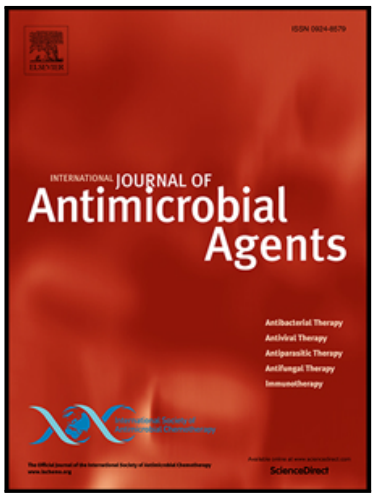

Phylogenetic analysis of resistance to ceftazidime/avibactam, ceftolozane/tazobactam and carbapenems in piperacillin/tazobactam-resistant Pseudomonas aeruginosa from cystic fibrosis patients

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HIGHLIGHTS

- Phylogenetic analysis of resistance in cystic fibrosis *P. aeruginosa*.
- AmpC variations are associated with high-level cloxacillin-insensitive ceftazidime resistance.
- AmpD variations are associated with ceftolozane/tazobactam and ceftazidime/avibactam double resistance.
- Mutational resistance emerged in phylogenetically separate lineages.
- Mutation-driven evolution in the population structure of *P. aeruginosa.*

Phylogenetic analysis of resistance to ceftazidime/avibactam, ceftolozane/tazobactam and carbapenems in piperacillin/tazobactam-resistant *Pseudomonas aeruginosa* from cystic fibrosis patients

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Key words: *Pseudomonas aeruginosa*, cystic fibrosis, ceftazidime/avibactam, ceftolozane/tazobactam, *ampC*, AmpD, *oprD,* mutation-driven evolution

ABSTRACT

Pseudomonas aeruginosa is one of the most important pathogens in cystic fibrosis. In this study we analysed the genetic basis and phylogenetic profile of resistance to ceftazidime/avibactam and ceftolozane/tazobactam as well as carbapenems in cystic fibrosis *P. aeruginosa* isolates. We conducted whole genome sequence analysis of a collection of isolates resistant to piperacillin/tazobactam from seven hospitals in Scotland since the introduction of these two cephalosporin/β-lactamase inhibitor combinations. Ceftazidime resistance was primarily related to AmpC induction, as tested by cloxacillin inhibition assays, while amino acid variations in AmpC were associated with high-level ceftazidime resistance not reversed by cloxacillin. Only isolates resistant to both ceftazidime/avibactam and ceftolozane/tazobactam carried AmpD mutations, likely resulting in *ampC* overexpression. All isolates resistant to ceftazidime/avibactam and/or ceftolozane/tazobactam were resistant to carbapenems and showed inactivating mutations in the chromosomal *oprD* gene. None of the isolates bore class A, B, D plasmid-encoded carbapenemases. Critically, we show that mutational resistance emerged in phylogenetically distant lineages suggesting that the mutations occur independently without conferring a selective advantage to any phylogenetic lineage. Our findings confirm the strong contribution of mutation-driven evolution to the population structure of *P. aeruginosa.*

1. INTRODUCTION

Pseudomonas aeruginosa remains one of the most important pathogens in cystic fibrosis. Being the biggest cause of morbidity and mortality in cystic fibrosis patients, *P. aeruginosa* status determines choices of prophylactic therapy as well as the treatment of pulmonary exacerbations (1, 2). Ceftazidime/avibactam and ceftolozane/tazobactam are cephalosporin/β-lactamase inhibitor combinations that have shown increased activity against *P. aeruginosa* in large multicentre studies (3, 4) and are now recommended in the UK as second line treatment for exacerbation of pulmonary infections where multidrug resistant strains are suspected.

Resistance to cephalosporins in *P. aeruginosa* is mainly related to chromosomally-encoded *ampC.* AmpC-mediated resistance may be non-mutational as a result of AmpC induction (5). However, the most commonly described mechanism of resistance to newer cephalosporins involves mutational derepression of *ampC* (6). In addition to *ampC* itself, genes involved in *ampC* overexpression include the *ampC* regulator *ampR*, *ampD* (amidase), *ampG* (muropeptide permease) and *dacB* (encoding PBP4) (7-10). On the other hand, resistance to ceftazidime/avibactam and ceftolozane/tazobactam requires deletions and mutations leading to structural modifications in AmpC, respectively (11, 12). Notwithstanding the higher stability of ceftolozane to hydrolysis by β-lactamases compared to piperacillin, variants of the *Pseudomonas*-derived cephalosporinase (PDC) have been associated with resistance to ceftolozane/tazobactam (13). A*mpC* derepression is also important for carbapenem resistance together with inactivation of porin protein D (OprD) (14). The efflux pump system MexAB-OprM has also been implicated in resistance to newer cephalosporins while MexXY-OprM is thought to exhibit a preferential role in resistance to carbapenems (15).

Development of multidrug resistance in *P. aeruginosa* lung isolates from cystic fibrosis patients has been mainly attributed to its ability to adapt to the cystic fibrosis airway microenvironment by multiple genotypic changes, hence the emphasis on intra-patient evolutionary isolate analyses (16). However, its ability to develop mutational resistance in the context of high selective pressure is also well known (17).

In this study we analysed the genetic basis and phylogenetic profile of resistance to ceftazidime/avibactam and ceftolozane/tazobactam as well as carbapenems in cystic fibrosis *P. aeruginosa*. The study focussed on a collection of multidrug resistant isolates from seven hospitals in Scotland since the introduction of these two cephalosporin/β-lactamase inhibitor combinations.

2. METHODS

2.1 Isolates

This study involved the analysis of twenty-four *Pseudomonas aeruginosa* received by the Cystic Fibrosis Antibiotic Susceptibility Testing Service (CFASS) at Aberdeen Royal Infirmary from four hospitals across Scotland. This nationally funded service performs antibiotic combination testing on multidrug resistant Gram-negative isolates from adult cystic fibrosis patients around Scotland. Isolates were purified and identified from sputum between May 2015 and November 2016. Isolates cultured from clinical samples received by the Medical Microbiology laboratory at Aberdeen Royal Infirmary underwent initial cytochrome C oxidase testing and were then formally identified using an automated mass spectrometry microbial identification system (VITEK® MS, Biomerieux, Marcy-l'Étoile, France). Isolates were randomly selected from our collection of piperacillin-tazobactam resistant isolates collected over the time period (resistance defined according to the EUCAST Clinical Breakpoint) and represented 31% of these isolates. Isolates 1600/1655, 1617/1618/1619 and 1663/1664 were from the same patient. Analysis focussed on piperacillin-tazobactam resistant isolates as piperacillin-tazobactam is the standard antipseudomonal agent used in Scottish hospitals, hence the anticipated higher multidrug resistance rates in such isolates.

2.2 Antimicrobial susceptibility testing and AmpC induction.

Isolates were cultured on Mueller-Hinton agar plates (Oxoid, Thermo Fisher Scientific, Loughborough, UK) and incubated aerobically at 37°C for 24 hours. From overnight culture, a suspension in normal saline (0.9%) was prepared for each isolate to reach a turbidity

equivalent to that of a 0.5 McFarland standard. The suspension was re-inoculated onto Mueller-Hinton agar plates to obtain a lawn culture. E-test strips (BioMérieux) of selected antimicrobial agents (ceftazidime, ceftazidime/avibactam, piperacillin/tazobactam, ceftolozane/tazobactam, ceftobiprole, imipenem, meropenem) were then placed on the plates which were incubated aerobically at 37°C for 24 hours. E-test MICs values for each antimicrobial agent were interpreted according to EUCAST 2018 breakpoint values. The inhibition zone was read from the edge of the strip showing no growth when viewed from the back of the plate against a light source.

To identify AmpC overproducers, Mueller-Hinton agar plates were prepared incorporating 1000 mg/L of cloxacillin in sterile agar media. The suspension with 0.5 McFarland standard for each isolate was inoculated on these plates. An E-test strip (BioMérieux, Marcy-l'Étoile, France) containing ceftazidime was placed in each plate and incubated at 37°C for 24 hours. Isolates were considered AmpC overproducers when ceftazidime MIC values decreased by at least two dilutions on Mueller-Hinton supplemented with cloxacillin (PMID: 19738025).

2.3 DNA extraction and sequencing

For RNA-free genomic DNA extraction, a single colony was sub-cultured in nutrient broth (Oxoid, Thermo Fisher Scientific, Loughborough, UK) and incubated aerobically at 37°C for 24 hours. Bacterial pellets were obtained by centrifuging 5 mL of an overnight bacterial broth culture for 5 minutes at 12,000–16,000 g. DNA was extracted using GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich Company Ltd., Dorset, UK) following manufacturer's instructions. DNA was quantified using Nano Drop Spectrophotometer disclosing 260/280 and 260/230 ratio values within the normal limits (Thermo Fisher Scientific). All samples ran on 0.7% agarose gel yielded distinct bands with no smearing. DNA samples were further purified using AMPure beads (Beckman Coulter, Brea, CA, USA).

Purified DNA was quantified using PicoGreen (Thermo Fisher Scientific) on the FLUOstar OPTIMA plate reader (BMG LABTECH Ltd. Bucks, UK). Input material was normalised to 500 ng prior to fragmentation and library preparation. Fragmentation was performed by

mechanical shearing to an average size of 350 bp using a MultiFunctional Bioprocessor (EpiSonic; amplitude 40, process time 3min 20sec, pulse on/off 20sec). Library preparation was performed using the NEBNext Ultra DNA library prep kit for Illumina (New England Biolabs) and standard Illumina multiplexing adapters with minor modifications to manufacturer's protocol. Libraries were PCR amplified (10 cycles) on a Tetrad (Bio-Rad) using in-house unique dual indexing primers as described previously (18). Post-PCR purification performed using Agencourt Ampure XP (Beckman Coulter; ratio 1:0.75). Individual libraries were normalised using PicoGreen (Thermo Fisher). Individual libraries were normalised and pooled together accordingly. The size profile of the pooled library was analysed on the 2200 or 4200 TapeStation. The pooled library was quantified using Qubit (Invitrogen) and diluted to ~10 nM for storage. The 10 nM library was denatured and further diluted prior to loading on the sequencer. Paired-end sequencing was performed using a HiSeq4000 150bp platform (Illumina, HiSeq 3000/4000 PE Cluster Kit and 300 cycle SBS Kit).

2.4. Genome assembly

The adapters and poor quality bases were removed from short paired-end reads using Trimmomatic (version 0.36) and trimmed reads were used for sequence assembly using the *de novo* assembly algorithm, SPAdes (version 3.9.0). The quality of the assembled genome was analysed using the program QUAST (version 4.3). The minimum size of output contiguous sequences (contigs) was 200 bp. We found one sample with a high number of contigs. This outlier has not been included in the following summary statistics. The average number of contigs for genomes sequenced in this study was 128 with an average total assembled genome size of 6,444,512 bp and an average N50 length of 178,878 bp and with a 110× depth coverage. Assembly statistics of genome sequences are shown in Supplementary Table S1.

2.5 Whole genome sequencing analysis

The draft genome was annotated using Prokka (version 1.11) and the accessory and core genes were identified by Roary (version 1.007001) using 80% of identity blastp. All core genes were aligned gene-by-gene using Muscle (version 3.8.31) and then concatenated using a custom script. The concatenated alignment was used to generate a ML core genome phylogenetic tree with a GTR model with gamma distribution of rate heterogeneity (GTR+G) using RAxML (version 8.2.12), and the pairwise SNP matrix was obtained with the program snp-dists version 0.6. The ggtree's version 1.13.1 R package was used for the visualization of the phylogenetic tree with integrated antimicrobial susceptibility data. The presence of resistant genes was scanned in the contigs in multifasta format using the program ABRicate version 0.8, which uses the database NCBI, resfinder and CARD, by setting identity at 80% in the blast. For the identification of single nucleotide polymorphisms (SNPs) in candidate genes, the trimmed reads were mapped to the PAO1 reference genome (NC_002516.2) using BWA-MEM (maximal exact match) (version 0.7.16a-r1181) with default parameters. SAM file-to-BAM file conversion was performed using SAMtools (version 1.3.1). SNPs and insertions or deletions (indels) were called using mpileup from Samtools and vcftools (version 0.1.15). The effect of the genetic variants (stop codon, missense or frameshift) was studied using SnpEff (version 4.3t). Additionally, predictive analyses of the impact of amino acid changes on gene functions were carried out using the PROVEAN web server tool which scores the impact as deleterious or neutral. The NCBI GenBank Bioproject ID for the genome data is PRJNA507097 (Table S1).

3. RESULTS AND DISCUSSION

3.1 Clonal structure

We have investigated a collection of twenty-four *P. aeruginosa* isolates from cystic fibrosis patients in Scotland selected on the basis of resistance to standard antipseudomonal agent piperacillin-tazobactam. Sequence typing and whole genome phylogenetic analysis shows isolate distribution over many lineages with few small clusters (Figure 1) (Table S1). Three of these clusters (1600/1655, 1617/1618/1619, 1663/1664) contained isolates from the same patient. Isolates 1600 and 1655 showed 29 SNPs between their core genomes (4,493

genes), while 1617, 1618, 1619 differed by 132 to 409 SNPs and 1663, 1664 by 282 SNPs. These differences are in line with intra-host evolution of *P. aeruginosa* clones during long-term carriage (19). Other strains co-localising on the phylogenetic tree included 1608/1622 (397 SNPs), 1442/1653/1713 (447, 937 and 1039 SNPs) and 1671/1689 (497 SNPs) but were all from unrelated patients.

3.2 Susceptibility to ceftazidime/avibactam and ceftolozane/tazobactam

Twenty out of the twenty-four isolates were resistant to ceftazidime and twelve were resistant to ceftazidime/avibactam and/or ceftolozane/tazobactam. None of the isolates resistant to ceftazidime/avibactam and ceftolozane/tazobactam were susceptible to carbapenems. All isolates were also resistant to ceftobiprole and piperacillin/tazobactam.

Notwithstanding the limited diversity captured in this study focussed on piperacillin/tazobactam resistant isolates only, the proportion of ceftazidime/avibactam resistant isolates (37.5%) was not higher than that reported by a review of overall activity of ceftazidime/avibactam against UK *P. aeruginosa* isolates where 56.7% of 410 multidrug resistant and non-carbapenemase/non-ESBL-producing *P. aeruginosa* isolates were resistant to ceftazidime/avibactam (20). Our data would suggest an at least equal ceftazidime/avibactam activity against cystic fibrosis multidrug resistant isolates. However, we found a higher proportion of isolates resistant to ceftolozane/tazobactam (37.5%) compared to the resistance rate to ceftolozane/tazobactam reported for the collection of isolates aforementioned (18.4%) (20). A more recent study targeted to 43 cystic fibrosis *P. aeruginosa* isolates shows even lower resistance rates to ceftolozane/tazobactam (4.3%), although the majority of the isolates included in this study were susceptible to meropenem (76.6%) (21).

3.3 Determinants of resistance to ceftazidime/avibactam and ceftolozane/tazobactam

We performed ceftazidime susceptibility testing in the presence of cloxacillin to identify AmpC overproducers amongst isolates resistant to ceftazidime, ceftazidime/avibactam and/or ceftolozane/tazobactam. Ceftazidime MIC reduction in the presence of cloxacillin was observed in 15 out of the 20 ceftazidime-resistant isolates (Table 1) suggesting that AmpC overproduction was the predominant resistance mechanism. AmpC missense mutations (A31V, Q155R, Q157R, V239T, V239A, G242D, G248S, S306T) were present in four out of the five isolates, the ceftazidime MIC of which was not inhibited by cloxacillin. Of the aforementioned mutations, Q157R and G248S have been described previously (11, 22, 23). Notably, variations in amino acid V239 occurred in multiple unrelated isolates. The *ampC* missense mutation T105A was present in 21 isolates but not associated with any resistance profile (data not shown). The contribution of T105A to extended-spectrum AmpC β-lactamase activity (ESAC) was suggested (24), but subsequently not confirmed (6). We speculate that missense mutations in *ampD*, *ampG* and *dacB* may contribute to ceftazidime resistance in isolate 1631 where *ampC* mutations were absent (Table 1). AmpC deletions previously associated with ceftazidime/avibactam resistance (12) were not detected in any of the isolates.

To further investigate molecular mechanisms of ceftolozane/tazobactam and/or ceftazidime/avibactam resistance we analysed mutations in *ampR (ampC* regulator)*, ampD* (amidase), *ampG* (muropeptide permease)*, mexR* (MexAB/OprM regulator) and *dacB* (encoding PBP4), all previously associated with *ampC* overexpression (8-10) (Table 1). All six double resistant isolates to both ceftolozane/tazobactam and ceftazidime/avibactam had *ampD* mutations - four isolates showed frameshifts and one isolate bore the H157R active site mutation (22, 25). Importantly ceftazidime resistance in all these isolates was not inhibited by cloxacillin, and it is therefore likely related to constitutive AmpC overproduction, in turn potentially driven by AmpD activity.

There was no definitive association with any particular mutations for the six isolates that were resistant to either ceftolozane/tazobactam or ceftazidime/avibactam. The three ceftazidime/avibactam resistant isolates showed *ampR* and *mexR* mutations (Table 1). We observed a total of six *ampR* missense SNPs - D135N (isolate 1713) and R86C (isolate 1663) were shown to contribute to upregulation of *ampC* in *P. aeruginosa* (11) and *Enterobacter cloacae* (26), respectively, while no role has been documented for A81S and A227V (Table 1). We have detected AmpR mutations M288R and G283E but omitted these from Table 1 as they are inconsequential to *ampC* expression (27).

Analyses of *mexR*, regulator gene of the MexAB/OprM ceftazidime-related efflux system, disclosed the following amino acid variations (Table 1): in-frame deletion R73-R82del, missense mutation A66V, the R83C mutation involved in DNA binding (28), frameshift G77fs and the V126E mutation (not shown) which plays no role in ceftazidime resistance (29). *MexR* in-frame deletion R73-R82del was observed in isolate 1638 which showed a MIC of 6 µg/ml for both ceftazidime/avibactam and ceftolozane/tazobactam as well as ceftazidime alone. Amongst the ceftolozane/tazobactam and/or ceftazidime/avibactam resistant isolates *dacB* mutations were only observed in isolate 1631, the only isolate without variations in AmpC where ceftazidime resistance was not reversed by cloxacillin (Table 1). Figure 1 shows the carriage profile of PDC β-lactamases (24) in this collection of isolates. PDC-3 was the most prevalent variant but none of the PDC-3 mutations associated with ceftolazone/tazobactam resistance (13) were observed.

Frameshift mutations in the MexXY regulator gene *mexZ* associated with resistance to ceftobiprole (30) were observed in nine isolates (Supplementary Table S2). However, all the isolates analysed in this study were ceftobiprole resistant regardless of the presence of *mexZ* mutations (Supplementary Table S2).

3.4 Genetic determinants of carbapenem resistance

Susceptibility to carbapenems is summarised in Table 1. Nineteen out of twenty-four isolates tested (79%) were resistant to meropenem and/or imipenem. Whole genome analysis using

the Abricate software, based on both the NCBI and CARD antimicrobial resistance gene databases, showed no evidence of carbapenemase genes in any of the isolates. All thirteen meropenem-resistant isolates (MIC >32 mg/L) showed either a frameshift or a stop codon in the *oprD* porin gene. This is consistent with other data showing that *oprD* mutations are a major mechanism of meropenem resistance in the UK (20). OprD missense mutations G55D (isolate 1442) and S278P (isolate 1631), the latter of which has been reported previously (31), were unique to these two imipenem/meropenem double -resistant isolates, although *oprD* frameshifts were also present in both cases (Table 1). Two identical SNPs were observed in different isolates, one of which (Q402fs) surprisingly occurred independently in two isolates 1638 and 1664 belonging to separate phylogenetic lineages (ST379 and ST885, respectively, Table1 and Figure 1). Six out of the eleven meropenem-sensitive isolates showed resistance to imipenem. Five of these six isolates showed intermediate susceptibility to meropenem but only two out of the six had frameshifts in *oprD* (P220fs and Q402fs) (Table 1). OprD missense mutations were not detected in the six imipenem-resistant meropenem-sensitive isolates.

The *mexT* gene was analysed in view of its role in negative regulation of *oprD* expression (32). Variations in MexT were seen in three carbapenem-resistant isolates that were also resistant to ceftozolane/tazobactam. However, all three isolates bore a frameshift in *oprD*, drawing uncertainty on the role of *mexT* (Table 1).

3.5 Resistance due to horizontal gene transfer

We identified only one mobile genetic element associated with resistance in three isolates from the same patient (1617, 1618, 1619). However, this class I integron carried only the aminoglycoside adenyltransferase gene *aadA1*, the sulphonamide resistance gene *sul1* and the efflux pump gene *qacΔE*, all of which are irrelevant to β-lactam resistance. It is important to highlight that none of the isolates bore class A, B, D plasmid-encoded carbapenemases, the carriage rate of which is notoriously lower in UK *P. aeruginosa* isolates as compared to Enterobacteriaceae (20).

4. CONCLUSIONS

Analyses of this collection of piperacillin/tazobactam cystic fibrosis *P. aeruginosa* isolates shows frameshifts and stop codons in *oprD* as the main mechanism of carbapenem resistance. AmpC induction was the most frequent mechanism of ceftazidime resistance, while amino acid variations in AmpC were associated with high-level ceftazidime resistance not reversed by cloxacillin. *AmpC* mutational derepression was likely associated with double resistance to ceftolozane/tazobactam and ceftazidime/avibactam in view of *ampD* missense mutations present only in these isolates. Importantly, we showed that these mutations occur independently without seeming to confer a selective advantage to any phylogenetic lineage. This is supported by the emergence of mutations (in some cases identical) in phylogenetically distant lineages against a backdrop of phenotypic variations in sequential isolates from the same patient. These observations confirm the strong contribution of mutation-driven evolution to the population structure of *P. aeruginosa* (17).

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DECLARATIONS

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Ethical Approval: Not required

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FIGURE LEGEND

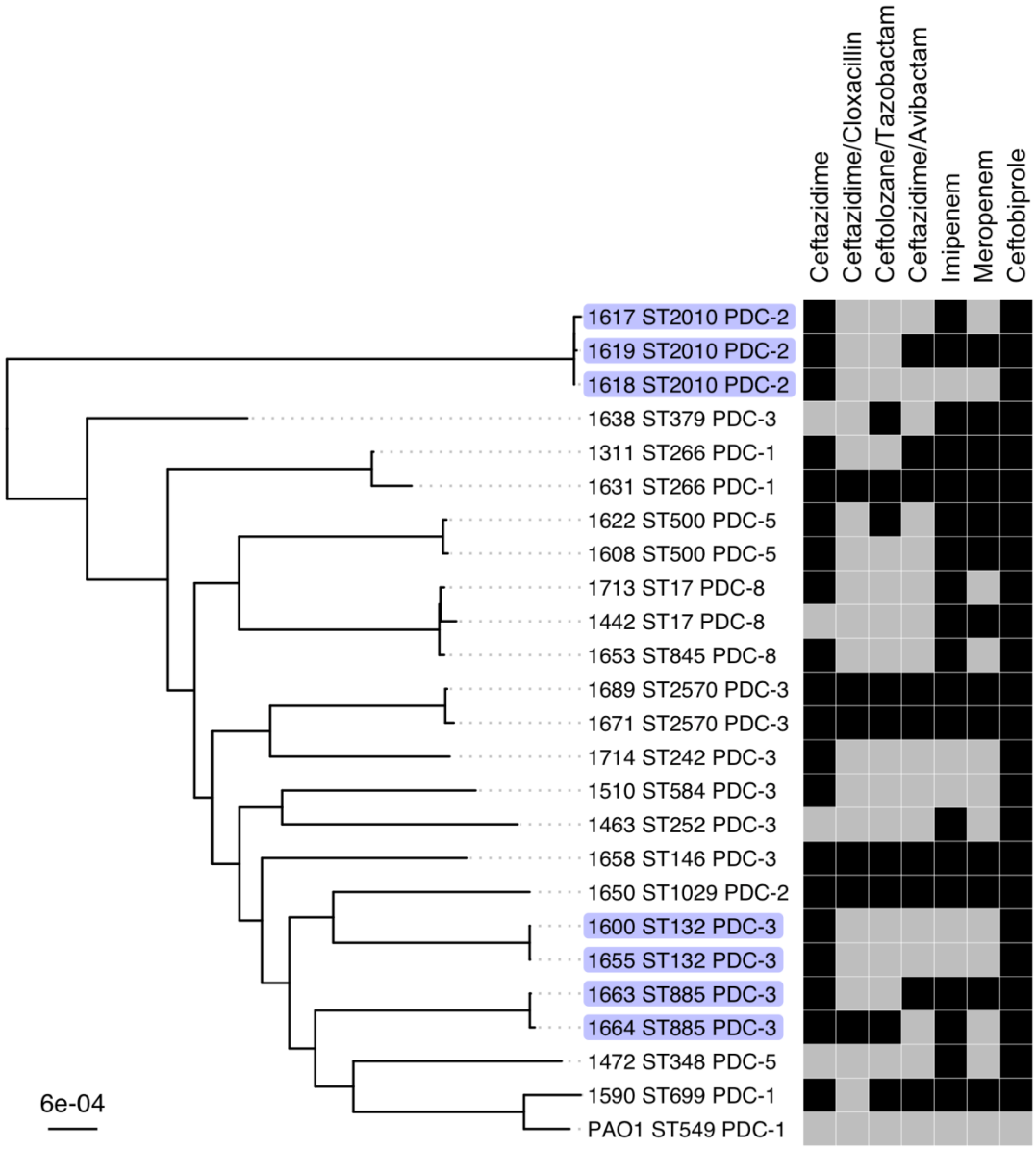

Figure 1: Core genome phylogenetic tree of Scottish *P. aeruginosa* isolates from cystic fibrosis. The core genome phylogenetic tree was generated using RAxML on 4,493 genes. The sequence type (ST) and the PDC variant (Pseudomonas-derived cephalosporinase) of all twenty-four isolates is shown along with reference strain PAO1 (NC_002516.2). The antimicrobial resistance phenotypes are shown in the heat map to the right (black = resistant, grey = susceptible). Isolates from the same patient are highlighted in

blue. The scale bar shown at the bottom left of the figure indicates the average number of substitution per site.

Table 1: Resistance-related phenotypes and genotypes in *P. aeruginosa* isolates

isolate	ST	ceftazidime	ceftazidime cloxacillin	ceftolozane tazobactam	ceftazidime avibactam	*ampC*	*ampR*	*ampD*	*ampG*	*mexR*	*dacB*	imipenem	meropenem	*oprD*	*mexT*
1442	17	2	1.5	2	8							>32	>32	G55D(n), Val127fs(d)	
1472	348	2	3	0.75	3							>32	4		
1463	252	3	0.75	2	2							>32	0.25		
1638	379	6	6	6	6					R73-R82del(d)		>32	>32	Q402fs(d)	
1510	584	12	1.5	0.38	0.5							1	1.5		
1618	2010	12	3	0.70	4					G77fs		1	4		
1655	132	16	2	3	3							4	0.75		
1311	266	16	4	3	12					A66V(n)		>32	>32	P220fs(d)	
1619	2010	16	4	2	24					G77fs(d)		>32	>32	W78fs(d)	
1622	500	24	6	6	1.5							>32	>32	W417fs(d)	
1663	885	32	8	1.5	24		R86C(d)					>32	>32	G104fs(d)	
1617	2010	32	2	1	4							>32	4		
1713	17	48	3	4	1		D135N(d); A227V(d)					16	6	P220fs(d)	
1600	132	96	0.13	3	1.5							1	0.25		
1653	845	96	6	0.75	2		A81S(n)					>32	6		
1608	500	256	0.5	1	1							>32	>32	W417stop	
1714	242	256	2	0.5	0.5							2	1		
1590	699	256	8	6	12			H157R(d)				>32	>32	W277stop	V57I(n)
1658	146	256	32	32	12			D105fs(d)		R83C(d)		>32	>32	N262fs(d)	M7V(n)
1631	266	256	256	256	256			T7A(n)	W336R(n)		P331L(n), T408I(d)	>32	>32	W138stop, S278P(d)	
1650	1029	256	256	256	256	Q155R(n), V239T(d), S306T(n)		G12fs(d)	H467R(n)			>32	>32	F69fs(d)	
1664	885	256	256	12	8	A31V(n); V239A(d); G242R(d); G248S(n)						>32	2	Q402fs(d)	A205V(d), G300A(n), S346R(n)
1671	25	256	256	256	256	Q157		F172				>3	>3	G152f	

	70					R(n); V239 A(d); G248 S(n)		fs(d)				2	2	s(d)	
1689	25 70	256	256	256	256	V239 A(d)		F172 fs(d)				>3 2	>3 2	G152f s(d)	

Grey shading for MICs above the respective breakpoints. Only *ampC*, *ampD, ampG, and dacB* variations potentially relevant to ceftolozane/tazobactam and/or ceftazidime/avibactam resistance are shown. Findings of predictive functional analyses using PROVEAN are shown in brackets (n=neutral; d=deleterious).