

First Identification of a Multidrug-Resistant *Pseudomonas putida* Co-Carrying Five β -Lactam Resistance Genes Recovered from a Urinary Tract Infection in China

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Abstract: The emergence of multidrug-resistant *Pseudomonas* spp. in the clinical settings has heightened public awareness. Here, we described the genomic characteristics of a *P. putida* isolate co-carrying five β -lactam resistance genes recovered from a urinary tract infection in China. Whole-genome sequencing was performed using Illumina NovaSeq 6000 and Oxford Nanopore MinION platforms. The genome sequence was annotated and further subjected to identify the sequence type (ST), antibiotic resistance and virulence genes. Phylogenetic analysis of 193 *P. putida* strains stored in NCBI public database based on core genome single nucleotide polymorphism (cgSNP) strategy were also performed and visualized. Our study indicated that *P. putida* PP_2463 was resistant to a wide range of antimicrobial agents tested, including aminoglycosides, carbapenems and fluoroquinolones. The complete genome sequence of *P. putida* PP_2463 is made up of one chromosome and two plasmids, which could be assigned to a new sequence type (ST) 148. The co-occurrence of β -lactam resistance genes *bla*_{IPM-15}, *bla*_{PME-1}, *bla*_{CARB-2}, and *bla*_{NDM-1} were first identified in *P. putida*, and a novel β -lactamase gene located in the chromosome were among the antimicrobial resistance genes discovered. The closest relative of *P. putida* PP_2463 was identified in 2012 from a urine sample in China, with a difference of 143 SNPs. Along with the presence of multiple β -lactamase genes and mobile genetic elements, the multidrug-resistant phenotype suggests a significant potential as an antibiotic resistance reservoir for *Pseudomonas* spp.

Keywords: *Pseudomonas putida*, whole genome sequencing, multidrug-resistance, *bla*_{PME-1}, *bla*_{NDM-5}, *bla*_{IPM-15}, *bla*_{CARB-2}, urinary tract infection

Introduction

In light of the high mortality rates associated with severe infections and the limited therapeutic treatment options available, the dissemination of carbapenem-resistant Gram-negative bacteria has been a significant concern in hospital settings worldwide. *Pseudomonas putida* is a non-fermenting Gram-negative bacillus that causes bacteremia and sepsis in newborns, neutropenic patients, patients with cancer, as well as urinary tract infections (UTIs). *P. putida* can cause nosocomial infections in hospitalized patients and its high adaptability to challenging environmental conditions enables it to survive in different ecological environments.¹ To date, several *P. putida* outbreaks have occurred in clinical settings, with some outbreaks being linked to the spread of contaminated water outlets.²⁻⁴ Although most *P. putida* are susceptible to a vast majority of antimicrobial agents, multidrug-resistant *P. putida* isolates harboring metallo- β -lactamase (MBL) genes and transposons have been identified already.^{5,6} Additionally, the horizontal transmission of antimicrobial resistance determinants between *P. putida* and *P. aeruginosa* constitutes a serious concern in clinical settings, although the definitive mechanisms remain largely undefined. In this study, we characterized the genomic features of a multidrug-

resistant *P. putida* isolate co-carrying several β -lactamase gene, including *bla*_{PME-1}, *bla*_{IMP-15}, *bla*_{NDM-1}, *bla*_{CARB-2} and a novel β -lactamase gene obtained from a urinary tract infection in China.

Materials and Methods

In March 2021, *P. putida* strain PP_2463 was recovered from the urine sample of a 65-year-old male patient with prostate cancer admitted to a tertiary hospital in Wenling, Zhejiang, China. The specimen was initially incubated overnight at 37°C on Columbia blood agar. Then, a single colony of the target strain was grown in Mueller Hinton broth overnight at 37°C (Oxoid Ltd, Basingstoke, UK). The strain was initially identified by VITEK 2 (bioMérieux, Marcy-l'Étoile, France) and Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS, Bruker, Billerica, MA, USA), and then subjected to 16S rRNA gene sequencing. Antimicrobial agents were purchased from Sigma-Aldrich (St Louis, Missouri, USA) and dissolved according to the manufacturers' instructions. Antimicrobial susceptibility testing was performed by DxM MicroScan WalkAway ID/AST system for the following antimicrobial agents: amikacin, gentamicin, tobramycin, piperacillin, ticarcillin/clavulanic acid, piperacillin/tazobactam, ceftazidime, cefepime, cefazolin, cefuroxime, cefoxitin, aztreonam, imipenem, meropenem, ciprofloxacin, and levofloxacin. The minimum inhibitory concentrations (MICs) were determined according to the Clinical and Laboratory Standards Institute (CLSI) 2020 guidelines. The MICs of the 17 tested antibacterial agents are shown in Table 1. *Escherichia coli* ATCC 25922 was used as a quality control strain.

A Genomic DNA Purification Kit (QIAGEN, Valencia, CA, USA) was used to extract genomic DNA from the isolate and NanoDrop™ spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to assess the purity and concentration of DNA. Whole genome shotgun sequencing of *P. putida* PP_2463 was undertaken using both short-read Illumina NovaSeq 6000 (Illumina Inc., San Diego, CA, USA) and long-read Oxford Nanopore MinION (Oxford Nanopore Technologies, Oxford, UK) platforms to study the mechanisms of antimicrobial resistance. Unicycler 0.4.8 was used to accomplish hybrid assembly of Illumina and Nanopore sequence reads.⁷ The genome sequence was automatically annotated by the NCBI Prokaryotic Genomes Annotation Pipeline (PGAP). *In silico* multilocus sequence typing (MLST) analysis was performed by PubMLST database.^{8,9} ABRicate 1.0.1 was used in conjunction with ResFinder 4.1 and CARD 2020 to identify the antimicrobial resistance genes (ARGs) in the genome.^{10,11} IslandViewer 4, ISfinder 1.0,

Table 1 Genotype and Phenotypic Resistance Profile of the *P. Putida* PP_2463

Antimicrobial Agents	MIC (mg/L)	Susceptibility	Antimicrobial Resistance Genes	
Aminoglycosides				
Amikacin	>32	R	<i>aph(3')-VIa</i> , <i>aph(6)-Id</i> , <i>aph(3'')-Ib</i> , <i>aadA2</i> , <i>rmtB</i> , <i>aph(3')-XV</i> , <i>aadA11</i>	
Gentamicin	>8	R		
Tobramycin	>8	R		
β-lactams				
Piperacillin	>64	R	<i>bla</i> _{PME-1} , <i>bla</i> _{NDM-1} , <i>bla</i> _{CARB-2} , <i>bla</i> _{IMP-15} , <i>bla</i> [*]	
Ticarcillin/clavulanic acid	>64	R		
Piperacillin/tazobactam	>64	R		
Ceftazidime	>16	R		
Cefepime	>16	R		
Cefazolin	>16	R		
Cefuroxime	>16	R		
Cefoxitin	>16	R		
Aztreonam	>16	R		
Imipenem	>8	R		
Meropenem	>8	R		
Fluoroquinolones				
Ciprofloxacin	>2	R		<i>qnrVC1</i>
Levofloxacin	>4	R		

Note: *Novel β -lactamase gene.

Abbreviation: MIC, minimum inhibitory concentration.

PHASTER 2016, CRISPRCasFinder 1.0, and antiSMASH 5.2.0 were used to predict genomic islands, insertion sequence (IS) elements, prophage sequences, clustered regularly interspaced short palindromic repeat (CRISPR) sequences, and secondary metabolite gene clusters, respectively.^{12–16}

Genomic sequences and the associated metadata of 192 *P. putida* strains stored in NCBI GenBank database were obtained. The bacterial core genome single nucleotide polymorphism (cgSNP) analysis between *P. putida* PP_2463 and 192 complete or draft genomic sequences of *P. putida* strains were determined to construct a maximum likelihood phylogenetic tree using CSI Phylogeny 1.4.¹⁷ This analysis was performed using the default parameters and *P. putida* NBRC 14164 (RefSeq ID: NC_021505.1) as the reference genome. SNP distance matrixes were calculated using SNP-dists. Phylogenetic tree were visualized and annotated by Interactive Tree of Life (iTOL) V5 web server.¹⁸ Easyfig was used to analyse genetic context of the antimicrobial resistance genes identified in *P. putida* PP_2463.¹⁹

Results and Discussion

The complete genome sequence of *P. putida* PP_2463 is made up of three contigs totaling 5,561,304 bp. The strain's overall G+C content was 62.5%, and a total of 5129 coding sequences (CDSs), 100 RNAs (74 tRNA, 22 rRNA, and 4 ncRNA) genes were found. Antimicrobial susceptibility testing showed that the isolate was resistant to all tested antimicrobial agents, including aminoglycosides, β -lactams, and fluoroquinolones (Table 1). The resistome of *P. putida* PP_2463 is made up of several genes that are responsible for resistance to β -lactams (*bla*_{PME-1}, *bla*_{IPM-15}, *bla*_{CARB-2}, *bla*_{NDM-1} and a novel β -lactamase gene), tetracyclines [*tet*(G)], aminoglycosides [*aadA2*, *aadA11*, *aph*(3')-VIa, *aph*(6)-Id, *aph*(3'')-Ib, *rmtB* and *aph*(3')-XV], fluoroquinolones (*qnrVc1*) and sulphonamide (*sul1*) (Table 1). The multidrug resistance phenotype to aminoglycosides, third-generation cephalosporins, carbapenems, quinolones and tetracyclines was adequately explained by these findings. Interestingly, all the antimicrobial resistant genes were located in the chromosome (contig 1) and three resistance gene clusters were formed (Figure 1). The first cluster contained the *bla*_{NDM-5} gene, two copies of *bla*_{PME-1}, and a novel β -lactamase gene. Analysis of the genetic environment revealed that *IS91* and *IS5* exists upstream and downstream of β -lactam resistance genes as a truncated fragment. The genetic environment of the second cluster revealed that *int11* and *Tn3* lies downstream of *bla*_{CARB-2}, while the *aadA*, *folP*, and *floR2* genes are located upstream of the *bla*_{CARB-2} gene. Besides, the second cluster also carried the *aph*(6)-Id, *aph*(3'')-Ib, *aadA2*, *sul1* and *tet*(G) genes. The third cluster carried several β -lactam resistance genes, including *bla*_{CARB-2}, *bla*_{IMP-15} and other resistance genes, including *rmtB*, *tet*(G), *aph*(3')-XV, *dfrA27*, *aph*(6)-Id, *aph*(3'')-Ib, *sul1*, *aadA11*, and *qnrVc1*. There are at least 65 genomic islands and multiple IS elements in the genome of *P. putida* PP_2463, and the majority of them belong to the *IS3*, *IS5*, and *IS66* families. Besides, a total of 10 prophages and just one CRISPR sequence can be predicted. Interestingly, we found a new β -lactamase gene (NCBI accession number OM585599) that

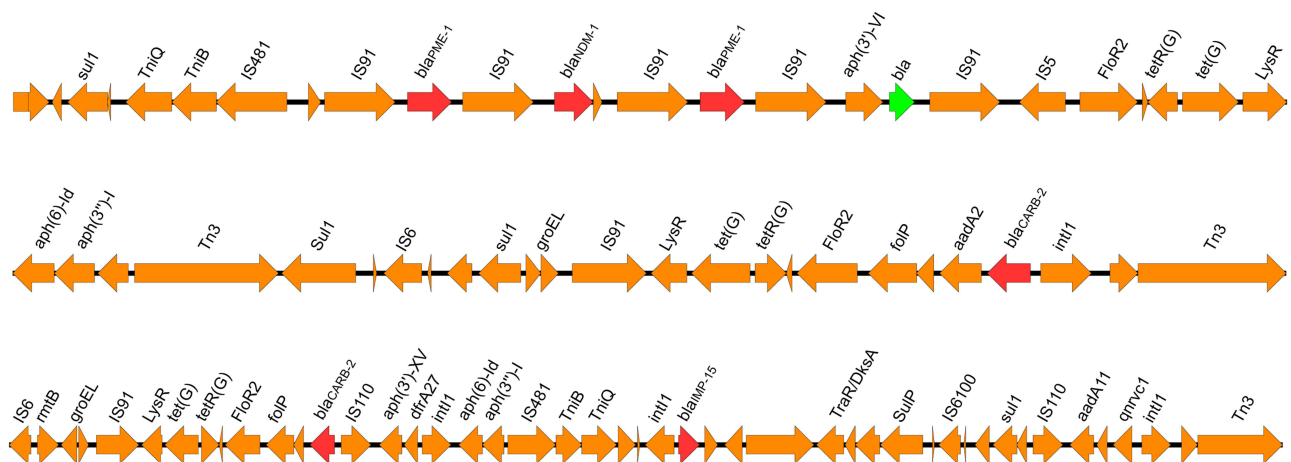


Figure 1 Genetic environment of three clusters of the β -lactam resistance genes in *P. putida* PP_2463. The red arrows represent the β -lactam resistance genes, the green arrow represents the novel β -lactamase gene discovered in this study, whereas the orange arrows represent additional coding sequences (CDSs).

has not even been named yet, and to our knowledge the co-occurrence of *bla*_{PME-1}, *bla*_{IPM-15} and *bla*_{NDM-1} genes was also firstly identified in *P. putida*.

In silico MLST analysis indicated that *P. putida* PP_2463 represent a novel sequence type, which has been assigned to ST148 after we submitted to the PubMLST database and curated. The phylogenetic connections between *P. putida* PP_2463 and a total of 192 *P. putida* strains currently deposited in the NCBI GenBank database were examined to evaluate the genomic epidemiological features of *P. putida* strains in a global context (Figure 2A). The 193 strains of *P. putida* in this study were recovered from various hosts, including human (n = 32, 16.5%), plant (n = 21, 10.8%), animal (n = 4, 2.0%) and other unknown resources (n = 136, 70.4%). The isolation dates ranged from 1977 to 2021, but most isolates were obtained between 2011 and 2021 (55.9%, n = 108). Europe (25.3%, n = 49) is the main separation continent, followed by Asia (24.3%, n = 47), North America (20.2%, n = 39), Africa (5.6%, n = 11), Antarctica (1%, n = 2), Oceania (1.0%, n = 2), South America (0.5%,

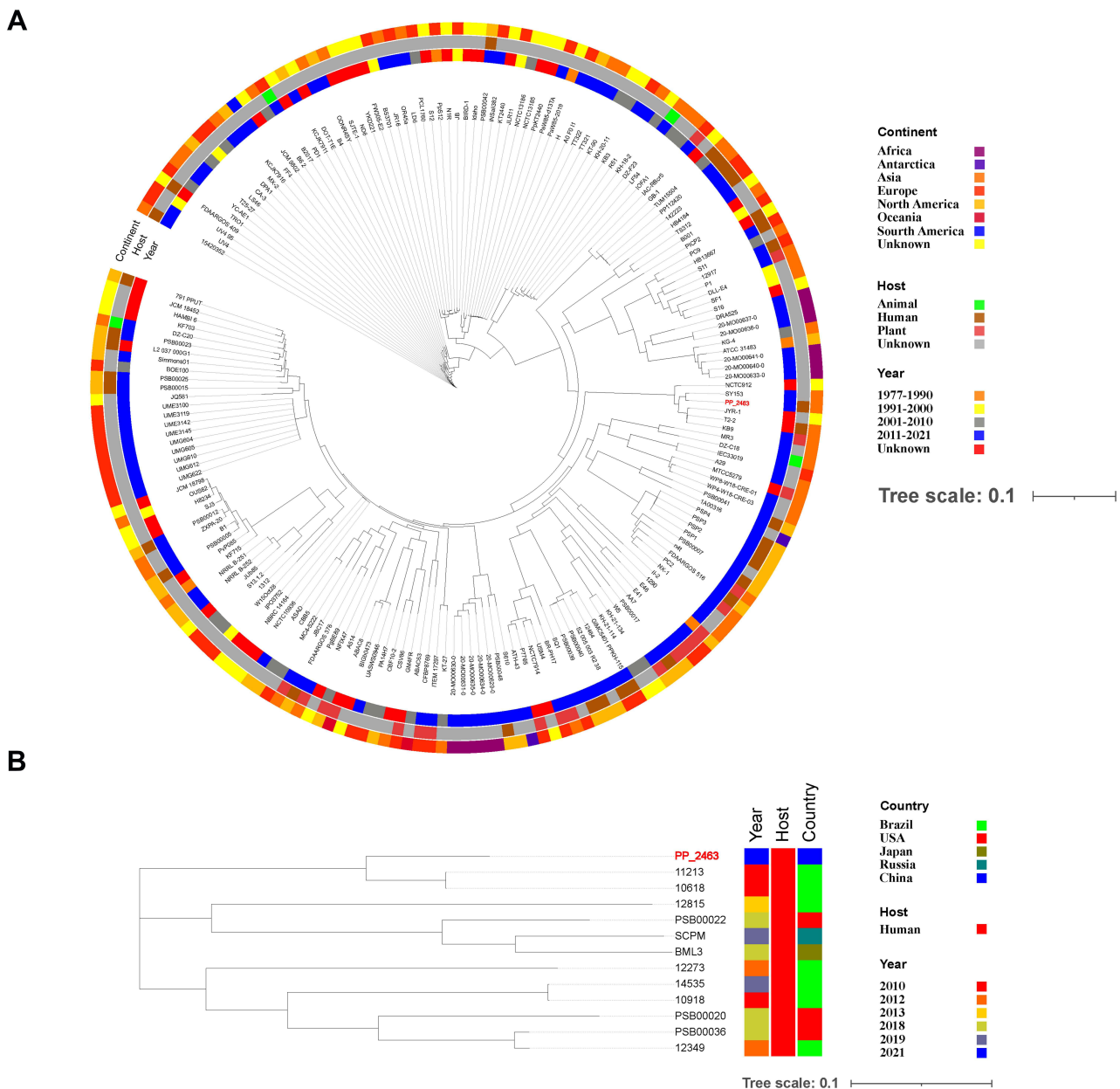


Figure 2 (A) The phylogenetic relationship between *P. putida* PP_2463 and a total of 192 *P. putida* strains currently deposited in the NCBI GenBank database. **(B)** The phylogenetic relationship between *P. putida* PP_2463 and a total of 12 *P. putendi* strains currently available in the NCBI GenBank database. The distance of SNPs is represented by the branch length. The positions corresponding to the three circles represent the continent, host and the isolation date, respectively.

n = 1). According to the results of phylogenetic analysis, the closest relative of *P. putida* PP_2463 was identified in 2012 from a urine sample in Sanya, China, with just 143 SNPs difference. Interestingly, after we submitted the genome sequence to NCBI for annotation, the curator used *P. juntendi* as the organism's name for this genome due to a higher level of similarity according to the current taxonomic nomenclature. We then further performed the average nucleotide identity (ANI) analysis of *P. putida* PP_2463 with the genome sequence of the type strains of *P. putida* and *P. juntendi*. The ANI results revealed that the genome of *P. putida* PP_2463 is > 99.9% identical to the type strain of both species. *P. juntendi* was a novel species firstly identified in hospital patients in Japan and Myanmar in 2019,²⁰ which is close to several species in the genus of *Pseudomonas*, including *P. putida*. Therefore, we also investigated the genomic epidemiological features of the currently available *P. juntendi* strains in a global context (Figure 2B). The closest relative of *P. putida* PP_2463, obtained from a blood sample in Brazil in 2010, with 10,607 SNPs difference. Ribosomal Multilocus Sequence Typing (rMLST) analysis also showed that *P. putida* PP_2463 could be identified as *P. putida* or *P. juntendi* with equal confidence score.

Conclusion

In summary, we firstly reported a ST148 *P. putida* strain in China that carrying multiple chromosome-borne *bla*_{PME-1}, *bla*_{IPM-15}, *bla*_{CARB-2}, *bla*_{NDM-1} and a novel β -lactamase genes. The prevalence of *P. putida* isolates carrying β -lactamase resistance determinants raises major concerns, emphasizing the critical need for alternate antibiotic therapy and continuing active surveillance. These findings will shed new light on the genomic epidemiological characteristics and the global transmission dynamics of *P. putida* and demonstrates its superior capacity for acquiring and maintaining foreign resistance determinants.

Data Sharing Statement

The genome sequences of the chromosome and plasmids of the isolate have been deposited in NCBI GenBank under accession numbers CP091088-CP091090.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the Declaration of Helsinki and obtained approval from Medical Ethics Committee at the Affiliated Wenling Hospital, Wenzhou Medical University, China. Written informed consent was provided by the patient to allow the case details to be published.

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Disclosure

The authors report no conflicts of interest in this work.

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