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1 **Disruption of *mpl* Activates β-Lactamase Production in *Stenotrophomonas maltophilia* and**

2 ***Pseudomonas aeruginosa* Clinical Isolates.**

3

4 **Karina Calvopiña[1], Matthew B. Avison[1]***

5 **[1]School of Cellular & Molecular Medicine, University of Bristol, Bristol, United Kingdom.**

6 ***Correspondence to: School of Cellular & Molecular Medicine, University of Bristol, Bristol,**

7 **United Kingdom. matthewb.avison@bris.ac.uk**

8

9 **RUNNING TITLE**

10 **Mpl loss and β-lactamase production**

11

12 **ABSTRACT**

13 **The hyperproduction of chromosomally encoded β-lactamases is a key method of acquired**

14 **resistance to ceftazidime, aztreonam, and when seen in backgrounds having reduced envelope**

15 **permeability, carbapenems. Here we show that loss of Mpl, a UDP-muramic acid/peptide ligase,**

16 **is a common and previously overlooked cause of chromosomally encoded β-lactamase**

17 **hyperproduction in clinical isolates of *Stenotrophomonas maltophilia* and *Pseudomonas***

18 ***aeruginosa*, important pathogens notorious for their β-lactam resistant phenotypes.**

19

20 **TEXT**

21 *Stenotrophomonas maltophilia* clinical isolates are resistant to almost all β-lactams because of the

22 production of two β-lactamases: L1, a subclass B3 metallo-β-lactamase and L2, a class A extended

23 spectrum β-lactamase (1) Production of L1 and L2 is co-ordinately controlled by AmpR, a LysR-type

24 transcriptional activator and induced during β-lactam challenge of cells (2). Where previously

25 characterised, AmpR regulators have been shown to bind two ligands in a competitive manner (3, 4).

26 As summarised in **Figure 1**, the AmpR activator ligand, an anhydro-muramyl-penta-peptide is produced

27 during β-lactam challenge via the concerted actions of lytic transglycosylases, which release

28 N-acetylglucosamine-anhydro-muramyl-peptides from peptidoglycan (5) and AmpG, a permease that

29 transports them into the cytoplasm (6, 7). NagZ, an enzyme that removes the N-acetylglucosamine

30 moiety is also necessary to release the AmpR activator ligand in some species (8), though not in

31 *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'-TCTCACATCCCGTGTAGGACT-3'). The product was blunt-end ligated into pBBRMCS-5 (Gm^R) (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

68   4 µg.mL^{-1} in KCAZ14(pBBRMCS-5::*mpl*), the same as the MIC against wild-type K279a. Production

69   of β-lactamase was also reduced to wild-type levels in KCAZ14(pBBRMCS-5::*mpl*) (**Table 1**) adding

70   further confirmation of successful complementation.

71   We have four ceftazidime resistant, β-lactamase hyperproducing clinical *S. maltophilia* clinical isolates

72   in our collection: isolates 49-6147, 3800 and 98 (19) and ULA-511 (28) **(Table1)**. Isolate 98 has an

73   Insertion Sequence element disrupting *ampD* (19). Whilst we also found a mutation causing an

74   Ala85Gly change in Mpl, the same mutation is carried by ~5% of *S. maltophilia* genomes in the

75   Genbank database so is probably insignificant. The other three clinical isolates have *mpl* mutations. In

76   49-6147, the mutation causes the deletion of amino acids 92-109, which disrupts the conserved

77   Ser-Gly-Pro region (29). In 3800, there is a frameshift at codon 368 and in ULA-511 there is a nonsense

78   mutation at codon 360.

79   The result of Mpl loss in KCAZ14 and these clinical isolates will be a build-up of penta-peptides

80   released by AmpD (**Fig. 1**). Even though there are other enzymes that can break down these penta-

81   peptides, it seems reasonable to hypothesise that this net accumulation of penta-peptide will affect

82   AmpD activity by feedback inhibition, increasing the concentration of its substrate, the AmpR activator

83   ligand, causing β-lactamase hyper-production (18).

84   This is the first report of *mpl* disruption causing β-lactamase hyperproduction in *S. maltophilia*, and to

85   find it in 3/4 clinical isolates was striking. It is also interesting to find that *mpl* loss of function mutations

86   have been seen to accumulate in *Pseudomonas aeruginosa* populations carried by people with Cystic

87   Fibrosis during long term colonisation in two separate studies (30, 31) and also in 3/4 patients with

88   *P. aeruginosa* mediated ventilator associated pneumonia (32). Indeed, *mpl* mutation has been identified

89   as a cause of AmpC β-lactamase hyperproduction in one *P. aeruginosa* PAO1 laboratory selected

90   transposon-insertion mutant (33). Whilst this did not dramatically increase β-lactam MICs (33), PAO1

91   is relatively permeable to β-lactams, because it lacks many of the efflux pump/porin altering mutations

92   seen in clinical isolates (34). Therefore, it would seem reasonable to propose that these clinically

93   acquired *P. aeruginosa mpl* mutations are being selected by β-lactam therapy. We have a small

94   collection of ceftazidime resistant *P. aeruginosa* clinical isolates, of which 2/5 have previously been

95   confirmed to hyperproduce AmpC (35). Both have a mutation in *mpl*, according to whole genome

96   sequencing. The mutations in isolates 86-14571 and 73-56826 cause Met297Val and an Arg103His

97   changes in Mpl, respectively. We conclude, therefore, that *mpl* loss in *S. maltophilia* and *P. aeruginosa*

98   is a clinically important and previously under-reported cause of β-lactamase hyperproduction and

99   acquired β-lactam resistance.

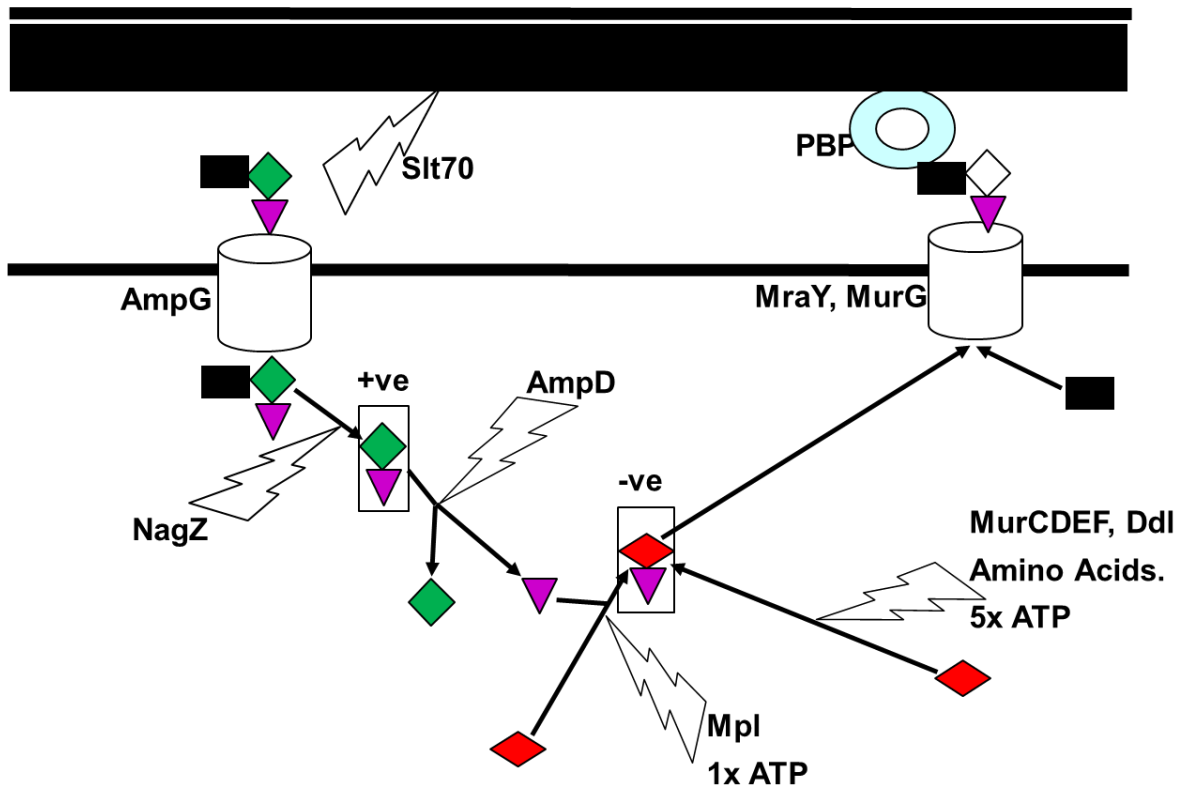100

101

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

109   **Fig 1**

110

111

112   **Fig 1 Role of Mpl in peptidoglycan recycling and AmpR activation.**

113   The schematic shows that N-acetylglucosamine (black square)-anhydro-muramyl (green diamond)-

114   penta-peptide (purple triangle) is removed from peptidoglycan by lytic transglycosylases such as Slt70

115   and enters the cytoplasm through the permease AmpG. NagZ removes the N-acetylglucosamine group

116   to produce the anhydro-muramyl-penta-peptide AmpR activator ligand ("+ve"). AmpD then releases

117   the penta-peptide ready to be linked to a UDP-muramic acid molecule (red diamond) by Mpl to produce

118   the UDP-muramyl-penta-peptide AmpR repressor ligand ("-ve"). This can then be further incorporated

119   into the biosynthetic pathway and processed by MurG and MraY, which add N-acetylglucosamine and

120   penicillin binding proteins, which add these high energy N-acetylglucosamine-muramyl (white

121   diamond)-penta-peptide substrates to the nascent peptidoglycan strand. UDP-muramyl-penta-peptide

122   formation can also occur without peptidoglycan recycling, through the sequential addition of amino

123   acids to UDP-Muramic acid. However, this requires five moles of ATP per mole of UDP-muramyl-

124   penta-peptide, whilst the recycling pathway only requires one.

125

126

127 **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.

Isolate	Mean β-lactamase activity ±SEM	Relevant amino acid changes (Relative to K279a)
K279a	0.02±0.004	WT
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* 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	

128

129 *Random Genetic Drift

130 WT: Wild type

131

132

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