonate was absent from the growth media (16). These transcriptome findings led us to investigate the roles of SsuD and TauD during Hex degradation.

In the present study, the functions of SsuD and TauD were evaluated using knockout strains (Δ *ssuD*, Δ *tauD*, and Δ *cbl*) of DR1. Furthermore, the roles of SsuD and TauD were determined using physiological and analytical experiments, including the measurement of sulfate consumption in sodium succinate (NaSc) and Hex-amended media, the assessment of H₂O₂ susceptibility under oxidative stress, and the quantification of hexadecanesulfonate during Hex metabolism. This report suggests that SsuD and TauD both play a defensive role in response to sulfate limitation and oxidative stress in *A. oleivorans* DR1, which possibly explains why their expression increases during Hex degradation.

RESULTS

Growth of *ssuD*-, *tauD*-, and *cbl*-defective mutants on organosulfonate-supplemented media. Growth experiments were conducted to identify the functions of SsuD and TauD and their substrate ranges in Hex-supplemented media. The magnesium sulfate (MgSO₄) in M9 medium was replaced with six different organosulfonates: ethanesulfonate (C₂SO₃H), hexanesulfonate (C₆SO₃H), hexadecanesulfonate (C₁₆SO₃H), taurine, 3-(*N*-morpholino)-propanesulfonic acid (MOPS), and HEPES as the sulfur source (see Materials and Methods). Of the six organosulfonates tested, only C₁₆SO₃H was consumed during Hex metabolism (Fig. S1 in the supplemental material). Compared to the DR1 wild-type (WT) strain, the Δ *ssuD* and Δ *tauD* mutant strains exhibited growth defects in SO₄-depleted and C₁₆SO₃H-supplemented media (Fig. 1A). However, similar growth patterns were observed for the WT and Δ *ssuD* or Δ *tauD* knockout strains when C₁₆SO₃H was added as the sole carbon source (–Hex, +SO₄) (Fig. 1A). The growth of the *cbl* knockout strain was completely impaired when C₁₆SO₃H

FIG 1 Growth of wild-type (WT), *ΔssuD*, *ΔtauD*, and *Δcbl* strains in C₁₆SO₃H- and taurine-supplemented media. Growth was measured in (A) C₁₆SO₃H- or (B) taurine-supplemented media. (A) +Hex (left)/−Hex (right) and +SO₄ (right)/−SO₄ (left) represent the presence/absence of *n*-hexadecane (Hex) and sulfate in M9 media, respectively. (B) +SO₄ (right)/−SO₄ (left) represent the presence/absence of sulfate in M9 media. NaSc denotes 10 mM sodium succinate. The colors indicate each strain (red, WT; blue, *ΔssuD*; green, *ΔtauD*; orange, *Δcbl*). All experiments were performed in triplicate, and the averages are presented here. The error bars represent the standard deviation.

was used as either a carbon or sulfur source, which confirmed that Cbl was responsible for the upregulation of both *ssuD* and *tauD*.

In sodium succinate (NaSc)-supplemented media instead of Hex, all of the tested strains (WT, *ΔssuD*, *ΔtauD*, and *Δcbl*) grew in the presence of taurine and sulfate (Fig. 1B). However, only the WT and *ΔssuD* strains grew when the sulfate was eliminated, indicating that the WT strain could utilize taurine as a sulfur source, while the *ΔtauD* and *Δcbl* strains could not when NaSc was added as the carbon source (Fig. 1B). Taken together, these results indicate that DR1 can utilize C₁₆SO₃H and taurine as a sulfur source, a process that involves the *ssuD* and *tauD* genes, in the presence of Hex and NaSc. Furthermore, it is likely that the Cbl regulator controls organosulfonate utilization.

Sulfate-limited conditions in the presence of *n*-hexadecane. In our previous study, we observed that growth of DR1 cells was negatively affected when the sulfate concentration was reduced in the Hex-supplemented medium, but the growth rate was similar regardless of the sulfate concentration in the NaSc-supplemented M9 medium (16). In the present study, we examined potential sulfate limitation in minimal salt basal (MSB) medium supplemented with either a succinate/acetate mixture (NaSc + NaAc) or Hex as a carbon source (Fig. 2). Although the DR1 cells exhibited similar cell densities during the stationary phase with either NaSc + NaAc- or Hex-supplemented MSB media (optical density at 600 nm [OD₆₀₀] = ~1.0), no growth defects were exhibited when using the NaSc + NaAc medium despite the lower sulfate concentration (Fig. 2). However, growth was inhibited when the sulfate concentration decreased during Hex metabolism (normal MSB maximum growth rate [$\mu_{\max}$] = 0.21 h^{−1}; 1/10 sulfate-reduced MSB $\mu_{\max}$ = 0.11 h^{−1}; Fig. 2). Further analysis showed that the DR1 cells consumed more sulfate (5-fold) in the presence of NaSc and NaAc (3.54 pmol/CFU) than with Hex (0.70 pmol/CFU) during the exponential growth phase. Thus, these results suggest that the DR1 strain experienced sulfate limitation during Hex metabolism.

Oxidative stress in *n*-hexadecane-supplemented media. Transcriptomic data suggested that the *ssu* and *tau* operons were also induced under oxidative stress. In

FIG 2 Growth of *A. oleivorans* DR1 under sulfate-limited conditions and the measurement of sulfate consumption. Growth was compared in normal (control, filled circles) and 1/10 sulfate-reduced (1/10 sulfate, white circles) MSB media when succinate and acetate (NaSc + NaAc) or Hex were added as a carbon source. Maximum growth rates ($\mu_{\max}$) were calculated during the exponential growth phase of the DR1 cells.

DR1, both operons were upregulated by red clay (Red), paraquat (PQ), phenazine methosulfate (PMS), and hydrogen peroxide (H_2O_2) treatment (Fig. S2A). Although the $\log_2$ -fold values of *ssuA1* and *ssuA2* were -0.5 and -0.2 , respectively, under H_2O_2 treatment, Red, PQ, PMS, and H_2O_2 all promoted *ssuD* and *tauD* gene expression (*ssuD* $\log_2$ value = 2.7 [Red], 4.9 [PQ], 4.5 [PMS], and 1.7 [H_2O_2]; *tauD* $\log_2$ value = 2.3 [Red], 4.8 [PQ], 5.8 [PMS], and 5.2 [H_2O_2]) (Fig. S2A). Furthermore, database analysis found that the upregulation of both operons under oxidative stress (H_2O_2) had been observed in *Acinetobacter baumannii*, *Pseudomonas aeruginosa* PAO1, *Pseudomonas putida* KT2440, *Corynebacterium glutamicum*, and *Escherichia coli* K-12 strains (Fig. S2B). In particular, *A. baumannii* experienced a dramatic upregulation of the *ssu* and *tau* operons (over 16.2-fold) following H_2O_2 treatment (*ssuD*, 114.3 ± 14.2 ; *tauD*, 88.6 ± 18.0). The expression of the two operons in *P. aeruginosa* was the lowest of those tested, but *ssuD* and *tauD* were still upregulated by 2.5- and 1.2-fold, respectively (Fig. S2B). However, the reasons for this upregulation have not yet been investigated.

Comparative growth assays of the WT, $\Delta ssuD$, $\Delta tauD$, and Δcbl strains showed that the growth of the mutant strains was inhibited during Hex metabolism with aeration (Fig. 3A). In particular, the growth of the $\Delta ssuD$ strain was significantly lower than that of the WT strain (Fig. 3A). In contrast, under static conditions, the $\Delta ssuD$ and $\Delta tauD$

FIG 3 Growth of WT, $\Delta ssuD$, $\Delta tauD$, and Δcbl strains under aeration or static conditions. OD_{600} was measured in Hex-supplemented M9 media under aeration (A) or static conditions (B). Colors indicate each strain (gray, WT; red, $\Delta ssuD$; blue, $\Delta tauD$; white, Δcbl). All experiments were performed in triplicate, and the average values are shown. Error bars indicate standard deviations.

strains grew at rates similar to that of the WT strain, while the Δcbl strain still did not grow (Fig. 3B). Thus, we examined whether the deletion of *ssuD* or *tauD* imposes greater oxidative stress on the cells using dihydrorhodamine 123 (DHR123) dye, a reactive oxygen species (ROS) indicator. The number of stained cells in $\Delta ssuD$ and $\Delta tauD$ was higher than that of the WT sample, indicating that a larger amount of ROS was generated in $\Delta ssuD$ and $\Delta tauD$ strains than WT (Fig. 4A).

In addition, compared with the WT strain, the expression levels of oxidative stress response genes such as *katG* (catalase G), *katE* (catalase E), *sodB* (superoxide dismutase), *trxC* (thioredoxin 2), and *grxC* (glutaredoxin 3) in the $\Delta ssuD$ strain were 1.6-, 3.6-, 1.9-, 2.4-, and 1.7-fold higher, respectively (Fig. 4B). Although the *katG* and *trxC* genes were not upregulated in the $\Delta tauD$ mutant (*katG*, 0.84-fold; *trxC*, 0.86-fold), the *katE*, *sodB*, and *grxC* genes were (Fig. 4B). The $\Delta ssuD$ and $\Delta tauD$ mutant strains also produced more biofilm after 24 and 48 h than did the WT strain. The fluorescence intensity of the mutant strains was three times higher than that of the WT strain after 24 h (Fig. 4C). Despite the significantly lower biofilm production in the mutant strains after 48 h, the fluorescence intensity was at least two times greater than that of the WT strain (Fig. 4C). Finally, we introduced a high concentration of hydrogen peroxide (50 mM H_2O_2) to the WT, $\Delta ssuD$, and $\Delta tauD$ cells during the exponential growth phase; the $\Delta ssuD$ and $\Delta tauD$ strains were subsequently found to be much more susceptible to oxidative stress than the WT strain (Fig. 4D).

SO_3^{2-} can be produced from the reduction of SO_4^{2-} via APS and PAPS or the oxidation of alkanesulfonate and taurine (Fig. 5). The SO_3^{2-} can then be reduced to sulfide ($-HS$) for cysteine synthesis or free hydrogen sulfide (H_2S). The examination of total H_2S concentration during Hex metabolism showed that initial H_2S production by the WT strain was much higher than that of the $\Delta ssuD$ and $\Delta tauD$ strains (Fig. 5). However, during the exponential phase, the total concentration of H_2S from the WT cells was not significantly higher than those from either mutant. Taken together, these results indicate that alkanesulfonate monooxygenase and taurine dioxygenase potentially play a role in oxidative stress defense and are responsible for the production of H_2S from sulfate or organosulfonate during Hex metabolism.

Spontaneous production of hexadecanesulfonate during *n*-hexadecane degradation. We hypothesized that the spontaneous generation of organosulfonate would induce the expression of the *ssu* and *tau* operons in the DR1 strain during Hex metabolism. Gas chromatography (GC) profiles revealed the appearance of a consistent hexadecanesulfonate peak (Fig. 6A) in the DR1 WT, $\Delta ssuD$, and $\Delta tauD$ strains grown in Hex-supplemented media, but no peak was observed in the WT cell extract cultured in NaSc-supplemented medium (Fig. 6A). Furthermore, mass spectra of peaks at 17.1 min in standard (10 mM hexadecanesulfone) and DR1 WT-grown Hex media (WT_Hex) confirmed that this peak is for hexadecanesulfonate (Fig. S3). The total amount of hexadecanesulfonate was nearly equal to that of intracellular hexadecanesulfonate at each time point (Fig. 6B). In addition, the hexadecanesulfonate concentration was highest in all three strains at 12 h (over 100 mM/OD₆₀₀) and gradually decreased over time (Fig. 6B). $\Delta ssuD$ and $\Delta tauD$ mutants had higher levels of intracellular hexadecanesulfonate than did the WT strain at 18, 24, and 48 h. Furthermore, the rate of decrease was slower in the mutant strains than in the WT strain (Fig. 6B). In conclusion, the total and intracellular hexadecanesulfonate levels observed in the WT, $\Delta ssuD$, and $\Delta tauD$ strains grown in Hex-supplemented medium during the exponential phase indicates that the efficiency of the $\Delta ssuD$ and $\Delta tauD$ mutants in degrading the hexadecanesulfonate produced from Hex metabolism was lower than that of the WT strain.

Evolutionary and ecological analysis of organosulfonate-metabolic bacteria. There were 1,976 completely sequenced bacterial genomes in the Integrated Microbial Genomes (IMG) database, and these were classified as *Proteobacteria* (*Alphaproteobacteria*, 11.0%; *Betaproteobacteria*, 8.3%; *Gammaproteobacteria*, 20.5%; *Deltaproteobacteria*, 2.6%; and *Epsilonproteobacteria*, 2.1%), *Firmicutes* (19.3%), *Actinobacteria* (15.4%), *Bacteroidetes* (6.3%), and other minor phyla (14.5%) (Fig. S4). Of these bacteria, 1,489 genomes did not contain *cysB*, *cbl*, *ssuD*, or *tauD* (Fig. S5), while 17 species carried all

FIG 4 Oxidative stress in $\Delta ssuD$ and $\Delta tauD$ mutants. (A) Quantification of reactive oxygen species-damaged cells using fluorescence-activated cell sorter (FACS) analysis and DHR123 fluorescent dye. The x axis represents the fluorescein isothiocyanate (FITC) intensity, and the y axis represents the cell count. The WT, $\Delta ssuD$, and $\Delta tauD$ strains are colored black, blue, and red, respectively. (B) Relative expression of oxidative stress response genes (*katG*, *katE*, *sodB*, *trxC*, and *grxC*) in *ssuD* and *tauD* knockout strains compared to the WT strain. The colors indicate each strain (gray, WT; blue, $\Delta ssuD$; red, $\Delta tauD$). (C, Left) CLSM image analysis of biofilm formation after 24 and 48 h. (C, Right) Biofilms were stained and quantified. (D) H_2O_2 sensitivity assays for the WT, *ssuD*, and *tauD* knockout strains. Spotted agar plates after H_2O_2 treatment and serial dilution.

four genes (e.g., *E. coli*), and 31 had one regulator (*cysB* or *cbl*) and two oxygenases (*ssuD* and *tauD*) (Fig. S5). In addition, 46 species had *ssuD* and *tauD* genes present in their genomes but neither a CysB regulator nor a Cbl regulator. The CysB and Cbl regulators were mainly found within *Betaproteobacteria* and *Gammaproteobacteria* (Fig.

FIG 5 Total H₂S concentration measurement. Total H₂S concentration was measured in the presence of *n*-hexadecane. (Left) Schematic of the sulfate assimilation pathway, which produces sulfide intermediates. (Right) H₂S microsensor measurements. The H₂S concentration was normalized by optical density (OD₆₀₀). The colors indicate each strain (gray, WT; red, Δ*ssu*D; blue, Δ*tau*D). All experiments were performed in triplicate, and the averages and their standard deviation are presented here.

S4), while *ssu*D and *tau*D genes were more widely distributed among the bacterial groups, including *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Firmicutes*, and *Actinobacteria*.

Of the bacterial species possessing the *ssu*D gene, 11.9% were found in soil, and only 5.3% were found in water (Fig. 7). In particular, 57.7% (15 species), 7.7% (2 species), and 3.8% (1 species) of the soil bacteria originated from territorial soil, the rhizosphere, and sediment environments, respectively. Likewise, *tau*D were more commonly found in soil-isolated bacteria (12.0%) than in those isolated from water (3.2%), with 66.0% of these from terrestrial soil, 8.3% from the rhizosphere, 4.2% from sediment, 8.3% from freshwater, 4.2% from marine environments, and 8.3% from hot springs when unclassified and other habitat bacteria were excluded (Fig. 7). Interestingly, 14.9% of the bacterial species harboring both *ssu*D and *tau*D were isolated from soil, but none were taken from water (78.6% terrestrial soil, 14.3%, rhizosphere, 7.1% sediment) (Fig. 7). Aerobic bacteria accounted for the highest proportion of bacteria that contained only *ssu*D, *tau*D, or both (*ssu*D, 29.8%; *tau*D, 23.4%; both, 25.5%), followed by facultative aerobic bacteria (*ssu*D, 15.9%; *tau*D, 15.2%; both, 13.8%) and anaerobic bacteria (*ssu*D, 12.6%; *tau*D, 14.6%; both, 12.8%) (Fig. S6). In conclusion, *cys*B, *cbl*, *ssu*D, and *tau*D were mainly found within the phylum *Proteobacteria*, with most of the bacterial species harboring *ssu*D and/or *tau*D genes being soil derived and/or aerobic.

DISCUSSION

Sulfur cycling in anaerobic alkane-degrading bacteria has been well studied due to its importance in carbon metabolism and energy production (12, 19). Sulfate-reducing bacteria produce energy by coupling the oxidation of hydrogen (H₂) or certain organic compounds with the reduction of sulfate (SO₄²⁻) to sulfide (H₂S, HS⁻) under anaerobic conditions (20). Recently, it has been revealed that an alkylsuccinate synthase homolog, designated pyruvate formate lyase (PflD), catalyzes C₁₀ to C₂₁ alkanes using thiosulfate or sulfate as a terminal electron acceptor in the sulfate-reducing archaeon *Archaeoglobus fulgidus* strain VC-16 (21). However, the sulfur metabolism of alkane utilizers has not previously been considered under aerobic conditions. Interestingly, in addition to the *ssu* and *tau* operons, other genes participating in sulfate assimilation were highly upregulated in Hex-supplemented media (16). Because M9 medium contains low concentrations of sulfur (M9 medium, 2 mM; other minimal media, 8 to 16 mM; LB, excess cysteine), the differential expression of sulfur metabolism-related genes can be observed depending on the carbon source (2).

FIG 6 Quantification of total and intracellular $C_{16}SO_3$. Total and intracellular $C_{16}SO_3$ levels were measured using GC-MS. (A) The $C_{16}SO_3$ peak was detected at 17.1 min in a 10 mM $C_{16}SO_3$ standard. For the same retention time, a peak was observed in the samples cultured in Hex (WT_Hex, $\Delta ssuD_Hex$, and $\Delta tauD_Hex$) but not in NaSc (WT_NaSc). (B) Total and intracellular $C_{16}SO_3$ concentrations were measured after 12, 18, 24, and 48 h of growth. The concentrations were normalized by cell density (OD₆₀₀). All experiments were performed in triplicate, and the averages are presented here. The error bars represent the standard deviation.

Genomic analysis revealed that *A. oleivorans* DR1 has multiple copies of *ssuD* and *tauD* (six and four copies, respectively) (Table S1). However, only one copy each of *ssuD* (AOLE_RS19315) and *tauD* (AOLE_RS11495) are part of the *ssuA1A2DCB* and *tauABCD* operons. Furthermore, the two genes showed the highest similarity with *ssuD* and *tauD* from the *E. coli* K-12 strain and were highly expressed in the presence of Hex (Table S1; Fig. S7A and C). Compared with the *ssu* and *tau* operons in the *E. coli* K-12 and *P. aeruginosa* PAO1 strains, the DR1 operons both had a similarity of at least 40%; however, *ssuE* (encoding FMN reductase) was located approximately 3.9 Mb away from the *ssu* operon and had a similarity of less than 35% (Fig. S7A). We also found that the binding sites of the potential regulators AlkR, Cbl, and OxyR were present upstream of the *ssuA1A2DCB* and *tauABCD* operons, suggesting that both operons are regulated by these global regulators; thus, these genes may be induced during alkane exposure, sulfate starvation, and oxidative stress (Fig. S7B). This information provided the basis for our hypothesis about the relationship between sulfate limitation, oxidative stress, and accidental alkanesulfonate production. Previous transcriptome sequencing (RNA-seq) data have demonstrated that the *ssu* and *tau* operons are upregulated when DR1 metabolizes Hex as opposed to NaSc (16). In particular, *ssuD* (AOLE_RS19315) and *tauD* (AOLE_RS11495) were significantly upregulated (13.2- and 7.4-fold, respectively), which was confirmed using quantitative real-time PCR (qRT-PCR) in the present study (Table

FIG 7 Distribution of *ssuD* and *tauD* genes among bacterial groups. Using the IMG database, the number and percentage of bacterial species that harbor *ssuD* and/or *tauD* genes are presented. The three pie charts on the right represent the ecological niche of the bacterial species (unclassified, others, water, and soil), and the pie charts on the left represent the specific niches within soil and water. The parentheses contain the number of bacterial species.

S1; Fig. S7C). Thus, we generated and analyzed knockout strains to identify the potential role of alkanesulfonate monooxygenase and taurine dioxygenase during Hex metabolism. In this study, *ssuD* and *tauD* refer only to AOLE_RS19315 and AOLE_RS11495, respectively.

In *E. coli*, SsuD can utilize a wide range of organosulfonate substrates [e.g., HEPES, MOPS, piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), and C_5SO_3H to $C_{14}SO_3H$] (10). However, SsuD in DR1 supported growth only when $C_{16}SO_3H$ was used as a sulfur source. This result might be due to a narrower substrate range of organosulfonate transporter (SsuABC) and/or SsuD in DR1 than those in *E. coli* (Fig. 1A and Fig. S1). The significantly lower growth in the *ssuD* knockout strain during Hex metabolism when $C_{16}SO_3H$ was the sulfur source suggests that the *ssuD* gene is directly related to $C_{16}SO_3H$ consumption (Fig. 1A). In addition, the growth of the *tauD*-knockout strain was slightly inhibited when $C_{16}SO_3H$ was the sulfur source, suggesting that *tauD* is also required for $C_{16}SO_3H$ metabolism. SsuD catalyzes alkanesulfonate to produce fatty aldehydes and sulfite (22). Thus, $C_{16}SO_3H$ degradation by SsuD can supply both carbon and sulfur. Unexpectedly, when $C_{16}SO_3H$ was used as a carbon source, the $\Delta ssuD$ and $\Delta tauD$ strains grew as much as the WT strain, possibly indicating that other *ssuD* or *tauD* genes compensate for the loss of these genes. In addition, 2 mM $C_{16}SO_3H$ appears to have been insufficient as a carbon source because the WT strain in the $C_{16}SO_3H$ -supplemented medium did not grow as much as in the medium to which Hex had been added (Fig. 1A). Taurine can be utilized as a sulfur source in the presence of NaSc but not Hex (Fig. 1B and S1). This might be because taurine dioxygenase requires 2-oxoglutarate (α -ketoglutarate), a tricarboxylic acid (TCA) cycle intermediate (11).

To adhere to lipophilic Hex, the bacterial cell surface becomes hydrophobic, creating a hydrophobic environment between the cell and the substrate (23, 24). This may make it difficult for polar substrates such as ions and water to enter the cells (25). On the other hand, *ssu* and *tau* are genes that are induced by sulfate starvation (26). DR1 cells exhibited sulfate-dependent growth when Hex was used as a carbon source (Fig. 2).

Although genes participating in SO_4^{2-} assimilation are highly upregulated in the presence of Hex (16), the amount of SO_4^{2-} consumed was lower when NaSc and NaAc were metabolized. In fact, DR1 cells may require high concentrations of sulfur for Hex metabolism because several oxidative stress-sensing proteins and detoxifying enzymes have Fe-S clusters or disulfide bonds (27, 28). Previous studies have suggested that alkane degradation generates intracellular oxidative stress (15, 16, 19). Therefore, this discrepancy between low SO_4^{2-} consumption and high expression of genes related to the sulfate assimilation in Hex-amended medium implies that DR1 cells experienced a limitation of sulfate during Hex degradation.

The transcriptomic results revealed that *ssuD* and *tauD* were upregulated under oxidative stress in DR1 and other species, suggesting that this is a general phenomenon (Fig. S2). In addition, the present study found that SsuD and TauD participated in the defense against oxidative stress, with the lower growth of ΔssuD and ΔtauD mutants under aeration conditions possibly due to oxidative stress-induced damage (Fig. S2A and B; Fig. 4A). *katG*, *katE*, *sodB*, *trxC*, and *grxC* were significantly upregulated during Hex degradation compared to succinate metabolism (16), and the expression of these genes was higher when ΔssuD and ΔtauD mutants metabolized Hex (Fig. 4B). Although OxyR-independent genes (e.g., *katE*, *sodB*, and *grxC*) are upregulated in both mutants (29), the predicted OxyR-binding site was located upstream of the *ssu* and *tau* operons (Fig. S7B). Furthermore, greater biofilm formation was observed in *ssuD*- or *tauD*-defective mutants than in the WT strain (Fig. 4C). It has been observed that OxyR binds to promoters of the K locus, which is one of polysaccharide-producing operons in *A. oleivorans* DR1, influences the expression of poly-*N*-acetylglucosamine (PNAG) synthesis operons, and functions as a repressor (30). It is unclear whether the OxyR regulator controls the *ssu* and *tau* operons, but both operons are upregulated under oxidative stress.

Both the *ssuD* and *tauD* knockout strains exhibited increased sensitivity to H_2O_2 compared to the WT strain (Fig. 4D). A past study has reported that *Bradyrhizobium japonicum* USDA110^T with a *ssuD* gene mutation was more susceptible to H_2O_2 -mediated oxidative stress than the WT strain, possibly due to the limitation of sulfur, which is important for the production of metabolites that defend against oxidative stress, such as glutathione (31). Hydrogen sulfide (H_2S) is important for the production of antioxidant materials (e.g., glutathione and cysteine) and also protects cells from oxidative stress (32). The total concentration of H_2S (normalized by OD_{600}) was higher in the WT strain than in the ΔssuD and ΔtauD mutants during the early exponential growth phase (Fig. 3A and Fig. 5). Even though high H_2S concentrations reduce bacterial growth, they may be the reason why mutant strains are more sensitive to H_2O_2 treatment (33).

The production and accumulation of $\text{C}_{16}\text{SO}_3\text{H}$ were detected in the presence of Hex, with the amounts of intracellular $\text{C}_{16}\text{SO}_3\text{H}$ in the ΔssuD and ΔtauD strains higher than in the WT strain at all measurement times (Fig. 6). One possible pathway for the production of $\text{C}_{16}\text{SO}_3\text{H}$ is that sulfite is incorporated into the carbon-carbon double bond in *trans*-2-keto-enoyl CoA produced during fatty acid β -oxidation, generating aliphatic alkanesulfonate products (C-sulfonation) (34). Another hypothesis is that hexadecanol, aided by alkane monooxygenase, reacts with PAPS, which is a sulfonate moiety donor (O-sulfonation) (35). Although both C-sulfonation and O-sulfonation require the transferases (2*R*)-phospho-3-sulfolactate synthase and sulfotransferase, respectively, the reaction may happen when high concentrations of unstable sulfur and carbon sources are present (36). Thus, the proposed mechanism is that *trans*-2-enoyl CoA or hexadecanol reacts with PAPS or sulfite, respectively, resulting in $\text{C}_{16}\text{SO}_3\text{H}$ production. Although the mechanism responsible for sulfonation is unclear, Hex may sequester the sulfur source and facilitate the accumulation of $\text{C}_{16}\text{SO}_3\text{H}$ in ΔssuD and ΔtauD strains. Sulfur sequestration also disturbs sulfate assimilation, resulting in lower H_2S production (Fig. 5 and 6).

Alkanesulfonate monooxygenase and taurine dioxygenase do not appear to be ubiquitous enzymes, and their regulators (CysB and Cbl) are mainly found in *Betapro-*

TABLE 1 Primers used in this study

Primer	Sequence (5'–3')
Knockout	
<i>cbl</i> -F	CGCGATTCCGCATGTTGGTTGATGCTGA
<i>cbl</i> -R	CGCGGTACCTGCGGTGTGATAATGCTGTG
<i>ssuD</i> -F	CGCGAGCTCATGGTGCAACATTGACCG
<i>ssuD</i> -R	CGCCCCGGGTGGGTACCTAAAGTGCTG
<i>tauD</i> -F	CGCGAATTCGATGGTGCTCGACAGTTGGA
<i>tauD</i> -R	CGCGGTACCGAGCAAAACGTTCAAGCGGA
qRT-PCR	
<i>katE</i> -F	CTGAATGGGAGTTGGGCGTA
<i>katE</i> -R	AGCCACCTGCTCTGTTTCTG
<i>katG</i> -F	TTAGGTAAACCCACGGGGG
<i>katG</i> -R	GCTCCATTGTGTTGGCGTTT
<i>sodB</i> -F	ACAACGCTGCTCAAGTTTGG
<i>sodB</i> -R	TCAGCAACTAACCAAGCCCA
<i>grxC</i> -F	GCGTAAAGGTGTTGCTTACAAA
<i>grxC</i> -R	GTCGAGTTTACCTTCACGCTC
<i>trxC</i> -F	TGTGCATCGTGTGGTGCTAA
<i>trxC</i> -R	CGCACCATTCTGCCCATAG

teobacteria and *Gammaproteobacteria* (Fig. S4 and S5). Although *E. coli* has both regulators (8), only one (AOLE_RS04605), which is more similar to Cbl than CysB, is present in *A. oleivorans* DR1 (amino acid identity for CysB, 45.7%; Cbl, 51.0%). *Firmicutes* and *Actinobacteria* do not have these regulators but do contain copies of the *ssuD* and *tauD* genes, pointing to the existence of other regulators (Fig. S4). From an ecological perspective, *ssuD* and *tauD* are primarily found within soil-isolated and aerobic bacteria (Fig. 7 and Fig. S6). Sulfur in the soil is mainly present in the form of organosulfur rather than sulfate (37, 38). Thus, soil-derived bacteria may require alkanesulfonate monooxygenase and taurine dioxygenase to obtain sulfur from their environment.

In this study, we considered the roles of SsuD and TauD during Hex degradation in *A. oleivorans* DR1. They were shown to have protective functions during sulfate limitation, oxidative stress, and accidental hexadecanesulfonate production. Their evolutionary lineage and ecological function were also discussed. To the best of our knowledge, SsuD and TauD have not received significant research attention from an ecological perspective. However, the genes encoding these enzymes potentially play a significant role in sulfur cycling in cells and within the environment.

MATERIALS AND METHODS

Bacterial strains, construction of mutant strains, and culture conditions. *A. oleivorans* DR1 (Korean Collection for Type Cultures [KCTC] deposition number KCTC 23045; strain JCM16667) was used in this study. To construct the mutants, each target gene was amplified using primers (Table 1). The PCR fragments for the mutants were digested with EcoRI and KpnI restriction enzymes for the Δcbl and $\Delta tauD$ strains or SacI and SmaI restriction enzymes for the $\Delta ssuD$ strain. These products were then separately ligated into a pVIK112 suicide vector. The recombinant plasmids were transformed into the *E. coli* S17–1 λ pir strain. After culturing in Luria-Bertani (LB-Km) medium containing 50 μ g/ml kanamycin (Km), the cloned plasmids were extracted and transformed into *A. oleivorans* DR1. The mutant strains were selected on LB-Km and confirmed based on PCR.

For growth testing using organosulfonates, a seed culture was washed twice with 1 $\times$ phosphate-buffered saline (PBS; pH 7.4) and inoculated into 1 ml of M9 medium at an initial concentration of 10^6 to 10^7 CFU per ml (CFU/ml). Then, 200 μ l of the inoculum was transferred into each well of a 96-well plate. Growth was monitored by measuring the optical density of the cultures at 600 nm (OD_{600}) using a multiplate reader (Tecan, Switzerland) at 30°C with intermediate intensity shaking between the measurement times (100 s) and before measurement (10 s). Because the original M9 minimal medium contains 2 mM magnesium sulfate ($MgSO_4$), the medium was modified to control the sulfur concentration (39). In addition, 0.5 and 1 mM organosulfonates (ethanesulfonate [C_2SO_3H], hexanesulfonate [C_6SO_3H], hexadecanesulfonate [$C_{16}SO_3H$], taurine, 3-(*N*-morpholino)-propanesulfonic acid [MOPS], and HEPES) were separately added to M9 medium instead of $MgSO_4$. To measure the CFU/ml and monitor growth under aeration conditions, the washed seeds were inoculated into 10 ml of M9 media in 50-ml flasks with the same biomass concentration (10^6 to 10^7 CFU/ml). Diluted samples were inoculated into nutrient broth agar (NA) plates or measured using a BioPhotometer (Eppendorf, Germany) at OD_{600} .

Quantification of sulfate consumption using ion chromatography. To quantify the sulfate (SO_4^{2-}) consumed by the cells, ion chromatography analysis was conducted. As the cells approached the

exponential growth phase (OD_{600} of ~ 0.6), they were centrifuged. The supernatant was collected and filtered with a pore size of $0.22\ \mu\text{m}$. The SO_4^{2-} concentration was measured using an ion chromatograph equipped with an autosampler (AS40; Thermo Scientific, USA). The samples (5 ml) were injected and passed through the column at a flow rate of 1.0 ml/min. A standard curve was established using MgSO_4 (Kanto Chemical, Japan). To calculate SO_4^{2-} consumption, the final SO_4^{2-} concentration was subtracted from the initial SO_4^{2-} concentration in the M9 medium (SO_4^{2-} concentration_{initial} – SO_4^{2-} concentration_{exponential phase}).

Quantification of reactive oxygen species-damaged cells using fluorescence-activated cell sorting analysis. The number of ROS-damaged cells was measured using dihydrorhodamine 123 (DHR123) as previously described (40). Briefly, cells in the exponential growth phase were washed in $1\times$ PBS and introduced to $5\ \mu\text{g/ml}$ DHR123. DHR123-treated samples were incubated at 30°C in darkness. After 1 h, the cells were washed twice with $1\times$ PBS and then resuspended in the same buffer. Damaged cells were counted using a FACSVerse flow cytometer (BD Biosciences, USA) using 10,000 cells per experiment. Three independent experiments were conducted and analyzed using BD Cell Quest Pro software (BD Biosciences, USA).

Quantitative real-time PCR. The total RNA from the cell cultures was extracted using an RNeasy minikit (Qiagen, USA) according to the manufacturer's instructions. cDNA synthesized from $1\ \mu\text{g}$ of each RNA sample and $20\ \mu\text{l}$ of the qRT-PCR mixture ($1\ \mu\text{l}$ of each primer [$0.5\ \mu\text{M}$], $2\ \mu\text{l}$ of 100-fold diluted cDNA, $10\ \mu\text{l}$ of Power SYBR green PCR master mix [Thermo Fisher Scientific, USA], and $6\ \mu\text{l}$ of distilled water) was prepared. qRT-PCR was conducted with target primers using the following process (Table 1): 95°C for 10 min at the holding stage, followed by 42 cycles of 15 s at 95°C and 60 s at 60°C at the cycling stage, and 15 s at 95°C , 60 s at 60°C , and 15 s at 95°C at the melting stage. The expression levels of each gene were normalized by 16S rRNA genes (41), and the experiment was conducted in triplicate.

Observation of biofilm formation using confocal laser scanning microscopy. Cells were cultured on confocal dishes for 24 h, and the biofilm was stained with FilmTracer Sypro Ruby biofilm matrix staining dye (Thermo Scientific, USA) for 30 min at room temperature. Confocal images of the stained biofilms were observed under red fluorescent light (excitation wavelength, 450 nm; emission wavelength, 610 nm) using confocal laser scanning microscopy (CLSM) (Carl Zeiss, Germany). The intensity of the fluorescence dye was measured using ZEN lite software.

H_2O_2 susceptibility testing of wild-type and mutant strains. For the H_2O_2 -sensitivity testing of the WT and mutant strains, overnight-cultured strains in NB were washed and diluted to 50 ml with M9 fresh media containing Hex. After 24 h of growth, the cells were treated with 50 mM H_2O_2 for 5 min. The samples were then washed twice with fresh $1\times$ PBS and serially diluted (10^1 to 10^5). The samples were then spotted onto NA plates.

Measurements of total H_2S concentration during Hex metabolism. To prepare the samples, 10 ml of Hex-supplemented M9 media in which the cells had been cultured was adjusted with 35% hydrogen chloride (HCl) so that the pH was < 2 . Subsequently, $700\ \mu\text{l}$ of zinc chloride stabilizer sulfide-absorbing solution (42) and $200\ \mu\text{l}$ of 4% NaOH were added to fix H_2S gas in the solution. The total H_2S concentration in each sample was measured using a microsensor equipped with an H_2S detection probe (Unisense, Denmark). The sensor was calibrated with a sodium sulfide (Na_2S) solution, and the values generated after 1 min were averaged. The measurements were conducted in triplicate, and each H_2S concentration was normalized by OD_{600} .

Gas chromatography-mass spectrometry analysis. The concentration of $\text{C}_{16}\text{SO}_3\text{H}$ was evaluated using a gas chromatograph (GC 6890N; Agilent) equipped with a mass spectrometer (MSD 5975; Agilent). Samples were prepared as previously described (15). To measure intracellular $\text{C}_{16}\text{SO}_3\text{H}$ concentrations, 10 ml of the culture was centrifuged, and the supernatant was removed. The cell pellets were resuspended in $1\times$ PBS and sonicated twice, and dichloromethane (DCM) was added to reach a 1:5 ratio (DCM:cell culture). The samples were vortexed for 5 min, and the DCM layer was transferred to GC vials. Dissolved $\text{C}_{16}\text{SO}_3\text{H}$ in DCM ($2\ \mu\text{l}$) was injected into the gas chromatograph equipped with an HP-5MS column. The injector and detector were maintained at 280°C and 300°C , respectively, and the oven temperature was held at 45°C for 2 min and then increased to 310°C at a rate of $10^\circ\text{C}/\text{min}$, after which it was held for 25 min.

Regulator-binding site prediction, transcriptomic analysis, and phylogenetic analysis. The similarity between genes was calculated using the protein BLAST (BLASTP) tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), and a hypothetical regulator binding site was searched and aligned using MUSCLE (43) based on a previous reference sequence (AlkR [44], Cbl [8], and OxyR [30]). The transcriptomic profiles were retrieved from the NCBI Gene Expression Omnibus (GEO) and Sequence Read Archive (SRA). The accession numbers were as follows: DR1 (RED and Hex, SRA accession no. [SRS804135](#); PQ and PMS, GEO accession no. [GSE70356](#); H_2O_2 , GEO accession no. [GSE108860](#)), *A. baumannii* (GEO accession no. [GSE114130](#)), *E. coli* (GEO accession no. [GSE20305](#)), *C. glutamicum* (GEO accession no. [GSE41232](#)), *P. putida* (GEO accession no. [GSE85475](#)), and *P. aeruginosa* (GEO accession no. [GSE3090](#)). The relative expression profile of DR1 was represented by the $\log_2$ -fold change, and heatmaps were constructed using Heatmapper (<http://www2.heatmapper.ca/expression/>).

To investigate the phylogenetic distribution of *cysB*, *cbl*, *ssuD*, and *tauD*, genomes were retrieved using the gene search function in the Integrated Microbial Genomes (IMG) database, which contained 1,976 completely sequenced bacterial genomes. For simplification, only one strain sequence was randomly chosen when multiple strains were available for a single species. The habitat and oxygen requirements of the bacterial species were determined based on the annotations in the IMG database. A cladogram was constructed using the NCBI Taxonomy Common Tree (<https://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi>) and visualized with the iTOL program (45).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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