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SmeOP-TolC$_{Sm}$ Efflux Pump Contributes to the Multidrug Resistance of *Stenotrophomonas maltophilia*

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A five-gene cluster, *tolC$_{Sm}$-pcm-smeRo-smeO-smeP*, of *Stenotrophomonas maltophilia* was characterized. The presence of *smeOP* and *smeRo-pcm-tolC$_{Sm}$* operons was verified by reverse transcription (RT)-PCR. Both operons were negatively regulated by the TetR-type transcriptional regulator SmeRo, as demonstrated by quantitative RT-PCR and a promoter-fusion assay. SmeO and SmeP were associated with TolC$_{Sm}$ (the TolC protein of *S. maltophilia*) for the assembly of a resistance-nodulation-cell-division (RND)-type pump. The compounds extruded by SmeOP-TolC$_{Sm}$ mainly included nalidixic acid, doxycycline, amikacin, gentamicin, erythromycin, leucomycin, carbonyl cyanide 3-chlorophenylhydrazone, crystal violet, sodium dodecyl sulfate, and tetrachlorosalicylanilide.

Multidrug efflux transporters capable of active extrusion of noxious compounds are classified into five families, including the resistance-nodulation-cell-division (RND) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the ATP binding cassette (ABC) family, and the multidrug and toxic compound extrusion (MATE) family (1). The RND systems generally form tripartite components composed of a periplasmic membrane fusion protein (MFP), an inner membrane RND transporter, and an outer membrane protein (OMP) (2).

Stenotrophomonas maltophilia is a nonfermentative Gram-negative bacillus. Eight RND-type efflux systems, SmeABC, SmeDEF, SmeGH, SmeIJK, SmeMN, SmeOP, SmeVWX, and SmeYZ, are postulated to be in the *S. maltophilia* genome (3). Among them, SmeABC, SmeDEF, SmeIJK, SmeVWX, and SmeYZ have been characterized (4–7). A possible promiscuous OMP, TolC$_{Sm}$, involved in multidrug resistance, has been proposed (8). Referencing the genome sequence of *S. maltophilia*, we noted that the *tolC* gene of *S. maltophilia* (*tolC$_{Sm}$*) and the *smeOP* system are located nearby (Fig. 1), implying that *smeOP* and *tolC$_{Sm}$* may be involved in a common mechanism for antibiotic extrusion. *smeO* and *smeP* were predicted to encode an MFP and an RND-type inner membrane transporter. A TetR-type transcription regulator (annotated as *SmeRo* here) was located immediately upstream of *smeOP* and divergently transcribed. The genes downstream of *smeRo* were the *pcm-tolC$_{Sm}$* operon, which has been known to contribute to multidrug resistance (8).

SmeRo acts as a regulator for the expression of *smeOP*. To evaluate the regulatory role of *smeRo*, a *smeRo* deletion mutant, KJΔRo, was constructed. A 359-bp DNA fragment (the 27-bp N terminus of *pcm*, the intergenic region of *pcm* and *smeRo*, and the 198-bp C terminus of *smeRo*) and a 434-bp DNA fragment (the 276-bp N terminus of *smeRo*, the intergenic region of *smeRo* and *smeO*, and the 33-bp N terminus of *smeO*) were amplified with the primers SmeRo3-F/SmeRo3-R and SmeRo5-F/SmeRo5-R (Fig. 1; see also Table S1 in the supplemental material), respectively, and sequentially cloned into pEX18Tc, yielding plasmid pΔRo for the construction of KJΔRo. The resultant in-frame deletion mutant, KJΔRo, included an internal deletion in the *smeRo* gene from nucleotide (nt) 273 to nt 494. The transcripts of *smeO*, *smeP*, *pcm*, and *tolC$_{Sm}$* in KJ and KJΔRo were determined by reverse transcription-quantitative PCR (qRT-PCR). The qRT-PCR was carried out at least in triplicate (6), and the primers we used are listed in Table S1. The transcript levels of *smeO*, *smeP*, *pcm*, and *tolC$_{Sm}$* in KJΔRo were greater than those in KJ by factors of 4.6 ± 2.1, 3.8 ± 1.9, 1.5 ± 0.6, and 1.4 ± 0.5, respectively (means and standard deviations). The effects of *smeRo* deletion on the expression levels of *pcm* and *tolC$_{Sm}$* were notably minor. Therefore, SmeRo plays a regulatory role, presumably as a repressor, in the expression of *smeOP*.

***smeRo*, *pcm*, and *tolC$_{Sm}$* form an operon.** Based on the genetic organization, we considered the possible presence of the *smeRo-pcm-tolC$_{Sm}$* operon. To test this possibility, an RT-PCR analysis was performed on the mRNA extracted from strains KJ and KJΔRo. Primer TolCQ-R (Fig. 1B; see also Table S1 in the supplemental material) was used to obtain cDNA. A 670-bp amplicon I (generated using primers 23-F/23-R) and a 1,123-bp amplicon II (generated with primers 123-F/23-R) were detected in KJΔRo, but only amplicon I was observed in KJ (Fig. 1B). KJΔRo is an in-frame deletion mutant with an internal deletion in the *smeRo* gene from nt 273 to nt 494. The primers 123-F and 23-R targeted nt 221 to 241 of the *smeRo* gene and nt 48 to 65 of the *tolC$_{Sm}$* gene (Fig. 1B). Therefore, a 1,123-bp amplicon II can be amplified only if *smeRo*, *pcm*, and *tolC$_{Sm}$* are cotranscribed. A *smeRo-pcm-tolC$_{Sm}$* transcript was observed in KJΔRo but not in KJ, even though the number of PCR cycles was increased to 40 (Fig. 1B). These observations support that a *smeRo-pcm-tolC$_{Sm}$* transcript was slightly

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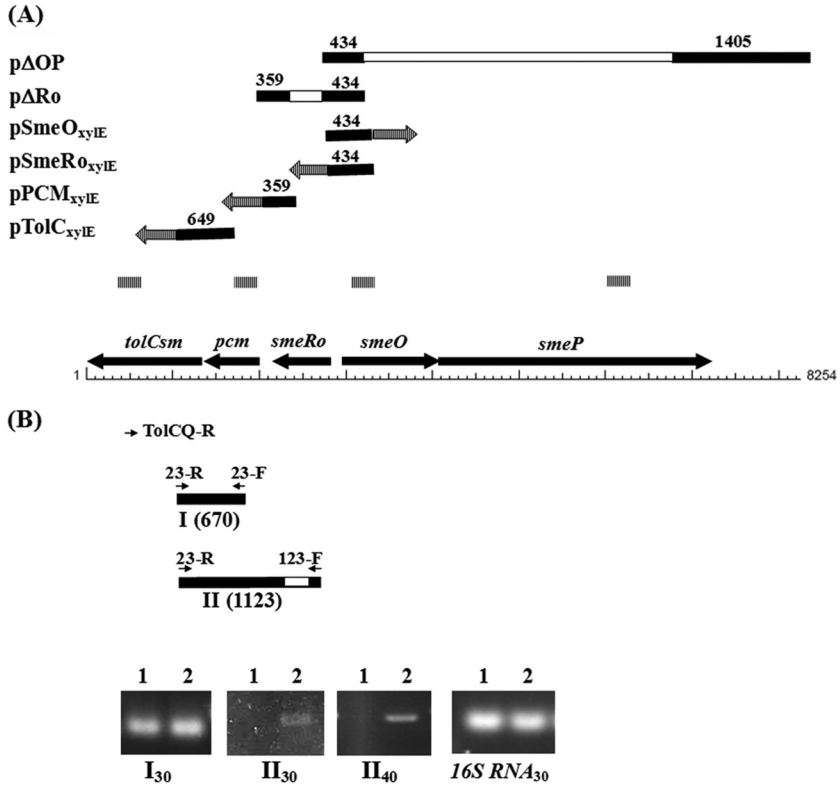

FIG 1 Schematic organization of the *smeOP* and *smeRo-pcm-tolC$_{Sm}$* operons from *S. maltophilia* strain KJ. The *smeOP* operon contains genes for a membrane fusion protein (SmeO) and an RND transporter (SmeP). A TetR-type transcriptional regulator gene, *smeRo*, is located immediately upstream of the *smeOP* operon and is divergently transcribed. The *smeRo*, *pcm*, and *tolC$_{Sm}$* genes form an operon. Solid arrows represent open reading frames (ORFs) and the direction of transcription. (A) Structures of recombinant plasmids. The solid lines represent the PCR amplicons, and each empty bar represents the deleted region for each plasmid construct. The numbers above the solid bars represent the PCR amplicon sizes (in bp). The arrows with vertical lines indicate the *xylE* gene. The vertical-line bars indicate the products of qRT-PCR. (B) Presence of the *smeRo-pcm-tolC$_{Sm}$* operon. The solid bars labeled I and II indicate the products of RT-PCR generated by primers 23-F/23-R and 123-F/23-R, respectively. The numbers in parentheses represent the PCR amplicon sizes (in bp). The arrows indicate the positions of primers. The empty bar represents the deleted region in strain KJΔRo. The agarose gels show the RT-PCR products of strains KJ and KJΔRo. Lanes 1, strain KJ; lanes 2, strain KJΔRo. The RT-PCR products, labeled I and II, were generated by primers 23-F/23-R and 123-F/23-R, respectively. The subscript numbers indicate the numbers of PCR cycles.

expressed in KJΔRo and that a *pcm-tolC$_{Sm}$* transcript was expressed in KJ and KJΔRo.

Assays of promoter activities of *smeRo-pcm-tolC$_{Sm}$* and *smeOP* operons. To further study the regulatory circuits, transcriptional *xylE* fusions to promoters of the *smeOP* operon (pSmeO$_{xylE}$), the *smeRo-pcm-tolC$_{Sm}$* operon (pSmeRo$_{xylE}$), the *pcm-tolC$_{Sm}$* operon (pPCM$_{xylE}$), and *tolC$_{Sm}$* (pTolC$_{xylE}$) were constructed (Fig. 1A), and each construct was introduced into strains KJ and KJΔRo. The expressed catechol 2,3-dioxygenase (C23O) activities were monitored as described previously (9). KJΔRo(pSmeO$_{xylE}$) exhibited a higher C23O activity than KJ(pSmeO$_{xylE}$) (Table 1), further confirming that SmeRo plays a repressor role in the expression of the *smeOP* operon. Compared to KJ(pSmeRo$_{xylE}$), KJΔRo(pSmeRo$_{xylE}$) had a slightly higher C23O activity (Table 1), which signifies the slightly negative autoregulation of *smeRo*. KJ(pPCM$_{xylE}$) and KJΔRo(pPCM$_{xylE}$) exhibited comparable C23O activities, indicating that the promoter of the *pcm-tolC$_{Sm}$* operon is constitutively active and not subjected to the regulation of SmeRo. No significant C23O activity was observed in KJ(pTolC$_{xylE}$) or KJΔRo(pTolC$_{xylE}$), supporting that there is no promoter activity in the 243-bp upstream region of the *tolC$_{Sm}$* gene.

Substrate spectrum of the SmeOP efflux pump. A 1,405-bp DNA fragment containing the partial C terminus of *smeP*, amplified with primers SmeP3-F and SmeP3-R, and the aforementioned 434-bp DNA fragment (generated with the primers SmeRo5-F/SmeRo5-R) were sequentially cloned into pEX18Tc, yielding plasmid pΔOP (Fig. 1A; see also Table S1 in the supplemental material). The SmeOP in-frame deletion mutant, KJΔOP, was obtained using the strategy described in reference 6. The

TABLE 1 Analysis of promoter transcription fusion constructs

Strain	C23O activity (Uc/OD$_{450}$)[a]
KJ(pSmeO$_{xylE}$)	18 ± 1.7
KJΔRo(pSmeO$_{xylE}$)	89 ± 9.2
KJ(pSmeRo$_{xylE}$)	20 ± 3.0
KJΔRo(pSmeRo$_{xylE}$)	54 ± 4.5
KJ(pPCM$_{xylE}$)	119 ± 17
KJΔRo(pPCM$_{xylE}$)	115 ± 14
KJ(pTolC$_{xylE}$)	5 ± 1
KJΔRo(pTolC$_{xylE}$)	8 ± 1

[a] One unit of catechol 2,3-dioxygenase (Uc) is defined as 1 nmol of catechol hydrolyzed per min. Results are expressed as the means ± standard deviations of three independent determinations. OD$_{450}$, optical density at 450 nm.

TABLE 2 Antimicrobial susceptibilities of *S. maltophilia* strain KJ and its derived deletion mutants

Compound class and/or agent[a]	MIC (μg/ml) for strain							
	KJ	KJΔOP	KJΔRo	KJΔRoΔOP	KJΔDEF	KJΔDEFΔOP	KJΔTolC	KJΔTolCΔOP
Chloramphenicol	8	8	16	8	4	4	4	4
Quinolones								
Nalidixic acid	8	4	16	4	4	2	2	2
Norfloxacin	16	16	16	16	16	16	16	16
Tetracyclines								
Doxycycline	1	0.5	2	1	0.5	0.25	0.5	0.5
Tetracycline	16	16	16	16	8	8	16	16
Aminoglycosides								
Amikacin	1,024	256	1,024	256	1,024	256	16	16
Gentamicin	1,024	256	1,024	256	1,024	256	8	8
Kanamycin	256	128	256	128	256	64	16	16
Macrolides								
Erythromycin	64	32	64	32	32	32	32	32
Leucomycin	256	32	256	64	128	16	16	16
Rokitamycin	512	512	512	512	256	256	128	128
Others								
Acriflavine	>1,024	1,024	>1,024	1,024	256	256	1,024	1,024
CCCP	16	8	128	8	16	8	8	8
CHH	32	32	32	32	32	32	32	32
Crystal violet	8	4	16	4	4	2	4	4
Fusaric acid	512	512	512	512	512	512	256	256
Menadione	64	64	64	64	64	64	32	32
Paraquat	1,024	1,024	1,024	1,024	1,024	1,024	512	512
Plumbagin	32	32	32	32	32	32	8	8
Proflavine	512	512	512	512	64	64	512	512
SDS	0.08	0.04	0.08	0.04	0.04	0.02	0.04	0.04
TCS	8	4	32	4	8	4	2	2

[a] CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CHH, 2-chlorophenylhydrazine hydrochloride; SDS, sodium dodecyl sulfate; TCS, tetrachlorosalicylanilide.

1,103-bp C terminus of the *smeO* gene and the 2,802-bp N terminus of the *smeP* gene in the mutant KJΔOP were deleted (Fig. 1A). The substrate spectrum of SmeOP was assessed by comparing the antimicrobial susceptibilities between KJ and KJΔOP and between KJΔRo and KJΔRoΔOP (Table 2). The susceptibility assay was performed by using the agar dilution method, as described previously (6), in at least three replicate experiments. The results indicated that SmeOP was responsible for the extrusion of nalidixic acid, doxycycline, aminoglycosides, macrolides, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), crystal violet, sodium dodecyl sulfate (SDS), and tetrachlorosalicylanilide (TCS). Among these compounds, the aminoglycosides, CCCP, and TCS were the most significant ones.

Complementation test for the *smeRo* mutant. To confirm that the phenotype observed for strain KJΔRo was due to inactivation of the *smeRo* gene, the plasmid pSmeRo (containing the full-length *smeRo* gene) was introduced into KJΔRo, yielding KJΔRo(pSmeRo). The transcript levels of *smeO*, *smeP*, *pcm*, and *tolC*$_{Sm}$ in KJΔRo(pSmeRo) were lower than those in KJΔRo(pRK415) by factors of 2.6 ± 1.1, 2.3 ± 1.0, 1.1 ± 0.4, and 1.1 ± 0.5, respectively. The susceptibilities of KJ(pRK415), KJΔRo(pRK415), and KJΔRo(pSmeRo) to aminoglycoside, CCCP, and TCS were tested by the agar dilution method and a disk diffusion assay. The complementation of KJΔRo with pSmeRo decreased resistance to aminoglycosides, CCCP, and TCS (see Table S2 in the supplemental material).

SmeOP requires TolC$_{Sm}$ for efflux pump function. Inactivation of the *smeOP* operon decreased the MICs to some antibiotics (Table 2), indicating that *smeOP* and its cognate OMP gene should be expressed constitutively in the wild-type KJ strain. Four possible OMP candidates, SmeC, SmeF, SmeX, and TolC$_{Sm}$, for the RND-type efflux pumps have been proposed (8). Of the four OMPs, the transcripts of *smeF* and *tolC*$_{Sm}$ were observed, and no significant transcripts of *smeC* and *smeX* were detected by RT-PCR (see Fig. S1 in the supplemental material). Therefore, SmeC and SmeX are less likely to be the cognate OMP of the SmeOP efflux pump. To assess the possibility of SmeOP-SmeF as the cognate OMP, the *smeDEF* operon was deleted from the chromosomes of strains KJ and KJΔOP, generating mutants KJΔDEF and KJΔDEFΔOP, respectively. Compared to KJΔDEF, KJΔDEFΔOP obviously had compromised resistance to aminoglycosides and leucomycin (Table 2), indicating that the SmeOP pump is still functional in the case of *smeF* inactivation. The possibility of SmeOP-TolC$_{Sm}$ as the cognate OMP was also evaluated. The susceptibility of KJΔTolC reported in our previous study (8) is also included in Table 2 for comparison. The introduction of Δ*smeOP* into KJΔTolC did not further compromise the resistance of KJΔTolC to any of the compounds tested (Table 2, KJΔTolC ver-

sus KJΔTolCΔOP), which lends support for $TolC_{Sm}$ being the cognate OMP for the SmeOP pump. Moreover, deletion of the *tolC*$_{Sm}$ gene was associated with greater decreases in MICs than those caused by deletion of *smeOP* (Table 2), signifying the promiscuous role of $TolC_{Sm}$. $TolC_{Sm}$ may participate not only in the function of the SmeOP pump but also in the function of other hitherto-uncharacterized efflux systems.

Distinct from the *tolC* orthologs reported for *Enterobacteriaceae* and *Pseudomonas aeruginosa*, *tolC*$_{Sm}$ of *S. maltophilia* is located in a *pcm-tolC*$_{Sm}$ operon. The protein L-isoaspartate *O*-methyltransferase (PCM), encoded by *pcm*, is an enzyme that recognizes and catalyzes the repair of damaged L-isoaspartyl and D-aspartyl groups in proteins. Thus, PCM may be involved in repairing damaged $TolC_{Sm}$ and keeping $TolC_{Sm}$ in a functional state. Recently, we verified that the *pcm* gene is less related to the $TolC_{Sm}$ function regarding antimicrobial extrusion (8). However, in addition to the antimicrobial efflux function, TolC-associated pumps are also known to play physiological roles for adaptation to stress, such as envelope stress or oxidative stress (10). Therefore, it cannot be immediately ruled out that PCM plays an important role in the physiological function of $TolC_{Sm}$.

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