



# Acquisition of Class C $\beta$ -Lactamase PAC-1 by Sequence Type 644 Strains of *Pseudomonas aeruginosa*

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**ABSTRACT** Four sequence type 644 (ST664) (serotype O:5) strains of *Pseudomonas aeruginosa* that were highly resistant to antibiotics, including ceftolozane-tazobactam and ceftazidime-avibactam, but were susceptible to colistin were found to harbor the gene encoding the rare class C  $\beta$ -lactamase PAC-1 on a chromosomally located Tn1721-like transposon. The *bla*<sub>PAC-1</sub> gene was associated with the 16S rRNA methylase determinant *rmtF2*, which confers pan-aminoglycoside resistance. These genotypically related strains were isolated in repatriated patients from Mauritius and Afghanistan and were close to a lineage reported in Nepal, Pakistan, and India.

**KEYWORDS** *Pseudomonas aeruginosa*,  $\beta$ -lactamase, PAC-1, antibiotics, mechanisms of resistance

In countries such as France, where the prevalence of extended-spectrum  $\beta$ -lactamases and carbapenemases in *Pseudomonas aeruginosa* is still low, resistance of the pathogen to the cephalosporins ceftazidime and cefepime mostly results from mutations upregulating the production of the intrinsic cephalosporinase AmpC. While transferable class C  $\beta$ -lactamases have spread significantly in *Enterobacteriales* strains over the past decades (1, 2), their occurrence in *P. aeruginosa* has not been reported in the literature.

The present work reports on the characterization of four multidrug-resistant strains of *P. aeruginosa* that harbor a class C enzyme, named PAC-1, that has been poorly characterized to date. These bacterial strains were isolated between January 2017 and April 2019, from three male patients repatriated from Mauritius (Indian Ocean) and one repatriated from Afghanistan after a bombing attack. One strain was present in a rectal swab and was considered nonpathogenic. The second strain was collected from multiple body sites, including blood, and was responsible for the patient's death. The third strain was involved in a diabetic foot infection. Finally, the fourth isolate was recovered from the urine of a patient suffering from renal colic.

The drug susceptibility (MICs) of the four isolates was determined by using customized Sensititre plates (Thermo Fisher Scientific), and results were interpreted according to the 2019 EUCAST breakpoints (3). As shown in Table 1, these strains were resistant to all of the antipseudomonal antibiotics tested except colistin and, for two of them, imipenem. They met the criteria of extensively drug-resistant *P. aeruginosa* (4). Their levels of resistance to ceftazidime (with or without 4  $\mu$ g/ml avibactam), ceftolozane with 4  $\mu$ g/ml tazobactam, cefepime, amikacin, and tobramycin were very high (MICs of >128  $\mu$ g/ml). Addition of 2,000  $\mu$ g/ml of the AmpC inhibitor cloxacillin to Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France) failed to restore the susceptibility of

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**TABLE 1** Characteristics of strains producing class C  $\beta$ -lactamase PAC-1

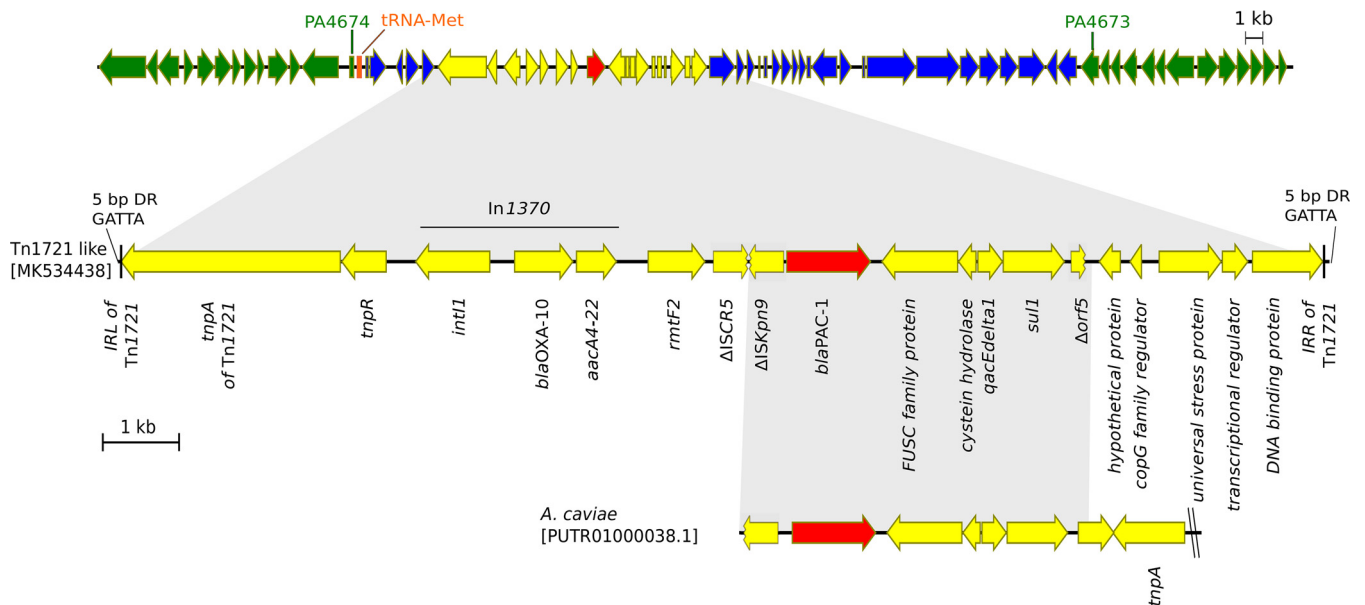
Strain	ST	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>														Acquired $\beta$ -lactamases
		TIC	TZP <sup>b</sup>	CAZ	FEP	CT <sup>b</sup>	CZA <sup>b</sup>	ATM	IPM	MEM	AMK	TOB	CIP	FOS	CST	
17.4313	ST664	>512	128	>128	>128	>128	>128	64	1	16	>128	>128	16	256	1	PAC-1, OXA-10
17.4319	ST664	>512	256	>128	>128	>128	>128	64	16	64	>128	>128	16	256	1	PAC-1, OXA-10
19.6465	ST664	>512	128	>128	>128	>128	>128	64	16	64	>128	>128	>16	128	1	PAC-1, OXA-10
19.6625	ST664	>512	128	>128	>128	>128	>128	128	2	32	>128	>128	>16	128	1	PAC-1, OXA-10
PAO1		16	4	1	1	$\leq 0.5$	1	4	1	$\leq 0.5$	4	0.5	$\leq 0.12$		1	
PAO1 $\Delta$ <i>dacB</i> <sup>c</sup>		64	32	16 (1)	8 (1)	1 (0.5)	2 (0.5)	8	1	$\leq 0.5$	$\leq 2$	1	$\leq 0.12$		1	
PAO1(pUCP24)		16	4	2	2	$\leq 0.5$	1	4	1	$\leq 0.5$	$\leq 2$	1	$\leq 0.12$		1	
PAO1(pUCP24 <i>bla</i> <sub>PAC-1</sub> )		32	16	>128 (64)	>128 (32)	>128 (64)	>128 (64)	8	1	$\leq 0.5$	$\leq 2$	1	$\leq 0.12$		1	

<sup>a</sup>MICs were determined by using customized Sensititre plates (Thermo Fisher Scientific). MIC values in parentheses were determined in the presence of 2,000  $\mu\text{g/ml}$  of cloxacillin. TIC, ticarcillin; TZP, piperacillin-tazobactam; CAZ, ceftazidime; FEP, cefepime; CT, ceftolozane-tazobactam; CZA, ceftazidime-avibactam; ATM, aztreonam; IPM, imipenem; MEM, meropenem; AMK, amikacin; TOB, tobramycin; CIP, ciprofloxacin; FOS, fosfomicin; CST, colistin.

<sup>b</sup>With a fixed concentration of 4  $\mu\text{g/ml}$  tazobactam or avibactam.

<sup>c</sup>PAO1 $\Delta$ *dacB* was a gift from Antonio Oliver.

the bacteria to penicillins and cephalosporins, as assessed by the disk diffusion method. Intriguingly, screening of class A and class B  $\beta$ -lactamases by double-disk synergy tests also yielded negative results, despite the use of various disk combinations (imipenem-EDTA, ceftazidime-EDTA, ceftazidime-clavulanate, and cefepime-clavulanate). PCR sequencing experiments targeting genes coding for various class A (CTX-M, GES, PER, SHV, TEM, and VEB types), class B (IMP, NDM, and VIM types), and class D (OXA-1, OXA-2, and OXA-10 variants)  $\beta$ -lactamases potentially produced by clinical *P. aeruginosa* strains were performed and revealed only the presence of the *bla*<sub>OXA-10</sub> gene in the four strains. Because production of penicillinase OXA-10 could not account for the resistance profiles of studied bacteria (5), the whole DNA content of two strains (17.4313 and 17.4319) was extracted and sequenced by hybrid *de novo* assembly of paired-end reads (2 by 75 reads), using the NextSeq Illumina technology (100 $\times$  coverage). The resultant reads were assembled with CLC Genomics Workbench 10.0.1 software (Qiagen Aarhus A/S) and then analyzed by using the CARD Resistance Gene Identifier 4.2.2 (<https://card.mcmaster.ca/analyze/rgi>). The two strains were determined to contain *rmtF2*, the gene that codes for 16S rRNA methylase RmtF2, and *aacA4-22*, the determinant of aminoglycoside modification. Previously identified in *P. aeruginosa* isolates from India and Nepal (6, 7), RmtF2 confers high levels of resistance to all aminoglycosides (8). A third gene, exhibiting 60% sequence homology to that of  $\beta$ -lactamase SRT-2, was finally identified by BLAST comparisons (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) as *bla*<sub>PAC-1</sub>, which is indicated in the GenBank database as encoding a putative class C  $\beta$ -lactamase (GenBank accession number [NG\\_052627.1](https://www.ncbi.nlm.nih.gov/nuccore/NG_052627.1)). Since no transconjugants could be obtained, despite several attempts (data not shown), we cloned the *bla*<sub>PAC-1</sub> gene in the broad-host-range vector pUCP24 (with gentamicin resistance) and transformed reference strain PAO1 with the resultant plasmid, pUCP24*bla*<sub>PAC-1</sub>. The resistance phenotypes of PAO1, PAO1(pUCP24::*bla*<sub>PAC-1</sub>), and the AmpC-overproducing mutant PAO1 $\Delta$ *dacB* (9) are presented in Table 1. Compared with PAO1 $\Delta$ *dacB*, PAO1(pUCP24::*bla*<sub>PAC-1</sub>) exhibited much higher levels of resistance to all of the cephalosporins tested, including ceftolozane-tazobactam and ceftazidime-avibactam (MICs of >128  $\mu\text{g/ml}$ ), while its levels of resistance to ticarcillin, piperacillin/tazobactam, and aztreonam were similar or lower. In contrast to AmpC, PAC-1 was poorly antagonized by cloxacillin even at the high concentration of 2,000  $\mu\text{g/ml}$  (cephalosporin MICs of  $\geq 32$   $\mu\text{g/ml}$ ) (Table 1). Since the MICs of carbapenems were unaffected by the expression of *bla*<sub>PAC-1</sub> in strain PAO1, we examined the sequence of the gene that codes for the carbapenem-specific porin OprD in the imipenem-resistant strains 17.4319 and 19.6465 (MICs of 16  $\mu\text{g/ml}$ ); we found a disruptive mutation (T209 deletion) in the *oprD* gene of 17.4319 and a premature stop codon (G831A, W277\*) in that of 19.6465. Analysis of the *nalB*, *nalC*, and *nalD* genes, whose alteration is known to upregulate the efflux operon *mexAB-oprM*, did not provide an explanation for the 4- to 16-fold higher levels of resistance of all strains to meropenem, compared with imipenem (i.e., only meropenem is a substrate of the efflux pump MexAB-OprM) (10). This MIC difference between the two carbap-



**FIG 1** Schematic representation of the genetic environment of *bla*<sub>PAC-1</sub>. The *bla*<sub>PAC-1</sub> gene (red) is located on a Tn1721-like transposon (yellow). The genetic environment of the transposon (indicated in blue; 4,549 bp on the left and 22,312 bp on the right) is 99% homologous to the genome of strain S04-90 (GenBank accession number CP011369.1); open reading frames indicated in green are homologous to a genomic region present in reference strain PAO1 (GenBank accession number AE004091.2).

enems is possibly due to OXA-10, because this  $\beta$ -lactamase was demonstrated to have stronger enzymatic activity with meropenem than with imipenem (11). The sequenced strains 17.4313 and 17.4319 both exhibited the canonical mutations T83I and S87L in the quinolone-resistance-determining regions of DNA gyrase subunit A and topoisomerase IV subunit C, respectively, accounting for the high levels of resistance of these bacteria to ciprofloxacin (MICs of 16  $\mu$ g/ml).

The enzyme PAC-1 is composed of 381 amino acids. This protein shares 65% sequence identity with the class C  $\beta$ -lactamase encoded by *Cronobacter turicensis* (formerly *Enterobacter sakazakii*) and 47% with the AmpC enzyme from *P. aeruginosa* strain PAO1 (PDC-1). As depicted in Fig. 1, the *bla*<sub>PAC-1</sub> gene was localized on a complex Tn1721-like transposon, a partial sequence of which was previously established from a Nepalese strain named IOMTU487 (GenBank accession number LC224309.1) (6). In our strains, *bla*<sub>PAC-1</sub> was found to be embedded in a 4,539-bp sequence containing a truncated insertion sequence (IS) element, *ISKpn9*, that was previously identified in an *Aeromonas caviae* strain (GenBank accession number PUTR01000038.1).

Analysis of the genetic environment of the Tn1721-like transposon indicated that this element was located on an  $\sim$ 27-kb DNA sequence showing 99% homology with the genome of a *P. aeruginosa* strain deposited in the NCBI database (strain S04-90; GenBank accession number CP011369.1). This long fragment was inserted between genes PA4673 and PA4674, according to the genome annotation of strain PAO1, next to a tRNA-Met-encoding gene. Attempts to identify an integrative and conjugative element (ICE) bearing the Tn1721-like transposon, by using the ICEberg website (<http://db-mml.sjtu.edu.cn/ICEberg>) or manual characterization of conserved features of ICEs (e.g., genes predicted to encode conjugative relaxases, type IV coupling proteins, and ATPases of type IV secretion systems), were unsuccessful (12, 13).

A whole-genome multilocus sequence typing analysis of 6,349 genetic loci with Bionumerics software package 7.6.3 revealed a close clonal relationship of the PAC-1-producing isolates with strain PA\_041 from Pakistan (GenBank accession number NZ\_RHSY00000000.1) (98% allelic identity), strain PA\_038 from Pakistan (GenBank accession number NZ\_RHSZ00000000.1) (97.7%), and strain VRFPA06 from India (92%) (14). Like the Nepalese strain IOMTU487, all eight isolates belonged to sequence type 664 (ST664) (serotype O:5).

In conclusion, PAC-1 is a cephalosporinase acquired by closely related strains of *P. aeruginosa* belonging to epidemic clone ST664 (15). Coinheritance of the *bla*<sub>PAC-1</sub>, *bla*<sub>OXA-10</sub>, and *rmtF2* genes is sufficient to provide *P. aeruginosa* with a high degree of resistance to major antipseudomonal penicillins, cephalosporins, and aminoglycosides. PAC-1 thus should be added to the growing list of  $\beta$ -lactamases produced by multidrug-resistant *P. aeruginosa* strains, especially those occurring in the Indian sub-continent.

**Accession number(s).** Genomic DNA sequences of the *P. aeruginosa* strains have been submitted to the GenBank database under accession numbers [SISN00000000](#) (strain 17.4313) and [SISM00000000](#) (strain 17.4319). The transposon sequence was deposited in the GenBank nucleotide database under accession number [MK534438](#).

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