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Disruption of *mpl* Activates β-Lactamase Production in *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* Clinical Isolates

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ABSTRACT The hyperproduction of chromosomally encoded β-lactamases is a key method of acquired resistance to ceftazidime, aztreonam, and, when seen in backgrounds having reduced envelope permeability, carbapenems. Here, we show that the loss of Mpl, a UDP-muramic acid/peptide ligase, is a common and previously overlooked cause of chromosomally encoded β-lactamase hyperproduction in clinical isolates of *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*, important pathogens notorious for their β-lactam-resistant phenotypes.

KEYWORDS beta-lactamases, ceftazidime, regulation

Stenotrophomonas maltophilia clinical isolates are resistant to almost all β-lactams because of the production of two β-lactamases: L1, a subclass B3 metallo-β-lactamase, and L2, a class A extended-spectrum β-lactamase (1). The production of L1 and L2 is coordinately controlled by AmpR, a LysR-type transcriptional activator, and is induced during β-lactam challenge of cells (2). Where previously characterized, AmpR regulators have been shown to bind two ligands in a competitive manner (3, 4). As summarized in Fig. 1, the AmpR activator ligand, an anhydro-muramyl-pentapeptide, is produced during β-lactam challenge via the concerted actions of lytic transglycosylases, which release *N*-acetylglucosamine-anhydro-muramyl-peptides from peptidoglycan (5), and AmpG, a permease that transports them into the cytoplasm (6, 7). NagZ, an enzyme that removes the *N*-acetylglucosamine moiety, is also necessary to release the AmpR activator ligand in some species (8), though not in *S. maltophilia* (9). The AmpR repressor ligand is a UDP-muramyl-pentapeptide (10). It is produced via the sequential addition of amino acids to a UDP-muramyl substrate, via four separate ligase enzymes, MurC (11), MurD (12), MurE (13), and MurF (14), with the last adding a D-alanine–D-alanine dipeptide made by a fifth ligase enzyme, Ddl (15). Mpl is an enzyme that can ligate a ready-made pentapeptide onto the UDP-muramyl substrate, skipping the MurC, MurD, MurE, Ddl, and MurF ligation reactions, each of which requires ATP hydrolysis (16). This Mpl-catalyzed reaction therefore saves considerable amounts of energy for the cell. Its pentapeptide substrate comes from the breakdown of anhydro-muramyl-pentapeptides by the peptide amidase AmpD. In this way, the breakdown of the anhydro-muramyl-pentapeptide AmpR activator ligand by AmpD is also directly linked to the production of the UDP-muramyl-pentapeptide AmpR repressor ligand by Mpl (2, 5, 17, 18) (Fig. 1).

Ceftazidime is a relatively weak substrate for both L1 and L2 β-lactamases from *S. maltophilia*, and so many clinical isolates remain ceftazidime susceptible (1). However, mutants that have acquired ceftazidime resistance can easily be identified in the laboratory, and ceftazidime-resistant isolates are commonly encountered in the clinic. In most cases, these mutants hyperproduce L1 and L2 (19). Mutations that reduce AmpD function are known to boost L1/L2 production, because the AmpR activator ligand is broken down much less if AmpD is damaged (20). Mutations that (presumably)

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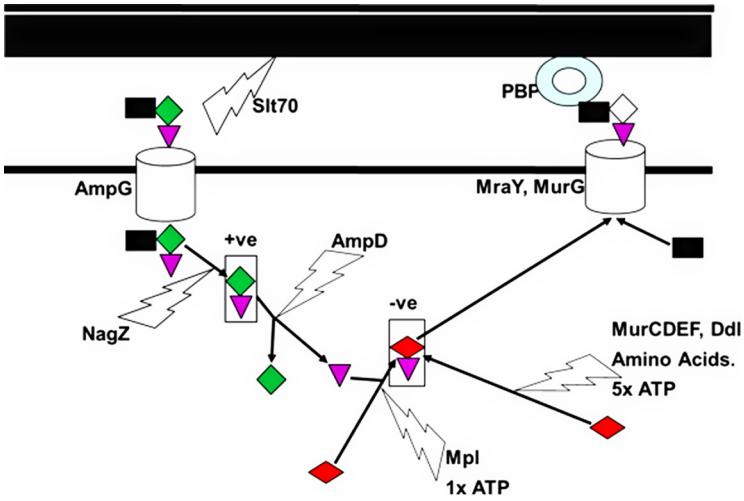

FIG 1 Role of Mpl in peptidoglycan recycling and AmpR activation. The schematic shows that *N*-acetylglucosamine (black square)-anhydro-muramyl (green diamond)-pentapeptide (purple triangle) is removed from peptidoglycan by lytic transglycosylases, such as Slt70, and enters the cytoplasm through the permease AmpG. NagZ removes the *N*-acetylglucosamine group to produce the anhydro-muramyl-pentapeptide AmpR activator ligand (+ve). AmpD then releases the pentapeptide ready to be linked to a UDP-muramic acid molecule (red diamond) by Mpl to produce the UDP-muramyl-pentapeptide AmpR repressor ligand (−ve). This can then be further incorporated into the biosynthetic pathway and processed by MurG and MraY, which add *N*-acetylglucosamine and penicillin-binding proteins, which add these high-energy *N*-acetylglucosamine-muramyl (white diamond)-pentapeptide substrates to the nascent peptidoglycan strand. UDP-muramyl-pentapeptide formation can also occur without peptidoglycan recycling through the sequential addition of amino acids to UDP-muramic acid. However, this requires five moles ATP per mole UDP-muramyl-pentapeptide, while the recycling pathway only requires one.

increase peptidoglycan turnover, releasing more muropeptides, also activate L1/L2 production, e.g., those in penicillin-binding protein 1A (PBP1A), encoded by *mcrA* (21), and in the lytic transglycosylase MltD, because this mutation stimulates the net production of lytic transglycosylase activity in the cell (22). Mutations in AmpR also activate L1/L2 production (4). We have previously characterized ceftazidime-resistant β-lactamase-hyperproducing laboratory-selected mutants derived from the extremely well-studied clinical isolate K279a. One of these mutants, KCAZ14, was wild type for *ampR*, *ampD*, and *mcrA* (19). To identify the mutation responsible, whole-genome resequencing was performed by MicrobesNG (Birmingham, UK) on a HiSeq 2500 instrument (Illumina, San Diego, CA, USA). Reads were trimmed using Trimmomatic (23) and assembled into contigs using SPAdes 3.10.1 (http://cab.spbu.ru/software/spades/). The assembled contigs were mapped to the reference genome for *S. maltophilia* K279a (24) obtained from GenBank (accession number NC_010943) using the progressive-Mauve alignment software (25). The only mutation identified in KCAZ14 was a deletion of 18 nucleotides in the *mpl* gene, deleting amino acids 141 to 146 of Mpl. The level of β-lactamase production, measured as described previously (19), was similar for the *mpl* mutant KCAZ14, for the *ampD* loss-of-function mutant KCAZ10 (19), and for KM11, an *ampR* activatory mutant (4) (Table 1). To confirm the involvement of *mpl* loss in the β-lactamase-hyperproducing ceftazidime-resistant phenotype of KCAZ14, we attempted complementation in *trans*. K279a *mpl* was amplified by PCR, as previously described (19), with primers mpl_F (5′-ACCAGATCCAGGTACCGCC-3′) and mpl_R (5′-TCTCACATCCCGTGTAGGACT-3′). The product was blunt-end ligated into pBBRMCS-5 (gentamicin resistance [Gm^r]) (26, 27) digested with SmaI, and the resulting recombinant plasmid was used to transform KCAZ14 to gentamicin resistance (15 $\mu g \cdot ml^{-1}$) via electroporation. The ceftazidime MIC against KCAZ14(pBBRMCS-5) was 64 $\mu g \cdot ml^{-1}$ and reduced to 4 $\mu g \cdot ml^{-1}$ in KCAZ14(pBBRMCS-5::*mpl*), the same as the MIC against wild-type K279a. The production of β-lactamase was also reduced to wild-type levels in

TABLE 1 β-Lactamase activity observed in *S. maltophilia* K279a and in ceftazidime-resistant K279a mutants and clinical isolates carrying different mutations

Isolate	β-Lactamase activity (mean ± SEM) (nmol · min^{-1} · μg^{-1} protein nitrocefin hydrolyzed in cell extracts)	Relevant amino acid changes (relative to K279a)
K279a	0.02 ± 0.004	WT[a]
KM11	0.99 ± 0.03	Asp135Asn in AmpR
KCAZ10	1.52 ± 0.04	159–168del in AmpD
KCAZ14	0.72 ± 0.01	140–146del in Mpl
49-6147	0.45 ± 0.12	92–109del Mpl
3800	0.73 ± 0.03	Truncation at 368 in Mpl
98	1.76 ± 0.07	IS insertion in *ampD*, Ala85Gly[b] in Mpl
ula-511	1.19 ± 0.01	Truncation at 360 in Mpl
KCAZ14(pBBRMCS-5)	1.14 ± 0.10	
KCAZ14(pBBRMCS-5::*mpl*)	0.03 ± 0.003	

[a]WT, wild type.
[b]Random genetic drift.

KCAZ14(pBBRMCS-5::*mpl*) (Table 1), adding further confirmation of successful complementation.

We have four ceftazidime-resistant β-lactamase-hyperproducing clinical *S. maltophilia* clinical isolates in our collection, isolates 49-6147, 3800, and 98 (19), and ULA-511 (28) (Table 1). Isolate 98 has an insertion sequence element disrupting *ampD* (19). While we also found a mutation causing an Ala85Gly change in Mpl, the same mutation is carried by ~5% of *S. maltophilia* genomes in the GenBank database and therefore is probably insignificant. The other three clinical isolates have *mpl* mutations. In 49-6147, the mutation causes the deletion of amino acids 92 to 109, which disrupts the conserved Ser-Gly-Pro region (29). In 3800, there is a frameshift at codon 368, and in ULA-511, there is a nonsense mutation at codon 360.

The result of Mpl loss in KCAZ14 and these clinical isolates will be a build-up of pentapeptides released by AmpD (Fig. 1). Even though there are other enzymes that can break down these pentapeptides, it seems reasonable to hypothesize that this net accumulation of pentapeptide will affect AmpD activity by feedback inhibition, increasing the concentration of its substrate, the AmpR activator ligand, causing β-lactamase hyperproduction (18).

This is the first report of *mpl* disruption causing β-lactamase hyperproduction in *S. maltophilia*, and to find it in 3/4 clinical isolates was striking. It is also interesting to find that *mpl* loss-of-function mutations have been seen to accumulate in *Pseudomonas aeruginosa* populations carried by people with cystic fibrosis during long-term colonization in two separate studies (30, 31) and in 3/4 patients with *P. aeruginosa*-mediated ventilator-associated pneumonia (32). Indeed, *mpl* mutation has been identified as a cause of AmpC β-lactamase hyperproduction in one *P. aeruginosa* PAO1 laboratory selected transposon-insertion mutant (33). While this did not dramatically increase β-lactam MICs (33), PAO1 is relatively permeable to β-lactams, because it lacks many of the efflux pump/porin-altering mutations seen in clinical isolates (34). Therefore, it would seem reasonable to propose that these clinically acquired *P. aeruginosa mpl* mutations are being selected by β-lactam therapy. We have a small collection of ceftazidime-resistant *P. aeruginosa* clinical isolates, 2/5 of which have previously been confirmed to hyperproduce AmpC (35). Both have a mutation in *mpl*, according to whole-genome sequencing. The mutations in isolates 86-14571 and 73-56826 cause Met297Val and an Arg103His changes in Mpl, respectively. We conclude, therefore, that *mpl* loss in *S. maltophilia* and *P. aeruginosa* is a clinically important and previously underreported cause of β-lactamase hyperproduction and acquired β-lactam resistance.

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We declare no conflicts of interest.

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