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

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Running Title: Induction of SmeYZ production in *S. maltophilia*

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.

Text

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 about 50% of clinical isolates (1,2). The chromosomal *aph*(3')-IIc gene is more widespread amongst *S. maltophilia* clinical isolates, and 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 over-expressed, which leads to hyper-resistance to all aminoglycosides, and, most importantly, *smeYZ* over-expressing mutants have been seen in the clinic (10). Disruption of *smeYZ* also affects *S. maltophilia* virulence (11), suggesting a more pleotropic 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 hyper-resistant mutant shown to over-express *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 of amikacin at 4 times MIC (14). In an attempt to identify the mutation responsible for *smeYZ* over-expression in this 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 the parental strain, K279a, according to LC-MS/MS envelope proteomics (**Figure 1**), which was carried out as described previously (18). Whole genome sequencing (WGS) was performed and analysed as 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'- CGCCTGCGGTCTTTGAC-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. A 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 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. In order 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 were used to transform K M5 to chloramphenicol resistance (30 $\mu g.mL^{-1}$). Using MIC and disc susceptibility testing, we confirmed that carriage of wild-type *rplA* by K M5 increased amikacin and gentamicin susceptibility relative to plasmid only control, though not to wild-type levels (**Table 2**, **Figure 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 over-expression of a different pump operon, *smeIJK* (10). Surprisingly, in fact, fluoroquinolone and tetracycline susceptibility reduced in KM5 pBBR1MCS::*rplA* compared with the plasmid only control (**Figure 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 (**Figure 2B**) being reduced in K M5 (pBBR1MCS::*rplA*) relative to plasmid only control (though not to wild-type levels, as seen for aminoglycoside MICs in **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 (**Figure 2A**).

In *P. aeruginosa*, MexXY is considered the most important efflux-pump involved in aminoglycoside resistance (23). It has been stated that at least two *P. aeruginosa* clinical isolates that hyperexpress *mexXY* have truncations in *rplA,* though the data were not presented and 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* (**Figure 1**) we next tested the inducibility of *smeZ* expression by the ribosomal acting antibiotics gentamicin and minocycline. Expression of *smeZ* was assessed by RT-qPCR using RNA prepared from a nutrient broth (NB) culture of K279a exposed to gentamicin (32 $\mu g.mL^{-1}$) or minocycline (0.5 µg.mL) versus untreated control. The method used was as described previously (18) with the primers *smeZ* RT F 5'-TGTCCAGCGTCAAGCACC-3' and *smeZ* RT R 5'-GCCGACCAGCATCAGGAAG-3'. Abundance of *smeZ was* normalised to *rrnB* abundance (as an RNA loading control) using the primers *rrnB* F 5'-GACCTTGCGCGATTGAATG-3' and *rrnB* R 5'-CGGATCGTCGCCTTGGT-3'. This experiment confirmed that, as predicted, the normalised 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 control **Figure 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 over-producing clinical isolates confirms that such a mutation does not impair fitness or virulence so much that the mutants cannot survive, colonise and cause infections in humans. Indeed, since reduced SmeYZ production reduces *S. maltiphilia* virulence, at least *in vitro* and in a mouse model of infection (11) it may be that the advantage of *rplA* mutation in *S. maltophila* is greater than simply raising aminoglycoside MICs. Given that *S. maltophilia* is frequently a coloniser of the lungs of cystic fibrosis patients, for which the aminoglycoside tobramycin is a regular therapy (28), the potential for selecting mutants with elevated SmeYZ production seems high.

Acknowledgements

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We declare no conflicts of interest.

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

	Antibiotic (µg)							
	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

Shaded values represent reduced zone diameters (≥5 mm relative to the parental clinical isolate K279a) in K M5 and in the clinical isolate 9189. Abbreviations: CAZ, ceftazidime; LEV, levofloxacin; CIP, ciprofloxacin; MH, minocycline; CN, gentamicin; AK, amikacin; C, chloramphenicol; SXT, sulphamethoxazole/trimethoprim. Numbers in brackets is the amount (µg) of antimicrobial found in each disc.

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

Strain	MIC	
	Amikacin	**Gentamicin**
K279a	16	8
K M5	>256	>256
9189	256	>256
K M5 (pBBR1MCS)	>256	>256
K M5 (pBBR1MCS::*rplA*)	128	32

Figure Legends

Figure 1 Production of SmeYZ in K M5.

Protein abundance data for whole envelope proteomics were normalised to the average 30S and 50S ribosomal protein abundance for each. Abundance of SmeY and SmeZ (Uniprot: B2FQ54 and B2FQ55) are reported as mean +/- Standard Error of the Mean (SEM), n=3. Differences between K279a and K M5 were statistically significant (p<0.05).

Figure 2 Effect of complementing K M5 with *rplA*

(A) Disc susceptibility testing for antimicrobials against K M5 carrying pBBR1MCS alone (white bar) or pBBR1MCS::*rplA* (black bar). Data are means +/- SEM, n=3. Abbreviations: 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, sulphamethoxazole/trimethoprim, 25 µg disc. **(B)** Abundance of SmeY normalised to average ribosomal protein abundance based on proteomics analysis is reported as mean +/- SEM, 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).

Figure 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 µg.mL^{-1}) or minocycline (MH) (0.5 µg.mL^{-1}). RNA was purified and the abundance of *smeZ* in each RNA preparation was assayed using RT-qPCR calculated using the 2^ΔΔCt method (18) using the *rrnB* gene as an internal control for RNA abundance. Values for *smeZ*/*rrnB* ratio from treated cells were normalised to untreated control (NB). Stars indicate values that are statistically significantly different from control (p<0.05). There were three biological and, for each, four technical replicates.

Figure 1

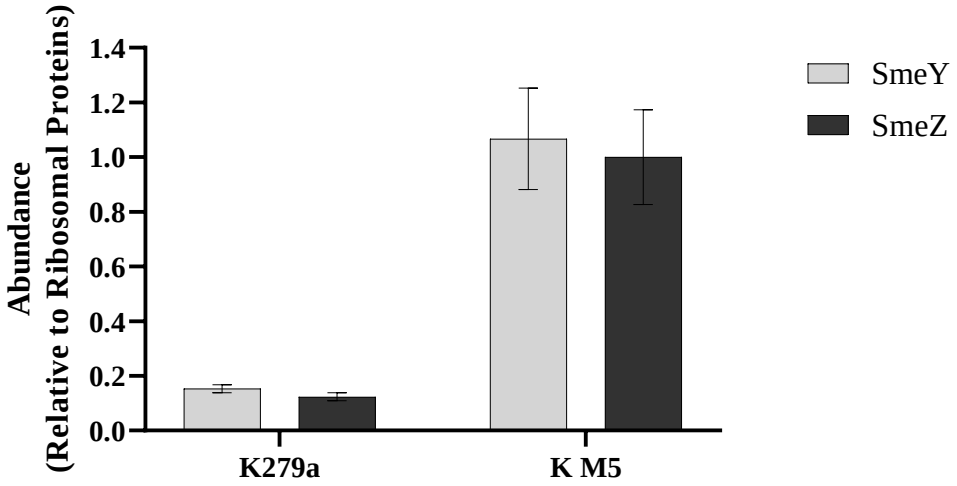
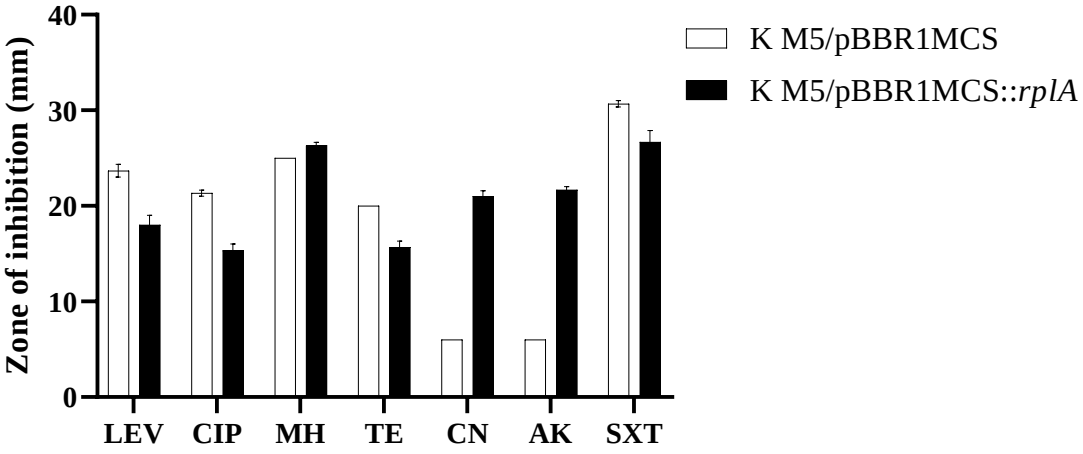

Abundance
(Relative to Ribosomal Proteins)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
K279a
K M5
SmeY
SmeZ

Figure 2

A


Zone of inhibition (mm)
40
30
20
10
0
LEV
CIP
MH
TE
CN
AK
SXT
K M5/pBBR1MCS
K M5/pBBR1MCS::rplA

B

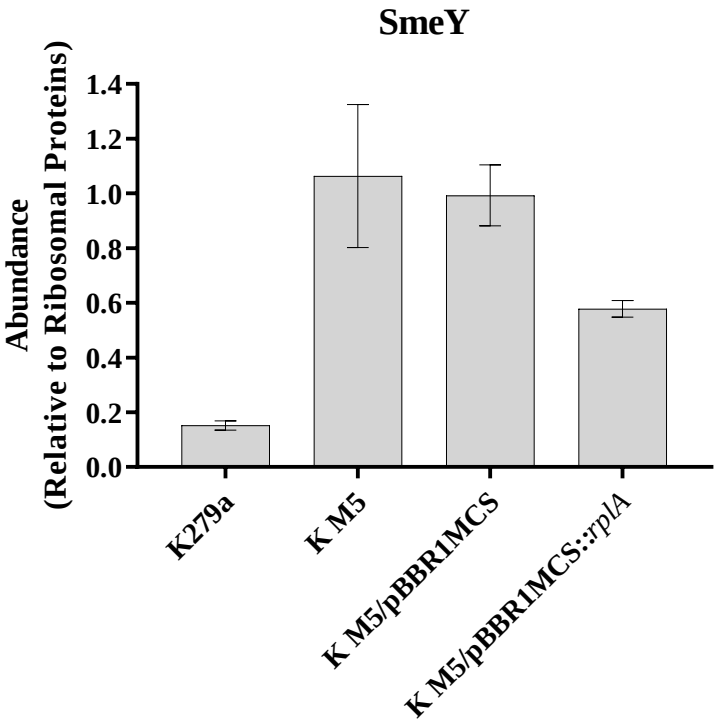
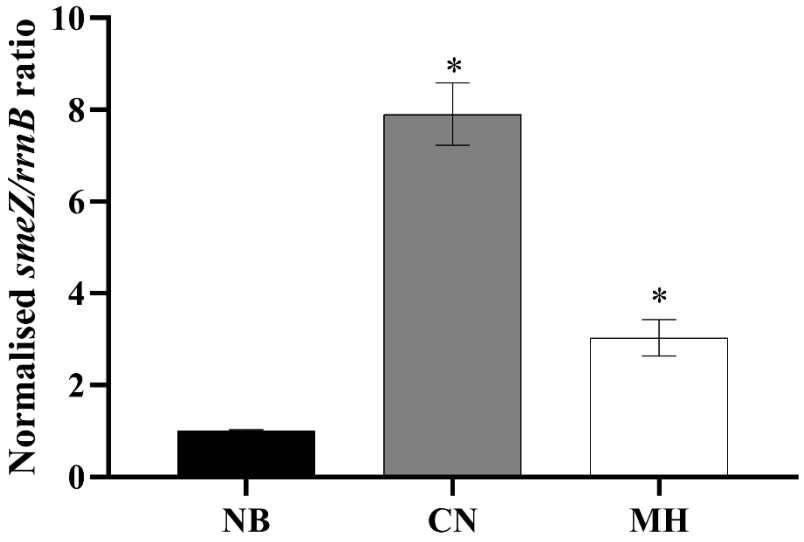

SmeY
Abundance
(Relative to Ribosomal Proteins)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
K279a
K M5
K M5/pBBR1MCS
K M5/pBBR1MCS::rplA

Figure 3



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