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Overexpression of SmeDEF Efflux Pump Decreases Aminoglycoside Resistance in *Stenotrophomonas maltophilia*

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ABSTRACT The SmeDEF pump of *Stenotrophomonas maltophilia* is negatively regulated by SmeT. In this study, strains KJΔT (*smeT* deletion mutant) and KJT-D^m (mutant with a defective SmeT-binding site) showed increased resistance to chloramphenicol/nalidixic acid/macrolides and susceptibility to aminoglycoside. Overexpression of the SmeDEF pump, in either KJΔT or KJT-D^m, downregulated *smeYZ* expression, which is responsible for the reduced aminoglycoside resistance. Furthermore, the SmeRySy two-component regulatory system was downregulated in response to SmeDEF overexpression, which supports its involvement in the regulatory circuit.

KEYWORDS antibiotic resistance, bacteria, efflux pump, two-component regulatory system

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Among the mechanisms known to be involved in multidrug resistance (MDR), the efflux pump is a major determinant, conferring resistance to various antibiotics simultaneously (1). Given the extrusion ability of efflux systems, it is generally accepted that the overexpression of efflux pumps is responsible for antimicrobial resistance. However, it has also been reported that overexpression of efflux pumps increases resistance to some antibiotics and reduces resistance to other antibiotics (2, 3). Accordingly, the overall expression of MDR pumps has been closely monitored.

Stenotrophomonas maltophilia, an opportunistic human pathogen, shows a significant degree of intrinsic resistance to a variety of antibiotics (4). Sequencing of the *S. maltophilia* genome revealed the presence of eight resistance-nodulation-cell division (RND) efflux systems, namely, SmeABC, SmeDEF, SmeGH, SmeIJK, SmeMN, SmeOP, SmeVWX, and SmeYZ (5). *smeDEF* expression is negatively regulated by *smeT*, which is located upstream and divergently transcribed from the *smeDEF* operon (6). The known substrates of the SmeDEF pump include chloramphenicol, quinolone, tetracycline, trimethoprim-sulfamethoxazole, and macrolides (7). The SmeYZ efflux system contributes mainly to resistance against aminoglycosides (AGs) and trimethoprim-sulfamethoxazole (8), and its expression is positively regulated by the SmeRySy two-component regulatory system (TCS), which is located upstream of the *smeYZ* operon (9). The expression of SmeDEF or SmeYZ has been reported to be linked to the MDR phenotype of clinical isolates (7, 8). The expression of the individual RND efflux pump has been extensively studied (6, 9, 10, 11); however, coordinated expression among these RND efflux systems in *S. maltophilia* has not been reported so far. In this

TABLE 1 Antimicrobial susceptibilities of *S. maltophilia* strain KJ and its derived mutants

Strain	MIC (μg/ml) for[a]:					
	SmeDEF pump substrates			SmeYZ pump substrates		
	CHL	CIP	ERY	AMI	KAN	GEN
KJ	8	1	64	1,024	256	1,024
KJΔDEF	4	0.5	32	1,024	256	1,024
KJΔYZ	8	1	128	16	8	8
KJΔYZΔT	32	8	512	16	8	8
KJΔT	32	8	512	128	64	128
KJΔTΔDEF	4	1	32	1,024	256	512
KJT-D^m	32	8	128	256	64	256
KJT-D^{m}ΔDEF	4	0.5	16	1,024	256	1,024

[a]CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; AMI, amikacin; KAN, kanamycin; GEN, gentamicin.

study, we demonstrated that the expression of the SmeYZ pump is attenuated in response to SmeDEF overexpression in *S. maltophilia*.

Inactivation of *smeT* increased susceptibility to AG. A *smeT* in-frame deletion mutant of *S. maltophilia* strain KJ, KJΔT, was prepared for our study (9). The susceptibility of KJΔT to antibiotics was assessed by the agar dilution method and interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (12). The MIC was defined as the lowest concentration of the antimicrobial agent that inhibited visible growth.

SmeT plays a negative regulatory role in the expression of the *smeDEF* operon (6). As expected, the MICs of chloramphenicol, ciprofloxacin, and erythromycin increased for KJΔT (Table 1), which is consistent with the reported substrate profile of the SmeDEF pump (7). However, an interesting observation attracted our attention; the susceptibility of KJΔT to AGs increased compared to that in the wild-type KJ strain (Table 1). This phenomenon was not expected, since overexpression of the efflux pump is generally related to a decrease in susceptibility.

Inactivation of *smeT* attenuated the expression of *smeYZ*. The resistance to AGs reported in *S. maltophilia* is attributable to aminoglycoside-modifying enzymes (AMEs), RND-type efflux pumps, and outer membrane permeability (8, 13, 14–16). Given that SmeT acts as a transcriptional repressor, we wondered whether SmeT also regulates the expression of AMEs, RND-type efflux pumps, and lytic transglycosidase genes, in addition to the *smeDEF* operon. To address this question, the transcripts of five annotated AME genes (aminoglycoside phosphotransferase gene [Smlt0191], *aac*(*2′*)-*Ic* [Smlt1669], *aph*(*3′*)-*IIc* [Smlt2120], streptomycin 3′-phosphotransferase gene [Smlt2336], and *aac*(*6′*)-*Iz* [Smlt3615]), eight RND-type transporter genes (*smeB*, *smeE*, *smeH*, *smeJ*, *smeN*, *smeP*, *smeW*, and *smeZ*), and six lytic transglycosylase genes (*mltA*, *mltB1*, *mltB2*, *mltD1*, *mltD2*, and *slt*) (5) in wild-type KJ and KJΔT strains were comparatively assessed by quantitative reverse transcriptase PCR (qRT-PCR) as described previously (3). The primers used for qRT-PCR are listed in Table S1 in the supplemental material. The 16S rRNA gene was chosen as the normalizing gene. All transcripts tested, except for *smeE* and *smeZ*, exhibited no significant difference between the KJ and KJΔT strains (Fig. 1). *SmeE* transcripts were indeed increased in KJΔT, which supports the theory that SmeT plays a role in repressing *smeDEF* expression. However, *smeZ* transcript levels were lower in the KJΔT strain compared to those in the wild-type KJ strain (Fig. 1). The plasmid pSmeY$_{xylE}$, which carries the promoterless *xylE* gene downstream of the *smeY* promoter, was constructed in our previous study (9). The plasmid pSmeY$_{xylE}$ showed lower C23O activity in KJΔT than in the wild-type KJ strain (Fig. 2A), which further confirms that inactivation of *smeT* attenuates the expression of *smeYZ*.

Considering that the major substrates of the SmeYZ pump are AGs (8), we wondered whether the decrease in Δ*smeT*-mediated AG resistance results from the downregulated expression of *smeYZ*. We assumed that knocking out *smeYZ* in a Δ*smeT* background would then bring the level of AG susceptibility to that found in *smeYZ* knockout

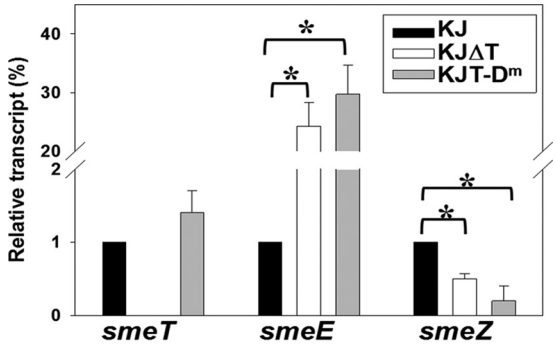

FIG 1 The amounts of *smeT*, *smeE*, and *smeZ* transcripts of wild-type KJ and its derived SmeDEF overexpression mutants, KJΔT and KJT-D^m. The mRNA expression levels of the indicated genes were analyzed by quantitative reverse transcriptase PCR (qRT-PCR). Error bars indicate the standard deviations of data from three independent experiments. *, $P \leq 0.05$ (significance calculated by Student's *t* test).

mutants, if the decrease in Δ*smeT*-mediated AG resistance resulted from *smeYZ* downregulated expression. A *smeYZ* and *smeT* double mutant, KJΔYZΔT, was constructed, and KJΔYZΔT had AG susceptibility comparable to that of KJΔYZ (Table 1), which suggests that the Δ*smeT*-mediated decrease in AG resistance involves SmeYZ.

Considering that the major substrates of the SmeYZ pump are AGs (8), we wondered whether the Δ*smeT*-mediated AG resistance compromise results from the downregulated expression of *smeYZ*. We assumed that the simultaneous inactivation of *smeT* and *smeYZ* would exacerbate the observed reduction in AG resistance if the decrease in Δ*smeT*-mediated AG resistance is independent of SmeYZ function. A *smeYZ* and *smeT* double mutant, KJΔYZΔT, was constructed; however, no further compromise in AG resistance was observed in KJΔYZΔT (Table 1), suggesting that the Δ*smeT*-mediated compromise in AG resistance involves SmeYZ.

SmeDEF overexpression downregulates *smeYZ*. It has been demonstrated that altering the expression of a single RND pump may have a downstream effect on any number of other RND efflux systems. Therefore, we further considered the possibility that *smeDEF* overexpression, rather than *smeT* inactivation, is the major cause for Δ*smeT*-mediated *smeYZ* downregulated expression. To test this, a *smeT* and *smeDEF* double mutant, KJΔTΔDEF, was constructed. The effect of SmeDEF overexpression on *smeYZ* expression in KJΔT was assessed by evaluating P_{smeY} activity in KJΔT and KJΔTΔDEF. As shown in Fig. 2A, the decrease in P_{smeY} activity in KJΔT reverted to the level of that in the wild-type strain once Δ*smeDEF* was introduced into the chromosome of KJΔT. However, inactivation of *smeDEF* in the wild-type KJ strain did not significantly affect the promoter activity of P_{smeY} (Fig. 2A). In addition, introduction of the Δ*smeDEF* allele into the chromosome of KJΔT reverted the level of AG susceptibility to that of wild-type KJ (Table 1), emphasizing the linkage between SmeDEF overexpression and a Δ*smeT*-mediated decrease in AG resistance.

Next, we sought to construct a *smeDEF* overexpression strain using an alternative mechanism in which the strain still harbored an intact SmeT protein. The intergenic (IG) region of *smeT-smeD* has been well characterized, and the inverted-repeat sequence (5′-ACAAACAAGCATGTATGT-3′) for SmeT binding was identified (6) (see Fig. S1 in the supplemental material). The double-crossover homologous recombination strategy was used to replace the SmeT-binding sequence (ACAAACAAGCATGTATGT) in the chromosomes of KJ cells with ACAAACAAGCATCTAGAT. An 863-bp DNA fragment containing a 349-bp *smeT* N terminus, complete *smeT-smeD* intergenic region, and 288-bp *smeD* N terminus was obtained by PCR using the primers TD-F and TD-R (see Table S1) and then cloned into pEX18Tc, which resulted in the plasmid pTD. The introduction of the three mutated nucleotides (Fig. S1) into the SmeT-binding sequence within plasmid pTD was carried out by site-directed mutagenesis PCR. The chromosomal SmeT-binding sequence of wild-type KJ was replaced with the mutated SmeT-binding sequence by the

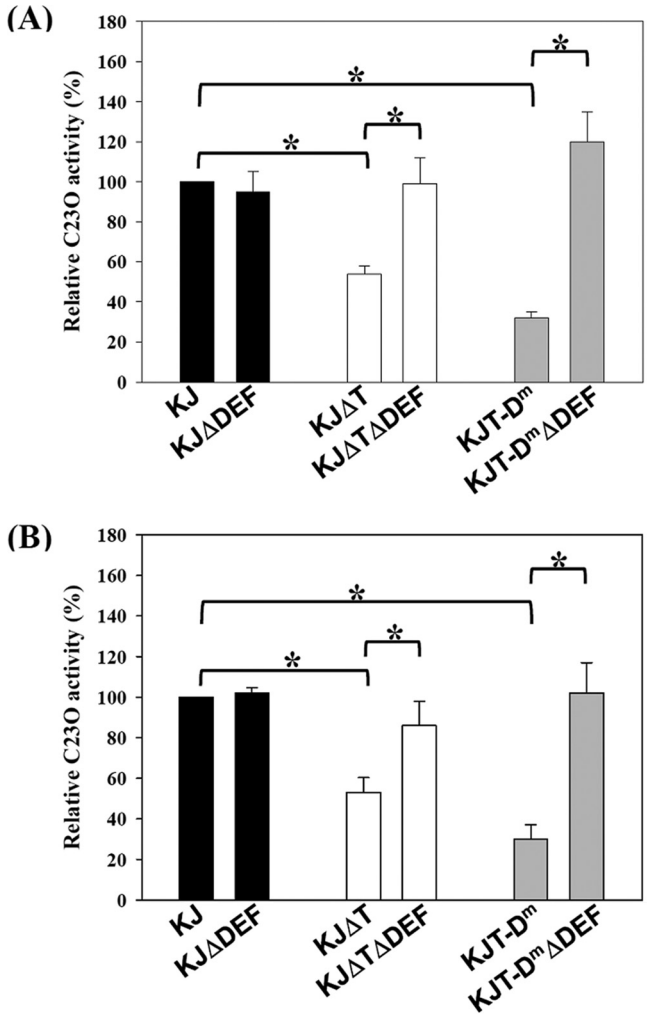

FIG 2 The impact of SmeDEF overexpression on the activity of promoters P_{smeY} and P_{smeRy}. The *smeY* and *smeRy* transcriptional fusion constructs, $pSmeY_{xylE}$ and $pSmeRy_{xylE}$, were transported into the assayed *S. maltophilia* strains by conjugation. The overnight-cultured plasmid-harboring strains were inoculated into fresh Luria broth with an initial optical density at 450 nm ($OD_{450\ nm}$) of 0.15 and cultured for 5 h, and the C23O activities were determined. Error bars indicate the standard deviations of data from three independent experiments. *, $P \leq 0.05$ (significance calculated by Student's *t* test). (A) C23O activities expressed by $pSmeY_{xylE}$. (B) C23O activities expressed by $pSmeRy_{xylE}$.

double-crossover homologous recombination strategy (8), yielding mutant KJT-D^m. Mutant KJT-D^m should have a defect in the interaction between SmeT and the operator region despite the presence of a wild-type SmeT, which, in turn, should derepress *smeDEF* expression. The *smeT* and *smeE* transcript levels and antibiotic susceptibility of strain KJT-D^m were determined by qRT-PCR and susceptibility testing, respectively. Compared to that in wild-type KJ, the level of *smeE* transcripts was higher in KJT-D^m (Fig. 1); the MICs of chloramphenicol, ciprofloxacin, and erythromycin were also elevated in KJT-D^m compared to those in the wild-type KJ strain (Table 1). This signifies the success of the KJT-D^m construct, which exhibits a phenotype of *smeDEF* overexpression and normal *smeT* expression. In addition, we noticed that the level of *smeZ* transcripts and the MICs of AGs were decreased in KJT-D^m compared to those in the control (Fig. 1 and Table 1), consistent with observations in strain KJΔT. Next, the Δ*smeDEF* allele was introduced into strain KJT-D^m, yielding KJT-D^mΔDEF. The promoter activity of *smeY* in KJT-D^m was lower than that in wild-type KJ but reverted to a wild-type level when the Δ*smeDEF* allele was introduced into the chromosome of KJT-D^m (Fig. 2A). A consistent conclusion was also obtained from the antibiotic susceptibilities of KJ, KJT-D^m, and

KJT-D^{m}ΔDEF (Table 1). Altogether, our results provide strong evidence that the decreased MICs of AG in KJΔT and KJT-D^m result from the overexpression of SmeDEF, independent of *smeT* deletion.

SmeDEF overexpression downregulates the SmeRySy TCS. Since the expression of the *smeYZ* operon is under the positive control of the SmeRySy TCS (9), which is divergently transcribed from the *smeYZ* operon, we examined whether SmeDEF overexpression-mediated *smeYZ* downregulated expression relies on the transcriptional activity of the *smeRySy* operon. To test this, the plasmid pSmeRy$_{xylE}$ was constructed for promoter transcriptional assays. A 574-bp DNA fragment upstream of the *smeRy* gene was amplified by PCR from the chromosome of *S. maltophilia* KJ using the primer sets SmeY5-F and SmeY5-R and cloned upstream of the promoterless *xylE* gene in plasmid pRKXylE, yielding plasmid pSmeRy$_{xylE}$. The promoter activity of the *smeRySy* operon in KJΔT was lower than that in wild-type KJ and reverted to a wild-type level when the Δ*smeDEF* allele was introduced into the chromosome of KJΔT (Fig. 2B). Similar results were observed in the strains KJ(pSmeRy$_{xylE}$), KJT-D^m(pSmeRy$_{xylE}$), and KJT-D^{m}ΔDEF (pSmeRy$_{xylE}$) (Fig. 2B). This was not due to a deletion of SmeDEF, since the promoter activities of the *smeRySy* operon were comparable in strains KJ and KJΔDEF (Fig. 2B). These data indicate that SmeDEF overexpression leads to a downregulation of the SmeRySy TCS, which ultimately results in decreased expression of the SmeYZ pump.

Concluding remarks. The phenomenon that overexpression of an RND-type efflux pump increases resistance to antimicrobials known to be substrates for the overexpressed pump, concomitant with a higher susceptibility to other antimicrobials, has been reported in *Pseudomonas aeruginosa* and *S. maltophilia*. MexCD-OprJ-overexpressing *P. aeruginosa* displays an increased resistance to fluoroquinolones, the fourth-generation cephalosporin cefepime, tetracycline, and chloramphenicol but shows reduced resistance to most other β-lactams (2). Although MexAB-OprM downregulated expression has been considered a critical determinant for the increase in β-lactam susceptibility (2), the exact link between MexCD-OprJ overexpression and MexAB-OprM downregulated expression remains unclear. In addition, the SmeVWX-overproducing *S. maltophilia* exhibits a phenotype characterized by elevated resistance to chloramphenicol, quinolone, and tetracycline and susceptibility to AG. The SmeVWX-overexpression-mediated increase in AG susceptibility results from SmeX overexpression rather than from inverse changes in the expression levels of the other efflux pump (3). In this article, we have described another example, where overexpression of the SmeDEF pump of *S. maltophilia* resulted in increased resistance to substrates of the SmeDEF pump and susceptibility to AG. We have further elucidated that SmeYZ downregulated expression is the key determinant for the SmeDEF overexpression-mediated increase in AG susceptibility and proposed the possibility of the involvement of the SmeRySy TCS in this regulatory circuit.

SmeSy and SmeRy of *S. maltophilia* exhibit protein identities of 30% and 38% with CpxA and CpxR of *Escherichia coli*, respectively. The CpxAR system of *E. coli* can sense and respond to envelope alterations, either the overexpression of the outer membrane protein NlpE or the disruption of phosphatidylglycerol homeostasis (17). Compared to the cytoplasmic environment, the bacterial envelope is a relatively crowded space, since about one-fourth to one-third of all bacterial genes encode membrane proteins (18). Therefore, overexpression of the SmeDEF pump may augment this challenging situation and stress the envelope environment. SmeSy may sense this stress and trigger a negative autoregulation, thereby attenuating the expression of the SmeYZ pump to alleviate the envelope stress. Another possibility is that overexpression of SmeDEF effluxes an inducing agent, which is normally sensed by the SmeRySy TCS, and decreases the expression of *smeRySy* and *smeYZ*. In addition, we also observed that SmeDEF was expressed at an appreciable basal level in wild-type KJ. Deletion of *smeDEF* from the chromosome of wild-type KJ caused a 2-fold decrease in MICs of chloramphenicol, ciprofloxacin, and erythromycin (Table 1) but did not affect *smeYZ* expression (Fig. 2A), suggesting that the basal expression level of *smeDEF* makes a mild contribution to antibiotic resistance without causing an envelope stress response.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02685-16.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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