ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2011, p. 5826–5833 Vol. 55, No. 12
0066-4804/11/$12.00 doi:10.1128/AAC.00317-11

Contribution of Resistance-Nodulation-Division Efflux Pump Operon *smeU1-V-W-U2-X* to Multidrug Resistance of *Stenotrophomonas maltophilia*▽

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Received 8 March 2011/Returned for modification 25 April 2011/Accepted 3 September 2011

KJ09C, a multidrug-resistant mutant of *Stenotrophomonas maltophilia* KJ, was generated by *in vitro* selection with chloramphenicol. The multidrug-resistant phenotype of KJ09C was attributed to overexpression of a resistance nodulation division (RND)-type efflux system encoded by an operon consisting of five genes: *smeU1*, *smeV*, *smeW*, *smeU2*, and *smeX*. Proteins encoded by *smeV*, *smeW*, and *smeX* were similar to the membrane fusion protein, RND transporter, and outer membrane protein, respectively, of known RND-type systems. The proteins encoded by *smeU1* and *smeU2* were found to belong to the family of short-chain dehydrogenases/reductases. Mutant KJ09C exhibited increased resistance to chloramphenicol, quinolones, and tetracyclines and susceptibility to aminoglycosides; susceptibility to β-lactams and erythromycin was not affected. The expression of the *smeU1-V-W-U2-X* operon was regulated by the divergently transcribed LysR-type regulator gene *smeRv*. Overexpression of the SmeVWX pump contributed to the acquired resistance to chloramphenicol, quinolones, and tetracyclines. Inactivation of *smeV* and *smeW* completely abolished the activity of the SmeVWX pump, whereas inactivation of *smeX* alone decreased the activity of the SmeVWX pump. The enhanced aminoglycoside susceptibility observed in KJ09C resulted from SmeX overexpression.

Multidrug efflux transport systems are an important mechanism for bacteria to combat antimicrobial agents (1). Among efflux transport systems, the resistance nodulation cell division (RND)-type multidrug efflux systems play a critical role in multidrug resistance (MDR), especially in Gram-negative bacteria. An RND-type efflux pump generally consists of three components: the inner membrane protein (IMP), which has homology to the RND family H^+ antiporter, and the outer membrane protein (OMP), which is involved in channel formation, along with the membrane fusion protein (MFP) to link IMP and OMP (19, 28). In some cases, regulatory genes are located adjacent to the operon encoding the pump components (2, 27). Some RND efflux pump operons are constitutively expressed (27), and others are quiescent (15).

Stenotrophomonas maltophilia is an important opportunistic human pathogen characterized by intrinsic resistance to a variety of antimicrobial agents (6). Genome sequence analysis reveals that *S. maltophilia* K279a encodes as many as eight putative RND efflux systems, including SmeABC, SmeDEF, SmeGH, SmeIJK, SmeMN, SmeOP, SmeVWX, and SmeYZ (4). Of these, SmeIJK and SmeYZ are reported to be constitutively expressed and contribute to intrinsic antibiotic resistance. SmeIJK slightly contributes to the intrinsic resistance to aminoglycosides, tetracyclines, and ciprofloxacin. SmeYZ is important in the intrinsic resistance to aminoglycosides in *S. maltophilia* (4). In addition, overexpression of *smeABC* (15) and *smeDEF* (2, 31, 36) pumps has been reported in multidrug-resistant mutants. Overexpression of the *smeABC* operon increases resistance to aminoglycosides, β-lactams, and quinolones (15). The SmeDEF efflux pump mainly confers resistance to quinolones, chloramphenicol, macrolide, novobiocin, and tetracyclines (36).

In the study described in this article, a novel RND-type efflux pump operon, *smeU1-V-W-U2-X*, from *S. maltophilia* KJ was identified and characterized. The *smeU1-V-W-U2-X* operon is distinct from known RND-type multidrug efflux pump operons and is composed of five genes. Regulator gene *smeRv* is divergently located upstream from the *smeU1-V-W-U2-X* operon; this work characterizes each operon component, the regulatory role of *smeRv*, and the significance of the operon to intrinsic and acquired resistance of *S. maltophilia*.

MATERIALS AND METHODS

Bacterial strains and primers. Table 1 lists the bacterial strains and plasmids used in this study. *S. maltophilia* KJ served as the parental wild-type strain (9). All PCR primers used in this study were designed on the basis of the *S. maltophilia* K279a genome sequence (4), as described in Table 2.

Selecting MDR mutants. An overnight culture of *S. maltophilia* KJ was plated onto LB agar containing 50 μg/ml chloramphenicol and incubated for 24 to 48 h at 37°C. The antimicrobial resistance profiles of the growing colonies were tested; colonies exhibiting resistance to at least three structurally unrelated antibiotics were selected for further study.

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▽ Published ahead of print on 19 September 2011.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or properties	Reference
S. maltophilia		
KJ	Wild type, a clinical isolate from Taiwan	9
KJΔL1ΔL2	*S. maltophilia* KJ double mutant of L1 and L2 genes; Δ*L1* Δ*L2*	This study
KJΔ5	*S. maltophilia* KJ *smeU1-V-W-U2-X* mutant; Δ*smeU1* Δ*smeV* Δ*smeW* Δ*smeU2* Δ*smeX*	This study
KJ09C	*S. maltophilia* KJ spontaneous chloramphenicol-selected mutant	This study
KJ09CΔL1ΔL2	*S. maltophilia* KJ09C with double mutations of *L1* and *L2* genes; Δ*L1* Δ*L2*	This study
KJ09CΔ5	*S. maltophilia* KJ09C *smeU1-V-W-U2-X* mutant; Δ*smeU1* Δ*smeV* Δ*smeW* Δ*smeU2* Δ*smeX*	This study
KJ09CΔRv	*S. maltophilia* KJ09C *smeRv* mutant; Δ*smeRv*	This study
KJ09CΔU1	*S. maltophilia* KJ09C *smeU1* mutant; Δ*smeU1*	This study
KJ09CΔVW	*S. maltophilia* KJ09C *smeV-smeW* mutant; Δ*smeV* Δ*smeW*	This study
KJ09CΔU2	*S. maltophilia* KJ09C *smeU2* mutant; Δ*smeU2*	This study
KJ09CΔX	*S. maltophilia* KJ09C *smeX* mutant; Δ*smeX*	This study
KJ09CΔ5L2::U1	*S. maltophilia* KJ09CΔ5 with *L2* gene replaced with *smeU1* gene; Δ*L2*::*smeU1*	This study
KJ09CΔ5L2::U2	*S. maltophilia* KJ09CΔ5 with *L2* gene replaced with *smeU2* gene; Δ*L2*::*smeU2*	This study
KJ09CΔ5L2::X	*S. maltophilia* KJ09CΔ5 with *L2* gene replaced with *smeX* gene; Δ*L2*::*smeX*	This study
Escherichia coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_k^- m_k^+) *phoA supE44* λ *thi-1 gyrA96 relA1*	Invitrogen
S17-1	λ pir-positive mating strain	34
Plasmids		
pEX18Tc	*sacB oriT* Tc^r	8
pRK415	Mobilizable broad-host-range plasmid cloning vector, RK2 origin; Tc^r	12
pX1918GT	Plasmid containing *xylE*-gentamicin resistance cassette; Amp^r Gm^r	33
pU1	pEX18Tc with a 1,341-bp DNA fragment of *S. maltophilia* KJ containing complete *smeRv-smeU1* intergenic region and *smeU1* gene; Tc^r	This study
pU2	pEX18Tc with a 1,074-bp DNA fragment of *S. maltophilia* KJ containing intact *smeU2* gene; Tc^r	This study
pX	pEX18Tc with a 1,591-bp DNA fragment of *S. maltophilia* KJ containing intact *smeX* gene; Tc^r	This study
pRv	pEX18Tc with a 1,308-bp DNA fragment of *S. maltophilia* KJ containing partial *smeRv* gene and upstream 227-bp fragment; Tc^r	This study
pΔU1	pEX18Tc with an internal deletion *smeU1* gene; Tc^r	This study
pΔU2	pEX18Tc with an internal deletion *smeU2* gene; Tc^r	This study
pΔX	pEX18Tc with an internal deletion *smeX* gene; Tc^r	This study
pΔRv	pEX18Tc with an internal deletion *smeRv* gene; Tc^r	This study
pΔVW	pEX18Tc with a 2,426-bp DNA fragment of *S. maltophilia* KJ containing complete *smeU1* gene, partial 3′ terminus of *smeW* gene, and *smeU2* gene; Tc^r	This study
pΔ5	pEX18Tc with a 1,982-bp DNA fragment of *S. maltophilia* KJ containing partial 5′ terminus of *smeU1* gene and partial 3′ terminus of *smeX* gene; Tc^r	This study
$p371Rv_{xylE}$	pRK415 with a 371-bp intergenic region of *smeRv* and *smeU1* genes and a transcriptional fusioned *xylE* gene; *smeRv*::*xylE* fusion construct	This study
$p371U1_{xylE}$	pRK415 with a 371-bp intergenic region of *smeRv* and *smeU1* genes and a transcriptional fusioned-*xylE* gene; *smeU1*::*xylE* fusion construct	This study
$p272X_{xylE}$	pRK415 with a 272-bp upstream of *smeX* gene and a transcriptional fusioned *xylE* gene; *smeX*::*xylE* fusion construct	This study
pYW	pEX18Tc with a 1,352-bp DNA fragment of *S. maltophilia* KJ containing 697 bp upstream of *L2* gene and 655 bp downstream of *L2* gene; Δ*L2* Tc^r	This study
pΔL2::U1	pYW with an inserted intact *smeU1* gene	This study
pΔL2::U2	pYW with an inserted intact *smeU2* gene	This study
pΔL2::X	pYW with an inserted intact *smeX* gene	This study

Allelic exchange method for construction of deletion mutants. Plasmids were constructed to obtain deletion mutants. The *smeU1*, *smeU2*, *smeX*, and *smeRv* genes were amplified by PCR, using primer sets SmeU1-F/SmeU1-R, SmeU2-F/SmeU2-R, SmeX-F/SmeX-R, and SmeRv-F/SmeRv-R, to yield plasmids pU1, pU2, pX, and pRv, respectively (Tables 1 and 2 and Fig. 1). Internal 173-bp, 103-bp, 313-bp, and 294-bp DNA fragments were removed from plasmids pU1, pU2, pX, and pRv, respectively, yielding plasmids pΔU1, pΔU2, pΔX, and pΔRv (Table 1 and Fig. 1). The 1,082-bp XbaI-SacI fragment retrieved from pU2 was ligated into plasmid pU1, to obtain pΔVW (Table 1 and Fig. 1). A 624-bp HindIII-PstI fragment and 1,364-bp PstI-EcoRI fragment were retrieved from pU1 and pX, respectively, and then sequentially cloned into pEX18Tc to obtain pΔ5 (Table 1 and Fig. 1).

Plasmids pΔU1, pΔVW, pΔU2, pΔX, pΔRv, and pΔ5 were mobilized into *S. maltophilia* via conjugation (17). The deleted allele was transferred to the *S. maltophilia* chromosome via double-crossover homologous recombination with selection by tetracycline (30 μg/ml)-norfloxacin (2.5 μg/ml) and then 10% sucrose. The correctness of the resultant mutants was verified by colony PCR amplification (16) and sequencing. Double mutants were constructed from single mutants by the same procedure.

Construction of promoter-*xylE* transcriptional fusions. To test whether the *smeX* gene has its own promoter, the 323-bp BamHI-SacI DNA fragment spanning nucleotides −272 to +51 relative to the *smeX* start codon was retrieved from pΔU2 and cloned into pRK415. Then, an *xylE* gene was inserted into the 52nd nucleotide of the *smeX* gene (Table 1 and Fig. 1). Two promoter-*xylE* transcriptional fusions, $p371U1_{xylE}$ and $p371Rv_{xylE}$, were constructed to assess the regulatory role of *smeRv*. A DNA fragment containing the 371-bp intergenic region and partial 5′ terminus of *smeRv* and *smeU1* was released by treating pU1 with HindIII and PstI and then cloned into pRK415. An *xylE* gene was inserted

TABLE 2. Primers used in this study

Primer name	Sequence (5′ → 3′)[a]	Length (bp)	Purpose
SmeRv-F	GATGGTACCGCCACGCTGCTGAC	1,308	Cloning
SmeRv-R	CAGAATAAGCTTGCCGCTGCTTTCC		
SmeU1-F	CGATTAAGCTTCGGCAATGAAG	1,341	Cloning
SmeU1-R	ACGTTCTAGAGTGGTATTGGGG		
SmeU2-F	GATGATATCCATCGCGTTCATCGCC	1,074	Cloning
SmeU2-R	TGACAGAGCTCGCCAGCACCAG		
SmeX-F	GGCTCTAGAGAAATCAGCGAAG	1,591	Cloning
SmeX-R	AGAAGAAAGGTACCGAAGCCAC		
YW5-F	GCAAGCTTCAAAGCGGATCGCCAG	697	Cloning
YW5-R	CAGCATCTAGAAGGCAAGCGAG		
YW3-F	ATCTAGACGGAAGTCCCCCATGAG	655	Cloning
YW3-R	GCCGGAATTCACCAGCAGCGAG		
SmeBQ-F	CGCCATCTCGCTGCTGTTC	198	qRT-PCR
SmeBQ-R	ATGCCGTTCTTCGCTGCC		
SmeCQ-F	GCGATGCCAACAGCGAGACC	190	qRT-PCR
SmeCQ-R	GTCGCCACTTCAGCCACCAG		
SmeEQ-F	TCCTGCCCAACGAAGACC	203	qRT-PCR
SmeEQ-R	CTTGACGAACGCCATGCC		
SmeFQ-F	CCCGAGCATCTCGCTGAC	207	qRT-PCR
SmeFQ-R	AAGCCCACCTGGATCGAC		
SmeHQ-F	GGCTACTCGGCGATCAAC	207	qRT-PCR
SmeHQ-R	CAGGCACAGGAACACCAC		
SmeJQ-F	GTCAGCCACCAGCAGCAG	192	qRT-PCR
SmeJQ-R	CAGCAGCCACACCACGTC		
SmeKQ-F	AACTCCGACCCCAGCGAC	191	qRT-PCR
SmeKQ-R	GCGATCATCGAGATCACCGAC		
SmeNQ-F	CAAGACCTCCACTGCCAAC	198	qRT-PCR
SmeNQ-R	AACAGCCAGATCACCGCC		
SmePQ-F	GTCAGCCAGTTCCTGTCC	191	qRT-PCR
SmePQ-R	TACTCCATCGTCGCCACC		
SmeZQ-F	TGTCCAGCGTCAAGCACC	218	qRT-PCR
SmeZQ-R	GCCGACCAGCATCAGGAAG		
SmeRvQ-F	TCGACGAACGCACGCACC	213	qRT-PCR
SmeRvQ-R	CCCGCTGATGACCGCCAAC		
SmeU1Q-F	CGGCGAGACCTCGATCAC	201	qRT-PCR
SmeU1Q-R	CAACCATCCAGCAGCGAC		
SmeVQ-F	GCGTGACAGCGAACTGCC	219	qRT-PCR
SmeVQ-R	TCATCGATCAGCAGCGCC		
SmeWQ-F	GCCCACACCATCTCGTTCCC	221	qRT-PCR
SmeWQ-R	TAGCCGTTGCCGTTGCCC		
SmeU2Q-F	GGTCGAGCAGGTACGCCAG	153	qRT-PCR
SmeU2Q-R	ACCGCCACCAGCGCATAG		
SmeXQ-F	TACGACCGCCGCAAGCAACC	219	qRT-PCR
SmeXQ-R	CAGCTCGAAGTAGTTGCGTGCC		
rDNA-F	GACCTTGCGCGATTGAATG	75	qRT-PCR
rDNA-R	CGGATCGTCGCCTTGGT		

[a] Underlining indicates the restriction sites introduced for cloning.

into the 174nd and 56nd nucleotides of the *smeU1* and *smeRv* genes, respectively, yielding plasmids p371U1$_{xylE}$ and p371Rv$_{xylE}$. All constructs were confirmed by PCR to ensure that the orientation of the *xylE* gene was opposite that of P_{lacZ} of the pRK415 vector.

Overexpression of *smeU1*, *smeU2*, and *smeX* by β-lactam-mediated system. The impact of the *smeU1*, *smeU2*, and *smeX* genes on antimicrobial sensitivity was evaluated by complementation. Due to a limitation of available selective antibiotics in KJ09C, the plasmid complementation assay is infeasible in MDR mutants. Alternatively, a β-lactam-mediated overexpression system has been developed. Our previous study described an *ampR-L2* regulon (9). When bacterial cells were treated with a β-lactam, the *L2* gene was inducibly expressed, dependent on an activated AmpR (17). Herein, we utilized the *ampR-L2* regulon to develop a β-lactam-mediated overexpression system in *S. maltophilia*. The target gene expected to be overexpressed was applied *in situ* to replace coding sequences of the *L2* gene and was overexpressed under the driving of β-lactam treatment. First, vector pYW was constructed for cloning the overexpressed gene intended. Two nonoverlapped DNA fragments of 697 bp and 655 bp, corresponding to regions upstream and downstream of the *L2* gene, were obtained by PCR, using primer sets YW5-F/YW5-R and YW3-F/YW3-R, respectively (Table 2). The two PCR amplicons were sequentially cloned into pEX18Tc, giving rise to plasmid pYW. To replace *L2* with *smeU1*, *smeU2*, and *smeX in situ*, the *smeU1*-, *smeU2*-, and *smeX*-containing DNA fragments from plasmids pU1, pU2, and pX were cloned into pYW, resulting in plasmids pΔL2::U1, pΔL2::U2, and pΔL2::X, respectively. The correctness of the inserted gene orientation in the construct was checked by sequencing. Plasmids pΔL2::U1, pΔL2::U2, and pΔL2::X were mobilized from *Escherichia coli* S17-1 into *S. maltophilia* KJ09CΔ5 by conjugation, and the correct double-crossover mutants, KJ09CΔ5L2::U1, KJ09CΔ5L2::U2, and KJ09CΔ5L2::X, were selected and checked as previously described (17).

Antimicrobial susceptibility testing. Antibiotic susceptibility testing was performed by agar dilution on Mueller-Hinton agar according to guidelines of the Clinical and Laboratory Standards Institute (CLSI) (3). MICs were defined as the lowest concentration of antimicrobial that completely inhibited growth. All antibiotics were purchased from Sigma. The MICs of imipenem and moxifloxacin were quantified using Etest strips (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions.

RNA preparation and qRT-PCR. Total RNA extracted from exponentially growing cells, using a PureLink total RNA purification system (Invitrogen, Carls-

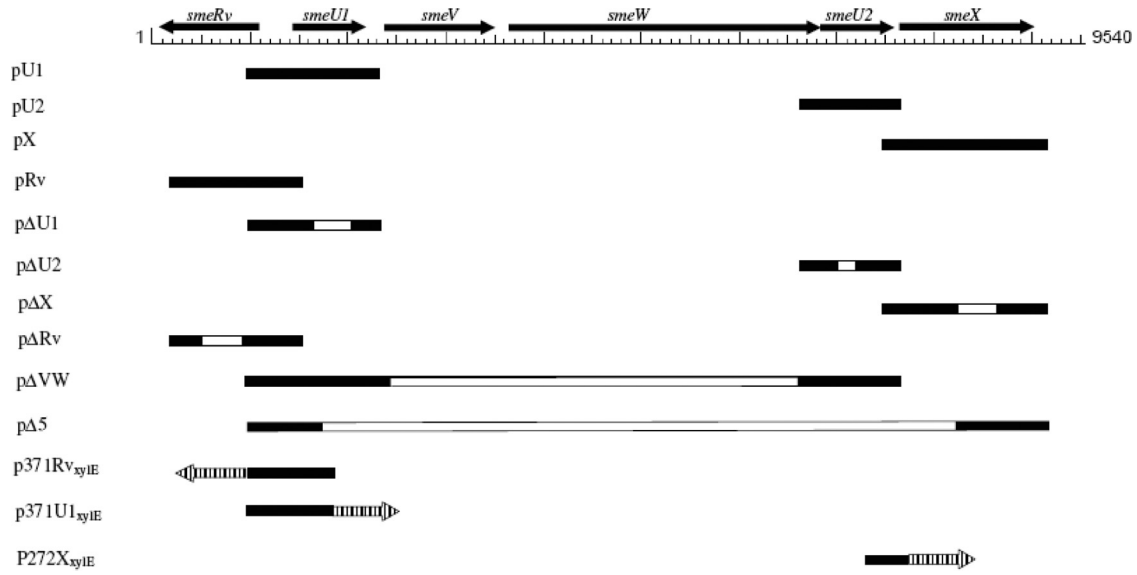

FIG. 1. Genomic organization surrounding *smeU1-V-W-U2-X* operon of *S. maltophilia* KJ and structure of recombinant plasmids used in this study. The gene orientation is indicated by arrows. Crosshatched arrows, *xylE* cassette; white boxes, deleted regions.

bad, CA), was treated with a unit of RNase-free DNase I (Invitrogen, Carlsbad, CA), and cDNA synthesis was performed with a Moloney murine leukemia virus reverse transcriptase first-strand cDNA synthesis kit (Epicentre Biotechnologies, Taiwan). The cDNA was then used directly as the template for quantitative real-time PCR (qRT-PCR) using a Smart Quant Green master mix (Protech Technology Enterprise Co., Ltd.) on an ABI Prism 7000 sequence detection system (Applied Biosystems). Normalized expression levels of the target gene transcripts were calculated relative to the 16S rRNA gene using the $\Delta\Delta C_T$ method, where C_T is the threshold cycle (18). Table 2 lists the primers used for qRT-PCR of each gene. Each result represents the average of three independent determinations.

Nucleotide sequence accession numbers. The nucleotide sequences of the *smeVWX* operon and *smeRv* gene of *S. maltophilia* KJ determined in the study described in this paper have been deposited in the GenBank database under accession numbers JN613813 and JN613814.

RESULTS AND DISCUSSION

Chloramphenicol selects for MDR mutants. Spontaneous chloramphenicol-selected mutant KJ09C was isolated by selecting strain KJ on LB medium containing 50 μg/ml chloramphenicol. Compared to its parental strain, KJ, the KJ09C mutant showed an MDR profile with increased resistance to chloramphenicol, quinolones, and tetracyclines; however, it was notably more susceptible to aminoglycosides, and its susceptibility to erythromycin was not affected (Table 3).

Overexpression of SmeVWX efflux system in MDR mutant KJ09C. The multidrug-resistant nature of mutant KJ09C is similar to that of mutants that overexpress multidrug efflux

TABLE 3. Antimicrobial susceptibilities of *S. maltophilia* KJ, its chloramphenicol-selected mutant KJ09C, and their derived constructs

Strain	MIC (μg/ml)[a]								
	CHL	Quinolones		Tetracyclines		Aminoglycosides			ERY
		NAL	MXF	TET	DOX	KAN	GEN	TOB	
KJ	8	8	0.094	16	1	256	1,024	512	64
KJ09C	128	256	0.25	64	2	64	256	256	64
KJΔ5	8	8	0.094	16	1	256	1,024	512	64
KJ09CΔ5	8	16	0.094	16	2	128	512	512	64
KJ09CΔRv	8	8	0.094	16	1	256	512	512	64
KJ09CΔU1	128	256	0.38	32	2	64	512	128	64
KJ09CΔVW	4	4	0.047	16	0.5	64	256	256	64
KJ09CΔU2	32	64	0.19	32	2	32	128	128	64
KJ09CΔX	32	32	0.064	32	2	512	1,024	512	64
KJ09CΔ5[b]	8	8	0.094	16	2	128	512	512	64
KJ09CΔ5L2::U1[b]	8	8	0.094	16	2	128	512	512	64
KJ09CΔ5L2::U2[b]	8	8	0.125	16	2	256	512	512	64
KJ09CΔ5L2::X[b]	4	4	0.064	8	1	8	32	8	64

[a] CHL, chloramphenicol; NAL, nalidixic acid; MXF, moxifloxacin; TET, tetracycline; DOX, doxycycline; KAN, kanamycin; GEN, gentamicin; TOB, tobramycin; ERY, erythromycin.

[b] Mueller-Hinton agar contains 30 μg/ml cefoxitin, in addition to the antibiotic indicated.

TABLE 4. Expression of genes of *smeRv-smeU1-V-W-U2-X* regulon for strains KJ, KJ09C and its derived mutant, determined by qRT-PCR

Gene	Relative fold change of transcript[a]						
	KJ	KJ09C	KJ09CΔRv	KJ09CΔU1	KJ09CΔVW	KJ09CΔU2	KJ09CΔX
smeRv	1	78.8 ± 6.1	0.36 ± 0.0	82.1 ± 7.7	174.4 ± 18.7	51.6 ± 4.1	47.17 ± 3.9
smeU1	1	34.3 ± 2.3	0.95 ± 0.1	2.1 ± 0.1	42.2 ± 5.7	47.5 ± 5.9	48.5 ± 2.4
smeV	1	22.5 ± 1.4	3.38 ± 0.4	10.4 ± 0.6	1.7 ± 0.1	21.5 ± 2.9	12.0 ± 0.5
smeW	1	58.9 ± 4.4	0.77 ± 0.2	41.9 ± 3.7	1.1 ± 0.9	19.2 ± 1.6	20.8 ± 1.8
smeU2	1	156.5 ± 13.4	1.82 ± 0.8	178.5 ± 15.4	179.7 ± 15.3	0.2 ± 0.0	108.4 ± 14.3
smeX	1	24.9 ± 2.7	0.31 ± 0.0	34.7 ± 4.0	26.7 ± 3.8	6.4 ± 0.7	0.62 ± 0.0

[a] Relative fold changes between wild-type KJ and its derived mutant were calculated by the $2^{-\Delta\Delta CT}$ method.

systems of the RND family. Genome analysis has revealed that *S. maltophilia* encodes as many as eight possible RND efflux systems: SmeABC, SmeDEF, SmeGH, SmeIJK, SmeMN, SmeOP, SmeVWX, and SmeYZ (4). Therefore, inner membrane RND-type transporter genes (*smeB*, *smeE*, *smeH*, *smeJ*, *smeK*, *smeN*, *smeP*, *smeW*, and *smeZ*) of each RND efflux system and three outer membrane protein genes (*smeC*, *smeF*, and *smeX*) were selected as target genes for qRT-PCR to evaluate transcript expression of each efflux pump. Expression of the genes assayed (except *smeW* and *smeX*) was equivalent between strains KJ and KJ09C (data not shown); *smeW* and *smeX* transcripts in strain KJ09C were approximately 59 and 25 times those in strain KJ (Table 4). The overexpression of the SmeVWX pump was thus considered putatively responsible for the MDR phenotype of mutant KJ09C.

β-Lactams are not substrates for SmeVWX pump. Because two inducible β-lactamase genes, the *L1* and *L2* genes, are present in *S. maltophilia* KJ (9), the contribution of the SmeVWX pump to β-lactam resistance may be shielded. To clarify this possibility, the β-lactam susceptibilities of mutants KJ09CΔL1ΔL2 (an *L1* and *L2* double mutant of KJ09C) and KJΔL1ΔL2 (an *L1* and *L2* double mutant of KJ) were compared. The β-lactams tested included piperacillin, carbenicillin, cefoxitin, cefuroxime, imipenem, and aztreonam. KJ09CΔL1ΔL2 generally showed an insignificant change in susceptibility to β-lactams compared with its parental strain, KJΔL1ΔL2 (data not shown), indicating that β-lactams are not substrates for the SmeVWX pump.

***smeU1*, *smeV*, *smeW*, *smeU2*, and *smeX* genes form an operon.** Sequence analysis surrounding the *smeVWX* operon was performed using the genome sequence of *S. maltophilia* K279a as a reference (4). Unlike the tripartite MDR efflux pump operon, the putative *smeVWX*-associated operon consisted of a membrane fusion protein gene (*smeV*), inner membrane RND transporter gene (*smeW*), outer membrane protein gene (*smeX*), and two additional genes annotated short-chain dehydrogenase/reductase (SDR) that are located upstream of the *smeV* gene (designated *smeU1*) and between the *smeW* and *smeX* genes (designated *smeU2*), respectively (Fig. 1). Sequence analysis revealed that the two SDR proteins encoded by *smeU1* and *smeU2* differed, with a protein identity of 21%. A putative LysR family transcriptional regulator gene, designated *smeRv* here, was oriented divergently from the *smeU1-V-W-U2-X* module and was located 371 bp from the *smeU1* gene start codon.

With the evidence of the higher expression of *smeW* and *smeX* transcripts in KJ09C (Table 4), transcripts of the *smeU1*, *smeV*, *smeU2*, and *smeRv* genes of strains KJ and KJ09C were also quantified by qRT-PCR. Table 4 shows each gene in the *smeRv-smeU1-V-W-U2-X* regulon to be concertedly highly expressed. To verify the possibility of the *smeU1-V-W-U2-X* operon, reverse transcription-PCR (RT-PCR) was performed to amplify each junction between two flanking genes, like *smeU1-smeV*, *smeV-smeW*, *smeW-smeU2*, and *smeU2-smeX*. The RT-PCR results (data not shown) further confirmed that the *smeU1*, *smeV*, *smeW*, *smeU2*, and *smeX* genes form an operon in *S. maltophilia*.

Sequence analysis of *smeU1-V-W-U2-X* operon. Comparison of the amino acid sequences of SmeV, SmeW, and SmeX with those of other RND-type efflux systems showed two most similar RND-type efflux systems, *dauE-mexE-mexF-Xcc1441-oprN* from *Xanthomonas campestris* pv. campestris (5) and *mexEF-oprN* from *Pseudomonas aeruginosa* (35).

The *dauE-mexE-mexF-Xcc1441-oprN* operon in *X. campestris* also consists of five genes. A five-member RND-type efflux pump operon in *S. maltophilia* is thus not unique. However, the function of the *dauE-mexE-mexF-Xcc1551-oprN* operon of *Xanthomonas campestris* pv. campestris has not been studied. Protein identities of corresponding components between the *smeU1-V-W-U2-X* and *dauE-mexE-mexF-Xcc1441-oprN* operons were 51% for SmeU1 and DauE, 80% for SmeV and MexE, 81% for SmeW and MexF, 74% for SmeU2 and XCC1441, as well as 74% for SmeX and OprN.

Another homologue of the *smeU1-V-W-U2-X* operon is the *mexEF-oprN* operon of *P. aeruginosa*. The protein identities of the SmeV/MexE, SmeW/MexF, and SmeX/OprN pairs were 53%, 58%, and 48%, respectively.

Because of the similarity in amino acid sequence and genetic organization, the SmeVWX pump in *S. maltophilia* appears to be the ortholog of the MexEF-OprN transporter in *P. aeruginosa* (10, 14). The overexpression of both systems has been shown to be readily selected by chloramphenicol (10, 14) and contribute to resistance to chloramphenicol, quinolones, and tetracyclines. Still, some different features exist between MexEF-OprN and SmeVWX. (i) Overexpression of MexEF-OprN in *P. aeruginosa* concomitantly decreases expression in the *oprD* gene, which is associated with resistance to imipenem (13). A similar mechanism does not seem to happen in the SmeVWX pump of *S. maltophilia*, since no homologue of *oprD* is found in the *S. maltophilia* K279a genome and the MICs of imipenem are undistinguishable in strains KJΔL1ΔL2 and KJ09CΔL1ΔL2. (ii) An MexEF-OprN overexpression mutant displays higher β-lactam susceptibility owing to reduced expression of MexAB-OprM (7). In contrast, the β-lactam sus-

TABLE 5. Determination of C23O activities of strain KJ and its derived mutants containing different transcriptional fusion constructs

Strain	C23O activity (Uc/OD_{450})[a]
KJ(p371Rv_{xylE})	5 ± 0.7
KJΔRv(p371Rv_{xylE})	58 ± 6.1
KJ09C(p371Rv_{xylE})	305 ± 41
KJ09CΔRv(p371Rv_{xylE})	21 ± 1.9
KJ(p371$U1_{xylE}$)	1 ± 0.4
KJΔRv(p371$U1_{xylE}$)	3 ± 0.7
KJ09C(p371$U1_{xylE}$)	25 ± 3.1
KJ09CΔRv(p371$U1_{xylE}$)	2 ± 0.5
KJ(p272X_{xylE})	4 ± 0.4
KJ09C(p272X_{xylE})	5 ± 0.5

[a] Uc is defined as 1 nanomole of catechol hydrolyzed per minute. Results are expressed as means ± SDs of three independent determinations. OD_{450}, optical density at 450 nm.

ceptibility is essentially unchanged in a *smeU1-V-W-U2-X* overexpression mutant, such as KJ09C.

Role of SmeVWX efflux pump in intrinsic and acquired resistance. To assess the contribution of SmeVWX pump activity to intrinsic and acquired antibiotic resistance, an unmarked deletion of the *smeU1-V-W-U2-X* operon was constructed in strains KJ and KJ09C, respectively. Strain KJ09CΔ5 showed MIC values within a 2-fold difference of those for wild-type strain KJ (Table 3), indicating that the SmeVWX pump is the major determinant contributing to the MDR phenotype of KJ09C. Meanwhile, the susceptibility profile of the KJΔ5 mutant was indistinguishable from that of the wild-type strain. The SmeVWX pump is apparently quiescent or its expression level is too low for detection of susceptibility differences from the wild-type strain.

Role of SmeRv regulator in *smeRv-smeU1-V-W-U2-X* regulon. Expression of a multidrug efflux pump is frequently regulated by both global and local regulators (26). The LysR-type regulator gene *smeRv* found in the vicinity of the *smeU1-V-W-U2-X* operon suggests that the *smeU1-V-W-U2-X* operon is specifically regulated by the contiguous *smeRv* gene. Furthermore, the qRT-PCR results (Table 4) indicated that *smeRv* expression elevated approximately 78-fold in the KJ09C mutant compared with that in wild-type strain KJ. The *smeRv* gene is therefore a putative candidate to positively regulate the expression of the *smeU1-V-W-U2-X* operon. To test this directly, a Δ*smeRv* allele was introduced into the KJ09C background and yielded mutant KJ09CΔRv. Transcripts of *smeU1*, *smeV*, *smeW*, *smeU2*, and *smeX* in KJ09CΔRv were quantified by qRT-PCR. Table 4 shows that transcripts of *smeU1*, *smeV*, *smeW*, *smeU2*, and *smeX* in KJ09CΔRv were as low as those in wild-type strain KJ. In addition, MICs for all antimicrobials tested in KJ09CΔRv returned to levels close to those in strain KJ (Table 3).

The regulatory role of SmeRv was further evaluated by transcriptional fusion assay. Transcriptional fusion constructs p371Rv_{xylE} and p371$U1_{xylE}$ were used to assess the expression of the *smeRv* promoter (P_{smeRv}) and the *smeU1-V-W-U2-X* operon promoter (P_{smeU1}). Catechol- 2,3-oxygenase (C23O) activity assays indicated that P_{smeRv} activity is 10-fold higher in the KJΔRv mutant than in wild-type strain KJ (Table 5). Therefore, the expression of *smeRv* was negatively autoregulated in the wild-type strain KJ, consistent with the feature of LysR family members (32). However, KJ09C(p371Rv_{xylE}) and KJ09C(p371$U1_{xylE}$) displayed markedly higher C23O activity than KJ09CΔR(p371Rv_{xylE}) and KJ09CΔR(p371$U1_{xylE}$) (Table 5), respectively, indicating a role for SmeRv in positive regulation for both promoters in the KJ09C background.

These observations suggested two possibilities about the SmeRv regulatory role: (i) it regulates *smeU1-V-W-U2-X* expression in a positively and quantitatively regulated way. In a wild-type strain, the expression of *smeRv* is too weak to start the expression of the *smeU1-V-W-U2-X* operon. In the KJ09C background, some unidentified mutations contribute to overexpression of *smeRv* (Table 4) and yield overexpression of the *smeU1-V-W-U2-X* operon. (ii) Like many LysR-type regulators (20), SmeRv could act as a negative or positive regulator, depending on the presence of an activator ligand. In wild-type strain KJ, owing to the absence of an activator ligand, SmeRv may act as a negative regulator. However, the presence of an activator ligand can allow SmeRv to serve as a positive regulator in the KJ09C background.

There is no mutation identified in *smeRv* or intergenic region of *smeRv* and *smeU1* in KJ09C. Mutations of regulator genes leading to overexpression of the RND-type efflux pump operon have been reported in the *mexAB-oprM* (29), *mexCD-oprJ* (25), and *smeDEF* (31) systems. The *smeRv* gene and intergenic region between *smeRv* and *smeU1* were amplified by PCR from strains KJ and KJ09C. Sequence analysis of these PCR fragments showed the absence of nucleotide changes. A similar result has been reported for *mexEF-oprN* in *P. aeruginosa* (13), meaning that the mutation responsible for the KJ09C phenotype is not located in *smeR*v or its regulatory region.

There is no individual promoter for the *smeX* gene. It has been reported that the outer membrane protein OprM in the *mexAB-oprM* operon of *P. aeruginosa* can be expressed independently of the promoter of *mexAB* (37). To ascertain whether the *smeX* gene has its own promoter, C23O activities expressed by the transcriptional fusion construct p272X_{xylE} were evaluated in strains KJ and KJ09C. No significant C23O activities were detected in KJ(p272X_{xylE}) and KJ09C(p272X_{xylE}) (Table 5). Therefore, the 272-bp region upstream of the *smeX* gene lacks significant promoter activity in wild-type strain KJ and mutant KJ09C.

Overexpression of SmeVWX pump contributes to resistance to chloramphenicol, quinolones, and tetracyclines. To examine the role of each *smeU1-V-W-U2-X* operon component in the antibiogram of KJ09C, the *smeU1*, *smeVW*, *smeU2*, and *smeX* genes were deleted. The MDR phenotype and polar effects of these derived mutants were evaluated by susceptibility testing and qRT-PCR (Tables 3 and 4). Table 4 shows that disruption of *smeU1* and *smeVW* of KJ09C alone (strains KJ09CΔU1 and KJ09CΔVW, respectively) did not affect the expression of genes located downstream of the disrupted gene. However, disruption of the *smeU2* gene caused a slight polar effect on expression of the *smeX* gene (Table 4).

Inactivation of *smeVW* of KJ09C (KJ09CΔVW) restored the susceptibility of chloramphenicol, quinolones, and tetracyclines to a level close to that for wild-type strain KJ. On the basis of the characteristics of the typical RND-type pump, we propose that SmeV and SmeW may couple with SmeX to form

an efflux pump for the extrusion of chloramphenicol, quinolones, and tetracyclines. When a Δ*smeX* allele was introduced into KJ09C, KJ09CΔX partially restored the susceptibility of chloramphenicol, quinolones, and tetracyclines, suggesting that another outer membrane protein(s) can assemble with SmeV and SmeW to form a functional efflux pump for the extrusion of chloramphenicol, quinolones, and tetracyclines. The substrate specificity of the RND-type efflux pump is generally dependent on the RND transporter and MFP, while both components can accommodate different OMPs to form functional efflux pumps with the same substrate profiles (23, 24).

Overexpression of SmeX increases susceptibility to aminoglycosides. Inactivation of *smeVW* did not affect the aminoglycoside MICs of KJ09C (Table 3, KJ09C versus KJ09CΔVW). However, the aminoglycoside MICs of KJ09CΔX were restored to the levels of wild-type strain KJ (Table 3, KJ09C versus KJ09CΔX). These observations indicate decreased aminoglycoside resistance of KJ09C via SmeX overexpression rather than as a direct consequence of SmeVWX simultaneous overexpression. To assess the roles of *smeX* further, overexpression of the chromosomally integrated *smeX* gene in the KJ09CΔ5 background was studied. Table 3 shows that overexpression of *smeX* in the KJ09CΔ5 background reduced the MICs of aminoglycosides but hardly affected the MICs of chloramphenicol, quinolones, and tetracyclines (Table 3, KJ09CΔ5 versus KJ09CΔ5L2::X), further attesting that SmeX overexpression is enough to increase aminoglycoside susceptibility in the mutant KJ09C background.

Increased antibiotic susceptibility resulting from overexpression of an RND-type efflux pump has been reported (7, 21). In *P. aeruginosa*, overexpression of the MexEF-OprN system is usually concomitant with downregulated expression of MexAB-OprM and MexXY-OprM systems, which in turn accounts for the increased β-lactam and aminoglycoside susceptibility in the *nfxc*-type MDR strain (7). However, it seems not to be the case in *S. maltophilia*, in that other putative RDN-type efflux pumps of strain KJ09C keep a constant level of expression like those of strain KJ except the *smeU1-VW-U2-X* operon (Table 4). On the basis of the present results, SmeX overexpression is enough to increase aminoglycoside susceptibility in the mutant KJ09C background. Three possibilities are proposed herein to explain the role of SmeX in aminoglycoside susceptibility: (i) aminoglycosides make up a class of highly cationic amphipathic antibiotics that do not easily diffuse spontaneously across a lipid bilayer. SmeX may independently serve as a porin for entrance of aminoglycosides. (ii) Surplus SmeX proteins may interact with lipopolysaccharides or phospholipids and alter outer membrane potential or structure, which in turn enhances outer membrane permeability to aminoglycosides. (iii) Overexpression of *smeX* causes the suppression of some non-RND-type efflux systems that pump aminoglycosides out.

Impact of SmeU1 and SmeU2 on antibiotic resistance. Inclusion of two distinct putative SDR genes in the *smeVWX* operon of *S. maltophilia* differs significantly from the situation for other known RND pumps. Compared to KJ09C, KJ09CΔU1 displayed equivalent susceptibility toward the antimicrobials tested (Table 3). However, deletion of *smeU2* from KJ09C (KJ09CΔU2) had an intermediate effect on the MICs of chloramphenicol, quinolones, and tetracycline, which had been reduced to a level below those of KJ09C but above those seen in wild-type strain KJ (Table 3). The MICs of aminoglycosides for KJ09CΔU2 were 2-fold lower than those for KJ09C (Table 3). The roles of SmeU1 and SmeU2 in antimicrobial susceptibility were also evaluated by overexpression of the *smeU1* and *smeU2* genes in the KJ09CΔ5 background, respectively. Overexpression of *smeU1* and *smeU2* in KJ09CΔ5 hardly influenced the MICs of the antimicrobials tested (Table 3, KJ09CΔ5, KJ09CΔ5L2::U1, and KJ09CΔ5L2::U2).

SmeU1 has been shown to be less relevant to the antimicrobial resistance of KJ09C, on the basis of results obtained with a deletion mutant construct (KJ09CΔU1) and overexpression system (KJ09CΔ5L2::U1). However, according to the results for KJ09CΔU2 and KJ09CΔ5L2::U2 (Table 3), two possible roles for SmeU2 in the antimicrobial resistance of KJ09C are proposed herein. First, SmeU2, like SmeU1, is irrelevant to antibiotic resistance. The change in antimicrobial susceptibility of KJ09CΔU2 (Table 3) is from the polar effect that deletion of *smeU2* exerts on *smeX* expression (Table 4) rather than from the *smeU2* deletion. Second, SmeU2 appears to enhance the activity of SmeVWX pump and to attenuate the aminoglycoside susceptibility of KJ09C associated with SmeX overexpression (Table 3, KJ09CΔU2). The activity of SmeU2 seems to be SmeX dependent (Table 3, KJ09CΔ5L2::U2). On the basis of characteristics of the SDR family (11), we propose a possible role for SmeU2 in processing the OMP SmeX, which in turn affects SmeVWX pump activity and aminoglycoside uptake (22, 30).

ACKNOWLEDGMENT

This research was supported by grant NSC 98-2320-B-039-011-MY3 from the National Science Council.

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