

# Inactivation of Lytic Transglycosylases Increases Susceptibility to Aminoglycosides and Macrolides by Altering the Outer Membrane Permeability of *Stenotrophomonas maltophilia*

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*Stenotrophomonas maltophilia* harbors six lytic transglycosylases (LTs): *mltA*, *mltB1*, *mltB2*, *mltD1*, *mltD2*, and *slt*. LT deletion increased susceptibility of *S. maltophilia* to aminoglycosides (AGs) and macrolides, and the underlying mechanisms were investigated. The expression of AG-modifying enzymes and efflux pumps was evaluated by quantitative reverse transcription-PCR (qRT-PCR). Susceptibility to 1-*N*-phenyl-naphthylamine, vancomycin, SDS, and bile salts was measured to assess outer membrane permeability. In conclusion, increased outer membrane permeability contributes to LT deletion-mediated increase in aminoglycoside and macrolide susceptibility.

Lytic transglycosylases (LTs) are an important class of bacterial enzymes recognized by their role in creating space within the peptidoglycan (PG) sacculus for its biosynthesis and recycling, cell division, and the insertion of macromolecular complexes spanning the cell wall, such as flagella, pili, and secretion systems (1). Many bacteria encode an array of different LTs that may be functionally redundant. With respect to their involvement in the biosynthesis and recycling of the PG sacculus, LTs are also linked to the expression of chromosomal  $\beta$ -lactamase genes in some *ampR*- $\beta$ -lactamase-bearing Gram-negative bacteria (2). Therefore, studies concerning LT-mediated antibiotic resistance primarily focus on  $\beta$ -lactams over other antibiotics.

*Stenotrophomonas maltophilia*, a nonfermentative, Gram negative, aerobic bacillus, is an opportunistic pathogen responsible for many nosocomial infections (3). It exhibits resistance to a broad array of antibiotics. According to the sequenced genomes, *S. maltophilia* harbors six putative LT genes: *mltA*, *mltB1*, *mltB2*, *mltD1*, *mltD2*, and *slt* (4). However, very little research has been published concerning the *S. maltophilia* LTs, except our recent report, in which we demonstrated that inactivation of *mltD1* confers a partial basal-level  $\beta$ -lactamase derepression phenotype (5). In this article, we assessed the relationship between LTs and susceptibility to antibiotics besides  $\beta$ -lactams in *S. maltophilia*.

*S. maltophilia* LT single deletion mutant ( $\Delta mltA$ ,  $\Delta mltB1$ ,  $\Delta mltB2$ ,  $\Delta mltD1$ ,  $\Delta mltD2$ , and  $\Delta slt$ ) strains and double mutant ( $\Delta mltD1 \Delta mltB1$  and  $\Delta mltD1 \Delta mltD2$ ) strains and a triple mutant ( $\Delta mltD1 \Delta mltB1 \Delta mltD2$ ) strain were prepared in our previous study (5). To rule out growth rate-mediated changes in susceptibility, first we assessed whether LT inactivation affected bacterial growth by monitoring the optical density at 450 nm (OD<sub>450</sub>) in 24 h with an interval of 3 h. No growth differences were observed between the wild-type KJ strain and its LT mutants (data not shown). The drug susceptibilities of wild-type KJ and its derived individual LT deletion mutants were assessed following Clinical and Laboratory Standards Institute (CLSI) guidelines (6). Inactivation of each LT did not significantly affect the susceptibility of *S. maltophilia* to chloramphenicol, nalidixic acid, or tetracycline, as the margin of error for the susceptibility test is in the 2-fold MIC value range (Table 1). It is worth mentioning that each LT mutant

was more susceptible to aminoglycosides (AGs); the  $\Delta mltB1$  and  $\Delta slt$  mutants were the most susceptible (Table 1). Furthermore, the  $\Delta mltB1$ ,  $\Delta mltD1$ ,  $\Delta mltD2$ , and  $\Delta slt$  mutants exhibited a prominent decrease in macrolide resistance. Since LT inactivation had never before been associated with an increased susceptibility to AGs and macrolides, we focused on elucidating the underlying mechanisms responsible for this phenotype.

Bacterial resistance to AGs can be mediated by target modification through mutation or methylation of 16S rRNA, drug modification by AG-modifying enzymes (AMEs), drug extrusion by efflux pumps, and changes in membrane permeability (7). The known mechanisms responsible for macrolide resistance include 23S rRNA modification, macrolide hydrolysis or modification, efflux pump extrusion, and changes in outer membrane permeability (8). In this article, knocking out LT genes by homologous recombination did not affect bacterial rRNA modification. Furthermore, no homologs of *ere*, *mph*, and *lin*, which encode proteins involved in macrolide hydrolysis and modification, were found in *S. maltophilia* by whole-genome searching (8). Therefore, we ruled out the possibility that rRNA and macrolide modification contributed to the LT deletion-mediated decrease in AG and macrolide resistance.

Five putative AMEs, aminoglycoside phosphotransferase (Smlt0191), AAC(2')-Ic (Smlt1669), APH(3')-IIc (Smlt2120), streptomycin 3'-phosphotransferase (Smlt2336), and AAC(6')-Iz (Smlt3615), and an array of efflux pumps were found in the se-

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

Strain	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>								
	CHL	NAL	TET	AMK	GEN	KAN	ERY	LM	VAN
KJ wild type	8	8	16	1,024	1,024	256	64	256	512
$\Delta mltA$ mutant	8	4	16	256	256	128	32	128	256
$\Delta mltB1$ mutant	8	4	16	64	128	64	8	16	128
$\Delta mltB2$ mutant	8	4	16	256	128	128	32	256	256
$\Delta mltD1$ mutant	8	4	16	128	512	128	32	32	64
$\Delta mltD2$ mutant	8	4	16	128	256	128	32	32	128
$\Delta slt$ mutant	8	8	16	64	128	64	32	64	128
$\Delta mltD1 \Delta mltB1$ mutant	8	4	16	256	512	64	32	64	128
$\Delta mltD1 \Delta mltD2$ mutant	8	8	16	128	512	128	32	64	128
$\Delta mltD1 \Delta mltB1 \Delta mltD2$ mutant	8	8	16	256	512	128	32	128	128

<sup>a</sup> CHL, chloramphenicol; NAL, nalidixic acid; TET, tetracycline; AMK, amikacin; GEN, gentamicin; KAN, kanamycin; ERY, erythromycin; LM, leucomycin; VAN, vancomycin.

quenced *S. maltophilia* K279a genome (4). Of them, expression of *aph(3')-IIc*, *aac(6')-Iz*, *smeDEF*, *smeIJK*, *smeOP*, *smeVWX*, *smeYZ*, and *macABCsm* has been proven to be related to AG and/or macrolide susceptibility in *S. maltophilia* (9–16). To assess whether the LT inactivation-mediated decrease in AG and macrolide resistance was linked to AMEs and efflux pumps, the transcripts of *aph(3')-IIc*, *aac(6')-Iz*, *smeE*, *smeJ*, *smeP*, *smeW*, *smeX*, *smeZ*, and *macBsm* from wild-type KJ and the LT mutants were quantified by quantitative reverse transcription-PCR (qRT-PCR). Total RNAs from wild-type KJ and the LT mutants were prepared from logarithmic-phase bacterial cultures and then reverse transcribed into cDNA as previously described (14). 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. The relative quantities of mRNA from each gene of interest were determined by the comparative cycle threshold method. The results revealed that the transcripts of AME and efflux pump-encoding genes in the LT mutants were comparable to that of the wild-type KJ, except *smeJ* (see Fig. S1 in the supplemental material). The *smeJ* transcript had a moderate increase in the  $\Delta mltD1$  and  $\Delta slt$  mutants compared to wild-type KJ (see Fig. S1); however, it cannot account for the AG resistance decrease of both mutants, since increased expression of the *SmeIJK* pump should enhance the AG resistance (12). The possible reasons for the *smeJ* increase in expression in  $\Delta mltD1$  and  $\Delta slt$  mutants will be discussed later. Therefore, at the moment, we cannot attribute the AG and macrolide resistance decrease of LT mutants to the decreased expression of the AME and efflux pump genes assayed.

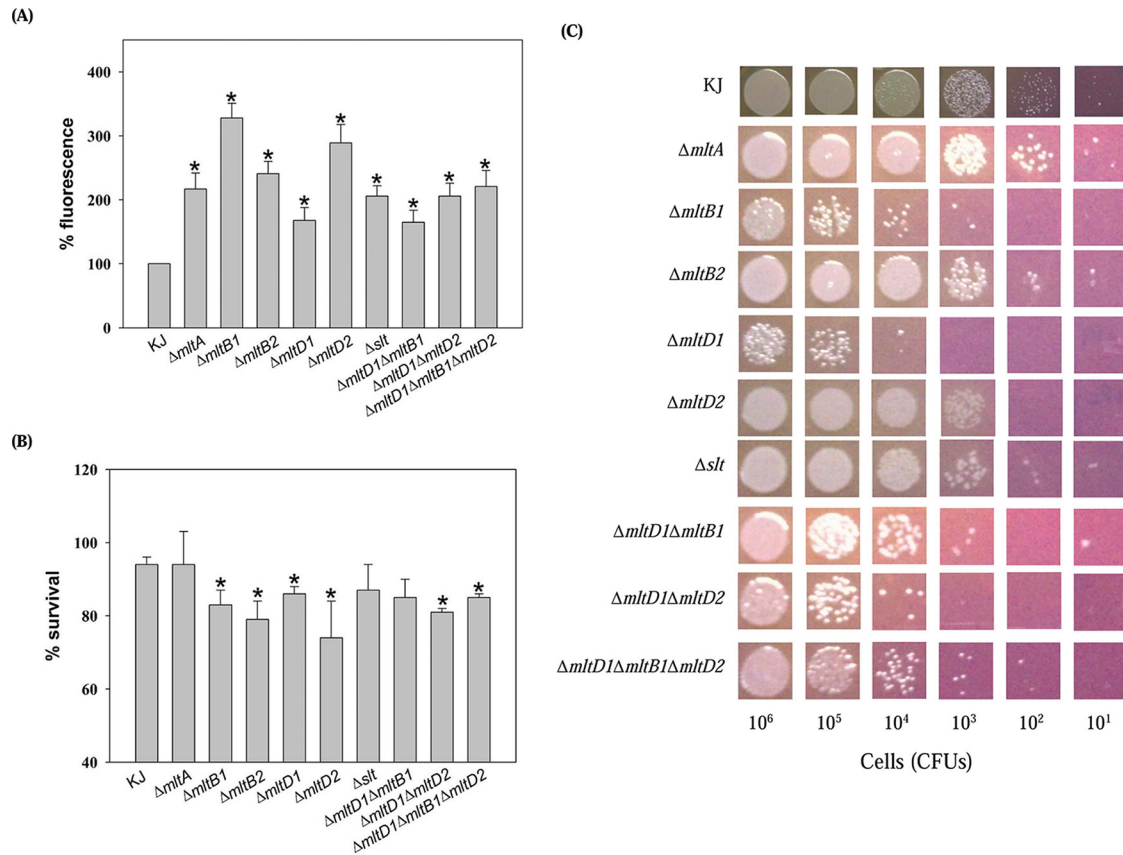
AGs and macrolides have been reported to enter the Gram-negative outer membrane by a self-promoted uptake mechanism (17), which relies on specific properties of the outer membrane. LTs are a family of enzymes that participate in peptidoglycan homeostasis and further affect the envelope integrity of bacteria. The loss of LTs has been reported to compromise membrane integrity (18). Therefore, we considered the possibility that LT deletion would have an impact on some aspect of the outer membrane. The hydrophobic fluorescent probe 1-*N*-phenyl naphthylamine (NPN) was used to study the permeabilizing effects of self-promoted uptake molecules on bacterial cells (19). Overnight cultures were subcultured into fresh LB broth and grown to mid-logarithmic phase. The cells were harvested by centrifugation, washed with 5 mM HEPES buffer (pH 7.2), and the suspension's optical density was adjusted to an OD<sub>450</sub> of 0.5 using the same buffer. Then, 100- $\mu\text{l}$  aliquots of cell suspension were pipetted into 96-well mi-

croter plates, and NPN was added to a final concentration of 15  $\mu\text{M}$ . Fluorescence was monitored after 5 min of incubation from three parallel wells per sample using a fluorescence spectrophotometer at excitation and emission wavelengths of 355 nm and 402 nm, respectively. The outer membranes of Gram-negative bacteria exclude the hydrophobic probe NPN. Fluorescence is emitted by NPN only after it partitions into the membrane; therefore, greater emission of fluorescence represents greater outer membrane permeability to NPN. LT inactivation caused higher uptake of NPN (Fig. 1A), suggesting there is a membrane defect and therefore AG may be gotten into cells better.

The outer membrane of Gram-negative bacilli is a general barrier for high-molecular-weight antibiotics such as vancomycin or macrolides. The vancomycin susceptibility can be used as an indicator for evaluating the outer membrane permeability of Gram-negative bacteria for high-molecular-weight substances. Next, we investigated whether LT inactivation altered the outer membrane permeability for high-molecular-weight substances. The susceptibility of LT mutants to vancomycin was evaluated. Compared to that of wild-type KJ, the MICs of the  $\Delta mltB1$ ,  $\Delta mltD1$ ,  $\Delta mltD2$ , and  $\Delta slt$  mutants to vancomycin decreased (Table 1). The elevated outer membrane permeability for high-molecular-weight substances in the  $\Delta mltB1$ ,  $\Delta mltD1$ ,  $\Delta mltD2$ , and  $\Delta slt$  mutants can provide an explanation for the increased macrolide susceptibility observed in the  $\Delta mltB1$ ,  $\Delta mltD1$ ,  $\Delta mltD2$ , and  $\Delta slt$  mutants.

In view of the outer membrane permeability alteration in LT mutants, we hypothesize that LT inactivation causes the membrane defect. To further support this hypothesis, the susceptibility of LT mutants to sodium dodecyl sulfate (SDS) and bile salts was assessed. The logarithmic-phase wild-type KJ and LT mutant cells were treated with 0.01% SDS or left untreated. CFU was determined after 10 min of incubation without shaking. The percentage of survival was defined as the CFU ratio of the SDS-additive group to the SDS-free counterpart. The SDS susceptibility increased in the  $\Delta mltB1$ ,  $\Delta mltB2$ ,  $\Delta mltD1$ , and  $\Delta mltD2$  mutants compared to that in wild-type KJ (Fig. 1B). For the bile salts susceptibility assay, the logarithmic-phase bacterial cells of  $5 \times 10^5$  CFU/ $\mu\text{l}$  were 10-fold serially diluted. Then, 2  $\mu\text{l}$  of bacterial cells was spotted onto the bile salts-containing MacConkey agars. The growth of bacterial cells was observed after 18 h of incubation at 37°C. The susceptibility of  $\Delta mltB1$ ,  $\Delta mltD1$ , and  $\Delta mltD2$  mutants to bile salts obviously increased (Fig. 1C). Taken together, these results further support that LT inactivations cause a membrane defect.

Since individual LT mutants display increased membrane sus-



**FIG 1** Outer membrane permeability and integrity of *S. maltophilia* KJ and its derived LT mutants. Values are averages from three replicates. *P* values were calculated using Student's *t* test comparing LT mutants to the wild-type KJ strain. \*, *P* < 0.01. (A) 1-*N*-Phenyl-naphthylamine (NPN) uptake assay. The bacterial cultures assayed were harvested, washed, and then adjusted to an OD<sub>450</sub> of 0.5 with 5 mM HEPES buffer (pH 7.2). Then 100  $\mu$ l of bacterial cells was pipetted into 96-well microtiter plates, and NPN was added to a final concentration of 15  $\mu$ M. Fluorescence was monitored by fluorescence spectrophotometer at excitation and emission wavelengths of 355 nm and 402 nm, respectively. (B) SDS survival assay. The survival of wild-type KJ and its derived LT mutants in LB broth without or with 0.01% SDS was determined by CFU counting. The percentage of survival was defined as the CFU ratio of the SDS-additive group to the SDS-free counterpart. (C) Bile acid susceptibility. The logarithmic-phase bacterial cells were collected, adjusted to a concentration of  $5 \times 10^5$  CFU/ $\mu$ l, and 10-fold serially diluted. Then 2  $\mu$ l of bacterial cells was spotted onto the bile salts-containing MacConkey agars. After 18 h of incubation at 37°C, the growth of bacterial cells was observed.

ceptibility to NPN, SDS, and bile salts (Fig. 1), it would be interesting to determine whether inactivation of more than one LT simultaneously would show an additive change in the membrane permeability. The  $\Delta mltD1 \Delta mltB1$ ,  $\Delta mltD1 \Delta mltD2$ , and  $\Delta mltD1 \Delta mltB1 \Delta mltD2$  mutants were thus assessed. Simultaneous inactivation of multiple LTs did not further increase the membrane susceptibility to aminoglycoside, macrolides, NPN, SDS, and bile salts (Table 1, Fig. 1).

Cavallari et al. have demonstrated that LT mutations in *Pseudomonas aeruginosa* do not affect membrane permeability and that these LT mutants exhibit no changes in susceptibility to tobramycin (2). In contrast to the observations in *P. aeruginosa*, LT inactivation in *S. maltophilia* increases the outer membrane permeability for self-promoted uptake compounds and high-molecular-weight substances, thus resulting in increased susceptibility to AGs and macrolides. The alteration in the outer membrane integrity of LT mutants can be an envelope stress to *S. maltophilia*. This may be the reason why *smeJ* transcripts of the  $\Delta mltD1$  and  $\Delta slt$  mutants are increased (see Fig. S1 in the supplemental material), since *smeJJK* is a member of  $\sigma^E$ -mediated envelope stress response regulon in *S. maltophilia* (12). To our knowledge, this is

the first report linking LT inactivation to a decrease in AG and macrolide resistance.

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