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ORIGINAL RESEARCH

PAU-1, a Novel Plasmid-Encoded Ambler Class A β-Lactamase Identified in a Clinical *Pseudomonas aeruginosa* Isolate

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Purpose: The aim of this work was to identify a novel β-lactamase gene bla_{PAU-1} encoded on the plasmid of a clinical *Pseudomonas a*eruginosa isolate.

Materials and methods: The clinical *P. aeruginosa* isolates were isolated from a hospital in southern China. Molecular cloning was performed to analyze the function of the resistance gene. The minimum inhibitory concentration (MIC) was determined by means of the agar dilution method to determine the antimicrobial susceptibilities of the strains. Whole-genome sequencing and comparative genomics analysis were performed to analyze the structures of the resistance gene-related sequences.

Results: PAU-1 is a molecular class A, Bush-Jacoby group 2be enzyme which encoded 293 amino acids and shared 74% amino acid identity with a putative class A β-lactamase from *Rhodoferax saidenbachensis*. Cloned bla_{PAU-1} in *Escherichia coli* and *P. aeruginosa* conferred resistance to piperacillin and ampicillin, and elevated the MIC with a 2–3 dilution for some oxyimino-β-lactams in *P. aeruginosa*. The genetic environment of bla_{PAU-1} is *tnp*A-*res-hp-relE-*bla_{PAU-1}*-lysR*, which is in accordance with the structure of a Tn*3* transposon. Epidemiological investigation of bla_{PAU-1} in the same district did not show any evidences of molecular dissemination associated with this determinant.

Conclusion: A novel class A β-lactamase gene, bla_{PAU-1}, associated with the mobile genetic element was identified on a transferable plasmid in a clinical *P. aeruginosa* isolate. Strict surveillance for the emergence of the new determinant should be established and an effort should be made to block the dissemination of this determinant.

Keywords: *Pseudomonas aeruginosa*, antimicrobial resistance, β-lactamase, PAU-1

Introduction

Production of the Ambler class A extended-spectrum beta-lactamase (ESBL) is an important cause of cephalosporin resistance in *Pseudomonas aeruginosa* which is one of the most common pathogens that causes burn wound infections, nosocomial pneumonia, and urinary tract infections.[1–4] As described previously, several novel ESBLs have been firstly identified from *P. aeruginosa*, and good examples are bla_{PER-1}[5] and bla_{PME-1}[6] which conferred resistance to extended-spectrum cephalosporins.

Most recently, a clinical *P. aeruginosa* isolate named *P. aeruginosa* PA1280 belonging to ST1119 with strong carbapenems resistance was characterized. The major mechanism of high-level carbapenems resistance in *P. aeruginosa* PA1280 is that it harboured an IncP-1β conjugative plasmid, termed pICP-4GES, where a class

1 integron containing four consecutive bla_{GES-5} gene cassettes was located.[7] In addition to four copies of bla_{GES-5}, the plasmid pICP-4GES also harboured a putative Ambler class A β-lactamase gene predictively encoding 303 amino acids (accession number APC57487), which was embedded in a Tn*3*-like transposon.[7] In this study, the function of this novel class A β-lactamase was partially characterised. In addition, comparative genome analysis was also performed to elucidate the potential origin of this new gene.

Materials and Methods

Clinical Strains

A total of 320 non-duplicate clinical *P. aeruginosa* isolates were used from the First Affiliated Hospital of Wenzhou Medical University in Zhejiang Province, Wenzhou, China ranging from 2009 to 2012 (about 80 strains from each year). The strains were identified using the Vitek-60 microorganism auto-analysis system (BioMerieux, France). The primary bacterial strains and plasmids used in this study are shown in Table 1. The ethics of this study were approved by the First Affiliated Hospital of Wenzhou Medical University (2017-BYS-0253) and the bacterial samples were also consented by the patients for anonymously scientific use.

Antimicrobial Susceptibility Test

The antimicrobial agents assayed were used immediately after their solubilisation. Minimum inhibitory concentrations (MICs) were determined by agar dilution method on Mueller-Hinton agar (Diagnostics Pasteur). All plates were incubated at 37°C for 18 h. The MICs of β-lactams were determined alone or in combination with a fixed concentration of tazobactam (2 μg/mL) or sulbactam (4 μg/mL) as previously described.[8] The results of the antimicrobial susceptibility tests for MICs were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2017). *P. aeruginosa* ATCC 27853 was used as the quality control strain. 13 β-lactams or their compounds used in antimicrobial susceptibility tests were ampicillin (AMP), piperacillin (PIP), piperacillin-tazobactam (TZP), cefoxitin (FOX), ceftriaxone (CRO), cefotaxime (CTX), ceftazidime (CAZ), cefoperazone (CFP), cefoperazone/sulbactam (CSL), cefepime (FEP), aztreonam (ATM), imipenem (IPM) and meropenem (MEM).

Table 1 Bacteria and Plasmids Used in This Work

Strain and Plasmid	Relevant Characteristic(s)	Source
Plasmid		
pUCP24	pUC18-derived broad-host-range vector, Gm^r	Our lab collection
pUCP24::bla_{PAU-1}	bla_{PAU-1} gene cloned into pUCP24 vector, Gm^r	This study
Strain		
E. coli JM109	*Escherichia coli* JM109 used as a host for the resistance gene cloning	Our lab collection
E. coli DH5α	*E. coli* DH5α used as a host for antimicrobial susceptibility test	Our lab collection
P. aeruginosa PA1280	A clinical *Pseudomonas aeruginosa* isolate	This study
PA1280ΔpICP-4GES	PA1280 with the plasmid pICP-4GES cured	This study
ΔPAO1	*P. aeruginosa* PAO1 deleted of *ampG* (PA4393)	Our lab collection
ΔPAO1[pUCP24]	PAO1Δ*ampG* carrying the expression vector of pUCP24, Gm^r	This study
ΔPAO1[pUCP24::bla_{PAU-1}]	PAO1Δ*ampG* carrying pUCP24 with bla_{PAU-1} gene, Gm^r	This study
E. coli DH5α[[pUCP24]	*E. coli* DH5α carrying the expression vector of pUCP24, Gm^r	This study
E. coli DH5α[pUCP24::bla_{PAU-1}]	*E. coli* DH5α carrying pUCP24 with bla_{PAU-1} gene, Gm^r	This study
P. aeruginosa ATCC27853	*P. aeruginosa* ATCC27853 is a FDA clinical isolate	Our lab collection

Genome Sequencing

Total genomic DNA was extracted from the bacterium using the AxyPrep Bacterial Genomic DNA Miniprep kit (Axygen Scientific, Union City, California). A 20-kb library was generated using the SMRTbell Template Prep Kit according to the PacBio standard protocol and sequenced on a PacBio RS II instrument (Pacific Biosciences, Menlo Park, California). In addition, a paired-end library with about 300-bp insert sizes was constructed and sequenced from both ends using Illumina technology (Illumina, San Diego, California). The PacBio long reads were initially assembled using Canu software.[9] The Illumina reads were then mapped onto the assembled

contigs to correct the primary assembly by using BWA and the Genome Analysis Toolkit.[10,11] Glimmer software (http://ccb.jhu.edu/software/glimmer) was used to predict protein-coding genes with potential open reading frames (ORF) >150 bp in length, and the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) program was used to annotate the predicted protein-coding genes.

Identification of Transcriptional Start Site Through Transcriptome Sequencing

P. aeruginosa PA1280 wild-type strain was started from a purified clone on LB agar plate. Overnight bacterial cultures were sub-cultured in LB medium and grown to OD_{600} of 1.0 with 100 μg/mL ampicillin. Total RNA was extracted using the RNAprep pure Cell/Bacteria Kit with on-column DNase I digestion (TIANGEN Biotech, Beijing, China). RNA was further treated by DNase I and purified by using the RNAclean Kit (TIANGEN Biotech, Beijing, China). The samples were then treated with the rRNA RiboMinus kit (Invitrogen, California) according to the manufacturer's instructions to remove 16S and 23S rRNAs. RNA concentration and purity were evaluated with NanoDrop 2000 spectrophotometer (Thermo Scientific, Madison, Wisconsin) and integrity was verified in denaturing agarose gel electrophoresis. The remaining RNAs were fragmented into short fragments and then subject to first-strand cDNA synthesis from fragments by random hexamer primers with dUTP during the second strand synthesis. Extremities of short fragments were processed by adding a single adenine after purification and connected with adapters. The second strand was degraded using UNG (uracil-N-glycosylase). The paired-end cDNA library was sequenced on the HiSeq 2500 platform (Illumina, San Diego, California). BWA was used to map the short reads onto the bacterial genome,[10] and Tablet was applied to view the mapping results.[12]

Processing and Retrieve of *bla*$_{PAU-1}$ Homologous Sequences

The *bla*$_{PAU-1}$ homologous gene was obtained from the NCBI nucleotide database using a *bla*$_{PAU-1}$ gene flanking region (an approximately 6 kb region surrounding the *bla*$_{PAU-1}$ gene from pICP-4GES sequence, accession number: MH053445). The resulting sequences were filtered, and only those containing the homologues of either *bla*$_{PAU-1}$ or Tn*3* transposon with an identity greater than 70% were retained.

Molecular Cloning of the *bla*$_{PAU-1}$ Gene

Total genomic DNA was extracted from the bacteria using the AxyPrep Bacterial Genomic DNA Miniprep kit (Axygen Scientific, Union City, California). A pair of PCR primers containing *Eco*RI and *Bam*HI restriction endonuclease adapters, respectively, was designed using the *bla*$_{PAU-1}$ gene of *P. aeruginosa* PA1280 as the template to amplify the *bla*$_{PAU-1}$ gene together with approximately 100 bp of its upstream promotor region. The primer sequences of PAU-F-*Eco*RI and PAU-R-*Bam*HI are 5′-CGGAATTCCGTTAAGCGGAAGGTCCATGATGA-3′ and 5′- CGGGATCCCCTTATCCCCGCTCCGACTTCAT-3′, respectively, which generate a 1,018 bp product. The PCR product was digested with *Eco*RI and *Bam*HI and was ligated into the vector pUCP24 (TaKaRa, Dalian, China).[13] The recombinant plasmid of the pUCP24::*bla*$_{PAU-1}$ was transformed into the recipient *E. coli* JM109 and was selected on LB agar plate containing 20 μg/mL gentamycin. The transformants were confirmed by PCR and Sanger sequencing. The pUCP24::*bla*$_{PAU-1}$ plasmid was extracted and further transformed into PAO1Δ*ampG* (an *ampG*-deleted *P. aeruginosa* PAO1 strain, abbreviated as ΔPAO1)[14] and standard *E. coli* DH5α.[15] The transformants were used for antimicrobial susceptibility tests. Cloning of *bla*$_{PAU-1}$ into pET-28b was performed by using the forward and reverse primers flanking the *Eco*RI and *Bam*HI restriction endonuclease adapters, respectively (Forward: 5′-CG GAATTCATGAAAAGACGCAACTTCTC-3′, Reverse: 5′-CGGGATCCTCATCGTATGCCTATAGAGGT-3′). The recombinant plasmids were then transformed into *E. coli* BL21 (DE3) to express PAU-1 enzyme induced by 1mM IPTG. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to detect the expression of PAU-1 enzyme.[16]

Results and Discussion

Identification of a Novel β-Lactamase-Encoding Gene on the *P. aeruginosa* PA1280 Plasmid Through Whole Genome Sequencing

A total of 91 among 320 *P. aeruginosa* isolates tested showed high resistance levels to ampicillin (≥4096 μg/mL). One strain (*P. aeruginosa* PA1280) showed the highest resistance to meropenem with an MIC of up to 256 μg/mL. It also had resistance to a wide range of

antimicrobials, spanning all types of β-lactam antibiotics (Table 2). To explore the molecular mechanism responsible for the observed resistance to the β-lactam antibiotics, the whole genome of *P. aeruginosa* PA1280 was determined, which consists of a 6.23 Mb chromosome encoding 5,785 CDSs and a 50,914 bp IncP-1β incompatibility group plasmid (pICP-4GES, MH053445) encoding 53 CDSs.

The *P. aeruginosa* PA1280 genome encoded six β-lactamase genes. With the exception of one gene ($bla_{OXA-129}$) encoded on the chromosome, the other five (a novel β-lactamase gene named bla_{PAU-1} and four bla_{GES-5} genes) were located on the plasmid pICP-4GES.[7] The bla_{PAU-1} gene was initially predicted to encode 303 amino acids. However, this result was not supported by transcriptome sequencing data. When we mapped the transcriptome reads to the pICP-4GES, it showed that the coding strand-specific reads possessed the same direction and all the most extremity reads around PAU-1-encoding gene harboured a T residue at the 5′ ends corresponding to the bla_{PAU-1} coding strand of pICP-4GES as indicated in the transcriptional start site (TSS) in the Figure S1. Moreover, there were no coding strand-specific reads spanning the junction between this T residue and nucleotides immediately upstream. This indicated that the exact TSS is the T residue. In addition, analyses of the upstream of the potential TSS (+1) revealed the presence of two conserved motifs with sequences of 5′-TATGAT -3′ near the −10 region and 5′-TTGAAG-3′ near the −35 region of the promoter. Thus, the start codon of the bla_{PAU-1} gene should be the ATG codon 18 bp downstream of the TSS, which generates a 293 amino acid enzyme. SDS-PAGE also showed that PAU-1 is about 33 kilo-Daltons which is similar to the theoretical molecular weight of 31.68 kD (Figure S2).

BLASTP search of PAU-1 against non-redundant protein database showed that the sequences sharing the highest similarity (72.7%, 213/293) with that of PAU-1 were two predicted class A β-lactamases from *Rhodoferax saidenbachensis* (accession numbers WP_029709665.1 and APW43006.1, the amino sequences of these two proteins are identical). One sequence (WP_029709665.1) was from *R. saidenbachensis* ED16, isolated from a drinking water reservoir in Germany, but the genome sequence was not available in the public database.[17] The other sequence (APW43006.1) was from *R. saidenbachensis* DSM 22,694, isolated from an environmental sample submitted by Korea University, with the gene encoded on the chromosome (Its complete genome sequence is available, accession number: CP019239.1). These PAU-1-like proteins are not well-characterised or documented. A multiple sequence alignment including PAU-1, the closest relative of PAU-1, and the two representatives of class A β-lactamases, BKC-1 and CTX-M-9, was performed to show the conserved motif of this newly identified enzyme (Figure S3). Furthermore, a BLASTN search against nucleotide collection database using bla_{PAU-1} as a query revealed that almost all of these enzymes are derived from the chromosomes of species belonging to the class Betaproteobacteria, including genera such as *Rhodoferax, Comamonas, Azoarcus, Hydrogenophaga* and *Collimonas*. To elucidate the evolutionary history of bla_{PAU-1} gene, more genotypes with higher identities need to be found.

PAU-1 Conferring Resistance to β-Lactams

Previous studies have shown that the major resistance mechanism to β-lactams in *P. aeruginosa* PA1280 was that it harboured a conjugative plasmid containing four copies of bla_{GES-5}. The plasmid cured *P. aeruginosa* PA1280 strain (PA1280ΔpICP-4GES) showed drastically decreased resistance to almost all the assayed β-lactams (Table 2). To assess the potential relevance of β-lactam resistance of PAU-1, the coding sequence of bla_{PAU-1} together with its promoter, was cloned into pUCP24 vector and then transformed into the *E. coli* DH5α and ΔPAO1. It showed that cloned bla_{PAU-1} in standard *E. coli* DH5α and ΔPAO1 conferred high-level resistance to piperacillin and ampicillin, and elevated the MIC with a 2–3 dilution for some oxyimino-β-lactams in ΔPAO1, such as ceftriaxone and cefepime (Table 2). PAU-1 was thus classified into molecular class A, Bush-Jacoby 2be group of the functional classification scheme.[18] The resistance profile of PAU-1 for β-lactams is in accordance with that of Ambler class A beta-lactamase.[19]

Comparative Analysis and Possible Origin of bla_{PAU-1}

After analyses of the bla_{PAU-1}-neighbouring region, we found that bla_{PAU-1} is located in a Tn*3*-like family transposon. In the transposon, the gene arrangement of *tnpA-res-hp-relE-*bla_{PAU-1}*-lysR* was observed (Figure 1). Flanking *tnpA* and *lysR*, a pair of 35 bp inverted repeat (IR) was identified.[7] This indicated that movement of bla_{PAU-1} was mediated by a Tn*3*-like transposon. The Tn*3*-like family transposons are widely distributed among prokaryotes.[20] However, the Tn*3*-like transposon structure most similar to the pICP-

Table 2 MICs of Nine Bacterial Strains Against 13 β-Lactams or Their Compounds

Antimicrobials	MIC(μg/mL)								
	PA1280	**PA1280ΔpICP-4GES**	**ΔPAO1 [pUCP24]**	**ΔPAO1[pUCP24::*bla*$_{PAU-1}$]**	**ΔPAO1**	***E. coli* DH5α**	***E. coli* DH5α [pUCP24]**	***E. coli* DH5α[pUCP24::*bla*PAU-1]**	***P. aeruginosa* ATCC27853**
AMP	>8192	1024	32	>1024	32	2	2	32	1024
PIP	1024	2	4	512	4	1	1	8	2
TZP	256	2	4	64	4	2	1	2	2
FOX	>128	>128	64	128	128	2	2	2	>128
CRO	>128	8	4	16	4	<0.125	<0.125	<0.125	8
CTX	>128	4	8	16	8	<0.125	<0.125	<0.125	4
CAZ	32	1	2	2	1	<0.25	<0.25	<0.25	1
CFP	>128	2	4	4	4	0.25	0.125	0.125	4
CSL	>128	2	2	1	2	<0.125	<0.125	<0.125	2
FEP	64	1	2	16	2	<0.125	<0.125	<0.125	2
ATM	16	1	2	4	4	<0.125	<0.125	<0.125	4
IPM	32	4	0.25	0.5	0.25	<0.125	<0.125	<0.125	16
MEM	256	1	1	1	1	<0.125	<0.125	<0.125	<0.125

4GES transposon in public databases was located on the *Acidovorax* sp. RAC01 chromosome (Figure 1). This transposon also includes a *lysR* family regulator, but does not harbour a bla_{PAU-1}-like gene in the neighbouring region or elsewhere in the genome (Figure 1). There are several examples of *lysR*-accompanied chromosomal class A β-lactamases. For instance, the *penA* gene is situated by a LysR-type transcriptional regulator, *penR*, on the *Burkholderia cepacia* 249 chromosome.[21] Another example is bla_{BOR-1}, which was identified in the *Bordetella bronchiseptica* and *Bordetella parapertussis* chromosomes.[22] The closest relative of PAU-1 is a putative class A β-lactamase (WP_029709665.1, 72.7% identity) on the *Rhodoferax saidenbachensis* DSM 22694 chromosome that is also situated close to a *lysR* family regulator. Interestingly, the *lysR* gene subtended by bla_{PAU-1} on pICP-4GES is extremely similar (82% amino acid identity) to the *lysR* next to the bla_{PAU-1}-like gene from the *Rhodoferax saidenbachensis* DSM 22694 chromosome. However, the second closest relative of bla_{PAU-1}, located on the chromosome of *Comamonas kerstersii* str. 8943 (with an amino acid identity of 68.9%, 202/293), did not harbour a *lysR* family regulator. All these close relatives of PAU-1 are derived from the class *Betaproteobacteria*. This indicated that bla_{PAU-1} was most likely originated from the chromosome of a bacterium belonging to *Betaproteobacteria* and was disseminated through horizontal gene transfer mediated by a Tn*3*-like family transposon. Many resistance genes first originated from chromosomes and then were captured by mobile genetic elements and subsequently appeared in plasmids.[23,24] Resistance plasmids could then be transferred to other bacteria by means of horizontal gene transfer and thus facilitate the spread of antibiotic resistance.[24,25] The conjugation experiment demonstrated that pICP-4GES is a transferable plasmid, suggesting that bla_{PAU-1} can potentially be transferred to other bacterial species or genera.

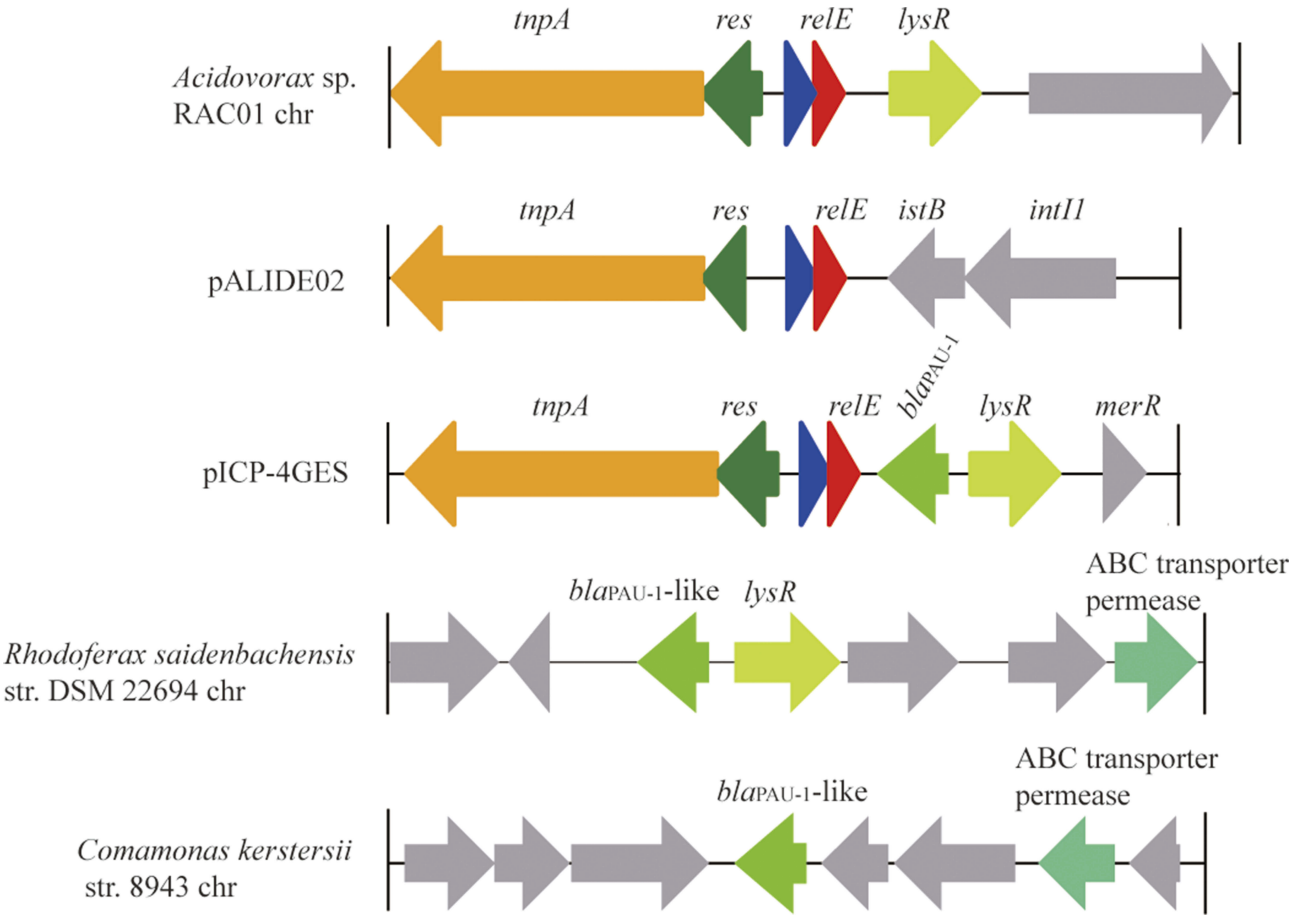

Figure 1 Comparative analysis of the genomic context of the bla_{PAU-1}-like gene related region. The bla_{PAU-1} gene and its closest relatives were compared. Homologous genes are filled with the same colour except for the genes which have no homologs in this context, which are coloured grey. The accession numbers of the sequences are: *Acidovorax* sp. RAC01 chromosome (CP016447), plasmid pALIDE02 (CP002451), plasmid pICP-4GES (MH053445), *R. saidenbachensis* DSM 22694 chromosome (CP019239), and *C. kerstersii* str. 8943 chromosome (CP020121).

To further investigate the prevalence of the bla_{PAU-1} gene in *P. aeruginosa* isolated from the same hospital, we screened back to 320 clinical *P. aeruginosa* isolates by PCR method. No bla_{PAU-1} homologue has been identified so far. It appears that the bla_{PAU-1} is rarely emerged and it is recently difficult to trace its precise ancestor. It also remains to determine the origin of bla_{PAU-1} and how it was transferred to *P. aeruginosa*.

Conclusions

In conclusion, a novel Ambler class A β-lactamase gene, bla_{PAU-1}, associated with a Tn*3* family transposon, was identified on a transferable plasmid from a clinical *P. aeruginosa* isolate. Cloned bla_{PAU-1} conferred resistance to ampicillin and piperacillin in *E. coli* background. Despite no close relatives of PAU-1 having been identified in the public database or in the clinical pathogens isolated in the same district where the novel gene was identified, strict surveillance for the emergence of the new determinant should be established and an effort should be made to block the dissemination of the resistance plasmid.

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Disclosure

The authors report no conflicts of interest in this work.

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