Mutations in Ribosomal Protein RplA or Treatment with Ribosomal Acting Antibiotics Activates Production of Aminoglycoside Efflux Pump SmeYZ in *Stenotrophomonas maltophilia*

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ABSTRACT Aminoglycoside resistance in *Stenotrophomonas maltophilia* is multifactorial, but the most significant mechanism is overproduction of the SmeYZ efflux system. By studying laboratory-selected mutants and clinical isolates, we show here that damage to the 50S ribosomal protein L1 (RplA) activates SmeYZ production. We also show that gentamicin and minocycline, which target the ribosome, induce expression of *smeYZ*. These findings explain the role of SmeYZ in both intrinsic and mutationally acquired aminoglycoside resistance.

KEYWORDS efflux, gentamicin, ribosomal mutations

Aminoglycoside resistance in the important opportunistic pathogen *Stenotrophomonas maltophilia* is multifactorial. Reduced amikacin and tobramycin susceptibility is conferred by a chromosomal *aac(6′)-Iz* gene, which is present in approximately 50% of clinical isolates (1, 2). The chromosomal *aph(3′)-IIc* gene is more widespread among *S. maltophilia* clinical isolates and is responsible for reduced kanamycin and neomycin susceptibility (3). A wide variety of other genes have been shown to play minor roles in intrinsic aminoglycoside susceptibility (4–9), but by far, the most significant inducible resistance mechanisms is the SmeYZ efflux system, where SmeZ is an RND-type efflux pump (10). Following mutation, *smeYZ* can be overexpressed, which leads to hyperresistance to all aminoglycosides, and, most importantly, *smeYZ*-overexpressing mutants have been seen in the clinic (10). Disruption of *smeYZ* also affects *S. maltophilia* virulence (11), suggesting a more pleiotropic role in physiology, as is common for RND-type efflux systems (12).

It has been shown that *smeYZ* expression is controlled by a two-component regulatory system encoded immediately upstream, named SmeSyRy (13). One *S. maltophilia* aminoglycoside hyperresistant mutant shown to overexpress *smeYZ* is K M5 (10, 14). This mutant is a derivative of the clinical *S. maltophilia* isolate K279a (15, 16) and was selected for its ability to grow on amikacin at 4 times the MIC (14). In an attempt to identify the mutation responsible for *smeYZ* overexpression in K M5, we resurrected it from the freezer and first confirmed using CLSI disc susceptibility testing (17) that it expresses a “resistance profile 3” phenotype, as previously defined (10, 14): particularly, reduced zone diameters for aminoglycosides, quinolones, and tetracyclines (Table 1). Both SmeY and SmeZ were found to be approximately 8-fold upregulated in K M5 relative to expression in the parental strain, K279a, according to liquid chromatography tandem mass spectrometry (LC-MS/MS) envelope proteomics (Fig. 1), which was carried out as described previously (18). Whole-genome sequencing (WGS) was performed and analyzed as described before (19) and showed that *smeSy* and *smeRy* are wild type in K M5. Instead, there is only one difference from K279a, confirmed by PCR sequencing using the primers *rplA* F, 5′-GCGAAGGAACCGGATCTGA-3′, and *rplA* R, 5′-CGCCTGCGG

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TABLE 1 Disc susceptibility profile of *S. maltophilia* derivatives and clinical isolates

Strain	Zone diameter (mm) for the following antibiotics ($\mu\text{g}/\text{disc}$): ^a							
	CAZ (30)	LEV (5)	CIP (5)	MH (30)	CN (10)	AK (10)	C (30)	SXT (25)
K279a	32	27	23	32	22	24	25	27
K M5	29	18	14	25	6	9	23	22
9189	31	24	18	31	6	11	6	6

^aShading indicates reduced zone diameters (≥ 5 mm relative to the parental clinical isolate K279a) in K M5 and in the clinical isolate 9189. CAZ, ceftazidime; LEV, levofloxacin; CIP, ciprofloxacin; MH, minocycline; CN, gentamicin; AK, amikacin; C, chloramphenicol; SXT, sulfamethoxazole-trimethoprim.

TCTTTGAC-3'. The single point mutation in K M5 is predicted to cause a Gly67Asp change in the largest protein from the 50S ribosomal subunit, L1, encoded by the *rplA* gene. The previously described clinical isolate 9189, which also has the resistance profile 3 phenotype (Table 1) and overexpresses *smeYZ* (10), was found by PCR sequencing to also carry differences in *rplA* relative to that in K279a: predicted to cause a Phe22Leu change in RplA and a frameshift mutation at codon 212 caused by the insertion of a single adenine base. To confirm a role for the observed *rplA* mutation in reduced aminoglycoside susceptibility in K M5, wild-type *rplA* was amplified alongside the overlapping *rplK* by PCR using the primers *rplAK* F, 5'-AAAGCGGCCGCATCCAGCTGTAGAGTCGAGC-3', and *rplAK* R, 5'-AAAGCGGCCGCCTGCGGTCTTTGACGGCTAC-3', cut with NotI (introduced site underlined) and ligated into the broad host range vector pBBR1MCS (20), and the recombinant or empty vector was used to transform K M5 to chloramphenicol resistance (30 $\mu\text{g}/\text{ml}$). Using MIC and disc susceptibility testing, we confirmed that carriage of wild-type *rplA* by K M5 increased amikacin and gentamicin susceptibility relative to that of the plasmid-only control, though not to wild-type levels (Table 2, Fig. 2A). The other markers of resistance profile 3, reduced susceptibility to fluoroquinolones and tetracyclines, were not reversed by complementation of the *rplA* mutation in K M5, and we have previously shown that this part of the phenotype is due to the overexpression of a different pump operon, *smeIJK* (10). Surprisingly, in fact, fluoroquinolone and tetracycline susceptibility was reduced in K M5(pBBR1MCS::*rplA*) compared to that for the plasmid-only control (Fig. 2A). It is known that there is an inverse correlation between *SmeYZ* production and *SmeDEF* production in *S. maltophilia* (21), and indeed, proteomics confirmed that, as well as *SmeY* production (Fig. 2B) being reduced in K M5(pBBR1MCS::*rplA*) relative to that for the plasmid-only control (though not to wild-type levels, as seen for aminoglycoside MICs [Table 2]), production of *SmeD*, *SmeE*, and *SmeF* increased 17.3-fold, 11.2-fold, and 17.3-fold, respectively ($P < 0.001$, $n = 3$ for all). *SmeDEF* is a known tripartite efflux pump for fluoroquinolones and tetracyclines in *S. maltophilia* (22), explaining our findings (Fig. 2A).

In *Pseudomonas aeruginosa*, MexXY is considered the most important efflux pump involved in aminoglycoside resistance (23). It has been stated that at least two *P.*

FIG 1 Production of *SmeYZ* in K M5. Protein abundance data for whole-envelope proteomics were normalized to the average 30S and 50S ribosomal protein abundance for each. Abundance of *SmeY* and *SmeZ* (UniProt B2FQ54 and B2FQ55) are reported as means $\pm$ standard errors of the means (SEMs), $n = 3$. Differences between K279a and K M5 were statistically significant ($P < 0.05$).

TABLE 2 Aminoglycoside MICs against *S. maltophilia* derivatives and clinical isolates

Strain	MIC ($\mu\text{g/ml}$)	
	Amikacin	Gentamicin
K279a	16	8
K M5	>256	>256
9189	256	>256
K M5(pBBR1MCS)	>256	>256
K M5(pBBR1MCS::rplA)	128	32

aeruginosa clinical isolates that hyperexpress *mexXY* have truncations in *rplA*, though the data were not presented and were reported as “unpublished” (24). Mutations in other ribosomal subunit genes have more conclusively been shown to cause *mexXY* overexpression (25). Given the similarities between *MexXY* and *SmeYZ*, this provides precedence for our experimental observation that *rplA* disruption is the cause of *smeYZ* overexpression in *S. maltophilia*.

Expression of *mexXY* in *P. aeruginosa* is inducible in response to ribosomal acting antibiotics (25, 26), and since ribosomal protein damage by mutation is associated with *SmeYZ* overproduction in *S. maltophilia* (Fig. 1), we next tested the inducibility of *smeZ* expression by the ribosomal acting antibiotics gentamicin and minocycline. Expression of *smeZ* was assessed by reverse transcriptase quantitative PCR (RT-qPCR) using RNA prepared from a nutrient broth (NB) culture of K279a exposed to gentamicin (32 $\mu\text{g/ml}$) or minocycline (0.5 $\mu\text{g/ml}$) versus untreated control. The method used was as described previously

FIG 2 Effect of complementing K M5 with *rplA*. (A) Disc susceptibility testing for antimicrobials against K M5 carrying pBBR1MCS alone or pBBR1MCS::rplA. Data are means $\pm$ SEMs, $n = 3$. LEV, levofloxacin, 5 μg disc; CIP, ciprofloxacin, 5 μg disc; MH, minocycline, 30 μg disc; TE, tetracycline, 30 μg disc; CN, gentamicin, 10 μg disc; AK, amikacin, 10 μg disc; SXT, sulfamethoxazole-trimethoprim, 25 μg disc. (B) Abundances of SmeY normalized to average ribosomal protein abundances based on proteomics analysis is reported as means $\pm$ SEMs, $n = 3$. The differences between K279a and K M5 and between K M5(pBBR1MCS) and K M5(pBBR1MCS::rplA) are statistically significant ($P < 0.05$).

FIG 3 RT-qPCR analysis of the effect of ribosomal acting antibiotics on *smeZ* expression in K279a. K279a cultures were diluted 1:100 from an overnight culture and each grown in NB for 3 h. The experimental group was grown in the presence of gentamicin (CN) (32 $\mu\text{g/ml}$) or minocycline (MH) (0.5 $\mu\text{g/ml}$). RNA was purified and the abundance of *smeZ* in each RNA preparation was assayed using RT-qPCR calculated using the threshold cycle ($2^{\Delta\Delta\text{CT}}$) method (18) using the *rrnB* gene as an internal control for RNA abundance. Values for the *smeZ/rnB* ratio from treated cells were normalized to those of the untreated control (NB). *, $P < 0.05$ versus control. There were three biological and, for each, four technical replicates.

(18) with the primers *smeZ* RT F, 5-TGTCCAGCGTCAAGCACC-3, and *smeZ* RT R, 5-GCCGAC CAGCATCAGGAAG-3. Abundance of *smeZ* was normalized to *rrnB* abundance (as an RNA loading control) using the primers *rrnB* F, 5-GACCTTGCGCGATTGAATG-3, and *rrnB* R, 5-CG GATCGTCGCCTTGGT-3. This experiment confirmed that, as predicted, the normalized expression of *smeZ* in the gentamicin- or minocycline-treated K279a cultures was significantly more (approximately 8-fold and 3-fold, respectively, $P < 0.05$, $n=3$ biological replicates, each with 4 technical replicates) than in the control (Fig. 3).

Based on our findings, we therefore conclude that RplA damage in *S. maltophilia* constitutively mimics the effects of treatment with ribosomal acting antibiotics and constitutively activates *SmeYZ* production, raising aminoglycoside MICs. Our finding that *rplA* mutations exist in *SmeYZ*-overproducing clinical isolates confirms that such a mutation does not impair fitness or virulence so much that the mutants cannot survive, colonize, and cause infections in humans. Indeed, since reduced *SmeYZ* production reduces *S. maltophilia* virulence, at least *in vitro* and in a mouse model of infection (11), it may be that the advantage of *rplA* mutation in *S. maltophilia* is greater than simply raising aminoglycoside MICs. Given that *S. maltophilia* is frequently a colonizer of the lungs of cystic fibrosis patients, for which the aminoglycoside tobramycin is a regular therapy (27), the potential for selecting mutants with elevated *SmeYZ* production seems high.

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We declare no conflicts of interest.

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