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

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RUNNING TITLE

Mpl loss and β-lactamase production

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 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.

TEXT

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) Production of L1 and L2 is co-ordinately controlled by AmpR, a LysR-type transcriptional activator and induced during β-lactam challenge of cells (2). Where previously characterised, AmpR regulators have been shown to bind two ligands in a competitive manner (3, 4). As summarised in Figure 1, the AmpR activator ligand, an anhydro-muramyl-penta-peptide 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-penta-peptide (10). It is produced
via 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 penta-peptide onto the UDP-muramyl substrate, skipping the MurC, D, E, Ddl and MurF ligation reactions, each of which requires ATP hydrolysis (16). This Mpl catalysed reaction therefore saves considerable amounts of energy for the cell. Its penta-peptide substrate comes from breakdown of anhydro-muramyl-penta-peptides by the peptide amidase AmpD. In this way, breakdown of the anhydro-muramyl-penta-peptide AmpR activator ligand by AmpD is also directly linked to production of the UDP-muramyl-penta-peptide 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) increase peptidoglycan turnover, releasing more muropeptides, also activate L1/L2 production, e.g. those in 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 characterised ceftazidime resistant, β-lactamase hyper-producing 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/). Assembled contigs were mapped to reference genome for S. maltophilia K279a (24) obtained from GenBank (accession number NC_010943) using progressiveMauve alignment software (25). The only mutation identified in KCAZ14 was a deletion of 18 nucleotides in mpl gene, deleting amino acids 141-146 of Mpl. The level of β-lactamase production, measured as previously (19) was similar for the mpl mutant KCAZ14, for an ampD loss of function mutant KCAZ10 (19) and for KM11, an ampR activatory mutant (4) (Table 1). To confirm involvement of mpl loss in the β-lactamase hyper-producing, ceftazidime resistant phenotype of KCAZ14, we attempted complementation in trans. K279a mpl was amplified by PCR as previously (19) with primers mpl_F (5’-ACCAGATCCAGGTACCGCC-3’), mpl_R (5’-TTCACATCCCCGTGAGACT-3’). The product was blunt-end ligated into pBBRMCS-5 (GmR) (26, 27) digested with SmaI and the resulting recombinant plasmid used to transform KCAZ14 to gentamicin resistance (15 µg.mL⁻¹) via electroporation. The ceftazidime MIC against KCAZ14(pBBRMCS-5) was 64 µg.mL⁻¹ and reduced to
4 µg.mL⁻¹ in KCAZ14(pBBRMCS-5::mpl), the same as the MIC against wild-type K279a. Production of β-lactamase was also reduced to wild-type levels in 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). Whilst 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 so is probably insignificant. The other three clinical isolates have mpl mutations. In 49-6147, the mutation causes the deletion of amino acids 92-109, 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 penta-peptides released by AmpD (Fig. 1). Even though there are other enzymes that can break down these penta-peptides, it seems reasonable to hypothesise that this net accumulation of penta-peptide will affect AmpD activity by feedback inhibition, increasing the concentration of its substrate, the AmpR activator ligand, causing β-lactamase hyper-production (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 colonisation in two separate studies (30, 31) and also 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). Whilst 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, of which 2/5 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 under-reported cause of β-lactamase hyperproduction and acquired β-lactam resistance.
ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.
Fig 1 Role of Mpl in peptidoglycan recycling and AmpR activation.

The schematic shows that N-acetylglucosamine (black square)-anhydro-muramyl (green diamond)-penta-peptide (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-penta-peptide AmpR activator ligand (“+ve”). AmpD then releases the penta-peptide ready to be linked to a UDP-muramic acid molecule (red diamond) by Mpl to produce the UDP-muramyl-penta-peptide 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)-penta-peptide substrates to the nascent peptidoglycan strand. UDP-muramyl-penta-peptide formation can also occur without peptidoglycan recycling, through the sequential addition of amino acids to UDP-Muramic acid. However, this requires five moles of ATP per mole of UDP-muramyl-penta-peptide, whilst the recycling pathway only requires one.
Table 1

β-Lactamase activity (nmol.min⁻¹.µg⁻¹ protein nitrocefin hydrolysed in cell extracts) observed in S. maltophilia K279a and in ceftazidime resistant K279a mutants and clinical isolates carrying different mutations.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mean β-lactamase activity ±SEM</th>
<th>Relevant amino acid changes (Relative to K279a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K279a</td>
<td>0.02±0.004</td>
<td>WT</td>
</tr>
<tr>
<td>KM11</td>
<td>0.99±0.03</td>
<td>Asp135Asn in AmpR</td>
</tr>
<tr>
<td>KCAZ10</td>
<td>1.52±0.04</td>
<td>159-168del in AmpD</td>
</tr>
<tr>
<td>KCAZ14</td>
<td>0.72±0.01</td>
<td>140-146del in Mpl</td>
</tr>
<tr>
<td>49-6147</td>
<td>0.45±0.12</td>
<td>92_109del Mpl</td>
</tr>
<tr>
<td>3800</td>
<td>0.73±0.03</td>
<td>Truncation at 368 in Mpl</td>
</tr>
<tr>
<td>98</td>
<td>1.76±0.07</td>
<td>IS insertion in ampD; Ala85Gly* in Mpl</td>
</tr>
<tr>
<td>ULA-511</td>
<td>1.19±0.01</td>
<td>Truncation at 360 in Mpl</td>
</tr>
<tr>
<td>KCAZ14 (pBBRMCS-5)</td>
<td>1.14±0.10</td>
<td></td>
</tr>
<tr>
<td>KCAZ14 (pBBRMCS-5::mpl)</td>
<td>0.03±0.003</td>
<td></td>
</tr>
</tbody>
</table>

*Random Genetic Drift

WT: Wild type
REFERENCES


