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Penicillin-binding protein 3 is a common adaptive target among *Pseudomonas aeruginosa* isolates from adult cystic fibrosis patients treated with β-lactams

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ABSTRACT

Objective: Determining the mechanisms that modulate β-lactam resistance in clinical *Pseudomonas aeruginosa* (*P. aeruginosa*) isolates can be challenging, as the molecular profiles identified in mutation-based or expression-based resistance determinant screens may not correlate with in vitro phenotypes. One of the lesser studied resistance mechanisms in *P. aeruginosa* is the modification of penicillin-binding protein 3 (*pbpB/ftsI*). This study reported that nonsynonymous polymorphisms within *pbpB* frequently occur among β-lactam resistant sputum isolates, and are associated with unique antibiotic susceptibility patterns.
Methods: Longitudinally collected isolates (n = 126) from cystic fibrosis (CF) patients with or without recent β-lactam therapy or of non-clinical origin were tested for susceptibility to six β-lactams (aztreonam, ceftazidime, cefsulodin, cefepime, meropenem, and piperacillin). Known β-lactam resistance mechanisms were characterised by polymerase chain reaction (PCR)-based methods, and polymorphisms in the transpeptidase-encoding domain of *pbpB* identified by sequencing.
Results: Twelve nonsynonymous polymorphisms were detected among 86 isolates (67%) from five CF patients with a history of β-lactam therapy, compared with one polymorphism in 30 (3.3%) from three patients who had not received β-lactam treatments. No nonsynonymous polymorphisms were found in ten environmental isolates. Multiple *pbpB* alleles, often with different combinations of polymorphisms, were detected within the population of strains from each CF patient for up to 2.6 years. Traditional patterns of *ampC* or *mexA* de-repression reduced expression of *oprD* or the presence of extended-spectrum β-lactamases were not observed in resistant isolates with nonsynonymous polymorphisms in *pbpB*.
Conclusion: This study's findings suggest that *pbpB* is a common adaptive target, and may contribute to the development of β-lactam resistance in *P. aeruginosa*.

1. Introduction

The Gram-negative bacterial pathogen *Pseudomonas aeruginosa* (*P. aeruginosa*) is the aetiological agent frequently responsible for a variety of difficult to treat infections, ranging from complicated urinary tract infections to chronic pneumonias [1,2]. Chronic pulmonary infections caused by *P. aeruginosa* are of particular importance, as they are associated with high rates of mortality and respiratory failure among individuals with the genetic disease cystic

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fibrosis (CF). Treating pseudomonal infections is a challenging aspect of CF patient care, as the increased frequency of antimicrobial therapy that accompanies lung disease progression can select for resistant *P. aeruginosa* variants within an infecting population [3-5].

β-lactam antibiotics (e.g. carbapenems, cephalosporins, monobactams) are commonly administered to suppress pseudomonal burden and treat episodes of pulmonary exacerbation in CF patients with *P. aeruginosa* infections [2]. The β-lactams target membrane-associated penicillin-binding proteins (PBPs) that are involved in the synthesis and recycling of peptidoglycan, a key structural component of both the Gram-positive and Gram-negative bacterial cell wall [6]. β-lactam binding to PBPs initiates their bactericidal activity by disrupting peptidoglycan synthesis and cell division, leading either to cell lysis, or if the bacterium survives, to the induction of a variety of adaptive processes [7]. Distinct β-lactam molecules have affinity for different PBPs, and their binding profiles vary between bacterial species [8,9]. To date, at least nine PBPs have been described in *P. aeruginosa* [10-12]. High molecular mass (HMM) PBPs (1a to 3x) possess transglycosylase and/or transpeptidase activity to elongate and cross-link muropeptide chains, while low molecular mass (LMM) PBPs (4–7) possess endopeptidase and carboxypeptidase activity and regulate cell wall recycling [6,12]. Penicillin-binding protein 3 (PBP3 or FtsI) of *P. aeruginosa*, an essential PBP encoded by the *pbpB* (or *ftsI*) gene [13], is the PBP to which many β-lactams, including the monobactams (e.g. aztreonam) and some later-generation cephalosporins (e.g. ceftazidime), have the highest affinity [8,14].

Pseudomonas aeruginosa commonly develops β-lactam resistance by: (i) acquiring mutations in the intragenic and/or regulatory regions of one or more intrinsically encoded resistance determinants, or (ii) the uptake of plasmid-borne, transposon-borne, or integron-borne β-lactam hydrolysing elements such as extended-spectrum β-lactamases (ESBLs) [1]. Resistance-associated mutations typically modulate the expression of the chromosomal cephalosporinase AmpC, multidrug efflux pump systems, particularly MexAB-OprM, and outer membrane porin channel proteins such as OprD [3,14,15]. However, predicting the mechanisms responsible for β-lactam resistance among *P. aeruginosa* on the basis of strain genotype is often challenging, particularly for CF isolates, as phenotype-genotype relationships and expression patterns of known resistance determinants can be highly discordant [16-18]. These discrepancies suggest that additional genetic determinants beyond traditional well-characterised loci are likely involved in the development of β-lactam resistance in *P. aeruginosa*.

A potential contributor to β-lactam resistance that is often overlooked in *P. aeruginosa* is mutation of the HMM PBPs. While characterising resistance-associated mutations among *P. aeruginosa* recovered from a CF patient treated aggressively with β-lactams, associations between *pbpB* alleles carrying novel nonsynonymous polymorphisms (leading to A244T, V465G and P527S substitutions) and distinct patterns of susceptibility to monobactam and cephalosporin antibiotics have previously been identified [4]. Modification of the HMM PBPs is an important mechanism of β-lactam resistance in many bacterial species [6,19-22]; however, the frequency and significance of such changes to *pbpB* in *P. aeruginosa* is unknown, as the sequence of *pbpB* (encoding PBP3) is often not directly interrogated in screens of resistance mechanisms among clinical isolates [3,5,15,23]. Previous speculation has suggested that PBP3 modifications may be modulators of β-lactam resistance in *P. aeruginosa* [11,24,25], although, associations between nonsynonymous polymorphisms in HMM PBPs such as PBP3 and elevated β-lactam minimum inhibitory concentrations (MICs) have yet to be examined in any detail.

The present study sought to: (i) investigate the prevalence of polymorphisms in *pbpB* among *P. aeruginosa* isolated from a population of adult CF patients; (ii) examine the relationship between *pbpB* genotype and in vitro susceptibility to β-lactam antibiotics; and (iii) measure the expression of other β-lactam resistance determinants to assess their relative contribution to *P. aeruginosa* MICs in these populations. It was hypothesised that nonsynonymous polymorphisms in *pbpB* are common among *P. aeruginosa* populations that have been exposed to β-lactam treatments and may contribute to β-lactam resistance.

2. Materials and Methods

2.1. Bacterial isolates and culture conditions

This study was conducted with Research Ethics Board approval from the University Health Network (Toronto, Canada) and St. Michael's Hospital (Toronto, Canada) (Protocols #09-0420-T and #09-289, respectively). Clinical *P. aeruginosa* isolates were previously cultured from sputum produced by adults with CF being followed at St. Michael's Hospital (Toronto, Canada) between 2010 and 2014. Patients who were *P. aeruginosa* positive were evaluated on the basis of having: (i) multiple *P. aeruginosa* isolates cultured from sequential sputum specimens and (ii) the administration (or lack thereof) of any β-lactam or β-lactam/β-lactamase inhibitor antibiotic combinations (both with and without anti-pseudomonal activity) within the study period and in the year preceding the initial study specimen. At least 10 *P. aeruginosa* isolates were randomly selected from the collection of isolates banked from each CF patient matching these criteria. Ten additional *P. aeruginosa* isolates of environmental origin were used as non-clinical comparators. All isolates were grown on Luria-Bertani (LB) agar (Wisent Inc., QC, Canada) for 48 hours at 37 °C.

2.2. Antimicrobial susceptibility testing

All *P. aeruginosa* isolates were susceptibility tested by agar dilution, in accordance with Clinical and Laboratory Standards Institute procedures as previously described [26]. The MIC, the lowest concentration of each antibiotic to inhibit *P. aeruginosa* growth, was determined for a panel of β-lactam antibiotics with different binding affinities for PBP3: aztreonam (ATM), ceftazidime (CAZ), cefsulodin (CFS), cefepime (FEP) (Alfa Aesar, MA, USA), meropenem (MEM) (TCI America, OR, USA) and piperacillin (PIP) (Sigma Aldrich, MO, USA), and was reported as the median of three biological replicates.

2.3. Sequencing of pbpB

Genomic deoxyribonucleic acid (DNA) was purified from *P. aeruginosa* isolates grown in LB broth (Wisent Inc., QC, Canada) using the DNeasy® Blood and Tissue kit (Qiagen, CA, USA). The full-length *pbpB* gene (PA4418) was amplified with Q5® High Fidelity DNA polymerase (New England Biolabs Ltd., MA, USA) using the PBP3F and PBP3R primers described previously [14], with a modified polymerase chain reaction (PCR) protocol (Table 1). Amplicons were gel purified using the Nucleospin® Gel and PCR Cleanup Kit (Machery-Nagel Inc., PA, USA).

Purified amplicons were sequenced using primers flanking nucleotides 975–1740 of *pbpB* (Table 1). Primers were designed using PrimerQuest® (https://www.idtdna.com/Primerquest/Home/Index). Sequencing reactions were performed using the Big Dye® Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, USA) with a modified cycling program (Table 1). Amplicons were Sanger sequenced on the 3730 Genetic Analyzer (Thermo Fisher Scientific, CA, USA) at the Centre for the Analysis of Genome Evolution and Function (Toronto, Canada).

Table 1
Primers used to interrogate sequence variation in *pbpB of Pseudomonas aeruginosa*.

Primer name	Length (bp)	Sequence (5′ → 3′)	Amplicon Size (bp)	Protocol	Reference
PCR amplification of *pbpB*					
PBP3F	19	GGCCGGTTGATTCTCGAGC	1,950	98 °C - 30 s; 35 cycles of	[14]
PBP3R	19	GGTCAGCTCGCGGATCAGC		98 °C - 10 s, 63 °C - 10 s,	
				72 °C - 20 s; 72 °C - 2 min	
Sanger sequencing of *pbpB* amplicons					
975	17	CGGCCGCTACACCATTC	N/A	96 °C - 1 min; 45 cycles of	This study
1276	17	GCCAACGACGGCAAGAG	N/A	96 °C - 10 s, 50 °C - 5 s, 60	This study
−1740	22	TCAGCCACGCCCTCCTTTTGCG	N/A	°C - 4 min; 60 °C - 4 min	This study
−1079	20	CGAAGGCGATCTTGCTGATG	N/A		This study
qPCR analysis of β-lactam resistance determinant expression					
ampC1	22	CGGCTCGGTGAGCAAGACCTTC	218	95 °C - 30 s; 40 cycles of	[27]
ampC2	22	AGTCGCGGATCTGTGCCTGGTC		95 °C - 20 s, 60 °C - 20 s	
mexA1	23	CGACCAGGCCGTGAGCAAGCAGC	316	and 72 °C - 30 s	[27]
mexA2	23	GGAGACCTTCGCCGCGTTGTCGC			
oprD-For	19	CGGCGACATCAGCAACACC	194		[18]
oprD-Rev	19	GGGCCGTTGAAGTCGGAGTA			
rpsL-F	21	GCAAGCGCATGGTCGACAAGA	201		[27]
rpsL-R	23	CGCTGTGCTCTTGCAGGTTGTGA			

2.4. Mutational analysis of pbpB sequences

Sequence traces were assembled with CodonCode Aligner v.6.0.2 (CodonCode Corporation, MA, USA), using the *pbpB* allele from the PAO1 strain as a reference. Multiple sequence alignments were performed by MUSCLE in the MEGA software (v 7.0.26) (http://www.megasoftware.net/). Synonymous and nonsynonymous polymorphisms, insertions and deletions were identified by sequence comparison with the reference allele.

The PROVEAN Protein software (http://provean.jcvi.org/index.php) was used to predict whether nonsynonymous *pbpB* variants could affect the function of the PBP3 protein. Amino acid changes were identified as neutral or deleterious if the PROVEAN score was above or below the default –2.5 threshold, respectively. All amino acid variants were mapped to the predicted PBP3 crystal structure in complex with ATM (PDB ID: 3PBS) using the UCSF Chimera software (v 1.12) (http://www.rbvi.ucsf.edu/chimera).

2.5. RNA isolation and qPCR

Total ribonucleic acid (RNA) was isolated from triplicate mid-logarithmic phase cultures ($OD_{600} = 0.5$) grown in Mueller-Hinton broth using the PureLink® RNA Mini kit (Life Technologies Inc., ON, Canada) according to the manufacturer's protocol. Five micrograms of each RNA preparation were converted to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA) as per the manufacturer's protocol.

Changes in the expression of the *ampC, mexA* and *oprD* genes were measured by qPCR. Triplicate cDNA preparations were amplified using the FastStart® Universal SYBR Green Master (Rox) kit (Roche Diagnostics GmbH, Mannheim, Germany) with a modified cycling protocol (Table 1). A CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., CA, USA) instrument was used for quantitation. Differences in relative gene expression, measured as fold change relative to PAO1, were determined by the $\Delta\Delta C_T$ method [28], with the *rpsL* gene used for normalisation. A $\geq$ 10-fold (*ampC*) or $\geq$ 2-fold (*mexA*) increase relative to PAO1 was considered overexpression, while $\leq$ 70% expression that of PAO1 was a reduction (*oprD*) [18,29].

2.6. PCR-based detection of extended spectrum β-lactamases and carbapenemases

Isolates were screened for the presence of ESBLs and carbapenemases using the Multiplex I ($bla_{TEM,SHV,OXA}$), II (bla_{CTX-M}), IV ($bla_{GES,PER,VEB}$), V (bla_{OXA-48}) and VI ($bla_{IMP,\ VIM,\ KPC}$) primer sets described previously [30]. Total DNA was amplified with modification using the Q5® High Fidelity DNA polymerase (New England Biolabs Ltd., MA, USA) following a protocol of denaturation at 98 °C for 30 seconds, followed by 30 cycles of 98 °C for 10 seconds, 60 °C for 20 seconds, 72 °C for 20 seconds, and elongation at 72 °C for 2 minutes and 30 seconds. Amplicons were purified as described earlier (Section 2.3) and Sanger sequenced using the corresponding forward and reverse primers for each enzyme where applicable [30].

2.7. Statistical analysis

Statistical analyses were performed in GraphPad Prism 7 (GraphPad Software Inc, CA, USA). Linear regression, Spearman correlation, χ^2 or Kruskal-Wallis tests with Dunn's Multiple Comparison correction were used to compare phenotypes, genotypes and gene expression patterns, as appropriate. Associations between polymorphisms and MIC were assessed by Fisher's exact tests with Bonferroni correction. A $P < 0.05$ was considered significant at a 95% confidence interval.

3. Results

3.1. Variations in minimum inhibitory concentrations among isolates

A total of 126 *P. aeruginosa* isolates were examined from chronically infected adult CF patients with diverse clinical treatment histories (n = 116) and the natural environment (n = 10) (Fig. S1, Table S1). Isolates were stratified into three study cohorts as follows: (i) β-lactam exposed (recovered from CF patients who were treated with one or more β-lactams within the study period) (n = 5 patients, n = 86 isolates); (ii) β-lactam naïve (from CF patients that had not received β-lactam therapy in the year prior to the initial isolate or during the study period) (n = 3 patients, n = 30 isolates); and (iii) environmental (non-clinical origin) (n = 10, n = 10 isolates). There was no difference in age ($P = 0.312$, Student's *t*-test) or lung function ($P = 0.7886$, Student's *t*-test) between patient cohorts (Fig. S1).

Pseudomonas aeruginosa strains recovered from β-lactam treated patients displayed higher median MICs for the six β-lactam antibiotics from four distinct sub-classes: ATM (monobactam), CAZ (third-generation cephalosporin), CFS (third-generation cephalosporin), FEP (fourth-generation cephalosporin), MEM (carbapenem) and PIP (ureidopenicillin) (Table 2). Isolates from the β-lactam exposed group displayed the highest median MICs for three PBP3-specific antibiotics, ATM, CFS and FEP, which

Table 2
Characteristics of Pseudomonas *aeruginosa* isolate collection.

	β-lactam exposed	β-lactam naïve	Environmental
Study cohort			
Number of patients	5	3	-
Isolates per patient	17.2 (15–20)	10 (10)	-
Total isolates	86	30	10
Median MIC (μg/mL)[a]			
ATM	64 (≤8–1024)	≤8 (≤8–512)	≤8 (≤8–16)
CAZ	32 (≤8–32)	≤8 (≤8–128)	≤8 (≤8)
CFS	64 (≤8–1024)	≤8 (≤8–32)	≤8 (≤8–16)
FEP	64 (≤8–1024)	16 (≤8–64)	≤8 (≤8)
MEM	4 (≤1–64)	2 (≤1–8)	1 (≤1–2)
PIP	128 (≤8–1024)	32 (≤8–512)	≤8 (≤8–16)
Type of polymorphism[b]			
Synonymous	15 (0.17)	13 (0.43)	13 (1.3)
Nonsynonymous	12 (0.14)	1 (0.03)	0 (0)
Total	27 (0.31)	14 (0.47)	13 (1.3)
Allelic variants of *pbpB*[b]			
pbpB alleles per patient	7.6 (0.09)	3.3 (0.11)	-
Total	35 (0.41)	10 (0.33)	9 (0.9)

[a] Median MICs are displayed as the median and (range).
[b] Polymorphisms and alleles are shown normalised by number of isolates in parentheses.

were elevated by at least three two-fold dilutions relative to β-lactam naïve ($P < 0.001$ for ATM and CFS, Kruskal-Wallis test) or environmental isolates ($P = 0.003$ for ATM and $P < 0.001$ for CFS, Kruskal-Wallis test). Variations in MIC were noted among isolates within patient and between patient cohorts; however, β-lactam exposed MICs were often at or above the agar dilution breakpoints defined by the Clinical & Laboratory Standards Institute for ATM, CAZ, FEP and PIP, whereas median MICs for the β-lactam naïve and environmental cohorts were characteristically low across the test panel.

3.2. Polymorphisms in the Pseudomonas aeruginosa pbpB gene

This study interrogated the transpeptidase-encoding domain to which antibiotics and peptidoglycan monomers bind (residues 225 to 579) [31] to identify all potential sequence variations. High-quality sequences were obtained for a 677 bp partial region spanning nucleotides 1018–1695 (residues 340–565). Sequence polymorphisms were found in *pbpB* alleles from all three study groups (Fig. S2). Fifty unique *pbpB* alleles were reported among all 126 *P. aeruginosa* isolates, with 28 synonymous and 13 nonsynonymous polymorphisms being noted at 41 independent nucleotides within the transpeptidase-encoding region (Table 2). Nonsynonymous polymorphisms were found nearly exclusively among strains exposed to β-lactams, with 35, 10, and 9 alleles defined by 12, 1, and 0 nonsynonymous polymorphisms among β-lactam exposed, β-lactam naïve, and environmental strains respectively ($P = 0.02$, χ^2 test). Multiple *pbpB* alleles were consistently detected within the population of *P. aeruginosa* strains from each patient (Table 2); however, three, including the reference allele, appeared in multiple patient isolates.

Pseudomonas aeruginosa from all five β-lactam treated patients carried *pbpB* alleles with up to four independent nonsynonymous polymorphisms segregating within the population of strains carried by a single patient (Fig. 1). Twelve of these polymorphisms were found only in β-lactam exposed isolates, with the average pool of *pbpB* alleles from each set of patient isolates carrying 2.8 nonsynonymous polymorphisms (range, 1–4 per population) (Table 2). Nonsynonymous polymorphisms varied in abundance within and between β-lactam exposed patients (Fig. 1); however, there was a high probability of isolating *P. aeruginosa* with a *pbpB* allele carrying a nonsynonymous variant from β-lactam exposed sputum specimens (Table S1). In four of the five β-lactam exposed populations, multiple nonsynonymous polymorphisms were found in at least one allele (range, 1–3). A positive statistical association was found between the number of nonsynonymous polymorphisms per allele and MICs for five of the six antibiotics by regression analysis (ATM $R^2 = 0.26$, $P < 0.001$; CAZ $R^2 = 0.11$, $P < 0.001$; CFS $R^2 = 0.38$, $P < 0.001$; FEP $R^2 = 0.10$, $P = 0.004$; PIP $R^2 = 0.20$, $P < 0.001$). The nonsynonymous polymorphisms of highest frequency in the dataset encoded substitutions between amino acid residues 454 and 527 of PBP3 (Fig. 1), with R504C identified in *pbpB* alleles in multiple β-lactam exposed patients. The remaining variants were unique to isolates from different patients and varied in abundance (range, 1.1–41.2% of the isolates) (Fig. 1).

Table 3
Amino acid substitutions detected in cystic fibrosis *Pseudomonas aeruginosa* isolates.

Variant	Median MIC (μg/mL)						Type[a]	PROVEAN Score
	ATM	CAZ	CFS	FEP	MEM	PIP		(Predicted Effect)[b]
N427S	8	8	8	16	2	8	S	−2.76 (D)
S368L	8	8	8	256	4	8	R-I	−3.01 (D)
S538L	8	16	64	64	4	32	R-II	−2.96 (D)
Q475R	16	16	128	64	4	256	R-III	−0.18 (N)
L434V	128	32	64	128	4	8	R-IV	0.454 (N)
A454V	512	32	512	16	4	512	R-IV	−3.25 (D)
L461V	64	32	32	8	4	128	R-IV	−2.35 (N)
R504C	16	32	512	64	4	256	R-IV	−3.05 (D)
P527S	1024	32	64	128	1	32	R-IV	−7.99 (D)
H394R	1024	32	64	256	1	128	R-V	−1.1 (N)
A419G	1024	32	64	128	8	64	R-V	−3.91 (D)
N427K	1024	32	64	128	8	64	R-V	−4.28 (D)
F507L	128	32	256	64	4	256	R-V	−5.94 (D)

[a] Elevated MICs for 0, 1, 2, 3, 4, 5 β-lactams designated as S, R-I, R-II, R-III, R-IV, and R-V.
[b] PROVEAN score of −2.5 was used to predict potential deleterious (D) or neutral (N) effects.

3.3. Polymorphisms in pbpB and elevated minimum inhibitory concentrations

Nonsynonymous polymorphisms in PBP3 were associated with elevated median MICs for all six β-lactams (Fig. 2). These polymorphisms were further categorised into one of six groups, with each group being defined by the susceptibility profile of the corresponding isolates. Subgroups were denoted as: (i) susceptible to all six β-lactams tested (Type S) or (ii) displaying reduced susceptibility for between one (Type R-I) and five (Type R-V) of ATM, CAZ, CFS, FEP, MEM or PIP (Table 3). The four nonsynonymous polymor-

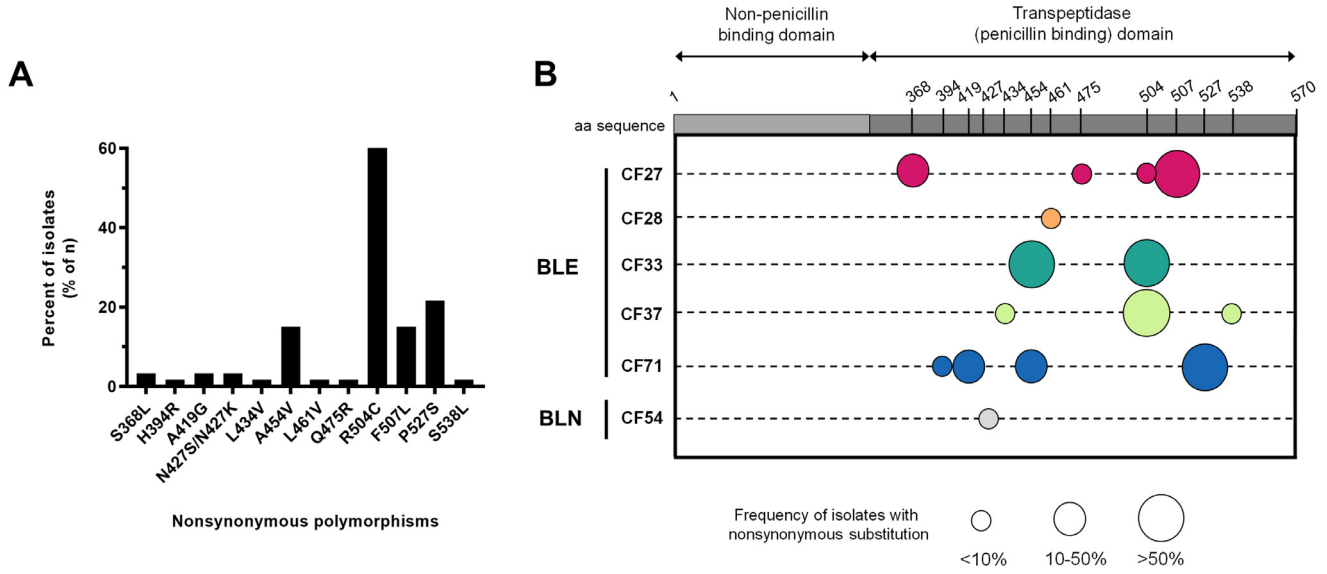

Figure 1. Frequency of nonsynonymous polymorphisms identified in *pbpB* among cystic fibrosis *P. aeruginosa*. Substitutions were found to vary in frequency both (A) within the sequenced isolate collection (n = 60 with amino-acid modifying polymorphisms) and (B) by patient of origin. Patients CF27, CF28, CF33, CF37 and CF71 formed the β-lactam exposed (BLE) group, while CF54 was one of three patients classified as β-lactam naïve (BLN).

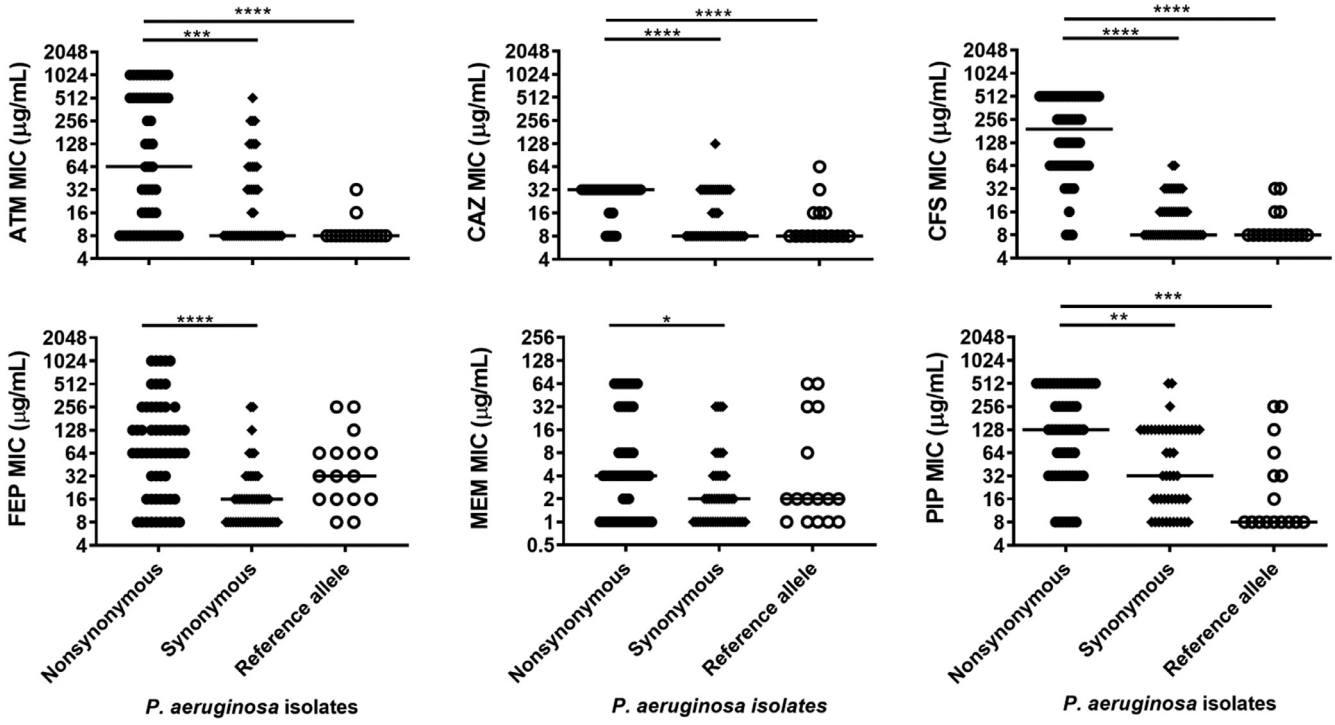

Figure 2. Polymorphisms in *pbpB* were found in isolates with increased median MICs for six β-lactams. Isolates carrying *pbpB* alleles with nonsynonymous polymorphism(s) display elevated median MICs (horizontal line) for (A) ATM, (B) CAZ, (C) CFS, (D) FEP, (E) MEM, and (F) PIP. The statistical significance of each comparison (Kruskal-Wallis test) is denoted by * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P < 0.001$. Isolates were grouped by whether they carried an allele with either nonsynonymous (n = 60), synonymous (n = 50) or no changes (reference *pbpB* allele) (n = 16) relative to that found in PAO1. Isolates where both nonsynonymous and synonymous polymorphisms were found in the same *pbpB* allele were included in the nonsynonymous category.

phisms of highest prevalence in the dataset encoded substitutions within the RIV and RV groups (A454V, R504C, F507L and P527S) and were detectable within the respective β-lactam exposed *P. aeruginosa* populations for up to 946 days from the initial specimen in which they were first reported (Table S1). The majority of these amino acid changes were predicted to alter protein function (i.e. be deleterious), with PROVEAN scores of ≤ -2.5 (Fig. 3). Six of the predicted deleterious substitutions were from the R-IV and R-V groups, with isolates carrying elevated MICs for nearly all of the β-lactams that were tested.

The binding of β-lactam antibiotics to PBP3 is mediated by interactions with three conserved motifs, SXXK (residues 294–297, α2), SXN (residues 349–351, α4-α5 turn), and KSGT (residues 484–487, β3) (Fig. 3). To examine whether regions of the transpeptidase domain involved in antibiotic-PBP3 interactions were more susceptible to mutational change, each polymorphism was

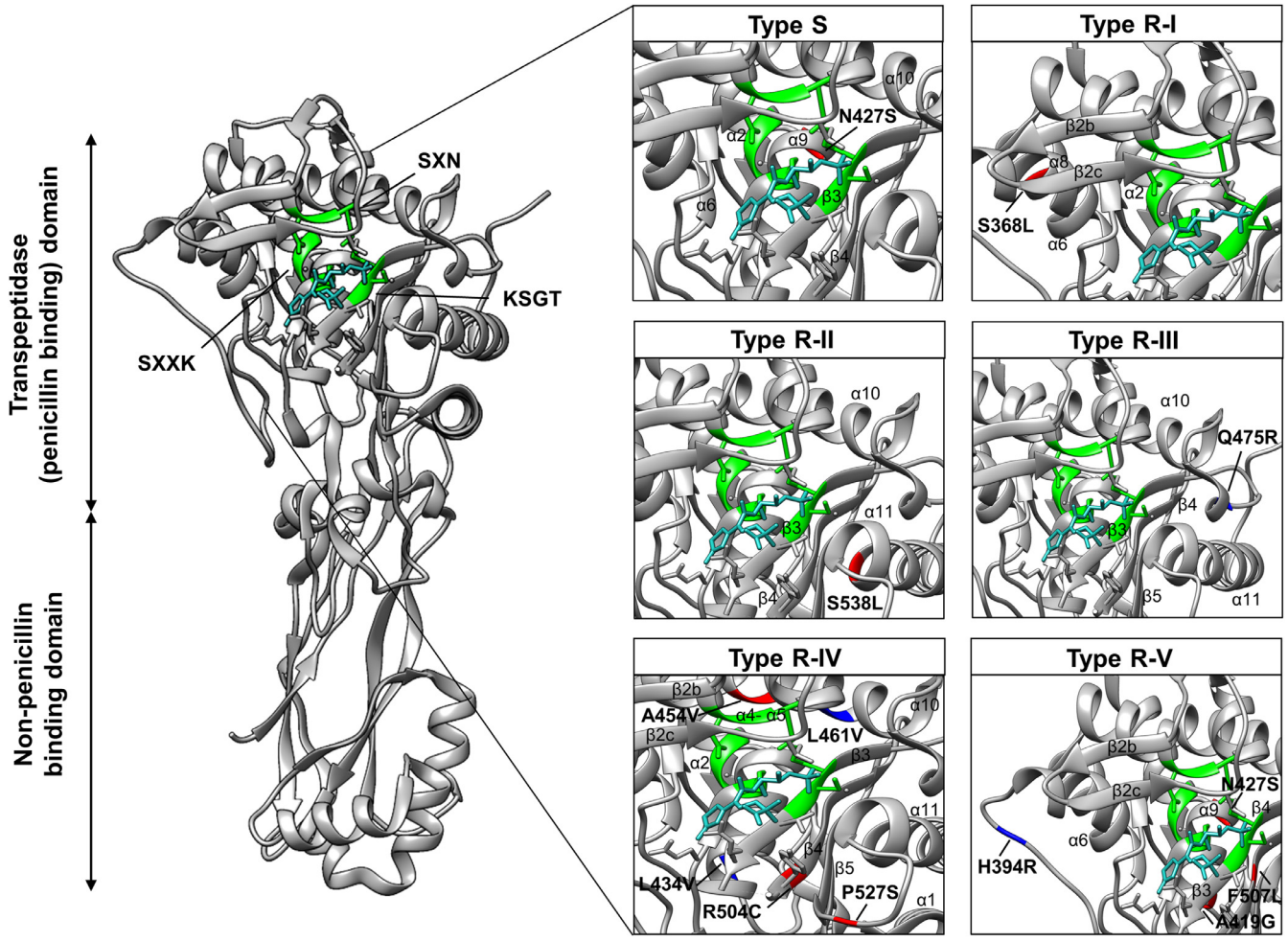

Figure 3. Amino acid residues affected by nonsynonymous polymorphisms surround the binding cleft of PBP3. The predicted substitutions resulting from the 13 nonsynonymous polymorphisms identified within the transpeptidase domain of PBP3 were grouped by their β-lactam susceptibility profiles of Type S or Type R-I to R-V representing susceptibility or resistance to 1–5 of the β-lactam antibiotics tested. Residues where polymorphisms were predicted to be deleterious by PROVEAN are shown in red, while those that are neutral are shown in blue. The conserved SXXK, SXN and KSGT motifs are highlighted in green. The X-ray crystal structure of PBP3 (PDB ID: 3PBS) [31] is shown in complex with a molecule of ATM (light blue).

superimposed onto the predicted crystal structure [31]. None of the 13 non-synonymous polymorphisms directly involved residues within the conserved motifs, but were rather located in features within close proximity (Fig. 3). The predicted amino acid changes from either the Type R-IV or R-V groups affected residues along the α9 and α10 helices, β4 strand or β5-α11 loop, with those of highest prevalence (A454V, R504C, F507L and P527S) being situated in regions known to experience antibiotic-induced conformational change [31,32]. A strong association with resistance to one or more β-lactams was identified for polymorphisms resulting in the R504C and P527S substitutions, with R504C being associated with elevated MICs for third-generation cephalosporins (CAZ, CFS, $P <$ 0.001, Bonferroni corrected Fisher's exact test), while the P527S variant displayed a similar trend with monobactams (ATM), as well as both third-generation and fourth-generation cephalosporins (CAZ, CFS, FEP) ($P <$ 0.001, Bonferroni corrected Fisher's exact test).

3.4. β-lactam resistance determinant expression is discordant from resistance

This study used qPCR to determine whether differences in the expression of other, better characterised resistance factors could explain MICs. While there were subtle differences in basal expression of *ampC, mexA* and *oprD* genes, which encode the primary intrinsic β-lactam resistance mechanisms AmpC, MexAB-OprM, and OprD, compared with the PAO1 laboratory strain, the altered transcription of these three targets was not associated with the presence of polymorphism(s) in *pbpB* (Fig. S3). Similarly, the expression of *ampC, mexA* or *oprD* was weakly correlated with isolate MIC for monobactams and cephalosporins (Table S2).

Of the seven acquired β-lactamase and four carbapenemase enzyme families screened by PCR, TEM and OXA class β-lactamases were only detected (Table S1). The same TEM β-lactamase of Type 2b, which was sequence-confirmed in a small number of isolates (data not shown), was carried in isolates from both β-lactam exposed (78%) and β-lactam naïve patients (7%). This enzyme was not associated with resistance to any of the six β-lactam antibiotics tested (ATM $P = 0.14$; CAZ $P = 0.40$; CFS $P = 0.93$; FEP $P = 0.99$; MEM $P = 0.71$; PIP $P = 0.28$, χ^2 test). A single isolate possessed an oxacillinase that is not naturally occurring in the *P. aeruginosa* genome, and was susceptible to CFS, FEP and PIP.

3.5. pbpB *is a common adaptive target among clinically and laboratory evolved* Pseudomonas aeruginosa

The relationship between *pbpB* status and reduced β-lactam susceptibility in *P. aeruginosa* has, to date, been poorly characterised. This study surveyed recent *P. aeruginosa* genomics stud-

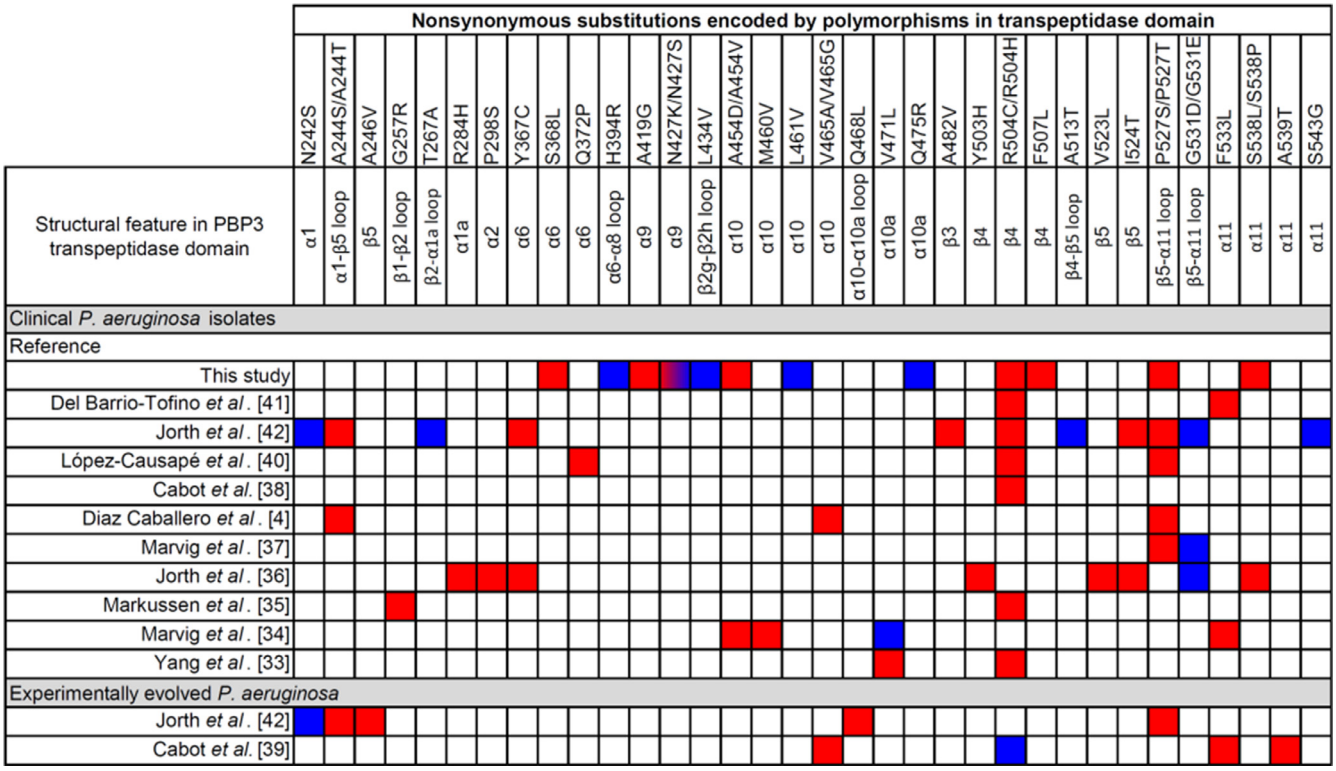

Figure 4. Catalogue of amino acid substitutions found in whole genome sequenced *P. aeruginosa*. Those polymorphisms that were predicted to encode deleterious substitutions by PROVEAN are shown in red, while those predicted to encode neutral substitutions are shown in blue.

ies to create a catalogue of polymorphisms from: (i) sequenced CF clinical isolates where limited colony sampling prevented statistical comparisons with MIC, or (ii) populations that were experimentally evolved under β-lactam selection. Eleven studies published between 2011 and 2017 identified polymorphisms in *ftsI/pbpB* isolates from sequenced *P. aeruginosa* populations (Fig. 4). Eleven amino acid residues were mutated in parallel across multiple independent studies both of CF patients and laboratory evolved strains (Fig. 4), with 28 additional nonsynonymous polymorphisms noted across studies [33-42]. In agreement with the current findings, the vast majority (74%) were also predicted to be deleterious by PROVEAN analysis and often affected the same functional structures of the PBP3 protein. In total, 34 residues within the PBP3 transpeptidase domain demonstrated 39 unique nonsynonymous polymorphisms across independent *P. aeruginosa* lineages.

6. Discussion

This study investigated the relationship between *pbpB* genotype and β-lactam susceptibility phenotype in *P. aeruginosa* populations from CF respiratory specimens. Nonsynonymous polymorphisms encoding substitutions in the transpeptidase domain of PBP3 were more frequently observed among *P. aeruginosa* that had been exposed to β-lactam antibiotics during the study period. This study confirmed that multiple *pbpB* alleles, often with unique polymorphism profiles, can be found within CF airway-adapted *P. aeruginosa* populations in moderately relative abundance, and some are maintained for extended periods of time.

The *pbpB* alleles carried by *P. aeruginosa* isolates from this collection could be distinguished by the β-lactam susceptibility profiles of the isolates, the residues affected by amino acid changes, and the predicted functional impact of these changes on β-lactam-PBP interactions. Mutations found in HMM PBPs in other bacterial species are typically found in residues that are in close proximity to the conserved SXXK, SXN and K(X)GT motifs, which can alter binding kinetics but nevertheless retain the ability to synthesise peptidoglycan [19-22]. Accordingly, it was noted that none of the 13 nonsynonymous polymorphisms identified in this study involved any of the three conserved PBP structural motifs. Instead, these polymorphisms affected residues in regions subject to antibiotic-induced conformational changes [31,32]. The main observations with respect to polymorphisms in *pbpB* in this isolate collection were that: (i) the number of nonsynonymous polymorphisms in an allele was directly related to MIC; (ii) many of the polymorphisms that were predicted to encode deleterious amino acid substitutions were typically present in isolates with elevated MICs and involved residues within structural elements that surround the PBP3 binding cleft; and (iii) the R504C and P527S variants were most strongly associated with reduced susceptibility for multiple different types of β-lactams.

The mechanism of PBP-mediated resistance involves reducing the affinity of β-lactam molecules for a PBP through alterations to the binding cleft, disrupting the interior network of chemical bonds and/or remodelling the active site conformation to occlude antibiotic binding [21,43]. In this study, many of the polymorphisms that were classified as Types R-IV and R-V surrounded the binding cleft. While PBP3-related MIC elevation in *P. aeruginosa* has been infrequently described, several studies have noted reduced binding of radiolabelled β-lactams or β-lactam analogs to PBP3 in PBP assays of strains that had been either serially passaged in vitro in the presence of cephalosporins [25] or recovered from a CF patient after high-dose tobramycin and piperacillin treatments [24]. Taken together, the location of the nonsynonymous polymorphisms, their predicted deleterious effects, and patterns of MIC elevation suggest that PBP3 modification has a similar role in the development of β-lactam resistance in *P. aeruginosa*.

Through the literature survey, it was noted that nonsynonymous polymorphisms occurring at the same positions, or in similar

regions of the transpeptidase domain, were identified in whole genome sequenced *P. aeruginosa* isolates from CF patients in Canada [4], Denmark [33-35,37] and the United States [36], as well as in laboratory strains that have been experimentally evolved in the presence of either ATM [42] or MEM [39]. Parallel patterns of mutation among different *P. aeruginosa* lineages suggest that specific genes or residues are functionally important [37]. It appears that polymorphisms leading to the R504C and P527S substitutions may be especially beneficial to these populations, given that they have been detected across several studies and were highly prevalent within the current longitudinal dataset. Both residues have known involvement in PBP3 antibiotic associated interactions, with R504 being situated next to T503, a β4 sheet residue whose positioning within the protein is affected by CAZ-induced conformational changes [31], while P527 is situated within the β5-α11 loop that has been shown to interact with both ATM and CAZ [31,32].

As β-lactams are a mainstay of antipseudomonal combination therapies, the current finding that *pbpB* modification may contribute to β-lactam resistance in *P. aeruginosa* is of great importance. Patterns of resistance in single clinical isolates (e.g. blood, sputum) are often characterised using a combination of disk-diffusion, transcript quantification, and sequence-based analysis of AmpC, OprD, MexAB-OprM and ESBLs [14,15,18,23]. As a result, the contribution of novel molecular targets such as *pbpB* would be missed by conventional approaches. In the current study, isolates with *pbpB* alleles carrying multiple nonsynonymous polymorphisms demonstrated elevated MICs in the absence of *ampC* and/or *mexA* overexpression and of ESBLs. Current data suggest that these previously uncharacterised determinants such as PBP3 could, in part, help to explain the discordance reported among some resistant strains, particularly in the absence of AmpC de-repression [18,38,39]. The implications of PBP-related polymorphisms in *P. aeruginosa* have only recently begun to be appreciated, with associations between mutation of LMM PBPs, increased AmpC expression and β-lactam resistance reported among various *P. aeruginosa* clinical isolates and antibiotic-passaged laboratory strains [14,23].

This study had several limitations. The patient cohorts that were examined were small and there may have been additional within-patient allelic diversity at the *pbpB* locus that was not captured at the current sampling depth. Similarly, it focused on CF-associated infections as a reservoir for PBP3-related polymorphisms, and their generalisability to other types of chronic *P. aeruginosa* infections is unknown. Further studies on larger cohorts of CF and non-CF *P. aeruginosa* from other body sites are needed to determine the prevalence of these polymorphisms across a broader range of infections. As PROVEAN offers only an in-silico prediction of the potential effects that these polymorphisms may have on PBP3 function, additional characterisation is required to delineate and quantify their influence on strain fitness under different antibiotic environments. Further in vitro manipulation is necessary to determine the effects of all PBP3 variants both alone and in combination, which to date has proven difficult as PBP3 is essential in *P. aeruginosa* [13]. However, the findings by others that PBP3 polymorphisms in *P. aeruginosa* strains experimentally evolved under selection by ATM or MEM [39,42] support the notion that PBP3 alterations may indeed be important contributors to β-lactam resistance in *P. aeruginosa*. On a broader level, this study adds support to the growing body of evidence suggesting that polymorphisms in loci outside of the traditional repertoire of resistance determinants in *P. aeruginosa* may have important roles in the clinical development of resistance.

In summary, *P. aeruginosa* populations sampled from the airways of β-lactam treated CF patients contain *pbpB* alleles with nonsynonymous polymorphisms in the transpeptidase domain that are associated with altered β-lactam susceptibility. Current data suggest that polymorphisms in the HMM PBP3 may play an important role in β-lactam resistance in *P. aeruginosa*.

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Competing Interests

Nil.

Ethical Approval

This study was conducted with Research Ethics Board approval from the University Health Network (Toronto, Canada) and St. Michael's Hospital (Toronto, Canada) (Protocols #09-0420-T and #09-289 respectively.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.01.009.

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