Mutations in pmrB confer cross-resistance to the LptD inhibitor POL7080 and colistin in Pseudomonas aeruginosa

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Pseudomonas aeruginosa is a major bacterial pathogen for which there is rising antibiotic resistance. We evaluated the resistance mechanisms of \textit{P. aeruginosa} against POL7080, a species-specific, first-in-class antibiotic in phase 3 clinical trials targeting the lipopolysaccharide transport protein LptD. We found resistance mutations in the two-component regulator \textit{pmrB}. Genome-wide transcriptomics and confocal microscopy studies together suggest that POL7080 is vulnerable to the same resistance mechanisms described previously for polymyxins, including colistin, that involve lipid A modifications to mitigate antibiotic cell surface binding.

\textit{P. aeruginosa} and other Gram-negative bacteria are becoming increasingly resistant to current antibiotics, and pose a major threat to patients with hospital-acquired infections, compromised immune systems, and chronic pulmonary infections\textsuperscript{1-4}. Unfortunately, the discovery of new agents targeting Gram-negative bacteria is especially challenging due to the exclusion of most small molecules by their impermeable cell wall\textsuperscript{5} and array of efflux pumps. Among the last-resort antibiotics currently used to treat severe multi-drug resistant pseudomonal infections are the polymyxin class of cationic antimicrobial peptides (cAMPs), including polymyxin B and colistin (polymyxin E). More recently, the first-in-class antibiotic POL7080, currently in phase 3 clinical trials, was reported with species-specific activity against \textit{P. aeruginosa} by inhibiting the lipopolysaccharide (LPS) transport protein LptD\textsuperscript{6-8}. The discovery of POL7080 (and its analogue POL7001) emerged from extensive chemical modifications of the cAMP protegrin-1 (PG-1), in which a beta-hairpin was introduced to create cyclized peptidomimetic analogues\textsuperscript{9-10}. The mechanism of action (MOA) of POL7080 and
its analogues differ from that of other cAMPs in several key ways. Polymyxins and PG-1 interact with LPS and exhibit broad spectrum anti-microbial activity through self-promoted uptake across the outer membrane, followed by cell lysis through ill-defined mechanisms\textsuperscript{11-13}. The LptD inhibitors POL7080 and POL7001, however, have been reported to exhibit a non-lytic MOA through LptD inhibition in \textit{P. aeruginosa} exclusively\textsuperscript{6-8}.

To investigate the resistance mechanisms to POL7080 and its analogues, we selected for spontaneously resistant \textit{P. aeruginosa} PA14 mutants by plating mid-log culture on Lysogeny Broth (LB) agar containing 1\(\mu\)M POL7001 (~15X the liquid MIC). Detailed experimental protocols are outlined in the supplemental materials section. We isolated six independent mutants and confirmed their resistance to POL7080, POL7001, and PG-1 by broth microdilution as described in the Clinical and Laboratory Standards Institute Guidelines\textsuperscript{14}. All clones were highly resistant with MIC shifts ranging 5–40 fold relative to PA14 (Table 1), with absolute MICs comparable to four POL7080-resistant clinical isolates reported previously\textsuperscript{15}. Whole-genome sequencing revealed 1 to 4 single nucleotide polymorphisms (SNPs) in each mutant relative to wildtype PA14, and all six carried a mutation in a common gene \textit{pmrB} (Table 1). PmrB is a histidine kinase and the membrane-bound sensor in the PmrA-PmrB two-component regulatory system in Gram-negative bacteria. In response to low Mg\textsuperscript{2+} levels and periplasmic AMPs, PmrB undergoes conformational changes in its histidine kinase and methyl-accepting (HAMP) domain, leading to autophosphorylation, phosphoryl group transfer to its cognate response regulator PmrA, and downstream activation of transcriptional programs regulating LPS modification\textsuperscript{16}. Interestingly, mutations in the PmrA-PmrB have been implicated in polymyxin resistance through upregulation of the lipid A deacylase \textit{pagL} and the \textit{arnBCADTEF-ugd} operon, resulting in LPS modifications that reduce polymyxin binding to the cell surface\textsuperscript{17-27}. Notably, one of our isolated mutants, PA14-\textit{pmrB}\textsubscript{G188S}, contained an amino acid substitution in the HAMP domain at exactly the same site previously implicated in colistin resistance in \textit{P. aeruginosa} isolates from patients with cystic fibrosis (\textit{pmrB}\textsubscript{G188D})\textsuperscript{21}. We thus decided to measure the MICs of colistin against all six resistant mutants and found cross-resistance in all cases with a 4–8 fold shifts in MIC (Table 1).

To confirm that alterations in \textit{pmrB} account for the observed resistance to POL7080 and colistin, we introduced a copy of the wildtype allele \textit{pmrB}\textsubscript{WT}, and the resistant alleles \textit{pmrB}\textsubscript{L172del} and \textit{pmrB}\textsubscript{G188S}, under the control of an arabinose promoter into PA14 at the neutral attTn7 chromosomal site using the pUC18-derived mini-Tn7 integration system\textsuperscript{28}. We also introduced the previously reported allele \textit{pmrB}\textsubscript{G188D}, conferring colistin...
resistance\textsuperscript{21}, into the wildtype background. MIC assays in the presence of 0.25\% (v/v) arabinose demonstrated
that all three mutated \textit{pmrB} alleles, but not the wildtype allele, conferred POL7080 and colistin resistance
\textit{(Table 2)}. Conversely, the introduction of the \textit{pmrB}\textsubscript{WT} allele into the resistant mutant PA14-\textit{pmrB}\textsubscript{L172del} did not
restore POL7080 susceptibility with the addition of arabinose, suggesting that resistant \textit{pmrB} alleles were
largely dominant over the wildtype allele.

We next performed expression analysis (RNA-seq) to investigate the role of \textit{pmrA}-\textit{pmrB} in response to
LptD inhibitors. After extracting total RNA from mid-log PA14 treated with 128nM POL7001 (2X the MIC), we
prepared RNA-seq libraries using the RNA TagSeq protocol\textsuperscript{29}, sequenced samples on an Illumina NextSeq
instrument, and analyzed the data using Burrows-Wheeler Aligner\textsuperscript{30} for alignment and DESeq2\textsuperscript{31} to determine
differential gene expression. We found that LPS modification genes were significantly upregulated in response
to POL7001, including the \textit{pmrA}-\textit{pmrB}, the lipid A deacylase \textit{pagL}, and entire \textit{arnBCADTEF-ugd} operon
responsible for adding 4-amino-4-deoxy-l-arabinose (L-Ara4N) to lipid A (\textit{Fig. 1A}). The aminotransferase \textit{arnB},
catalyzing the final step in L-Ara4N addition, was among the most highly upregulated genes in the entire
dataset. We confirmed these findings with quantitative reverse transcription PCR (qRT-PCR) after treating
mid-log PA14, or resistant PA14-\textit{pmrB}\textsubscript{L172del}, with either POL7080 or vehicle control (\textit{Fig. 1B}). Relative to
control, POL7080 significantly induced \textit{pmrA}, \textit{arnB} and \textit{pagL} expression in PA14. Notably, \textit{arnB} and \textit{pmrA}
transcript levels in untreated PA14-\textit{pmrB}\textsubscript{L172del} exceeded those in POL7080-treated PA14. Together these data
reveal that a signature transcriptional response, the upregulation of key LPS modification genes\textsuperscript{17-27,32}, is
constitutively present in the resistant PA14-\textit{pmrB}\textsubscript{L172del} based on upregulation of key LPS modification genes.
These results highlight a common cellular response to LptD inhibitors and polymyxins, and support a shared
mechanism by which \textit{pmrB} mutations confer cross-resistance to POL7080 and colistin.

Finally, we investigated whether \textit{pmrB} resistance mutations mitigate POL7080 binding to the cell
surface. We synthesized TAMRA-L27-11 (\textit{Fig. S1}), a red-fluorescent analogue of POL7080 with retained
inhibitory activity (\textit{Fig. S2}), to probe for differential uptake in PA14-\textit{pmrB}\textsubscript{L172del} relative to PA14 using confocal
microscopy. After treating mid-log PA14 or PA14-\textit{pmrB}\textsubscript{L172del} with 500nM TAMRA-L27-11, cells were washed,
fixed with 4\% paraformaldehyde, and stained with 4′,6-diamidino-2-phenylindole (DAPI) for nucleic acid
visualization. Red-field and blue-field confocal microscopy showed comparable DAPI staining but over 3-fold
reduction in TAMRA-L27-11 uptake in PA14-\textit{pmrB}_{L172del} relative to PA14 (Fig. 2), indicating less efficient drug binding at the cell surface of the resistant mutant.

In summary, we report a series of \textit{pmrB} mutations that confer high-level resistance to POL7080 and moderate cross-resistance to colistin. Expression analysis and confocal microscopy data support a resistance mechanism in which \textit{pmrB} mutations result in LPS modifications by transcriptionally regulating the \textit{arnBCADTEF-ugd} operon, known to result in L-Ara4N addition to LPS, to reduce drug binding to the cell surface. These data align well with known resistance mechanisms to polymyxins, in which LPS modification with L-Ara4N reduces drug binding to the cell surface\textsuperscript{20-22}. Our findings suggest that pre-existing colistin resistance may limit the utility of POL7080 in a subset of highly resistant cases of \textit{P. aeruginosa}, and that if successfully developed, POL7080 exposure could inadvertently drive cross-resistance to colistin and other polymyxins.

**Acknowledgements**

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### Table 1: Summary of resistant mutants sequenced after selection with POL7001.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PA14_ID</th>
<th>Gene</th>
<th>SNPs</th>
<th>Protein Change</th>
<th>Function</th>
<th>POL7001</th>
<th>POL7080</th>
<th>PG-1</th>
<th>Colistin</th>
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<td>Wildtype PA14</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>0.064</td>
<td>0.064</td>
<td>1.25</td>
<td>0.625</td>
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<td>PA14-\textit{pmrB}_{L172del}</td>
<td>63160</td>
<td>\textit{pmrB}</td>
<td>CGCT506C</td>
<td>L172del\textsuperscript{†}</td>
<td>Two component system</td>
<td>1.25</td>
<td>1.25</td>
<td>&gt;20</td>
<td>5</td>
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<td>PA14-\textit{pmrB}_{G188S}</td>
<td>63160</td>
<td>\textit{pmrB}</td>
<td>G562A</td>
<td>G188S</td>
<td>Two component system</td>
<td>0.62</td>
<td>0.31</td>
<td>&gt;20</td>
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<td></td>
<td>36020</td>
<td>\textit{pqIB}</td>
<td>C1341A</td>
<td>H447Q</td>
<td>Superoxide function</td>
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<td>0.31</td>
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<td></td>
<td>08680</td>
<td>\textit{tufB}</td>
<td>C910A</td>
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<td>Elongation factor</td>
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<td></td>
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<td>hypo\textsuperscript{‡}</td>
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<td>\textit{pmrB}</td>
<td>G406C</td>
<td>V136L</td>
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<td>1.25</td>
<td>&gt;20</td>
<td>2.5</td>
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<tr>
<td></td>
<td>23460</td>
<td>\textit{orfN}</td>
<td>TG138T</td>
<td>G47fsL53X\textsuperscript{±}</td>
<td>O-polysaccharide addition</td>
<td>1.25</td>
<td>1.25</td>
<td>&gt;20</td>
<td>2.5</td>
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<tr>
<td></td>
<td>43080</td>
<td>\textit{vgrG14}</td>
<td>C1325A+C1330G</td>
<td>A442E + H444D</td>
<td>Type VI secretion</td>
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<td>1.25</td>
<td>&gt;20</td>
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<td>\textit{pmrB}</td>
<td>A394C</td>
<td>T132P</td>
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<td>1.25</td>
<td>1.25</td>
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<td>\textit{pmrB}</td>
<td>G464A</td>
<td>R155H</td>
<td>Two component system</td>
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<tr>
<td></td>
<td>23460</td>
<td>\textit{orfN}</td>
<td>TG139T</td>
<td>G47fsL53X\textsuperscript{±}</td>
<td>O-polysaccharide addition</td>
<td>1.25</td>
<td>1.25</td>
<td>&gt;20</td>
<td>2.5</td>
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\textsuperscript{†}: in-frame deletion at L172
\textsuperscript{‡}: hypothetical protein
\textsuperscript{±}: frame shift with premature stop at L53

### Table 2: MICs in 0.25% arabinose after introduction of second \textit{pmrB} alleles into PA14 and \textit{pmrB}_{L172del} backgrounds.

<table>
<thead>
<tr>
<th>Background strain</th>
<th>attTn7 Allele</th>
<th>POL7080 MIC (µM)</th>
<th>Colistin MIC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA14</td>
<td>---</td>
<td>0.064</td>
<td>0.50</td>
</tr>
<tr>
<td>PA14</td>
<td>\textit{pmrB}_{WT}</td>
<td>0.10</td>
<td>0.50</td>
</tr>
<tr>
<td>PA14</td>
<td>\textit{pmrB}_{L172del}</td>
<td>0.60</td>
<td>&gt;4.0</td>
</tr>
<tr>
<td>PA14</td>
<td>\textit{pmrB}_{G188S}</td>
<td>0.20</td>
<td>4.0</td>
</tr>
<tr>
<td>PA14</td>
<td>\textit{pmrB}_{G188D}</td>
<td>0.60</td>
<td>&gt;4.0</td>
</tr>
<tr>
<td>PA14-\textit{pmrB}_{L172del}</td>
<td>---</td>
<td>1.25</td>
<td>5</td>
</tr>
<tr>
<td>PA14-\textit{pmrB}_{L172del}</td>
<td>\textit{pmrB}_{WT}</td>
<td>0.60</td>
<td>ND</td>
</tr>
</tbody>
</table>
Figure 1. LPS modification genes are upregulated in response to POL7001 and POL7080 treatment and constitutively expressed in the resistant PA14-\textit{pmrB}_{L172del} strain. (A) RNAseq data shows log$_2$ (fold change) in sequencing reads of PA14 after treatment with POL7001 (37°C, 100 minutes) relative to vehicle control. Bracketed genes are located within the same operon. Upregulated genes include the \textit{pmrA}-\textit{pmrB} two-component regulatory genes, lipid A deacylase \textit{pagL}, and the \textit{arnBCADTEF-ugd} operon. (B) After treatment of PA14 and resistant PA14-\textit{pmrB}_{L172del} with POL7080 or DMSO control (37°C, 100 minutes), qRT-PCR data shows log$_2$ (fold change) in LPS modification gene transcript levels (normalized to \textit{rpoD} expression) relative to PA14 vehicle control. Asterisks indicate paired t-test p-values < 0.03 between POL7080-treated PA14 and PA14-\textit{pmrB}_{L172del} vehicle control. In all experiments, error bars represent S.E.M of three biological replicates.
**Figure 2.** Differential uptake of TAMRA-L27-11 by PA14 versus PA14-pmrB<sub>L172del</sub>. (A) Red-field and blue-field confocal microscopy images of PA14 (top panels) and PA14-pmrB<sub>L172del</sub> (bottom panels) show reduced TAMRA-L27-11 uptake in PA14-pmrB<sub>L172del</sub> relative to PA14. All cells were DAPI stained after treatment with 500nM TAMRA-L27-11 for 120 minutes. (B) Average fluorescence intensities of TAMRA (red bars) and DAPI (blue bars) were calculated for PA14 (solid bars) and resistant PA14-pmrB<sub>L172del</sub> (striped bars) using ImageJ software. Error bars represent S.E.M, and asterisks indicate unpaired t-test p-value < 0.0001 between PA14 and PA14-pmrB<sub>L172del</sub> after TAMRA-L27-11 treatment.
References:


