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A novel sulfonamide resistance mechanism by two-component flavin-dependent monooxygenase system in sulfonamide-degrading actinobacteria

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ABSTRACT

Sulfonamide-degrading bacteria have been discovered in various environments, suggesting the presence of novel resistance mechanisms via drug inactivation. In this study, *Microbacterium* sp. CJ77 capable of utilizing various sulfonamides as a sole carbon source was isolated from a composting facility. Genome and proteome analyses revealed that a gene cluster containing a flavin-dependent monooxygenase and a flavin reductase was highly up-regulated in response to sulfonamides. Biochemical analysis showed that the two-component monooxygenase system was key enzymes for the initial cleavage of sulfonamides. Co-expression of the two-component system in *Escherichia coli* conferred decreased susceptibility to sulfamethoxazole, indicating that the genes encoding drug-inactivating enzymes are potential resistance determinants. Comparative genomic analysis revealed that the gene cluster containing sulfonamide monooxygenase (renamed as *sulX*) and flavin reductase (*sulR*) was highly conserved in a genomic island shared among sulfonamide-degrading actinobacteria, all of which also contained *sul1*-carrying class 1 integrons. These results suggest that the sulfonamide metabolism may have evolved in sulfonamide-resistant bacteria which had already acquired the class 1 integron under sulfonamide selection pressures. Furthermore, the presence of multiple insertion sequence elements and putative composite transposon structures containing the *sulX* gene cluster indicated potential mobilization. This is the first study to report that *sulX* responsible for both sulfonamide degradation and resistance is prevalent in sulfonamide-degrading actinobacteria and its genetic signatures indicate horizontal gene transfer of the novel resistance gene.

1. Introduction

Antibiotic resistance has become one of the most serious global health issues because of the dissemination of pre-existing resistance from many known pathogens (Surette and Wright, 2017), limited drug discoveries (Adu-Oppong et al., 2017) and emergence of novel resistance mechanisms (Liu et al., 2016). Sulfonamides are synthetic antimicrobial agents that have been widely used in human and veterinary medicines (Huovinen, 2001). Extensive use of sulfonamides worldwide not only causes environmental pollution but also threatens public health because of the potential development and dissemination of antibiotic resistance (Larcher and Yargeau, 2012).

Bacterial resistance to sulfonamides mainly occurs because of mutations in *folP* gene encoding dihydropteroate synthase (DHPS) involved in nucleotide biosynthesis or through acquisition of alternative DHPS genes (*sul1*, *sul2*, and *sul3*), the products of which have low affinity to sulfonamides (Perreten and Boerlin, 2003; Skold, 2000; Yun et al., 2012). Recently, the fourth mobile sulfonamide resistance gene *sul4* was found to be widespread across Asia and Europe (Razavi et al., 2017). Thus, *sul* genes commonly located in plasmids are the most common mechanism of sulfonamide resistance and have been detected in a wide range of bacterial species from many different environments, including agricultural soils and wastewaters (Byrne-Bailey et al., 2009; Phuong Hoa et al., 2008).

Bacterial catabolism of sulfonamides is important not only for antibiotic degradation to clean up pollutants in the environment, but also for antibiotic resistance, considering that enzymes involved in degradation can be regarded as a potential resistance mechanism (Yang et al., 2004). Sulfonamides were regarded as recalcitrant chemicals (Ingerslev and Halling-Sørensen, 2000) until several sulfonamide-degrading bacteria were isolated from various environmental sites such as acclimated membrane reactors, agricultural soil, seawater, and

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activated sludge in recent years. Many of these bacteria were found to utilize these drugs as a sole carbon and energy source (Deng et al., 2016; Jiang et al., 2014; Mao et al., 2018; Reis et al., 2018; Ricken et al., 2013; Tappe et al., 2013; Topp et al., 2013; Wang and Wang, 2018), but there was limited information available on the genes responsible for sulfonamide degradation and exact mechanism involved in degradation. Recently, an $FMNH_2$-dependent monooxygenase was found to initiate the catabolism of sulfonamides in *Microbacterium* sp. strain BR1 (Ricken et al., 2017). Sulfonamide monooxygenase (SadA) and flavin reductase (SadC) were responsible for the initial *ipso*-hydroxylation and the subsequent cleavage of sulfonamides and proposed to be related to sulfonamide resistance (Ricken et al., 2013; Ricken et al., 2017), but their roles in the resistance have never been elucidated.

Here we report the isolation of *Microbacterium* sp. CJ77 from a sediment sample near a composting facility which was capable of degrading sulfonamides as a sole carbon source. Using genomic and proteomic approaches, we identified the conserved gene cluster and analyzed expression profiles of the gene cluster in response to sulfonamide treatment. The reaction mechanism and biochemical properties were elucidated using purified enzymes. We also demonstrated that the acquisition of the genes encoding these enzymes conferred resistance to sulfonamides and their genetic signatures were associated with mobile genetic elements.

2. Materials and methods

2.1. Chemicals and culture media

Sulfamethoxazole, sulfamethazine, sulfadiazine, and sulfanilamide were purchased from Sigma. Sulfathiazole was purchased from TCI (Tokyo, Japan). Minimal medium contained the following components per liter: 7 g of $Na_2HPO_4·12H_2O$, 1 g of KH_2PO_4, 10 mg of $CaCl_2·2H_2O$, 2 mg of ferric citrate, 20 mg of $MgSO_4·7H_2O$, and 53 mg of NH_4Cl. Sulfonamides (0.5 to 5 mM) were added as a sole carbon source for growth. Culture media were supplemented with 1% (v/v) BME vitamins solution 100× (Sigma Aldrich, St. Louis, MO, USA), 0.001 g *p*-aminobenzoic acid and 0.05% yeast extract (Difco Laboratories, Detroit, MI, USA) to enhance growth if necessary.

2.2. Isolation and identification of sulfonamide-degrading bacterium

A sulfonamide-degrading bacterium was isolated from sulfonamide-contaminated sediment samples near a swine manure composting facility in Gangwon province, South Korea. Isolation was conducted by enrichment culture using sulfathiazole (100 μg/mL) as a sole carbon source in the above minimal medium at 30 °C for four weeks. After subculture once in a week, colonies were isolated by spreading on agar plates of the same medium as enrichment culture. Identification of the isolate was performed by PCR amplification of the 16S rRNA gene and sequencing at Macrogen (Seoul, Korea). The 16S rRNA gene was aligned with the nearest sequences obtained from the database of the EzBioCloud server (http://www.ezbiocloud.net) (Yoon et al., 2017a).

2.3. Sulfonamide degradation assay

Microbacterium sp. CJ77 was grown in 50 mL of the minimal medium described above at 30 °C. Cultures without cells were used as controls to examine abiotic degradation. Heat-killed cells were used to monitor adsorption of sulfonamides. Culture supernatants were subject to HPLC analysis after centrifugation at 13,000 ×*g* for 20 min. For the crude extract assay, sulfonamide-grown cells were disrupted by sonication in 50 mM Tris-HCl buffer (pH 7.5). Cell debris was removed by centrifugation at 13,000 ×*g* at 4 °C for 1 h and filtered (0.2 μm) to obtain cell-free protein extracts. The reaction mixture contained 250 μg of protein, 200 μM sulfonamide, 1 mM NADH, and 5 μM FMN in 1 mL of 50 mM Tris-HCl buffer (pH 7.5) and incubated at 30 °C. The reaction was stopped by adding 12% phosphoric acid. Samples taken from the reaction mixture were analyzed by HPLC. To detect metabolites in the reaction, the reaction mixture was extracted with an equal volume of ethyl acetate three times. The ethyl acetate extract was evaporated to dryness under nitrogen gas. The residue was dissolved in methanol for HPLC analysis. For the activity assay for recombinant strains, *E. coli* BL21(DE3) harboring appropriate plasmid constructs were grown at 37 °C overnight in 5 mL of LB medium supplemented with 100 mg/L of ampicillin. The overnight culture was transferred into 50 mL of fresh LB medium containing ampicillin and incubated at 30 °C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM when the cells reached an OD_{600} of 0.4–0.6 and the culture was induced for 3 h. Sulfamethoxazole was added to the culture at a final concentration of 0.5 mM and the culture was further incubated for 16 h. The culture supernatant was taken at intervals for HPLC analysis.

2.4. HPLC and LC-MS/MS analyses

Degradation of sulfonamides and detection of metabolites were analyzed by HPLC using an Atlantis dC-18 column (4.6 × 250 mm; Waters) and Varian ProStar HPLC (Varian Inc., Walnut Creek, CA, USA) system with a diode-array detector. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) both of which contained 0.1% (v/v) formic acid. The following gradient was applied at a flow rate of 1 mL/min; 5% solvent B for 1 min, solvent B from 5% to 95% for 11 min, 95% solvent B for 1 min, and 5% solvent B for 2 min. An LTQ Velos mass spectrometer (Thermo Scientific, Waltham, MA, USA) with Accela PDA detector (Thermo Scientific) was used for liquid chromatographic/electrospray ionization mass spectrometric (LC/ESI-MS) and tandem MS (LC/ESI-MS/MS) analyses. The column, mobile phase and gradient conditions were same as used for HPLC analysis. Survey full-scan MS spectra (*m/z* 50 to 500) were acquired to determine the precursor ions and charge states, and MS/MS spectra from the survey scan were acquired with options of normalized collision energy of 35% and dynamic exclusion duration for 20 s. Mass spectral data were analyzed with Xcalibur software v. 2.1 (Thermo Scientific). Chemical structures were confirmed by comparison with those of authentic compounds.

2.5. Genome sequencing and annotation

Genomic DNA of the isolate was extracted using the DNEasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Genome sequencing was performed at Chunlab (Seoul, Korea). The draft genome sequence of strain CJ77 was determined by a combination of Illumina MiSeq (250-bp paired end) and Roche 454 (8-kb insert paired end) sequencing platforms. Generated paired-end sequencing reads were assembled using CLC genomics workbench 6.5 (CLC bio). The contigs were assembled using CodonCode Aligner 3.7.1 (CodonCode Corp., Centerville, MA, USA). Coding sequences (CDS) were predicted by Glimmer 3.02 (Delcher et al., 2007). For functional annotation, the predicted CDS were compared to those from catalytic families (catFam), the COG database, NCBI reference sequences (RefSeq), and SEED subsystem (Overbeek et al., 2005; Pruitt et al., 2009; Tatusov et al., 2000; Yu et al., 2009). The genome sequence has been deposited in the NCBI GenBank database under the accession number NZ_PQBR00000000.1.

2.6. Proteome analysis

Cells were grown in the minimal medium described above using four different substrates as a carbon source for growth; glucose, sulfamethoxazole, sulfamethazine and sulfanilamide. Detailed methods for the preparation of proteome samples and LC-MS/MS analysis by a linear ion trap mass spectrometer (LTQ Velos, Thermo Scientific) coupled with a nano sprayer (Thermo Scientific) were described previously

(Kim et al., 2017). MS/MS data were acquired and deconvoluted using Xcalibur 2.1 (Thermo Scientific), and the whole dataset was searched by the SEQUEST algorithm implemented in Proteome Discoverer 1.3 software (Thermo Scientific). The genome sequence of *Microbacterium* sp. CJ77 was used as database for protein identification. Filter parameters for peptide identification (high peptide confidence of $\Delta Cn >$ 0.1 and false discovery rate of < 5%) and protein identification (more than two peptides per protein) were applied to the spectra searched by SEQUEST. The shared proteome of biological duplicate samples was used for further analysis, and protein expression level was determined by normalized spectral counts.

2.7. Cloning, expression, and purification of monooxygenase and flavin reductases

Cloning and expression of genes were conducted using pET28-(a) vector for single gene expression and pETDuet-1 vector for co-expression of two genes in *E. coli* BL21 (DE3). Monooxygenase and flavin reductase genes were amplified by PCR using appropriate primers (Table S1). *E. coli* BL21(DE3) harboring appropriate plasmid constructs were cultivated at 37 °C. At an OD_{600} of 0.5, cultures were induced with IPTG at a final concentration of 0.1 mM, and then further incubated at 37 °C for 3 h or at 20 °C for 12 h as required. Cells were harvested and re-suspended in 20 mM Tris-HCl buffer (pH 7.5). Cell-free protein extracts were obtained as described previously. The recombinant His-tagged proteins were purified using a His GraviTrap column (GE Healthcare, Chicago, IL, USA) according to the manufacturer's instructions.

2.8. Enzyme assay for kinetic studies

Sulfonamide degradation activity by purified enzymes was determined by HPLC analysis as described above. The reaction mixture contained 50 μM sulfamethazine, 0.75 μM sulfonamide monooxygenase, 0.5 μM, flavin reductase, 1.0 μM FMN and 400 μM NADH in 50 mM Tris-HCl buffer (pH 7.5) and was incubated at 25 °C. The reaction was stopped by adding 12% phosphoric acid and sulfamethazine and its metabolites were quantified at every 1 min for 5 min. Steady state kinetic parameters were obtained by fitting initial velocity data to the standard Michaelis-Menten equation. The initial velocities for various concentrations of sulfonamides were obtained with sulfonamide monooxygenase (0.5, 5.0 and 2.5 μM), the equivalent amounts of flavin reductase and FMN, and 200 μM NADH at 25 °C for 1 min.

2.9. Antibiotic susceptibility test

The minimum inhibitory concentration (MIC) was determined by the broth microdilution method according to CLSI recommendations (Wiegand et al., 2008). LB broth medium containing 0.1 mM IPTG was used for susceptibility testing. The susceptibility of *E. coli* BL21(DE3) harboring appropriate plasmid constructs was tested against sulfamethoxazole. The test was performed in duplicate. For disk diffusion assay, *E. coli* BL21(DE3) harboring appropriate plasmid constructs was grown at 37 °C overnight in 5 mL of LB medium supplemented with 100 mg/L of ampicillin. The overnight culture was transferred into fresh medium containing ampicillin and incubated at 37 °C up to an $OD_{600} = 0.4$. The bacterial suspension was spread on LB agar supplemented with ampicillin and IPTG (0.2 mM final concentration). Filter paper disks with sulfamethoxazole (20 μg) were overlaid onto the *E. coli* lawn and plates incubated at 30 °C overnight.

2.10. Phylogenomic analysis

Genome assembly data of 173 *Microbacterium* strains, *Micrococcus luteus* NCTC 2665, *Arthrobacter* sp. D2 and *Arthrobacter* sp. D4 were downloaded from GenBank and compared with the genome of strain CJ77. Average nucleotide identity (ANI) between the genomes was calculated by OrthoANIu tool (Yoon et al., 2017b). Phylogenetic tree of all available genomes of the genus *Microbacterium* was reconstructed using 697 orthologous CDS shared by 95% of the strains. Substitution model selection and maximum likelihood phylogenetic analysis were performed by concatenated alignment using IQ-TREE (Nguyen et al., 2015). For the selected *Microbacterium* genomes and two *Arthrobacter* genomes, 453 orthologous CDS shared by the selected strains were used for phylogenetic analysis.

2.11. Comparative genomic analysis

Blastn was performed against the genomes of 38 representative *Microbacterium* spp. and five sulfonamide-degrading strains for every 500-bp fragment of the contigs of strain CJ77 genome to detect homologous genomic sequences. The resulting identity values were visualized as circular heat maps using Circos software (Krzywinski et al., 2009). Orthologs of the CDS of strain CJ77 were determined by searching for bi-directional blastp best hits (Wolf and Koonin, 2012).

2.12. Codon usage analysis

Codon usage was analyzed for CDS in the strain CJ77 genome. Relative synonymous codon usage (RSCU) of each CDS was calculated, and correspondence analysis was performed based on 59-dimensional vectors of RSCU values. A distance matrix of the genes based on their RSCU values was used for permutated multivariate analysis of variance (Adonis) with 999 permutations. Calculation of RSCU values, their distance matrix and corresponding analysis were performed using the GCUA program (McInerney, 1998). The Adonis test was performed using vegan R package (vegan: Community Ecology Package. R package version 2.4-4.2017. https://CRAN.R-project.org/package=vegan).

3. Results

3.1. Sulfonamide-dependent expression of a gene cluster in sulfonamide-degrading Microbacterium sp. CJ77

A bacterial strain capable of degrading sulfonamides was isolated from a sulfonamide-contaminated site (Ok et al., 2011) by enrichment culture using sulfathiazole as a sole carbon source. The isolate designated *Microbacterium* sp. CJ77 was Gram-positive, rod-shaped and yellow-pigmented bacterium. It was able to utilize various types of sulfonamides as a carbon source for its growth (Fig. S1). Abiotic degradation and adsorption of sulfonamides were not observed. Degradation of sulfamethazine, sulfamethoxazole, sulfathiazole, and sulfadiazine was followed by accumulation of the corresponding dead-end metabolites, 2-amino-4,6-dimethylpyrimidine, 3-amino-5-methylisoxazole, 2-aminothiazole, and 2-aminopyrimidine, respectively (Figs. S1 and S2), while the benzyl ring parts of sulfonamides were not detected in the culture supernatants. When the expression levels of proteins from cells grown on glucose (control), sulfanilamide, sulfamethoxazole and sulfamethazine were compared by proteome analysis, several genes in a gene cluster were highly up-regulated in cultures containing each sulfonamide as a carbon source (Fig. 1 and Table S2). The gene cluster was found to contain homologs of sulfonamide monooxygenase (SadA) and flavin reductase (SadC) which were previously identified to be responsible for the initial cleavage of sulfonamides in *Microbacterium* sp. BR1 (Ricken et al., 2013, 2017).

3.2. Reaction mechanism of sulfonamide degradation

In the presence of NADH and flavin cofactor (FMN or FAD), the heterologously expressed and purified sulfonamide monooxygenase and flavin reductase of strain CJ77 (Fig. S3) resulted in the rapid degradation of sulfonamides with concomitant production of the dead-end

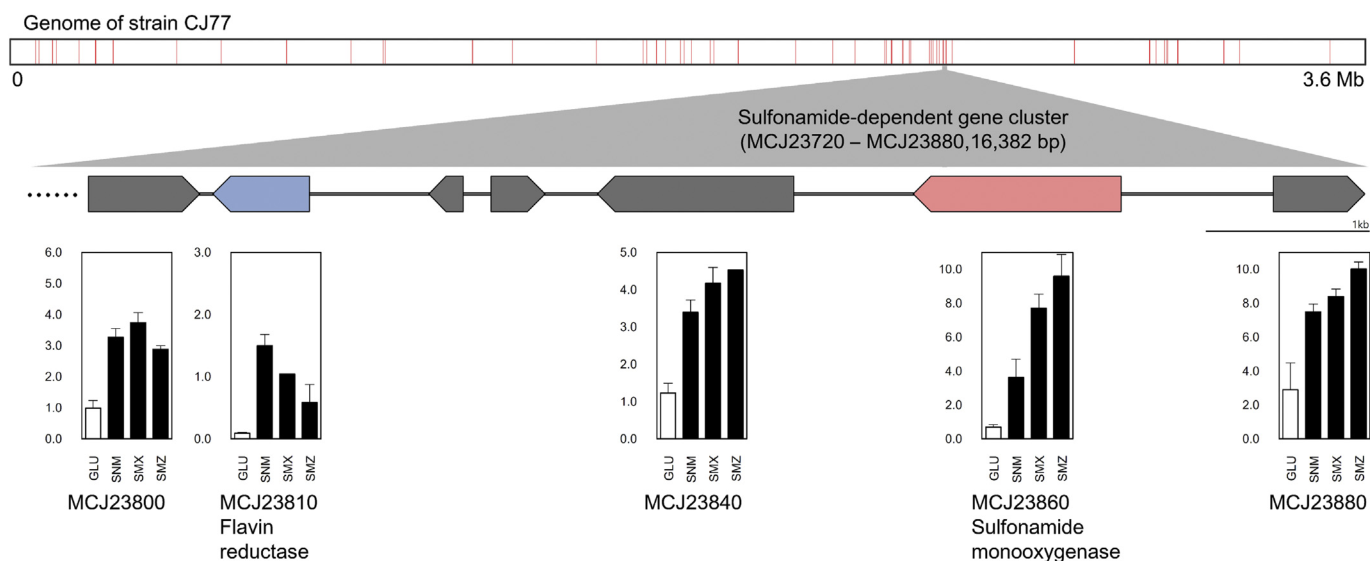

Fig. 1. Genetic organization and expression profiles of a gene cluster for sulfonamide degradation. The locations of transposases and integrases are shown as red bars in the genome of *Microbacterium* sp. CJ77. Expression levels are displayed by normalized spectral counts below the genetic map of the cluster. GLU, SNM, SMX, and SMZ indicate glucose, sulfanilamide, sulfamethoxazole and sulfamethazine, respectively, used as a sole carbon source. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

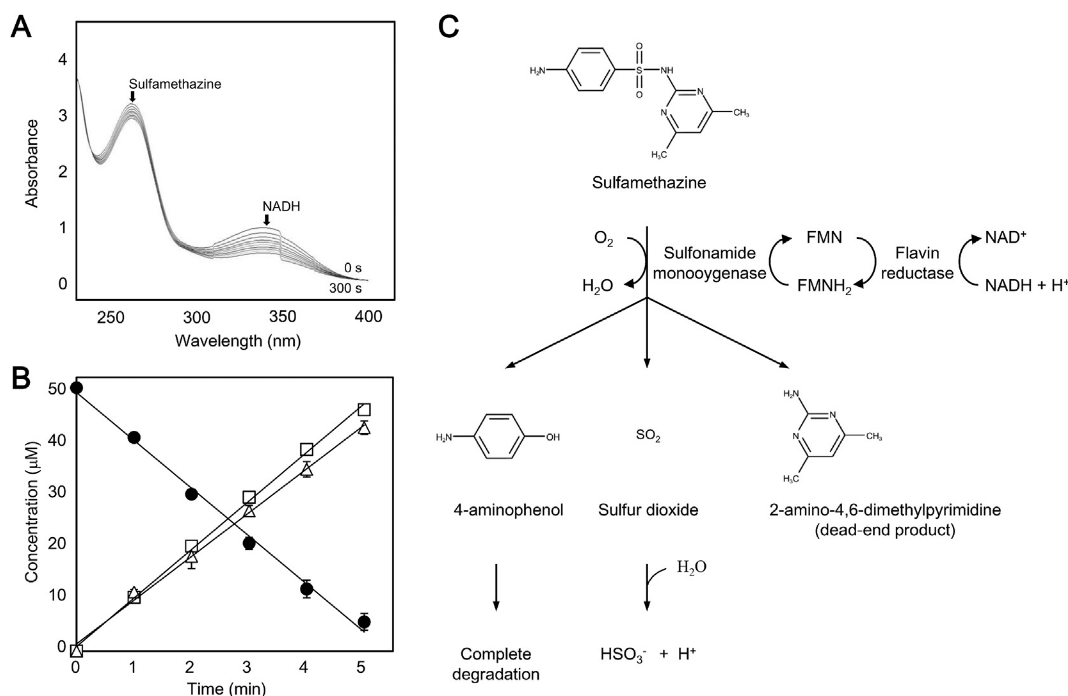

Fig. 2. Sulfamethazine degradation by purified sulfonamide monooxygenase and flavin reductase and its reaction mechanism. (*A*) UV–visible spectrum during the sulfamethazine degradation by purified proteins. The reaction mixture contained 50 μM sulfamethazine, 2 μM sulfonamide monooxygenase, 0.1 μM flavin reductase, 2.0 μM FMN, and 200 μM NADH in 50 mM Tris-HCl buffer (pH 7.5) and was incubated at 25 °C. Scan was taken at every 30 s for 5 min. (*B*) Kinetics of the sulfonamide degradation reaction. The reaction mixture contained 50 μM sulfamethazine, 0.75 μM sulfonamide monooxygenase, 0.5 μM flavin reductase, 1.0 μM FMN, and 400 μM NADH in 50 mM Tris-HCl buffer (pH 7.5) and incubated at 25 °C. Sulfamethazine (closed circle), 2-amino-4,6-dimethylpyrimidine (open square) and 4-aminophenol (open triangle) were analyzed over time. (*C*) Proposed sulfonamide degradation mechanism for the initial cleavage reaction mediated by sulfonamide monooxygenase and flavin reductase.

products and 4-aminophenol in stoichiometric manners (Fig. 2A and B). Our results are consistent with the reaction mechanism previously proposed in *Microbacterium* sp. BR1 (Ricken et al., 2013), where SadA and SadC were reported to initiate the catabolism of sulfonamides in *Microbacterium* sp. BR1, but the reaction was conducted using partially purified enzymes (Ricken et al., 2017). In the present study, the mechanism of the initial cleavage reaction of sulfonamide drugs was demonstrated using purified enzymes (Fig. 2C); Flavin reductase reduces the oxidized form of flavin cofactor (FMN) through the oxidation of NADH. The reduced flavin cofactor ($FMNH_2$) functions as electron donor for the *ipso*-hydroxylation of sulfonamide substrates by sulfonamide monooxygenase. Subsequently, the hydroxylation of sulfonamides results in the cleavage of the drugs, releasing 4-aminophenol, sulfite and the corresponding dead-end metabolites. Purified monooxygenase

Table 1
Steady state kinetic parameters for the initial cleavage reaction by sulfonamide monooxygenase.

Substrate*	K_m (μM)†	V_{max} (U/mg protein)†	V_{max}/K_m
Sulfamethazine	16.83 ± 2.27	0.5809 ± 0.0530	0.03452
Sulfamethoxazole	23.44 ± 0.95	0.0634 ± 0.0025	0.00270
Sulfathiazole	9.76 ± 1.15	0.0321 ± 0.0033	0.00329
Sulfadiazine	24.35 ± 1.81	0.0111 ± 0.0007	0.00046

* 200 μM NADH, 50 mM Tris- HCl (pH 7.5) and an equal amount of FMN to sulfonamide monooxygenase concentration were used in the reactions.

† Kinetic values are shown with standard deviations of fit of the data to the Michaelis-Menten equation.

and flavin reductase showed the degradation activities towards several sulfonamides with different substrate specificities (Table 1). Kinetic studies indicated that the highest V_{max} was observed with sulfamethazine, while the substrate affinity was highest (lowest K_m value) for sulfathiazole (Table 1). The order of catalytic efficiency (V_{max}/K_m) for these substrates is as follows: sulfamethazine, sulfathiazole, sulfamethoxazole and sulfadiazine (Table 1). In addition to flavin reductase (MCJ23810) in the gene cluster, four other paralogous flavin reductases present in the genome of strain CJ77 displayed sulfonamide degradation activities when combined with sulfonamide monooxygenase (Table S3), indicating that flavin reductase is not specific for the reaction.

3.3. Sulfonamide monooxygenase as a novel class D flavin-dependent monooxygenase

Sulfonamide monooxygenase was considered to belong to the two-component flavin-dependent monooxygenase (FDM) family (Huijbers et al., 2014; Ricken et al., 2017). Interestingly, sulfonamide monooxygenase from strain CJ77 exhibited relatively low sequence similarities (< 50%) with other known monooxygenases available in the GenBank database, except for homologs found in the genomes of previously reported sulfonamide-degrading actinobacteria *Microbacterium* spp. BR1, SDZm4, and C448, and *Arthrobacter* spp. D2 and D4, many of which were initially annotated as hypothetical proteins. Phylogenetic analysis based on amino acid sequences from all classes of FDMs from the RCSB protein data bank (PDB) and class D FDMs from the Unitprot database (Huijbers et al., 2014; Mascotti et al., 2016) revealed that sulfonamide monooxygenases from these sulfonamide-degrading actinobacteria formed a distinct lineage of class D FDM within other known FDMs (Fig. 3). Antibiotic-inactivating monooxygenases such as TetX, Rox, and Baeyer-Villiger monooxygenase, which conferred resistance to tetracycline, rifamycin, and imipenem (Hoshino et al., 2010; Koteva et al., 2018; Minerdi et al., 2015; Yang et al., 2004), respectively, were previously characterized to be single-component FDMs belonging to class A or B (Fig. 3). Sulfonamide monooxygenases identified in this study are distinguished in that they are two-component FDMs in class D. Structural homology modeling with the closest characterized protein (HsaA from *Mycobacterium tuberculosis*) revealed that several residues at the flavin-binding site were well-conserved in sulfonamide monooxygenase of strain CJ77, while residues at the substrate-binding site varied (Fig. S4).

3.4. Two-component monooxygenase system as a novel sulfonamide resistance determinant

Like other known antibiotic-inactivating monooxygenases (Forsberg et al., 2015; Hoshino et al., 2010; Koteva et al., 2018; Minerdi et al., 2015; Yang et al., 2004), the decomposition of sulfonamides indicate a potential resistance mechanism via inactivation of the drugs. To clarify their roles in resistance, genes encoding sulfonamide monooxygenase and flavin reductase were introduced into a sulfamethoxazole-susceptible *E. coli* strain. Both genes were successfully expressed in *E. coli* cells, which exhibited sulfonamide degradation activity (Fig. 4). When antibiotic susceptibility was tested, *E. coli* cells harboring both of two component genes showed a significant increase in resistance compared to the control *E. coli* cells (Fig. 4). *E. coli* cells harboring only the monooxygenase gene also displayed a lower level of resistance (Fig. 4), suggesting that indigenous flavin reductases present in *E. coli* contribute to the slight increase in resistance, as also shown in the degradation activity, and both genes were required for the acquisition of resistance to the drugs. In conclusion, our results demonstrate that the two-component system consisting of sulfonamide monooxygenase and flavin reductase is key enzymes for both sulfonamide degradation activity and novel resistance mechanism via drug inactivation. Therefore, we propose that the heretofore unrecognized monooxygenase responsible for sulfonamide resistance should be renamed as SulX in analogy to TetX, which is distinguished from previously known sulfonamide resistance genes (*sul1234*) (Perreten and Boerlin, 2003; Razavi et al., 2017; Skold, 2000). Flavin reductase as a two-component system is renamed as SulR.

3.5. Comparative genomic analysis of sulfonamide-degrading actinobacteria

To date, genes homologous to *sulX* have been found only in the genomes of sulfonamide-degrading actinobacteria including *Microbacterium* spp. BR1, SDZm4 and C448, and *Arthrobacter* spp. D2 and D4. Phylogenetic analysis of the genomes of *Microbacterium* spp. placed the four sulfonamide-degrading strains in a distinct lineage (Fig. 5A and Fig. S5). Based on average nucleotide identity (ANI) values (Richter and Rossello-Mora, 2009), strains CJ77 and BR1 (99.2%), and strains C448 and SDZm4 (97.8%) belong to the same species respectively. In addition, genomic comparison showed that the four sulfonamide-degrading *Microbacterium* strains had higher similarities compared to other non-sulfonamide-degrading strains (Fig. 5B). Particularly, two genomic island regions were highly conserved among the four sulfonamide-degrading *Microbacterium* strains in the genome comparison map (Fig. 5B). Interestingly, the two regions contained the *sulX/sulR*-containing gene cluster (genomic island 1) and *sul1*-carrying class 1 integron (genomic island 2), respectively. Codon usage and G + C content of the protein-coding sequences (CDSs) in genomic island 1 were significantly different from those of *Microbacterium* core genes (Fig. S6), suggesting that these sequences were acquired from different origins. The occurrence of horizontal gene transfer was also demonstrated by the presence of tRNA genes at the 5′ and 3′ ends of genomic island 1 (Boyd et al., 2009) (Fig. S7).

3.6. Comparative analysis of sulX gene clusters and sul1-carrying class 1 integrons associated with mobile genetic elements

The *sulX* gene cluster (29,680 bp) containing genes encoding sulfonamide monooxygenase (SulX) and flavin reductase (SulR) in the strain CJ77 genome was highly conserved in sulfonamide-degrading actinobacteria (shown in red; Fig. 6A). It remains ambiguous whether this gene cluster was present in *Arthrobacter* sp. D4 or missing during genome sequencing. In the genomes of *Arthrobacter* spp. D2 and D4, another gene homologous to *sulX* (73.4%) was detected in other regions of the genomes. The *sulX* gene cluster of strain CJ77 was unique compared to those of other sulfonamide-degrading strains in that the cluster was located in a transposase-rich region (Figs. 1 and 6A). Three intact insertion sequences (IS) of the IS*3* family whose sequences were identical except for direct repeat sequences were detected around the gene cluster (Fig. 6A and Fig. S8). These features suggest that three insertion events occurred independently after strain CJ77 had acquired the *sulX* gene cluster. Similar IS elements were found to be prevalent in the genomes of various actinobacteria (Table S4). Interestingly, repeated insertion of the identical IS resulted in the formation of three possible genetic structures of composite transposon, two of which contained the *sulX* gene cluster (Fig. 6A). The presence of composite transposons harboring the intact *sulX* gene cluster indicates the possibility of

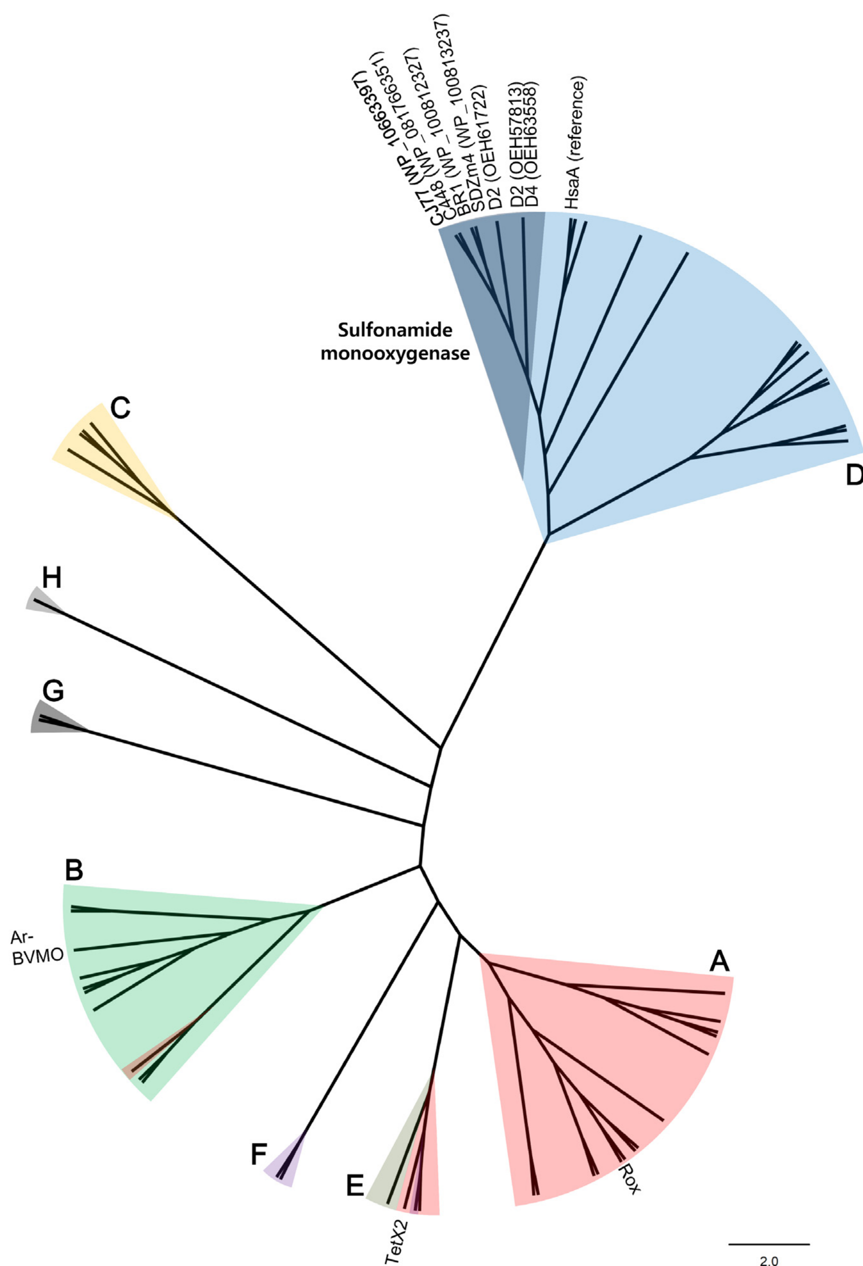

Fig. 3. Phylogenetic relationship of sulfonamide monooxygenase of *Microbacterium* sp. CJ77 (WP_103663397) and its homologous proteins with representative flavin-dependent monoxygenases (FDMs) of eight different classes. Proteins homologous to sulfonamide monooxygenase are SadA from *Microbacterium* sp. BR1 (WP_100812327, 95.5% identity) and *M. lacus* SDZm4 (WP_100813237, 95.7% identity), and hypothetical proteins from *Microbacterium* sp. C448 (WP_081766351, 99.1% identity) and *Arthrobacter* spp. D2 (OEH61722 and OEH57813, 91.7% and 74.6% identities, respectively) and D4 (OEH63558, 78.2% identity). HsaA (3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione monooxygenase) from *Mycobacterium tuberculosis* is the most closely related protein among the structurally characterized class D FDMs. Sulfonamide monooxygenase and its homologs forming distinct branches within class D FDMs are shaded in dark blue. Representative FDMs are all classes of FDMs from the RCSB protein data bank (PDB). Class D FDMs are obtained from the Uniprot database. TetX2, Rox and Ar-BVMO indicate tetracycline-degrading monooxygenase, rifamycin monooxygenase and imipenem Baeyer-Villiger monooxygenase, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

transposition events and subsequent emergence of sulfonamide resistance in the clinical settings.

Mobilization of the *sulX* gene cluster was indicated by two insertion events that may have occurred in *Arthrobacter* spp. strains D2 and D4 (Fig. 6A). First, compared with *Arthrobacter* sp. ATCC 21022 as a reference genome (Deng et al., 2016), a larger transposon structure (region 1) with intact direct repeats and imperfect inverted repeats was identified to be inserted into a gene encoding amino acid permease (shown in green) of strains D2 and D4 (Figs. 6A and S9A). A part of structure and sequence of the transposon were highly similar to those of the previously reported Tn*552* transposon of megaplasmid pAO1 from *A. nicotinovorans* ATCC 49919 (Fig. S9A) (Igloi and Brandsch, 2003). Another insertion event (region 2) was detected inside region 1 in strain D2 (Fig. 6*A*). The *sulX* gene cluster and an intact IS element belonging to the IS21 family were inserted into a gene encoding ATP-binding protein (Fig. S9B). The presence of transposon-associated *sulX* gene cluster in both *Microbacterium* and *Arthrobacter* strains indicated that horizontal gene transfer occurred among these groups of bacteria.

In the genomes of sulfonamide-degrading *Microbacterium* strains, another sulfonamide resistance gene, *sul1*, was found to be located in a typical structure of the clinical class 1 integron (Gillings, 2014), including *qacE*Δ*1*, *sul1*, *orf5* and *tni* module (Fig. 6B). Class 1 integrons have been regarded to play an important role in disseminating antibiotic resistance genes (Gillings, 2014) and proposed as a proxy for anthropogenic pollution (Gillings et al., 2015). In these bacterial genomes, the class 1 integron and IS element IS*1326* were carried in a Tn*402*-like transposon (Fig. 6B). IS*1326*-inserted class 1 integrons have been reported to be prevalent in proteobacteria (Jones-Dias et al., 2016) but not in actinobacteria. The prevalence of *sul1* associated with the class 1 integron has been reported in many bacterial isolates from manured agricultural soils (Byrne-Bailey et al., 2009; Wang et al., 2014), suggesting that sulfonamide resistance evolved under sulfonamide selective pressures through horizontal gene transfer of *sul1*-carrying class 1 integron among disparate taxa.

4. Discussion

As the environmental resistome has been regarded as a reservoir of

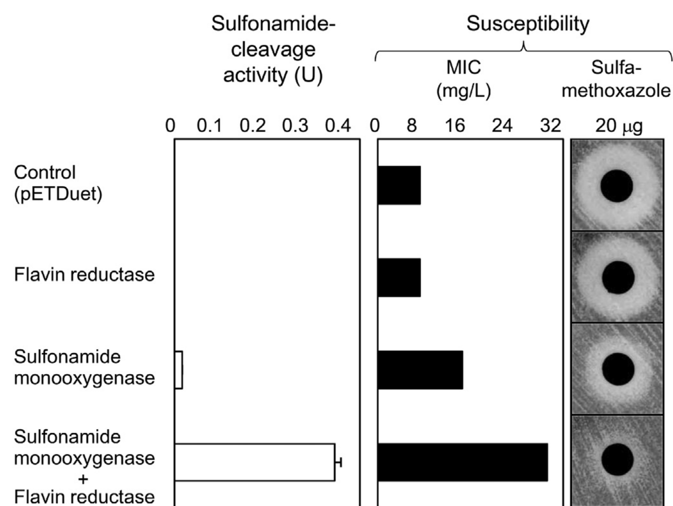

Fig. 4. Sulfonamide-cleavage activity associated with resistance of *E. coli* cells where sulfonamide monooxygenase and flavin reductase were heterologously expressed. Activity was assayed using cells of *E. coli* strains harboring the plasmid pET-Duet derivatives. Susceptibility of *E. coli* cells against sulfamethoxazole was tested by broth dilution assay and disk-diffusion assay.

novel antibiotic resistance genes (Perry et al., 2014), a more extensive understanding of the resistome gained in the past few decades has enabled studies of the evolution and dissemination of antibiotic resistance. Among the various antibiotic resistance mechanisms (Crofts et al., 2017), the enzymatic inactivation mechanism remains relatively unexplored and should be rigorously examined to identify undiscovered resistance determinants in the environment (Morar and Wright, 2010), considering the enormous bacterial diversity and their functional versatility (Morar and Wright, 2010; Wright, 2007). Several novel resistance mechanisms by antibiotic-inactivating enzymes have been discovered in environmental bacteria (Pawlowski et al., 2016; Spanogiannopoulos et al., 2012). Furthermore, recent advances in metagenomics revealed previously unrecognized sequences that were functionally demonstrated to confer novel resistance (Forsberg et al., 2015; Kim et al., 2018).

Because sulfonamides are synthetic antibiotics, naturally occurring enzymes that degrade or modify these drugs may not be readily developed compared to antimicrobials of natural origin (Morar and Wright, 2010). Sulfonamide-degrading bacteria were relatively recently discovered mainly in sulfonamide-contaminated sites and all of those strains whose genome sequences are available contained both *sulX* and *sul1*, suggesting that sulfonamide degradation is associated with sulfonamide resistance. Notably, two genomic islands shared only among the genomes of sulfonamide-degrading *Microbacterium* strains contained the *sulX* gene clusters and *sul1*-carrying class 1 integrons respectively (Fig. 5): two independent sulfonamide resistance determinants co-existed and were distantly located in the genomes of sulfonamide-degrading actinobacteria. Among the sulfonamide-degrading bacteria reported, the genome sequences of three

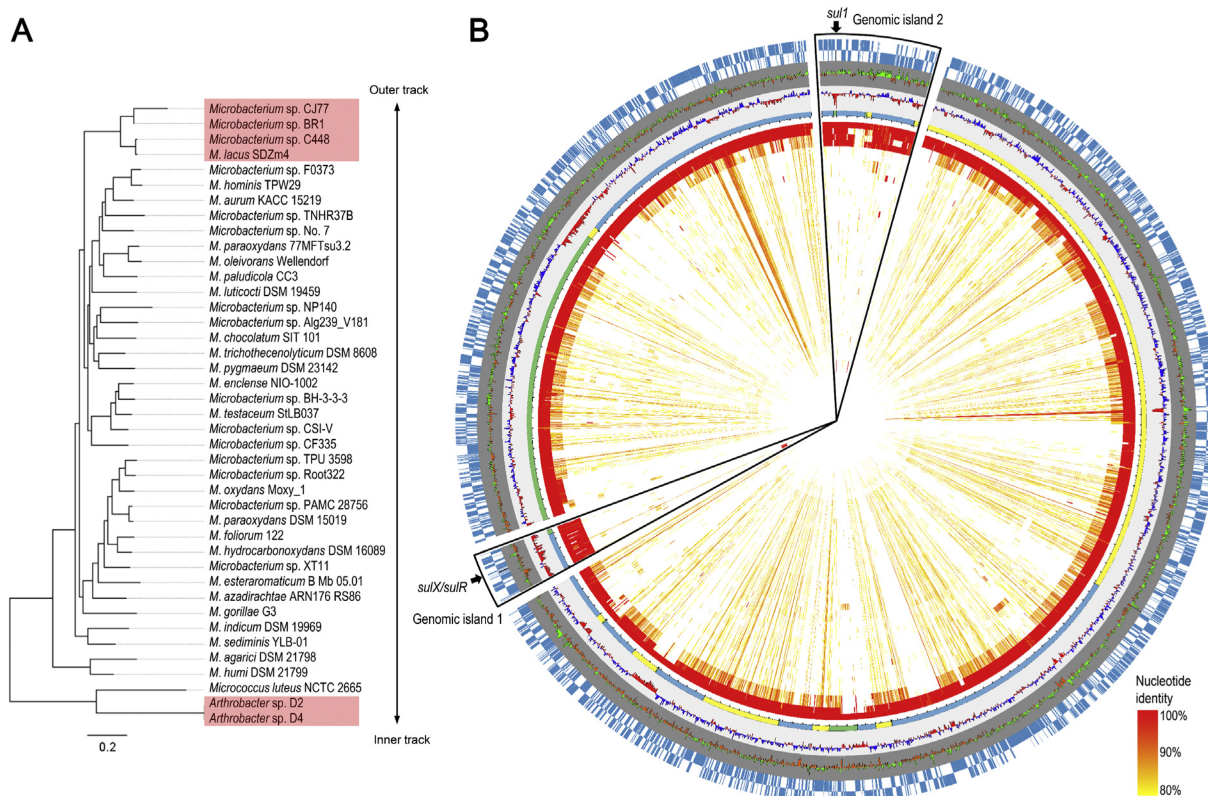

Fig. 5. Two genomic islands harboring sulfonamide-resistant genes *sulX/sulR* and *sul1* shared among sulfonamide-degrading *Microbacterium* strains. (*A*) Maximum-likelihood phylogenetic tree of 34 representative *Microbacterium* spp. and six sulfonamide-degrading bacteria based on their core gene sequences. Sulfonamide-degrading strains are highlighted with a red background. (*B*) Genome maps of *Microbacterium* spp. compared to CJ77 as a reference. The tracks from outside to inside represent: 1st, forward CDS on CJ77 contigs; 2nd, reverse CDS on CJ77 contigs; 3rd, GC skew calculated for 1000-bp windows; 4th, % G + C content of 1000-bp windows; 5th, CJ77 contigs in three alternating colours; 6th to the last tack, heat maps (red to yellow) of nucleotide sequence identity obtained from blastn search of every 500-bp fragment of the CJ77 genome. The genomes are displayed in the same order as the phylogenetic tree shown in (*A*). The genomes of four sulfonamide-degrading *Microbacterium* strains on the upper side of the tree are placed in the outer track and two *Arthrobacter* genomes in the inner track. Genomic islands are highlighted with black border line. Arrows indicate the locations of *sulX/sulR* and *sul1* genes, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

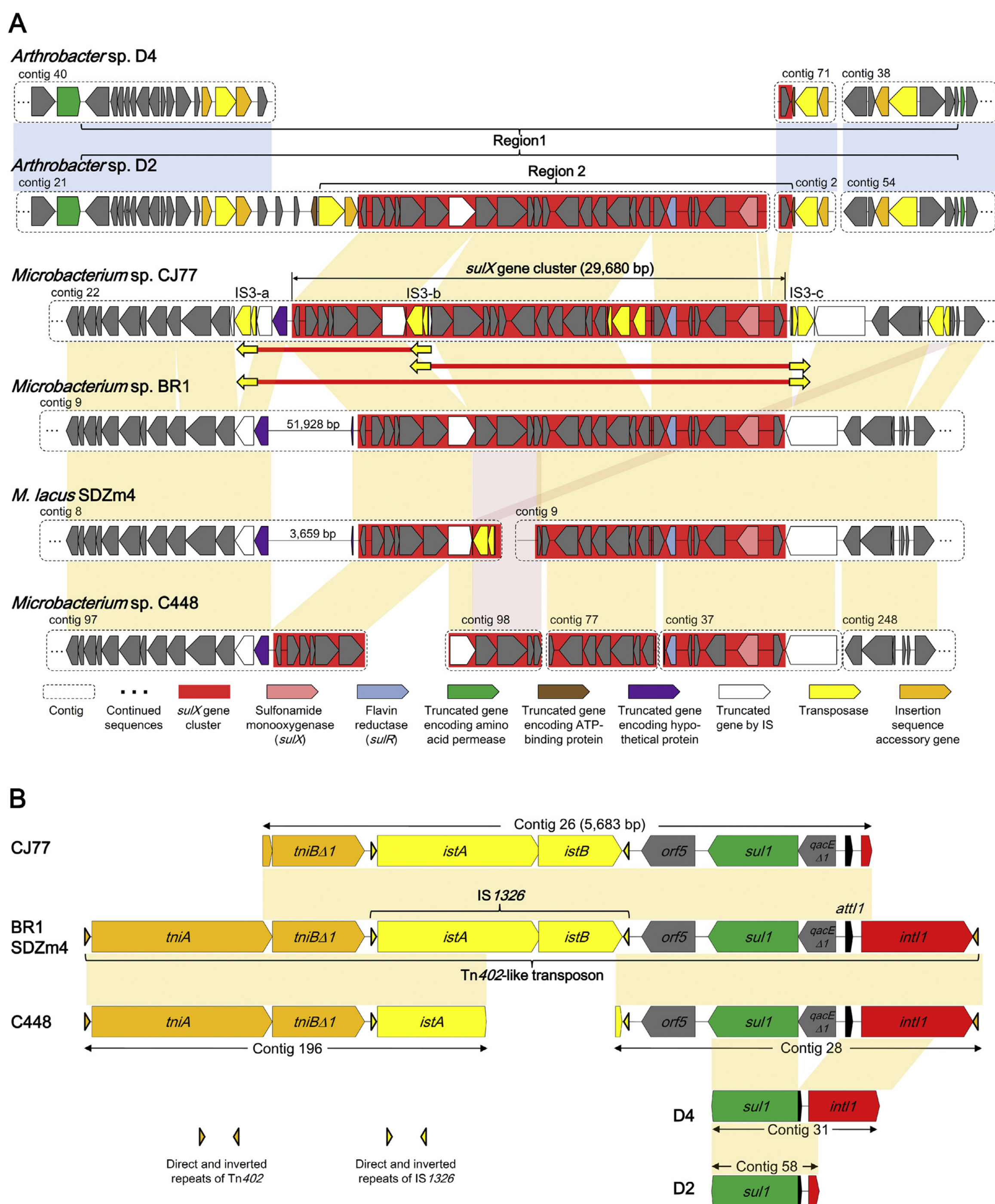

Fig. 6. Comparative analysis of the *sulX* gene clusters in the genomic island 1 (A) and class 1 integrons in the genomic island 2 (B) of sulfonamide-degrading actinobacteria. Shades indicate conserved regions displaying higher than 98% identity. Red lines with yellow arrows indicate potential composite transposon structures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proteobacterial species *Pseudomonas psychrophila* HA-4 (Jiang et al., 2014), *Shewanella oneidensis* MR-1 and *Shewanella* sp. strain MR-4 (Mao et al., 2018) are available in addition to the six actinobacteria analyzed in this study. These proteobacteria were not found to possess *sulX* gene, suggesting that different mechanisms may be involved in the sulfonamide degradation. The co-existence of *sul1* and sulfonamide degradation genes in sulfonamide-degrading actinobacteria is consistent with the prevailing idea of resistance to antibiotics as a condition for degradation (Islas-Espinoza et al., 2012). To evaluate the contribution of these two genes to sulfonamide resistance, gene deletion studies were performed using strain CJ77 but knock-out mutants for *sulX* or *sul1* have yet to be isolated. However, we observed that mutant strains which lost sulfonamide degradation activity were still highly resistant to sulfonamides, suggesting that *sul1* plays a major role in sulfonamide resistance in strain CJ77.

Codon usage and GC content of the *sulX* gene cluster distinguished from those of *Microbacterium* core genes suggest that the gene clusters were acquired at later stages of species evolution. Clearly defined insertion events observed in *Arthrobacter* spp. D2 and D4 provide strong evidence of mobilization. Particularly, in strain CJ77, the presence of multiple IS elements and putative composite transposon structures containing the *sulX* gene cluster also indicate potential mobilization of

sulfonamide resistance. Many studies have reported composite transposons carrying metabolic gene clusters that may have been acquired under certain selection pressures (Clark et al., 2013; Mei et al., 2014). Considering that the sulfonamide-dependent expression of *sulX* gene cluster can provide a selective advantage for the use of sulfonamides as carbon sources, sulfonamide metabolism may have evolved in sulfonamide-resistant bacteria that had already acquired the *sul1*-carrying class 1 integron under sulfonamide selection pressures. Currently, *sulX* has been found in only a few sulfonamide-degrading actinobacteria. This may be because of the low number of sulfonamide-degrading bacteria reported or relatively recent evolution of this gene. The presence of the *sulX* gene cluster at geographically distant locations including Europe, North America and Asia suggests that evolution of the gene cluster occurred independently (Bouju et al., 2012; Deng et al., 2016; Tappe et al., 2013; Topp et al., 2013) and it was much more globally widespread than discovered so far, as acquisition of the gene cluster confers selective advantages in sulfonamide-contaminated environments. Furthermore, the emergence of sulfonamide-degrading bacteria in a particular ecological niche may lead to elimination of the selective pressure which can allow sulfonamide-susceptible bacteria to survive, influencing the microbial community structure in the niche (Deng et al., 2018).

Since the tetracycline-degrading monooxygenase (TetX) conferring resistance was first identified in the transposons of commensal *Bacteroides* spp. (Speer et al., 1991; Whittle et al., 2001), *tetX* gene has been discovered in environmental *Sphingobacterium* sp. (Ghosh et al., 2009), the duck pathogen *Riemerella anatipestifer* (Chen et al., 2010), *Myroides* sp. from a meat processing plant (Li et al., 2016), clinical isolates of *Enterobacteriaceae* and *Pseudomonadaceae* (Leski et al., 2013), and in sequence data of uncultured bacteria. Remarkably, transposon structures (Tn*4351* and CTnDOT) harboring *tetX* gene were significantly conserved in commensal, environmental and clinical isolates (Ghosh et al., 2009; Leski et al., 2013; Speer et al., 1991; Whittle et al., 2001), indicating widespread horizontal gene transfer between disparate taxa (Ghosh et al., 2015). As sulfonamides have been extensively used worldwide, *sulX* associated with mobile genetic elements as well as *sul1*-carrying class 1 integron may be now under mobilization and subsequently emergent in animal and clinical isolates as shown for *tetX*.

5. Conclusions

Although sulfonamide monooxygenase was first identified to catalyze the initial cleavage of sulfonamides in *Microbacterium* sp. BR1, the role of this protein in the resistance was never demonstrated. Furthermore, the association of sulfonamide-degrading genes with mobile genetic elements was not elucidated in detail. In the present study, through a combination of proteomics, heterologous protein expression, and in vitro enzyme assays, we successfully identified the flavin-dependent monooxygenase SulX in non-pathogenic environmental actinobacteria, which not only catalyzed the degradation of sulfonamides but also conferred resistance to these antibiotics. Comparative genomic analysis revealed that *sulX* orthologs were prevalent in sulfonamide-degrading actinobacteria and contained genetic contexts for mobilization. Our study suggests that much wider diversity of resistome might be present in the environment than previously thought, which may be associated with the bacterial metabolism of antimicrobials. Indeed, numerous antibiotic-resistant bacterial strains subsisting on antibiotic chemicals were isolated from the natural environment (Dantas et al., 2008). Therefore, exploring microbial metabolic versatility related to the degradation of antimicrobials is important for expanding our knowledge of antibiotic resistance mechanisms, recollecting the concept “microbial infallibility” which states that most organic chemicals including antimicrobials have been degraded and recycled on the planet throughout history (Alexander, 1965).

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Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.03.046.

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