Title: A novel mechanism of ceftolozane-tazobactam resistance in *Pseudomonas aeruginosa* mediated by L2 β-lactamase[‡]

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31

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Abstract (unstructured):

The prevalance of non-susceptibility to ceftolozane-tazobactam (C/T) among *Pseudomonas aeruginosa* remains low but novel mechanisms of C/T resistance are of concern. Herein, we describe a novel *Pseudomonas aeruginosa* genotype associated with high-level C/T resistance (>256/4 µg/mL) in a single patient. Whole genome sequencing of the isolate was compared to that of a susceptible isolate cultured from the same patient two months earlier. Analysis of the sequences revealed two different *P. aeruginosa* high-risk clones: ST111 followed by ST235. The C/T-resistant ST235 isolate contained five copies of a genetic element comprised of an L2 β-lactamase gene (bla_{L2}) and a truncated $ampR_{L2}$ transcriptional regulator gene, which are commonly found together in *Stenotrophomonas maltophilia* strains and have not been reported to mediate resistance to C/T. Comparative genomic analysis with other *P. aeruginosa* isolates failed to identify alternative explanations for the observed C/T resistance. We found that exogenous expression of bla_{L2} modestly increased C/T MICs in genetically distinct *P. aeruginosa* strains. A screen of our archived isolates identified two *P. aeruginosa* clinical isolates, PS2045 and PS2046, with one and two copies, respectively, of the genetic element containing bla_{L2} and truncated $ampR_{L2}$. Interestingly, disruption of the gene bla_{L2} but not the truncated $ampR_{L2}$ in PS2045 led to a significant decrease in C/T MIC. Thus, we report a novel mechanism of C/T resistance in *P. aeruginosa* mediated by an L2 β-lactamase independently of its canonical regulator $AmpR_{L2}$.

54 **Introduction:**

55 Rising rates of antibiotic resistance in the bacterial pathogen *Pseudomonas aeruginosa* pose a serious
56 threat to public health. Infections caused by antibiotic-resistant strains of *P. aeruginosa* are extremely
57 difficult to treat and are associated with severe morbidity and high mortality rates. Ceftolozane-
58 tazobactam (C/T), a combination of an antipseudomonal cephalosporin, ceftolozane, and a β-lactamase
59 inhibitor, tazobactam (1, 2), has been efficacious in treating *P. aeruginosa* infections (2-5). The high
60 activity of C/T against multi-drug resistant (MDR), extensive-drug resistant (XDR) and difficult to treat
61 (DTR) strains of *P. aeruginosa* (2, 6-10) is attributable to the ability of C/T to evade several antibiotic
62 resistance mechanisms employed by *P. aeruginosa* that inactivate first-line antipseudomonal antibiotics
63 (11, 12).

64

65 The prevalence of *P. aeruginosa* non-susceptibility to C/T remains low among clinical isolates from
66 critically ill patients, ranging from 3 to 18 percent (4, 13-15), but the evolution of C/T resistance during
67 therapy is concerning. While the most common mechanism of C/T resistance in *P. aeruginosa* involves
68 mutations leading to overexpression or increased hydrolytic activity of the class C β-lactamase AmpC
69 (16-18), other mechanisms such as the acquisition of extended-spectrum β-lactamase (ESBL) genes and
70 over-expression of efflux pumps have been occasionally reported (17, 19-24). Given the results of the
71 recent multicenter CACTUS study of hospitalized patients in the USA, which found greater clinical
72 efficacy (OR=2.07, 95% CI 1.16-3.70) with C/T vs. ceftazidime-avibactam (CZA) across a variety of *P.*
73 *aeruginosa* infections (25), it is expected that C/T use and resistance will increase in the near term. Thus,
74 it is crucial to understand emerging C/T resistance mechanisms in *P. aeruginosa*. Herein, we describe a
75 novel mechanism of C/T resistance mediated by acquisition of the L2 β-lactamase gene (bla_{L2}) by a *P.*

76 *aeruginosa* strain cultured from a patient who had received multiple courses of C/T and other
77 antimicrobials.
78
79 **Materials and Methods:**
80 *Patient case and clinical course*
81 All clinical data were obtained from the Northwestern Memorial Hospital (NMH), Chicago electronic
82 medical record. A case report exemption was obtained from the Northwestern University and
83 Midwestern University Institutional Review Boards.
84
85 A 51-year-old male with a history of quadriplegia, autonomic hyperreflexia, neurogenic bladder and
86 chronic foley catheterization complicated by *Enterococcus faecalis* urinary tract infections (UTIs),
87 decubitus ulcers complicated by chronic osteomyelitis, chronic respiratory failure with tracheostomy
88 complicated by recurrent *P. aeruginosa* pneumonia, and *Clostridioides difficile* infection presented from
89 a skilled nursing facility with reports of low oxygen saturations, increased respiratory secretions, and
90 hyponatremia. Six months prior to the current admission, the patient was admitted with multidrug-
91 resistant *P. aeruginosa* pneumonia and was treated with a 7-day course of intravenous (IV) C/T (3 grams
92 every 8 hours as 1-hour infusion) and inhaled polymyxin B. Two months prior to the current admission,
93 the patient was admitted with recurrent pneumonia due to multidrug-resistant *P. aeruginosa* cultured
94 from an endotracheal tube (ET) aspirate (saved as PA-NM-055, **Table 1**) that was treated with C/T and IV
95 polymyxin B for 5 days. One month prior to the current admission, the patient was admitted with
96 suspected pneumonia and urosepsis treated empirically with linezolid and aztreonam, with the latter

97 agent changed to C/T plus inhaled polymyxin B for 5 days based on susceptibilities of the *P. aeruginosa*
98 isolate cultured from the ET aspirate (isolate not saved - **Table 1**).

99

100 During the index admission, the patient experienced new fevers to 101°F (38.3°C), prompting an
101 evaluation for infection, and was empirically treated with meropenem and linezolid. The patient was
102 found to have methicillin-resistant *Staphylococcus aureus* (MRSA) growing from two of two blood
103 cultures on hospital day 2 and sputum cultures grew *P. aeruginosa* (isolate not saved - **Table 1**). The
104 patient was then switched from meropenem to C/T for possible pneumonia vs. tracheitis due to *P.*
105 *aeruginosa,* and linezolid was changed to daptomycin for continuation of MRSA bacteremia treatment.
106 After 7 days of *P. aeruginosa*-targeted treatment, the patient's pulmonary symptoms improved, and C/T
107 was discontinued; however, the patient continued to be intermittently febrile during the hospitalization.
108 Diagnostic imaging studies suggested the cause of intermittent fevers was a new acute sacral
109 osteomyelitis. On hospital day 16, a bone biopsy was performed, which grew ESBL-positive *Klebsiella*
110 *pneumoniae*, *Morganella morganii*, MRSA, and *P. aeruginosa* (saved as PA-NM-086) demonstrating
111 elevated minimum inhibitory concentrations (MICs) for meropenem (**Table 1**). The patient was
112 continued on daptomycin, and meropenem (2 grams IV every 8 hours) was added as a continuous
113 infusion for treatment of acute osteomyelitis. Additional susceptibility testing for C/T, CZA, and
114 polymyxin B were requested. Four days after the biopsy, results of the MIC testing on PA-NM-086
115 revealed high-level C/T resistance but susceptibility to CZA (**Table 1**); therefore, IV meropenem was
116 changed to IV CZA, IV polymyxin B, and metronidazole, with a planned 6-week course of therapy. On
117 hospital day 85, the patient was discharged to an acute inpatient rehabilitation center after completing
118 6 weeks of IV antibiotics for osteomyelitis.

119

120 *Microbiology and Susceptibility Testing*

121 Antibiotic susceptibility was tested for *P. aeruginosa* isolates collected from the patient across multiple
122 admissions, comparator *P. aeruginosa* clinical isolates PS2045 and PS2046 collected previously from
123 blood of patients hospitalized at NMH, and other *P. aeruginosa* strains constructed during the study. As
124 part of routine patient care, antibiotic susceptibility testing for all clinical isolates from the patient was
125 performed by the clinical microbiology laboratory using Vitek II (bioMérieux, Marcy l'Étoile, France),
126 Kirby Bauer, or Etest (bioMérieux, Marcy l'Étoile, France or Liofilchem), depending on the drug. These
127 results were verified for PA-NM-055 and PA-NM-086 by repeat testing in the lab (**Table 1**). All strains
128 used in the study, including reference *P. aeruginosa* strains PA14 and PAO1, and *E. coli* strains used for
129 cloning, are described in **Table S1**.

130

131 Confirmatory susceptibilities were determined for archived *P. aeruginosa* strains by microbroth dilution
132 on Sensititre plates (Thermo Fisher Scientific). *P. aeruginosa* ATCC 27853 was used for quality control
133 purposes. Briefly, isolates were grown overnight on Luria-Bertani (LB) agar at 37°C. The following day, 3-
134 5 colonies were picked and re-suspended in water and diluted to an optical density at 600 nm (OD_{600}) of
135 0.8-1.0. A total of 30 µL of the bacterial suspension was inoculated into 11 mL of cation-adjusted
136 Mueller-Hinton Broth (CA-MHB). The positive control and wells containing antibiotic were inoculated
137 with 50 µL of the suspension, and the negative control was inoculated with 50 µL of CA-MHB only. The
138 plate was incubated at 37°C for 20 hours and read manually for growth. Antibiotic concentrations that
139 led to >95% inhibition of growth (i.e. no visible growth) were noted as MICs.

140

141 C/T MICs for the clinical isolates and constructed strains were determined using antibiotic strips
142 (bioMérieux, Marcy l'Étoile, France or Liofilchem). Bacterial strains were streaked onto LB agar plates

143 and grown overnight at 37°C. A few individual colonies were resuspended in CA-MHB to achieve an
144 OD_{600} of 0.2-0.3 that corresponded to 10^8 CFU/mL. The bacterial suspension was plated onto cation-
145 adjusted Mueller-Hinton agar (CA-MHA) using cotton swabs. A C/T antibiotic strip (Liofilchem) was
146 placed on the plates and incubated for 16-20 hours at 37°C. Assessment of MIC was done as per the
147 manufacturer's instructions (i.e., the intersection of the strip resulting in >95% growth inhibition i.e. no
148 visible growth was read as the C/T MIC).

149

150 *Whole Genome Sequencing*

151 Whole genome sequencing (WGS) of the isolates PA-NM-055 and PA-NM-086 was performed as follows.
152 Isolates from frozen stocks (-80°C) were streaked onto LB agar plates, and a single colony was cultured
153 in LB broth overnight at 37°C. Genomic DNA was extracted using the Promega Maxwell 16 Cell DNA
154 Purification Kit (Promega, Madison, WI, USA). Libraries were prepared using the Nextera XT DNA Library
155 Preparation Kit (Illumina, Inc., San Diego, CA, USA). The clinical isolates were sequenced on a NextSeq
156 500 instrument (150 base pair reads, paired-end, mid-output flow cell, 133 M read pairs). Sequences
157 were trimmed using Trimmomatic v0.32 (26) and then *de novo* assembly was performed with SPAdes
158 3.9.1 (27). Contigs shorter than 200 bp or with an average read coverage of less than 5x were excluded.

159

160 For long read sequencing of PA-NM-086, a sequencing library was prepared from unsheared and non-
161 size-selected genomic DNA using the Oxford Nanopore ligation sequencing kit SQK-LSK110 and
162 sequenced on a FLO-MIN106 flow cell on a MinION sequencing device. Guppy v3.4.5 was used to base
163 call reads with the R9.4.1 high-accuracy model. Assembly of long reads was performed using the
164 Trycycler v0.5.0 pipeline (28). Reads were filtered using Filtlong v0.2.1 to remove reads shorter than
165 1000 bases and the bottom 5% of the reads based on quality. Reads were then subsampled into 24

166 subsets using the Trycycler “subsample” function. Eight read subsets were assembled using Flye v2.8.3
167 (29), eight read subsets were assembled using Raven v1.5.3, and eight read subsets were assembled
168 using minimap2 v2.21, miniasm v0.3, and minipolish v0.1.3 (30, 31). Clustering, reconciliation,
169 circularization, multiple sequence alignment, and consensus sequence generation from the resulting 24
170 assemblies were performed using Trycycler. The consensus assembly was polished with medaka v1.4.3
171 using the “r941_min_hac_g507” model. Illumina reads were aligned to the assembly using BWA v0.7.17
172 (32), and assembly errors were corrected using Pilon v1.23 (33) with a minimum depth setting of 0.1.
173 Read alignment and Pilon correction were performed sequentially until no further assembly corrections
174 were identified. Polypolish v0.4.3 (34) was used for final assembly correction using the Illumina short
175 reads. Sequencing reads and genome assemblies are deposited in NCBI under BioProject accession
176 PRJNA547625.

177

178 *Phylogenetic Analysis*

179 To characterize the prevalence of L2 β lactamases among publicly available *P. aeruginosa* genome
180 sequences, all *P. aeruginosa* genome assemblies (n=46,236, accessed Oct 14, 2025) in the NCBI assembly
181 database were searched for the bla_{L2} gene. All sequence type 235 genomes (n=2996, accessed Oct 14,
182 2025) were downloaded and whole genome phylogenetic relationships were estimated using Mashtree
183 v1.2.0 (35). The presence of the bla_{L2} gene sequence was identified in the genome sequences using
184 nucleotide BLAST. Ten ST235 genome assemblies without a full-length bla_{L2} gene were selected
185 randomly. Illumina read sequences were downloaded from NCBI for all L2-containing sequences
186 identified by nucleotide BLAST against the NCBI WGS database with results limited to *P. aeruginosa*
187 (taxid 287). For the four ST235 isolate sequences for which sequence reads were not available in the
188 Sequence Read Archive (SRA) database of NCBI, pseudoreads were generated from the genome

189 assemblies as previously described (36). Short read and pseudoread sequence alignment, phylogenetic
190 analysis, and tree visualization were performed as previously described (37). Sequence reads and
191 pseudoreads were aligned to the PA-NM-086 genome assembly.

192

193 *Construction of strains expressing* bla_{L2}

194 The *P. aeruginosa* reference strains PA14 and PAO1 were genetically modified to exogenously express
195 bla_{L2} (**Table S1**). The gene bla_{L2} was amplified from the genomic DNA of PA-NM-086 using Q5 high
196 fidelity polymerase (New England Biolabs) and primers 1 and 2 (**Table S2**). The amplicon was integrated
197 at the EcoRI site downstream to the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lac*UV5
198 promoter in the plasmid pPSV37-Gen^R (38) by Gibson assembly (Thermo Fisher Scientific). The resulting
199 recombinant plasmid (designated "pbla_{L2}") was introduced into the conjugative *Escherichia coli* strain
200 SM10 (39) by calcium chloride heat shock method and the transformants were selected on LB agar
201 containing gentamicin (15 µg/mL). Conjugation was performed to transfer the recombinant plasmid
202 from the transformant SM10 strain to the *P. aeruginosa* strains, and the exconjugants were selected on
203 LB agar containing gentamicin (30 µg/mL) and irgasan (triclosan, 5 µg/mL). The plasmid construct was
204 isolated (Qiagen) from the exconjugant *P. aeruginosa* strains and verified by Sanger sequencing using
205 internal primers recognizing the bla_{L2} gene and an adjacent plasmid pPSV37 sequence (**Table S3**). The
206 vector control strains were constructed by introducing the empty vector pPSV37 into PA14 and PAO1
207 using the same method. These strains were grown on CA-MHA containing gentamicin (15 µg/mL) with or
208 without 1 mM IPTG for determining C/T MICs.

209

210 *RNA extraction and reverse-transcriptase-quantitative-PCR (RTqPCR)*

211 Overnight cultures of bacterial strains grown in LB medium were subcultured in LB medium and grown
212 to an OD_{600} of 0.4. IPTG (1 mM) and imipenem (1 µg/mL) were added to test for the induction of gene
213 expression in the indicated experiments. Negative control cultures were left untreated. All bacterial
214 cultures were then incubated for an additional 2 hours. A volume of bacterial culture corresponding to 1
215 x 10^8 CFU was centrifuged, and the pellet was resuspended in RNA protect reagent (Qiagen). After
216 incubation for 5 min at room temperature, samples were centrifuged again, and bacterial cells in the
217 pellet were treated with lysozyme (15 µg/mL in Tris-EDTA). RNA was extracted using an RNEasy kit
218 (Qiagen), and cDNA was synthesized with Superscript IV VILO reverse transcriptase kit (Invitrogen) using
219 master mix with and without reverse transcriptase, the latter used as a negative control to assess for
220 DNA contamination. RT-qPCR was performed by using qPCR probe assays (Integrated DNA Technologies)
221 on a CFX-96 real-time thermocycler (Biorad). Expression level was determined by normalizing the
222 threshold cycle (Ct or Cq) value of each target gene (Ct_{target}) for every sample to that of the respective
223 housekeeping gene *rpoD* ($Ct_{reference}$) generating ΔCt values and plotted as fold change in gene expression
224 with respect to the mean ΔCt of multiple biological replicates of the control sample. Each experiment
225 was carried out at least twice (i.e., at least 2 biological replicates). The primers and probes used for the
226 RT-qPCR are listed in **Table S2**.

227

228 *Generation of deletion mutants*

229 Gene deletion mutants in the comparator *P. aeruginosa* strain PS2045 background were generated using
230 a suicide-vector mediated allelic replacement method (40). Fwd-up and Rev-up primers were used to
231 generate a ~500 bp fragment homologous to 5' end ('up' fragment), whereas Fwd-down and Rev-down
232 primers were used to generate a ~500 bp fragment homologous to 3' end ('down' fragment) of the
233 target gene (**Table S3**). However, a 936 bp transposon gene immediately 3' to the bla_{L2} gene in PS2045 is

234 duplicated elsewhere in the genome of PS2045, preventing amplification of an 'up' fragment. To avoid
235 the duplicated region, we targeted deletion of only a 477 bp internal segment of bla_{L2} instead of the
236 entire gene. This segment encodes the S-X-X-K motif and SDN loop, which are required for the activity of
237 the L2 β-lactamase (41). The 'up' and 'down' fragments were combined using the Fwd-up and Rev-down
238 primers to generate the full fragment, which was integrated at the HindIII site of the plasmid pex18-Hyg^R
239 (40) by Gibson assembly (Thermo Fisher). The resulting plasmid was introduced into the conjugative *E.*
240 *coli* strain SM10 (39) by calcium chloride heat shock method. The transformants were selected on LB
241 agar containing hygromycin (100 µg/mL) and used for conjugation with the strain PS2045 at 37°C for 1
242 hour. The resulting PS2045 merodiploids with the entire plasmid integrated into the genome were
243 selected using LB agar supplemented with hygromycin (500 µg/mL) and irgasan (5 µg/mL). These
244 merodiploids were resolved by counter-selection on LB agar supplemented with sucrose (10 ug/mL) to
245 select for mutants that lost the *sacB*-containing vector backbone along with the target gene. The
246 deletion of the target gene in the selected colonies was verified by PCR amplification with confirmatory
247 primers (**Table S3**) followed by Sanger sequencing of the PCR product. Genomic DNA from mutant
248 isolates was sequenced using a commercial service (SEQCENTER) to produce paired-end 150 bp reads.
249 Sequencing reads were aligned to the complete genome assembly of isolate PS2045 and assessed for
250 mutations relative to the reference using Breseq v0.39.0 (42).

251

252 *Statistical analysis*

253 Each experiment was carried out at least twice and results were plotted using GraphPad prism version
254 10. Statistical significance of the difference observed between datasets, i.e. the median MICs or the
255 mean fold changes in gene expression, was determined by one-way ANOVA with Tukey's multiple

256 comparisons test for comparing multiple samples and Student's t test for comparing 2 samples,
257 generating p values, where $p < 0.05$ was considered statistically significant.

258

259 **Results:**

260 *Antibiotic resistance phenotypes of P. aeruginosa isolates collected from the patient*

261 The respiratory isolate collected 2 months prior to the index admission (PA-NM-055) and the respiratory
262 isolate collected on day 2 of the index admission were both susceptible to C/T and CZA (**Table 1**). In
263 contrast, the bone biopsy isolate collected on hospital day 16 (PA-NM-086) was found to be highly
264 resistant to C/T while remaining susceptible to CZA, giving the clinical impression that C/T resistance
265 emerged after treatment with repeated courses of C/T. However, genomic analysis revealed that PA-
266 NM-055 belonged to the sequence type (ST) 111, whereas PA-NM-086 belonged to ST235, indicating
267 that the patient had experienced infections by at least two distinct high-risk clones of *P. aeruginosa* over
268 time. Due to the limited number of isolates available for analysis, we were unable to determine whether
269 PA-NM-086 represented a new infection that contributed to acute osteomyelitis observed in the patient
270 or whether the PA-NM-086 lineage had been present in this patient for an extended period and at some
271 point replaced the more susceptible PA-NM-055 after multiple treatment courses.

272

273 *Characterization of antimicrobial resistance (AMR) genes in the C/T resistant strain PA-NM-086*

274 To investigate possible mechanisms of C/T resistance in PA-NM-086, we performed WGS. Long read *de*
275 *novo* assembly with short read correction yielded a single circular 6.92 Mb chromosome. Interestingly,
276 mutations known to be associated with C/T resistance in *P. aeruginosa* (15-24) were absent in PA-NM-
277 086. In particular, mutations in the class C β-lactamase gene bla_{AmpC}, which encodes the Pseudomonas-
278 derived cephalosporinase (PDC) member of the AmpC family, were not observed in PA-NM-086. The
279 AmpC family of beta-lactamases are involved in peptidoglycan recycling and are commonly associated

280 with C/T resistance (4, 16, 21-24, 43-53). However, an Ambler Class A β-lactamase gene *bla*$_{L2}$ with 100%
281 identity with the L2 β-lactamase gene found in many *Stenotrophomonas maltophilia* strains was
282 identified (41). This gene was not present in the respiratory isolate PA-NM-055, which was C/T sensitive.

283

284 *Frequency of bla*$_{L2}$ in *P. aeruginosa*

285 We next examined whether *bla*$_{L2}$ was present in other *P. aeruginosa* strains. We first searched a publicly
286 available *P. aeruginosa* whole-genome sequence repository (~46,000 genomes) for other ST235 isolates
287 that contained the *bla*$_{L2}$ gene. Only six of the 2,995 ST235 genomes examined contained this gene,
288 suggesting that the presence of the *bla*$_{L2}$ gene in *P. aeruginosa* is quite rare even within this high-risk
289 clonal lineage (**Fig. 1A**). Across all the *P. aeruginosa* genomes (~46,000) available in NCBI, *bla*$_{L2}$ was
290 identified in 12 isolates, 11 of which were collected in the USA (**Fig 1B**). Further, this gene was
291 predominantly confined to just 5 lineages (**Fig. 1B**), suggesting that the transfer of the *bla*$_{L2}$ gene from *S.*
292 *maltophilia* to *P. aeruginosa* was a globally rare event.

293

294 *Genetic context of bla*$_{L2}$

295 We used the complete genome sequence of PA-NM-086 to determine the genomic context of *bla*$_{L2}$. In *S.*
296 *maltophilia,* the *bla*$_{L2}$ gene is adjacent to the *ampR*$_{L2}$ gene, which encodes a transcriptional regulator of
297 *bla*$_{L2}$ (54). In PA-NM-086, we found that the 912 bp *bla*$_{L2}$ gene is adjacent to an identical *ampR*$_{L2}$ gene
298 except that the length of the *ampR*$_{L2}$ gene is reduced from 867 bp to 507 bp (**Fig. 2**). We designated this
299 truncated *ampR*$_{L2}$ allele as "*ampR*$_{L2}^{tr}$" that encodes an open reading frame of 168 amino acids of which
300 the first 129 amino acids are identical to that of AmpR $_{L2}$ of *S. maltophilia*. Of note, the genomes of
301 nearly all *P. aeruginosa* isolates contain an *ampR* gene (here designated as "*ampR*$_{AmpC}$") directly adjacent

302 to *bla*$_{AmpC}$ (53), which is distinct from *ampR*$_{L2}$ in *S. maltophilia* (71.6% identity over 67% of the *ampR*$_{AmpC}$

303 gene). The truncated *ampR*$_{L2}^{tr}$ gene in PA-NM-086 has only 72% identity over 41% of *ampR*$_{AmpC}$. These

304 findings indicate that *ampR*$_{L2}^{tr}$ likely originated from *S. maltophilia* rather than from gene duplication of

305 *ampR*$_{AmpC}$. The genes *bla*$_{L2}$ and *ampR*$_{L2}^{tr}$ are part of a larger 9,034 bp element that contains 7 other genes

306 and is repeated in tandem 5 times in the genome of PA-NM-086 (**Fig. 2**). Additional AMR genes

307 identified in this element include two tetracycline resistance genes [*tetR* and *tetA,* coding for the

308 tetracycline repressor protein class G and tetracycline efflux pump, respectively (55, 56)] and an

309 aminoglycoside resistance gene [*aph*, coding for an aminoglycoside 3' phosphotransferase (57)]. This

310 genomic region contains IS91 family transposases, suggesting that it is part of a composite transposon or

311 insertion sequence (IS)-associated resistance cassette.

312

313 *Impact of exogenous expression of the L2 β-lactamase on C/T resistance in P. aeruginosa*

314 Since *S. maltophilia* strains are relatively resistant to C/T (58-60) and *bla*$_{L2}$ was originally found in *S.*

315 *maltophilia* (61, 62), we hypothesized that the gain of *bla*$_{L2}$ contributed to C/T resistance in the strain

316 PA-NM-086. To test this hypothesis, we constructed a plasmid p*bla*$_{L2}$ to exogenously express *bla*$_{L2}$ gene

317 in the presence of IPTG and introduced it in the *P. aeruginosa* reference strains PA14 and PAO1. We

318 compared the C/T MICs in the presence and absence of IPTG and found that the addition of IPTG

319 increased the C/T MIC in PA14-p*bla*$_{L2}$ (**Fig. 3**) and PAO1-p*bla*$_{L2}$ (**Fig. S1**) by approximately four-fold (i.e.,

320 two doubling dilutions), which was statistically significant. In contrast, the addition of IPTG had no effect

321 on C/T resistance in strains containing the empty vector (PA14-EV and PAO1-EV).

322

323 *Associations between the bla*$_{L2}$ *expression and C/T resistance in P. aeruginosa*

324 We next examined whether differences in *bla*$_{L2}$ gene copy number were associated with different
325 degrees of C/T resistance. As mentioned, PA-NM-086 contains five copies of the *bla*$_{L2}$ gene. A search of
326 *P. aeruginosa* WGSs in our collections identified 2 clinical isolates, PS2045 and PS2046 (**Table S1**),
327 containing 1 and 2 copies, respectively, of the 9,034 bp *bla*$_{L2}$-containing genetic element (**Fig. 1B**). We
328 determined the C/T MICs of these two isolates. Whereas PS2045 had a low C/T MIC of ~4 µg/mL, PS2046
329 was highly resistant to C/T, with an MIC of >256 µg/mL, consistent with a relationship between *bla*$_{L2}$
330 gene copy number and MIC.

331

332 We reasoned that strains with higher *bla*$_{L2}$ copy numbers may express more L2 β-lactamase, which
333 would in turn lead to higher C/T MICs. To examine this, we performed RT-qPCR on PA-NM-086, PS2045,
334 and PS2046. The expression levels of the *bla*$_{L2}$ genes in these strains correlated with *bla*$_{L2}$ copy number
335 (**Fig. S2A**). Notably, expression of *bla*$_{L2}$ in PA14-p*bla*$_{L2}$ under uninduced (C/T MIC ~0.19 µg/mL) and
336 induced (C/T MIC ~0.75 µg/mL) conditions correlated with C/T resistance of PA14-p*bla*$_{L2}$ in these
337 conditions (**Fig. 3** and **Fig. S2A**). However, expression of *bla*$_{L2}$ in the clinical isolates PS2045 (C/T MIC ~4
338 µg/mL) and PS2046 (C/T MIC >256 µg/mL) was lower than that in strain PA14-p*bla*$_{L2}$ in the presence of
339 IPTG induction (C/T MIC ~0.75 µg/mL). This suggested that the expression level of *bla*$_{L2}$ was not solely
340 responsible for C/T MICs in these strains. Because over-expression of *bla*$_{AmpC}$ has been linked to C/T
341 resistance (16), we measured *bla*$_{AmpC}$ expression but found similar *bla*$_{AmpC}$ transcript levels in all strains
342 (**Fig. S2B**). We also found that the sequences of *bla*$_{AmpC}$ gene in PS2045, PS2046 and PA-NM-086 are
343 identical. Further, known mutations associated with increased C/T resistance in *P. aeruginosa* were
344 absent in PS2045 and PS2046 like PA-NM-086. Therefore, difference in C/T resistance levels among
345 these strains do not appear to be related to the inherent levels of *bla*$_{AmpC}$ expression in these strains.
346 Overall, these findings indicate that exogenous expression of a *bla*$_{L2}$ gene leads to a modest but

347 statistically significant increase in resistance to C/T in *P. aeruginosa* and that other mechanisms may
348 augment this resistance.

349

350 *Loss of L2 β-lactamase decreases C/T resistance in P. aeruginosa*

351 To confirm the role of L2 β-lactamase in C/T resistance in PA-NM-086, we sought to examine the effect
352 of deletion of the bla_{L2} gene on C/T MICs. This was difficult in PA-NM-086 because it contains five copies
353 of the bla_{L2} gene. Since PS2045 contains only one copy of bla_{L2} (**Fig. 2**), it allowed the targeted deletion
354 of the bla_{L2} gene. We deleted approximately 50% of the $bla_{L2,}$ open reading frame, including the region
355 coding for the active site of L2 β-lactamase, to construct the mutant strain PS2045Δbla_{L2}^{act} (see
356 Methods). The C/T MIC value of PS2045Δbla_{L2}^{act} (~0.75 µg/mL) was significantly lower than that of the
357 parental strain (~4 µg/mL) (**Fig. 4**). This indicated that a functional L2 β-lactamase contributes to a
358 modest level of C/T resistance in *P. aeruginosa*.

359

360 *L2 β-lactamase in P. aeruginosa has activity against other β-lactam antibiotics*

361 The L2 β-lactamase of *S. maltophilia* has broad activity against β-lactam antibiotics (41, 61, 63, 64). We
362 assessed the effect of disruption of bla_{L2} on the resistance of the strain PS2045 to other β-lactams. The
363 mutant strain PS2045Δbla_{L2}^{act} displayed reduced levels of resistance to aztreonam, cefepime,
364 ceftazidime and piperacillin/tazobactam in comparison to the parental strain (**Table 2**). However, this
365 was not observed for the carbapenems imipenem, meropenem, and doripenem, as the parental PS2045
366 strain was susceptible to all three carbapenems despite the presence of an intact bla_{L2} gene. The
367 antibiotic resistance profiles of the clinical isolates examined in this study with one, two, and five copies
368 of the bla_{L2} gene (PS2045, PS2046, PA-NM-086, respectively) were also consistent with a role for the L2

369 β-lactamase in resistance to multiple β-lactams (**Table 2**). These findings demonstrated that the L2 β-
370 lactamase increases detectable resistance to multiple β-lactams but probably not to carbapenems.

371

372 *$AmpR_{L2}^{tr}$ does not regulate expression of the L2 β-lactamase in PA-NM-086*

373 In *S. maltophilia,* $AmpR_{L2}$ regulates expression of bla_{L2}, and induces bla_{L2} expression in the presence of
374 the β-lactam antibiotic imipenem (54). As $ampR_{L2}^{tr}$ is substantially truncated (867 bp reduced to 507 bp)
375 in PA-NM-086, we hypothesized that bla_{L2} expression in this strain may not be regulated by $AmpR_{L2}^{tr}$. To
376 test this, we determined the expression levels of bla_{L2} in PA-NM-086 in the presence and absence of a
377 low concentration of imipenem (1 µg/mL). We used PA14-pbla_{L2} strain as a negative control because
378 bla_{L2} expression in this strain is controlled by IPTG induction of the *lac*UV5 promoter (**Fig. S2A**). As a
379 positive control, we examined the expression of bla_{AmpC}, which has been reported to be induced by
380 imipenem (65). As expected, bla_{L2} expression in PA14-pbla_{L2} was induced by IPTG and not imipenem
381 (**Figs. S2A and 5A**), and bla_{AmpC} was induced by imipenem in PA-NM-086 as well as PA14-pbla_{L2} (**Fig. 5B**).
382 In PA-NM-086, no significant difference was observed in bla_{L2} expression levels in the presence or
383 absence of imipenem (**Fig. 5A**). This indicated that bla_{L2} expression in PA-NM-086 occurs constitutively,
384 consistent with the interpretation that $ampR_{L2}^{tr}$ does not modulate bla_{L2} expression in the absence of
385 imipenem. We also generated a deletion mutant of $ampR_{L2}^{tr}$ in PS2045 and found that deletion of
386 $ampR_{L2}^{tr}$ did not affect the C/T MIC of PS2045 (**Fig. 4**). This agreed with the lack of $AmpR_{L2}^{tr}$-dependent
387 regulation of bla_{L2} expression in PA-NM-086 (**Fig. 5A**). Taken together, these results suggest that L2 β-
388 lactamase contributes to C/T resistance in *P. aeruginosa* independently of the product of the $ampR_{L2}^{tr}$
389 gene.

390

391 **Discussion:**

392 Our investigation suggests that the L2 β-lactamase gene, usually associated with *S. maltophilia*, has been
393 transferred to *P. aeruginosa* on more than one occasion, though this remains a globally rare event. The
394 L2 β-lactamase, along with L1 β-lactamase, is produced by many *S. maltophilia* strains and is largely
395 responsible for the widespread resistance of this species to most β-lactams, including third-generation
396 cephalosporins (61, 66, 67). *S. maltophilia* demonstrates C/T MIC range of 16/4 to >128/4 µg/mL (58-
397 60). The L2 β-lactamase may contribute to this relatively high level of C/T resistance. In our study, we
398 found that introduction of bla_{L2} into the *P. aeruginosa* strains PA14 and PAO1 caused a modest
399 approximately four-fold increase in C/T MICs. Likewise, disruption of the L2 β-lactamase encoding gene
400 in PS2045 resulted in an approximate four-fold reduction in the C/T MIC. The L2 β-lactamase is therefore
401 yet another example of cross-species transfer of an antibiotic resistance mechanism. Although PS2045,
402 PS2046, and PA-NM-86 are very closely related, they have 1, 2, and 5 copies of the ~9 kb element,
403 respectively, suggesting that selective pressures exist that cause repeated duplication of this element.
404 The bone-biopsy isolate (PA-NM-086) had 5 copies of the bla_{L2} gene and a C/T MIC >256 µg/mL, which
405 matched with C/T MIC of a blood isolate, PS2046, that contained only 2 copies of bla_{L2}. In contrast, the
406 blood isolate, PS2045, containing only one copy of bla_{L2}, displayed a low MIC of ~4 µg/mL, which
407 suggests non-linear scaling of resistance (as measured by MIC) as copy number of bla_{L2} gene increases or
408 that the C/T MICs of these strains are influenced by additional resistance determinants. We hypothesize
409 that exposure to C/T concentrations in excess of 4X MIC may result in eradication of strains with 1 copy
410 of bla_{L2}. Such PK/PD exposures are achievable for strains with C/T MICs of 1 µg/mL (68), but would be
411 much more difficult to achieve for strains with an MIC of 4 µg/mL or greater and even more so when
412 site-of-infection exposures are lower than those attained in plasma with maximal labeled dosing (69).

413

414 The 100 percent identity of the bla_{L2} gene sequences in *P. aeruginosa* and *S. maltophilia* suggests that
415 the former acquired the gene from the latter. The gene appears to have been obtained from *S.*

416 *maltophilia* along with a truncated *ampR*$_{L2}$ gene as part of a 9,034 bp genetic element. L2, a class A
417 serine β-lactamase, is encoded by a chromosomal gene in *S. maltophilia* (41). Chromosomally encoded
418 genes are less readily transferred across species than those encoded on plasmids, which may explain
419 why *bla*$_{L2}$ was identified in only a handful of *P. aeruginosa* genomes. Both *P. aeruginosa* and *S.*
420 *maltophilia* are ubiquitous environmental organisms, found in soil, plant material, rivers, lakes, and
421 streams (70, 71) , which would provide ample opportunities for genetic exchange. Interestingly, both
422 bacteria are also commonly cultured from the lungs of individuals with cystic fibrosis (72), which may be
423 another site at which genetic exchange between these bacteria occurred.

424

425 This is the second report of the presence of an L2 β-lactamase gene in *P. aeruginosa* but appears to
426 represent a more recent transfer of the gene. A previous report by Tian and colleagues described in *P.*
427 *aeruginosa* the acquisition of the *bla*$_{PME-1}$ gene, which is similar to *bla*$_{L2}$ (73). However, several
428 observations suggest that the *bla*$_{PME-1}$ gene is distinct from *bla*$_{L2}$. While the L2 β-lactamase of PA-NM-086
429 has 100% amino acid identity to the corresponding protein of *S. maltophilia,* PME-1 has only 50% amino
430 acid identity (73). (This is substantially less than the 68% amino acid identity observed between SHV-1
431 and TEM-1, which are 2 distinct β-lactamases.). The *bla*$_{PME-1}$ gene is carried on a 9-kb plasmid and was
432 not adjacent to an *ampR*$_{L2}$ gene. Although PME-1 conferred resistance to third-generation
433 cephalosporins including ceftazidime, it was inhibited by tazobactam and would therefore be predicted
434 to be inactive against C/T. We therefore suggest that PME-1 and L2 β-lactamase be considered distinct
435 β-lactamases.

436

437 Several lines of evidence indicate that the L2 β-lactamase contributes to C/T resistance. First, multiple
438 *bla*$_{L2}$-containing isolates of *P. aeruginosa* had elevated MICs to C/T. Second, upon exogenous expression

439 of *bla*$_{L2}$ in two distinct *P. aeruginosa* strains (PA14 and PAO1) that did not naturally contain it, we
440 observed a modest but significant increase in the C/T MIC levels. Third, the disruption of *bla*$_{L2}$ in PS2045
441 caused a modest but significant reduction in C/T MICs. Together, these findings support the contribution
442 of *bla*$_{L2}$ to C/T resistance in *P. aeruginosa*. A number of exogenously acquired β-lactamases can enhance
443 the resistance of *P. aeruginosa* to C/T, including GES-6, SHV-2a, VIM-2, and IMP-28 (49, 51, 74, 75). Our
444 findings indicate that the L2 β-lactamase should be added to this list.

445

446 Our findings suggest that the L2 β-lactamase contributes to C/T resistance independently of the *ampR*$_{L2}^{tr}$
447 gene in *P. aeruginosa*. In *S. maltophilia,* AmpR$_{L2}$-dependent induction of *bla*$_{L2}$ expression occurs in the
448 presence of β-lactam antibiotics such as imipenem (54). However, in the absence of β-lactam antibiotics,
449 some expression of *bla*$_{L2}$ can occur independently of AmpR$_{L2}$ in *S. maltophilia* (76). Therefore, it is
450 possible that the *P. aeruginosa* strains used in this study, which naturally contain *bla*$_{L2}$ gene, express
451 *bla*$_{L2}$ constitutively irrespective of the presence of an inducer antibiotic and independently of regulation
452 by AmpR$_{L2}^{tr}$. In *S. maltophilia,* induction of *bla*$_{L1}$ and *bla*$_{L2}$ is dependent on AmpR$_{L2}$; disruption of the
453 regulator results in reduced activity of the L2 β-lactamase and corresponding reductions in the MICs of
454 several β-lactams (54). We observed that in the presence of AmpR$_{L2}^{tr}$, L2 expression was not affected by
455 the inducer imipenem, suggesting a loss of the context-dependent repressor and activator effects
456 typically associated with AmpR (77). Although our study shows that L2 β-lactamase contributes to C/T
457 resistance independently of a functional AmpR$_{L2}$ in the strain PS2045, it remains to be tested whether
458 the presence of a functional AmpR$_{L2}$ would further influence L2 β-lactamase-dependent C/T resistance
459 in *P. aeruginosa*. AmpR$_{L2}$ has been suggested as a potential drug target due to its role in resistance to
460 multiple β-lactams (78); however, since L2 β-lactamase contributes to C/T resistance even in the
461 absence of regulation by AmpR$_{L2}$, such an approach would be ineffective against L2-expressing strains of
462 *P. aeruginosa*.

463

464 To date, much work on C/T resistance in *P. aeruginosa* has been focused on the core genome
465 chromosomal bla_{AmpC} gene, a class C β-lactamase (16, 17, 21, 24, 44-47, 50). Mutations leading to bla_{AmpC}
466 de-repression can increase the MIC of C/T to 16 ug/mL for *P. aeruginosa* (75). The range of mutations
467 that predispose clinical strains to overproduce bla_{AmpC} remains incompletely understood (79). We found
468 that bla_{AmpC} in PA-NM-086 was induced in the presence of the β-lactam imipenem, suggesting that it
469 could also be contributing to C/T resistance. A second mechanism of bla_{AmpC} mediated resistance to C/T
470 is the structural alteration of AmpC itself. Paired isolate studies (comparing isolates collected pre- and
471 post-C/T treatment) have found that alterations within the bla_{AmpC} open reading frame are major drivers
472 of increased C/T MICs and phenotypic resistance (17, 80). *P. aeruginosa* isolates with a deletion of a 21
473 bp region of the bla_{AmpC} have increased C/T MIC values of >256 µg/mL (4). Additionally, alterations
474 within the omega-loop of AmpC appear to widen the enzyme's binding pocket, leading to increased
475 ceftolozane and ceftazidime hydrolysis and reduced affinity for avibactam (50). However, the bla_{AmpC}
476 gene present in PA-NM-086 did not exhibit any of the mutations associated with C/T resistance, making
477 it unlikely that structural changes in AmpC were directly responsible for the elevated C/T MIC in this
478 strain.

479

480 Our study has several limitations. First, we were not able to ascertain what portion of PA-NM-086 C/T
481 resistance was due to the L2 β-lactamase. Our MIC analysis indicated that the five copies of the bla_{L2}
482 gene in PA-NM-086 may cause higher C/T MICs, as strains with more copies of the bla_{L2} gene had higher
483 C/T MICs. Because we were unable to delete all five copies of this gene in PA-NM-086, we could not
484 verify this. However, the non-linear impact of bla_{L2} copy number on C/T resistance level observed in the
485 *P. aeruginosa* strains used in this study (zero to one: 2 doubling dilutions and one to two copies: > 2

486 doubling dilutions) suggests that there is an association between *bla*$_{L2}$ gene copy numbers and C/T MICs
487 in our isolates. Confirmatory studies are needed. Second, we lacked an antecedent C/T-susceptible
488 strain of the same lineage as PA-NM-086 from our patient to strengthen a comparative genomics
489 analysis. Third, our copy number experiments and deletion-expression analyses used different strains of
490 *P. aeruginosa,* so we were unable to take into account the effects of other strain background differences
491 on C/T resistance. Fourth, the induction of expression of *bla*$_{L2}$ in PA14 and PAO1 did not result in the
492 same C/T MIC as observed in PS2046, which expressed *bla*$_{L2}$ lower than PA14-p*bla*$_{L2}$ with IPTG induction.
493 However, the log-scale changes in C/T MIC were similar upon exogenous expression and deletion of
494 *bla*$_{L2}$, suggesting that the L2 β-lactamase can have a measurable impact on C/T resistance even at low
495 copy numbers.

496

497 **Conclusion**

498 In conclusion, mutational and expression analyses suggest that acquisition of the class A β-lactamase
499 gene *bla*$_{L2}$ contributed to C/T resistance in *P. aeruginosa*. Additional studies are needed to better
500 understand the processes that select for the transfer of resistance genes between *P. aeruginosa* and *S.*
501 *maltophilia*. This study reports a rare but important case of antibiotic resistance that extends our
502 understanding of C/T resistance in *P. aeruginosa*.

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769 Avibactam and Ceftolozane-Tazobactam in Multidrug-Resistant Pseudomonas aeruginosa.
770 Antibiotics (Basel) 11.

771

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796 *Data Analysis: Rhodes, Ozer, Garai*

797 *Obtained Funding: Rhodes, Hauser*

798 *Administrative, technical, or material support: Nozick, O'Donnell*

799 *Supervision: Rhodes, Ozer, Hauser*

800

801 **Data availability:**

802 The source data associated with the manuscript are available on the Prism repository of Northwestern

803 University and can be accessed through the digital object identifier (doi)

804 https://doi.org/10.18131/8g2gz-7zt62.

805 **Table 1.** Susceptibility profiles of *P. aeruginosa* isolates collected longitudinally from a single patient

Time of sample collection	*Two months prior to admission*		*One month prior to admission*	*Hospital day 2*	*Hospital day 16*	
Sample	*ET[b] aspirate*	*ET[b] aspirate (repeat testing[c])*	*ET[b] aspirate*	*Sputum*	*Bone biopsy[d]*	*Bone biopsy[d] (repeat testing[c])*
Isolate ID:	PA-NM-055		Not available		PA-NM-086	
Antibiotic[a] / MIC (µg/mL)						
Amikacin	≥64	32	≥64	≥64	16	8
Aztreonam	Resistant (KB)	8	Susceptible (KB)	Intermediate (KB)	Resistant (KB)	≥32
Cefepime	≥64	≥32	≥64	≥64	≥64	≥32
Ceftazidime	Resistant (KB)	≥32	Susceptible (KB)	Resistant (KB)	Resistant (KB)	≥32
Ciprofloxacin	≥4	≥4	≥4	≥4	≥4	≥4
Gentamicin	≥16	≥16	≥16	≥16	≥16	≥16
Meropenem	≥16	8	≥16	≥16	8	4
Piperacillin-tazobactam	Resistant (KB)	≥128/4	-- [e]	Resistant (KB)	Resistant (KB)	≥128/4
Polymyxin B	1	1[f]	-- [e]	0.75	1.5	1[f]
Tobramycin	≥16	≥16	≥16	≥16	≥16	≥16
Ceftolozane-tazobactam[g]	0.75/4	0.75/4	0.75/4	0.5/4	192/4	≥256
Ceftazidime-avibactam[g]	-- [e]	2/4[c]	2/4	1.5/4	1.5/4	1/4[c]

806

807 **Footnotes:** a) MICs from Vitek II unless otherwise stated; b) respiratory sample collected from
808 endotracheal tube; c) Sensititre MICs unless otherwise stated;d) bone biopsy sample collected from
809 sacral bone e) indicates result not available; f) colistin MIC; g) MICs from antibiotic strip test unless
810 otherwise stated. ET, endotracheal aspirate; KB, Kirby-Bauer. The 'repeat testing' column represents
811 MIC values from multiple experiments. If MICs values varied between 2 experiments by one dilution, the
812 higher value was chosen and if they varied by more than one dilution, the value in the middle was
813 chosen. If the experiment was done more than twice, the median or average of 2 medians was chosen.
814

815

816 **Table 2.** Susceptibility profiles of *P. aeruginosa* isolates containing different copy numbers of bla_{L2} and
817 the mutant containing a disrupted bla_{L2} gene (PS2045Δbla_{L2}^{act}).
818

Antibiotic	**PA-NM-086 (5 bla_{L2})**	**PS2046 (2 bla_{L2})**	**PS2045 (1 bla_{L2})**	**PS2045 Δbla_{L2}^{act} (No bla_{L2})**
Aztreonam[a]	>=32	≥32	≥32	**4**
Imipenem[a]	≥16	<=1	<=1	<=1
Cefepime[a]	≥32	≥32	16	**4**
Meropenem[a]	4	<=1	<=1	<=1
Ceftazidime[a]	≥32	≥32	16	**<=1**
Ampicillin/Sulbactam[a]	≥128/64	≥128/64	≥128/64	≥128/64
Doripenem[a]	4	<=0.5	<=0.5	<=0.5
Piperacillin/Tazobactam[a]	≥128/4	≥128/4	16/4	**<=8/4**
Ticarcillin/Clavulanic acid[a]	≥256/2	≥256/2	32/2	32/2
Ceftolozane-tazobactam[b]	≥256/4	≥256/4	4/4	**1/4**
Ceftazidime-avibactam[b]	Not measured[c]	Not measured[c]	Not measured[c]	Not measured[c]

819

820 **Footnotes:** a) MICs from microbroth dilutions (Sensititre); b) MICs from antibiotic strip test (Liofilchem)
821 unless otherwise stated; c) indicates result not available. MICs were determined for each isolate in 2
822 different experiments. If the values differed between 2 experiments by one dilution, the higher value
823 was chosen and if they varied by more than one dilution, the value in the middle was chosen. **I**f the
824 experiment was done more than twice, the median or average of 2 medians was chosen. Values
825 highlighted in bold indicate reduction in MIC by more than one dilution factor in the mutant strain
826 PS2045 Δbla_{L2}^{act} in comparison to the parental strain PS2045.

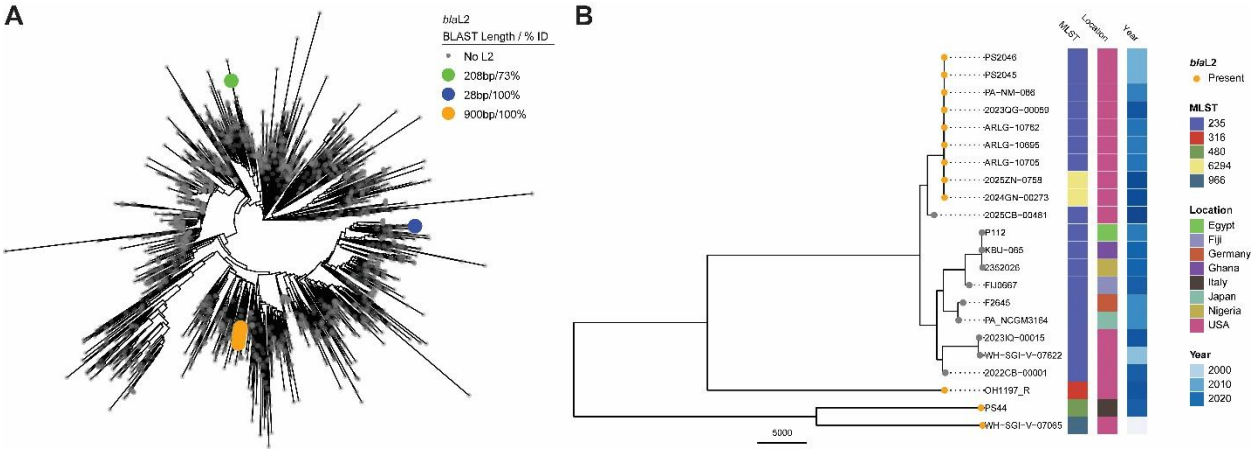
827

828

829 **Figure legends**

830

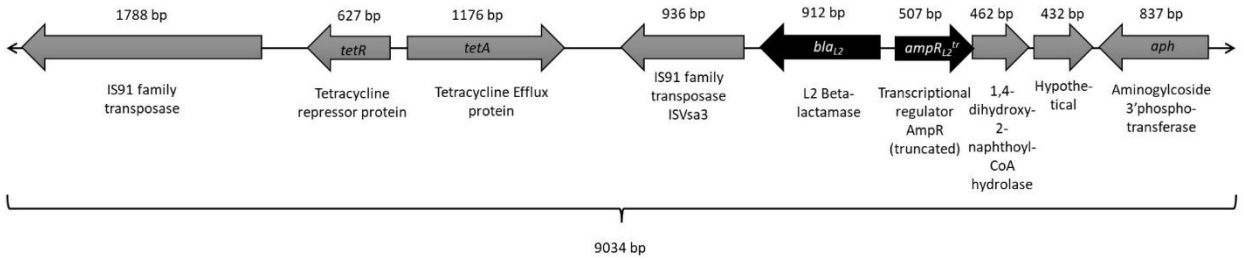
831 **Fig. 1. The presence of the *bla*$_{L2}$ gene among publicly available *P. aeruginosa* genomes.**



832

833 **Figure legend: A)** Whole- genome phylogenetic analysis of ST235 genome assemblies in NCBI (n=2995,
834 one outlier removed) using Mashtree. Isolates with blastn hits for the *bla*$_{L2}$ gene are highlighted. Taxa are
835 colored by the alignment length and percent identity of the BLAST results. Six genomes had identical genes
836 (orange), and two genomes contained portions of the *bla*$_{L2}$ gene (blue and green). **B)** Phylogenetic
837 relationships of all *bla*$_{L2}$-positive genome sequences in NCBI by Mashtree analysis. Isolates carrying *bla*$_{L2}$
838 gene (orange) with > 88% blastn sequence identity across > 95% length of the *bla*$_{L2}$ gene and relative to
839 the 10 randomly selected non-*bla*$_{L2}$ ST235 isolates from NCBI (gray) are shown. Bars show the multilocus
840 sequence type (MLST), geographic location and decade of collection of each isolate.

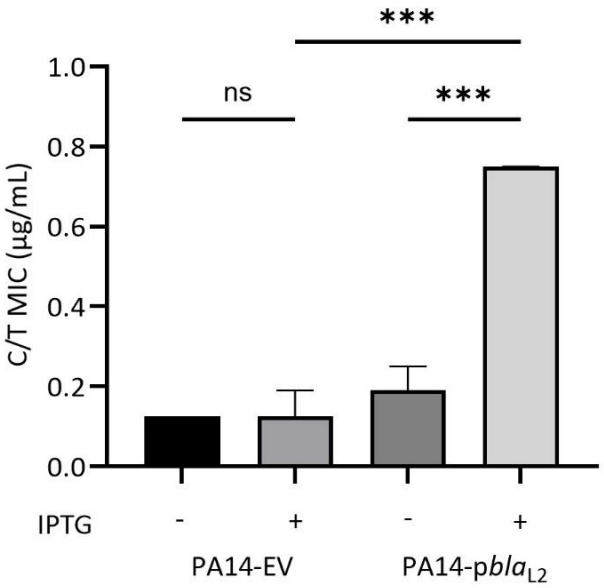
841 **Fig. 2. Genomic element containing *bla*$_{L2}$.**



842

843 **Figure legend:** Five tandem copies of the genomic element (9,034 bp) shown in the figure were found in
844 PA-NM-086. The length and annotation of each open reading frame is indicated.

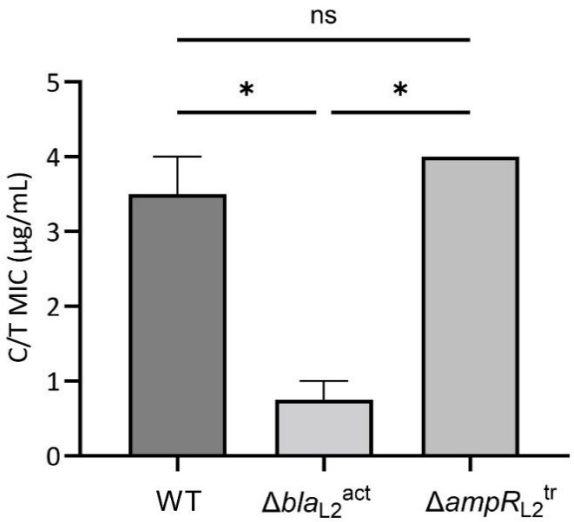
845 **Fig. 3. Effect of exogenous expression of *bla*$_{L2}$ in *P. aeruginosa* strain PA14 on C/T MIC.**



846

847 **Figure legend:** C/T MICs are shown for the strains PA14-EV and PA14-*pbla*$_{L2}$ with (+) or without (-) IPTG
848 as determined by antibiotic strip tests. The bars represent median MIC values of 3 biological replicates
849 with confidence intervals shown as error bars. One-way ANOVA was used to determine statistical
850 significance, indicated as *p* values, where ns = $p > 0.05$ and *** = $p < 0.001$. Abbreviations- EV, empty
851 vector; MIC, minimal inhibitory concentration; IPTG, isopropyl-β-D-thiogalactopyranoside.

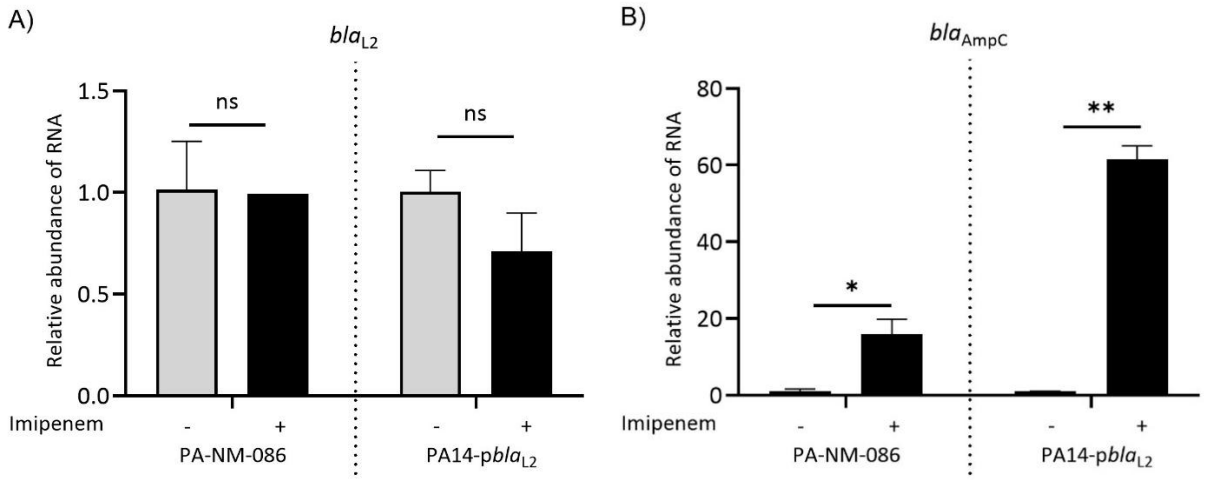
852 **Fig. 4. C/T MICs for a parental PS2045 strain compared to PS2045 strains with disruptions of the *bla*$_{L2}$**
853 **or *ampR*$_{L2}^{tr}$ genes.**



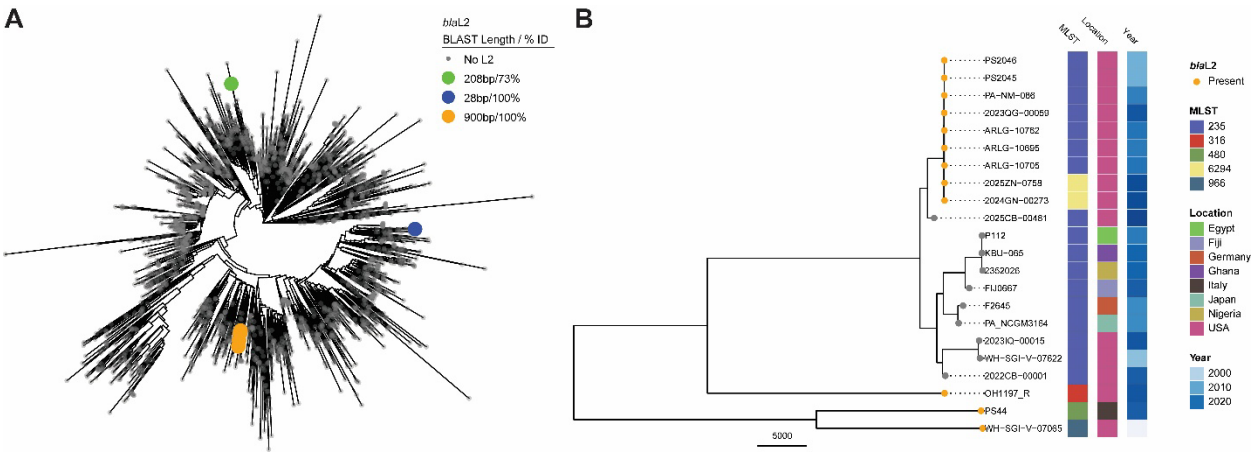
854

855 **Figure legend:** C/T MICs in the parental (WT) and mutant strains of the clinical isolate PS2045 were
856 determined by antibiotic strip tests. Disruption of the *bla*$_{L2}$ gene (Δ*bla*$_{L2}^{act}$) significantly reduced C/T
857 MICs, whereas deletion of the *ampR*$_{L2}^{tr}$ gene (Δ*ampR*$_{L2}^{tr}$) had no effect. The bars represent median MIC
858 values from 2 biological replicates, and error bars represent confidence intervals. One-way ANOVA was
859 used to determine statistical significance, indicated as *p* values, where ns = $p > 0.05$ and * = $p < 0.05$.

860 **Fig. 5. Expression of *bla*$_{L2}$ and *bla*$_{AmpC}$ in the presence and absence of imipenem.**



861
862 **Figure legend:** Expression of the genes **A)** *bla*$_{L2}$ and **B)** *bla*$_{AmpC}$ in the strains PA-NM-086 and PA14-*pbla*$_{L2}$
863 in the presence (+) or absence (-) of imipenem was determined by RT-qPCR. Gene expression is shown
864 as relative abundance in RNA levels in comparison to the respective untreated control (-) after
865 normalizing to the house keeping gene *rpoD*. The bars represent means of 2 biological replicates. Error
866 bars represent standard deviations. Student's t test was used to determine statistical significance,
867 indicated as *p* values, where ns = $p > 0.05$, * = $p < 0.05$, and ** = $p < 0.01$.



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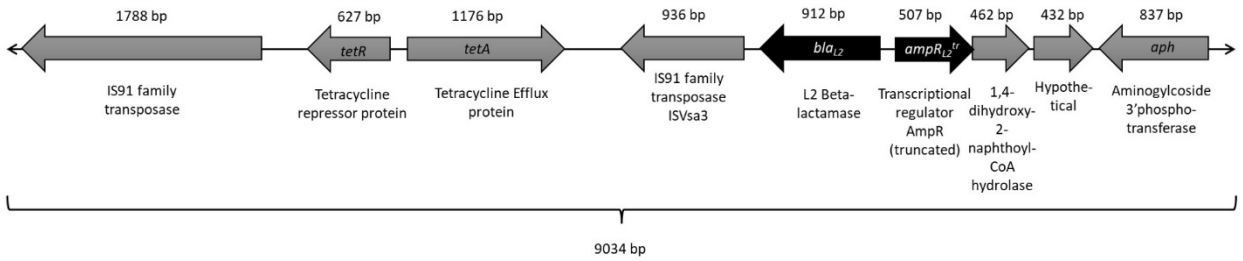

Fig. 2. Genomic element containing *bla*$_{L2}$. Five tandem copies of the genomic element (9,034 bp) shown in the figure were found in PA-NM-086. The length and annotation of each open reading frame is indicated.

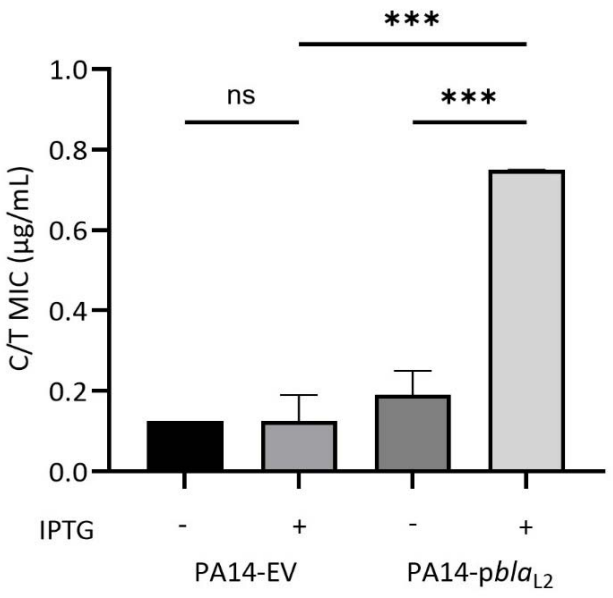

Fig. 3. Effect of exogenous expression of *bla*$_{L2}$ in *P. aeruginosa* strain PA14 on C/T MIC. C/T MICs are shown for the strains PA14-EV and PA14-*pbla*$_{L2}$ with (+) or without (-) IPTG as determined by antibiotic strip tests. The bars represent median MIC values of 3 biological replicates with confidence intervals shown as error bars. One-way ANOVA was used to determine statistical significance, indicated as *p* values, where ns = $p > 0.05$ and *** = $p < 0.001$. Abbreviations- EV, empty vector; MIC, minimal inhibitory concentration; IPTG, isopropyl-β-D-thiogalactopyranoside.

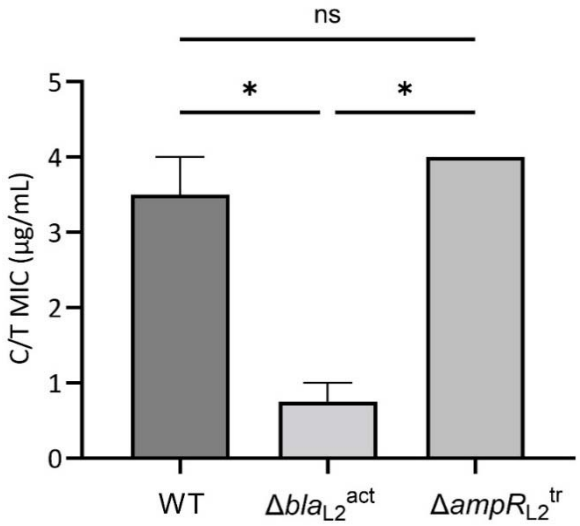

Fig. 4. C/T MICs for a parental PS2045 strain compared to PS2045 strains with disruptions of the *bla*$_{L2}$ or *ampR*$_{L2}^{tr}$ genes. C/T MICs in the parental (WT) and mutant strains of the clinical isolate PS2045 were determined by antibiotic strip tests. Disruption of the *bla*$_{L2}$ gene (Δ*bla*$_{L2}^{act}$) significantly reduced C/T MICs, whereas deletion of the *ampR*$_{L2}^{tr}$ gene (Δ*ampR*$_{L2}^{tr}$) had no effect. The bars represent median MIC values from 2 biological replicates, and error bars represent confidence intervals. One-way ANOVA was used to determine statistical significance, indicated as *p* values, where ns = $p > 0.05$ and * = $p < 0.05$.

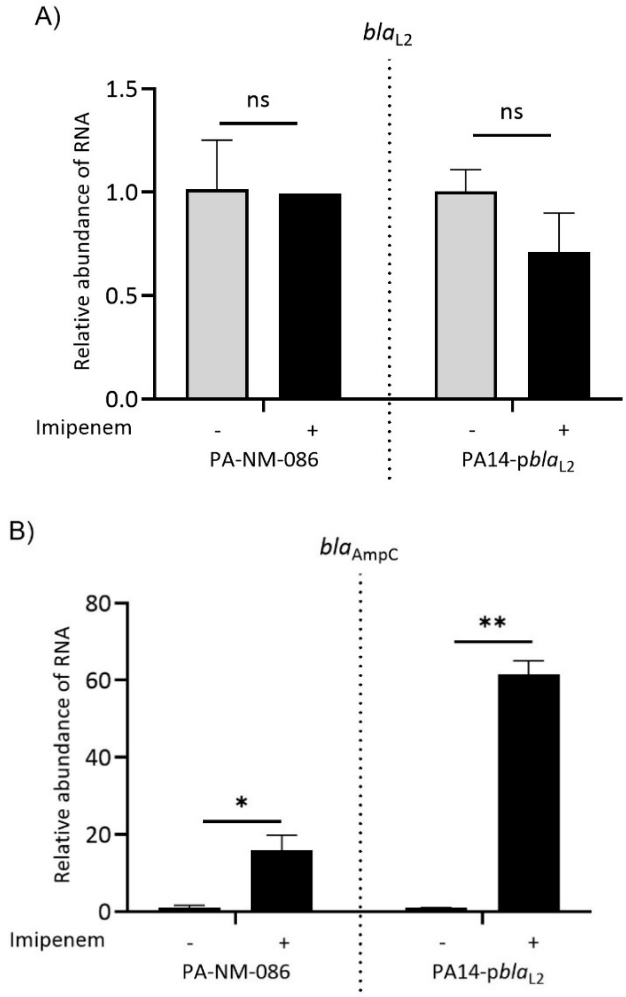

Fig. 5. Expression of *bla*$_{L2}$ and *bla*$_{AmpC}$ in the presence and absence of imipenem. Expression of the genes **A)** *bla*$_{L2}$ and **B)** *bla*$_{AmpC}$ in the strains PA-NM-086 and PA14-*pbla*$_{L2}$ in the presence (+) or absence (-) of imipenem was determined by RT-qPCR. Gene expression is shown as relative abundance in RNA levels in comparison to the respective untreated control (-) after normalizing to the house keeping gene *rpoD*. The bars represent means of 2 biological replicates. Error bars represent standard deviations. Student's t test was used to determine statistical significance, indicated as *p* values, where ns = $p > 0.05$, * = $p < 0.05$, and ** = $p < 0.01$.