

# Emergence and Genetic Characterization of Plasmid-Encoded VIM-2-Producing *Pseudomonas stutzeri* with Novel Integron In1998 Isolated from Cerebrospinal Fluid

Shuxiu Liu<sup>1,2</sup>  
Hao Xu<sup>2</sup>  
Xiaobing Guo<sup>1</sup>  
Shuang Li<sup>3</sup>  
Qian Wang<sup>1,2</sup>  
Yuan Li<sup>4</sup>  
Ruishan Liu<sup>1,2</sup>  
Jianjun Gou<sup>1</sup>

<sup>1</sup>Department of Laboratory Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, 450052, People's Republic of China; <sup>2</sup>State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, 310003, People's Republic of China; <sup>3</sup>Department of Obstetrics and Gynecology, Zhengzhou Central Hospital Affiliated to Zhengzhou University, Zhengzhou, 450052, People's Republic of China; <sup>4</sup>Department of Nuclear Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, 450052, People's Republic of China

Correspondence: Jianjun Gou  
Tel +86 371 6627 8237  
Fax +86 371 6691 3569  
Email jianjung@zzu.edu.cn

**Purpose:** To investigate the genomic and plasmid characteristics of a newly discovered *Pseudomonas stutzeri* strain with a *bla*<sub>VIM-2</sub>-carrying plasmid and novel integron In1998 isolated from a cerebrospinal fluid specimen in a teaching hospital.

**Methods:** Species identification was performed by MALDI-TOF MS, and *bla*<sub>VIM-2</sub> was identified by PCR and Sanger sequencing. Whole-genome sequencing analysis was conducted using the Illumina NovaSeq 6000 and Oxford Nanopore platforms. Integron detection was performed using INTEGRALL. The phylogenetic tree was constructed by using kSNP3.0. Plasmid characteristics were assessed by S1-pulsed-field gel electrophoresis (S1-PFGE), Southern blotting, conjugation experiments, and whole-genome sequencing analysis. Comparative genomics analysis of the plasmid and genetic context of *bla*<sub>VIM-2</sub> were conducted by using BLAST Ring Image Generator (BRIG) and Easyfig 2.3, respectively.

**Results:** ZDHY95, an MDR strain of *P. stutzeri* harboring *bla*<sub>VIM-2</sub>, was identified. It was sensitive only to amikacin and was resistant to carbapenems,  $\beta$ -lactams, aztreonam, fluoroquinolones, and aminoglycosides. Joint S1-PFGE, Southern blot, conjugation assay, and whole-genome sequencing experiments confirmed that the *bla*<sub>VIM-2</sub> gene was located within class I integron In1722 of the plasmid and that the surrounding genetic environment was 5'CS-*aacA4*'-30-*bla*<sub>VIM-2</sub>-*aacA4*'-3'CS. The novel class I integron In1998 was detected on the chromosome of *P. stutzeri* ZDHY95, and the gene cassette array was 5'CS-*aacA3*-*aadA13*-*cmlA8*-*bla*<sub>OXA-246</sub>-*arr3*-*dfrA27*-3'CS. Phylogenetic analysis showed that antimicrobial resistance gene-carrying *P. stutzeri* isolates were divided into two clusters, mainly containing isolates from the USA and Pakistan.

**Conclusion:** A novel *bla*<sub>VIM-2</sub>-carrying conjugative plasmid, pZDHY95-VIM-2, was reported for the first time in *P. stutzeri*, elucidating the genetic environment and transfer mechanism. The gene structure of the novel class I integron In1998 was also clarified. We explored the phylogenetic relationship of *P. stutzeri* with drug resistance genes and suggested that *Pseudomonas* with metallo- $\beta$ -lactamases (MBLs) in the hospital environment may cause infection in patients with long-term intubation or after interventional surgery.

**Keywords:** *Pseudomonas stutzeri*, *bla*<sub>VIM-2</sub>, In1998, In1722, Tn5563, whole-genome sequencing, bacterial genomics, antibiotic resistance

## Introduction

*Pseudomonas stutzeri* is an aerobic, nonfermenting, active, Gram-negative oxidase-positive bacterium with unique colony morphology.<sup>1,2</sup> Burri and Stutzer first described it in 1985,<sup>3</sup> and the specific metabolic properties, such as

denitrification, degradation of aromatic compounds, and nitrogen fixation, distinguish it from other pseudomonads species.<sup>2,4</sup> Historically, *P. stutzeri* was not commonly isolated clinically and rarely caused human disease,<sup>5</sup> exist mainly in environmental, occupying diverse ecological niches.<sup>2</sup> However, the disease spectrum associated with its infections is broad, including endocarditis, bacteremia, pneumonia, osteomyelitis, arthritis, and ocular infections.<sup>6</sup> *P. stutzeri* has been increasingly considered an opportunistic pathogen responsible for human infections.<sup>6–8</sup>

Carbapenem-resistant *P. stutzeri* was first isolated from Taiwan's hospital environment in 2001, with three isolates carried IMP-1 and one harbored VIM-2.<sup>9</sup> Subsequently, Netherlands identified a clinical *P. stutzeri* isolate with a single 70-kb plasmid carrying the *bla*<sub>DIM-1</sub> gene in 2010,<sup>10</sup> and Brazil reported *bla*<sub>IMP-16</sub>-carried *P. stutzeri* the same year.<sup>11</sup> In 2017, class I integron containing *bla*<sub>VIM-2</sub> emerged in *P. stutzeri* in Bangladesh.<sup>12</sup> However, the carbapenemase resistance genes detected in *P. stutzeri* (except *bla*<sub>DIM-1</sub>) were identified as chromosomal located. The discovery of transferable plasmid carrying *bla*<sub>VIM-2</sub> gene in *P. stutzeri* has not so far been reported.

The VIM-type Metallo- $\beta$ -lactamases were first identified in *Pseudomonas aeruginosa* in Europe and have subsequently been reported worldwide in *Enterobacteriaceae*, *Pseudomonas*, and *Acinetobacter*.<sup>13,14</sup> VIMs have a broad substrate hydrolysis profile, which can degrade almost all classes of  $\beta$ -lactams apart from the monobactams.<sup>13</sup> The *bla*<sub>VIM</sub> is usually integrated into class I integron in the gene cassette and spreads among bacteria through mobile genetic elements.<sup>13</sup> Besides, the coexistence of *bla*<sub>VIM</sub> and one or more aminoglycoside resistance genes, such as *aacA4*, *aacA7*, *aadA1*, *aadA2*, *aadB*, and *aacC1*, is very common.<sup>15</sup> VIM-2 was first identified in southern France in 1996,<sup>16</sup> which exhibits 93% amino acid identity to VIM-1<sup>17</sup> and sequence heterogeneity primarily observed in the NH<sub>2</sub>- and carboxy-terminal regions.<sup>13</sup> Worldwide, VIM-2 is the most comprehensive distributed MBLs in Gram-negative bacteria.<sup>18</sup>

We first identified a clinical *P. stutzeri* isolate ZDHY95 with *bla*<sub>VIM-2</sub>-harboring plasmid and novel integron *In1998* and implemented phylogenetic analysis in this study. Additionally, we elucidated the resistance mechanism of isolate and characterized the genetic environment and transfer mechanism of the plasmid.

## Materials and Methods

### Sample Collection and Bacterial Culture

Strains ZDHY95 and ZDHY372 were isolated from two cerebrospinal fluid specimens collected from the same patient with an interval of one month. The patient experienced intubation and drainage treatment after a cerebral hemorrhage in the Department of Neurosurgery of a teaching hospital of Zhengzhou University in April 2019. Subsequently, the patient developed a severe intracranial infection and persistent high fever. Therefore, the drained cerebrospinal fluid was collected for microbiological culture overnight at 37°C in 5% CO<sub>2</sub> after inoculation on Columbia blood agar plates.

### Phenotype Confirmation and Antimicrobial Susceptibility Testing

The species of the isolates ZDHY95 and ZDHY372 were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Bruker, Bremen, Germany).<sup>19</sup> Antimicrobial susceptibility testing (AST) was conducted by agar dilution, and standard strains of *Escherichia coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) were used as quality controls. The results of AST were interpreted following the Clinical and Laboratory Standards Institute (CLSI) 2020 standards.<sup>20</sup>

### Carbapenemase Gene Identification and Pulsed-Field Gel Electrophoresis

The carbapenemase-encoding gene was identified using PCR and DNA sequencing. The detailed technique for PCR amplification was as described previously.<sup>21</sup> The homology analysis of the strains ZDHY95 and ZDHY372 was determined via pulsed-field gel electrophoresis (PFGE) as previously described, with a slight modification.<sup>22</sup> Briefly, DNA plugs with whole-cell genomic DNA of culture-lysed cells were digested using the restriction enzyme SpeI (Takara Bio Inc., Japan). PFGE was undertaken on a CHEF-DR III system (Bio-Rad, Hercules, CA, USA), and the patterns were visually assessed and interpreted following published guidelines.<sup>23</sup>

### Plasmid Characterization and Conjugation Assays

The number and sizes of the plasmids of the strains were characterized by S1-PFGE.<sup>24</sup> The location of the *bla*<sub>VIM-2</sub> gene was confirmed by Southern blotting and hybridization

with a digoxigenin-labeled *bla*<sub>VIM</sub> probe using a DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics). Conjugation transfer experiments were conducted to explore plasmids' transferability with rifampin-resistant *P. aeruginosa* PAO1Ri as recipients, as described previously.<sup>25</sup> Subsequently, Mueller-Hinton agar (OXOID, Hampshire, United Kingdom) plates that contained both 200 mg/L rifampicin and 2 mg/L meropenem were used to select transconjugants. The final identification of transconjugants includes MALDI-TOF/MS identification, *bla*<sub>VIM</sub> gene detection, and AST.

## Whole-Genome Sequencing and Bioinformatics Analysis

The Bacterial DNA kit (Omega, Biotek, Norcross, USA) was utilized to extract the strains' genomic DNA. Subsequently, the DNA was sequenced using Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) and Oxford Nanopore (Oxford Nanopore Technologies, Oxford, UK) platforms. Assembly of sequencing fragments was performed via Unicycler v0.4.2, and finally obtained the strains' complete genomic sequences. The genome sequences were annotated by RAST2.0 (<http://rast.nmpdr.org/>). Insertion sequence (IS) elements and integrons were detected via the ISfinder database (<https://www-is.biotoul.fr/>) and INTEGRALL (<http://integrall.bio.ua.pt/>). The identification of acquired antimicrobial resistance genes (ARGs) was carried out by Resfinder (<https://cge.cbs.dtu.dk/services/ResFinder/>). BLAST Ring Image Generator (BRIG) was used to generate a circular map to compare multiple plasmid genome sequences. The figures comparing the genetic context of the antibiotic resistance genes were developed with Easyfig 2.3.

## Phylogenetic Analysis

The fasta files of all *P. stutzeri* strains were downloaded from the NCBI genome database and conducted ARGs analysis by Resfinder. The phylogenetic tree was constructed on core genome single nucleotide polymorphisms (SNPs) in WGS data via kSNP3.0 with *P. stutzeri* strain 40D2 as the reference. The maximum likelihood tree was visualized and modified using online tools iTOL (<https://itol.embl.de/>).

## Accession Numbers and Ethical Approval

The complete nucleotide sequences of both the *P. stutzeri* ZDHY95 chromosome and plasmid pZDHY95-VIM-2

were deposited in GenBank with accession numbers CP063358 and CP063359. The ethical protocol was approved by the Ethics Committee of First Affiliated Hospital of Zhejiang University (no. 2018–752).

## Results

### Species Confirmation and Homology Analysis

Bacteriological culture of the cerebrospinal fluid was positive, Gram-negative bacilli ZDHY95 and ZDHY372 were isolated, and the species were confirmed as the opportunistic pathogen *P. stutzeri*. The PFGE results of the two strains are shown in [Figure S1](#). Genetic relatedness based on the PFGE patterns and interpreted according to the criteria proposed by Tenover et al<sup>23</sup> showed that ZDHY95 and ZDHY372 are of the same clone. Therefore, isolate ZDHY95 was selected for further detailed investigation.

### AST of *Pseudomonas Stutzeri* ZDHY95

The results of AST of *P. stutzeri* ZDHY95 are shown in [Table 1](#). According to the MIC breakpoints for other non-Enterobacterales in CLSI 2020 standards, isolate ZDHY95 exhibited resistance to piperacillin-tazobactam, ceftazidime, cefepime, cefotaxime, ceftriaxone, meropenem, imipenem, gentamicin, ciprofloxacin, and levofloxacin, intermediate resistance to aztreonam, with sensitivity only to amikacin.

**Table 1** MIC Values of Antimicrobials for *P. stutzeri* ZDHY95, Recipient Strain PAO1Ri, and Transconjugant ZDHY95-PAO1Ri

Antimicrobials	MIC Values (mg/L)		
	<i>P. stutzeri</i> ZDHY95	ZDHY95-PAO1Ri	PAO1Ri
Piperacillin-tazobactam	128/R	64/I	1/S
Ceftazidime	32/R	32/R	1/S
Cefepime	32/R	16/I	1/S
Cefotaxime	128/R	128/R	8/S
Ceftriaxone	128/R	128/R	4/S
Meropenem	32/R	32/R	0.25/S
Imipenem	32/R	32/R	4/S
Aztreonam	16/I	1/S	1/S
Gentamicin	32/R	32R	1/S
Amikacin	8/S	8/S	2/S
Ciprofloxacin	64/R	4/R	1/S
Levofloxacin	64/R	4/I	1/S

**Abbreviations:** R, resistant; S, susceptible; I, intermediate.

## Location of *bla*<sub>VIM-2</sub> and the AST of Transconjugant

S1-PFGE and Southern blot hybridization indicated that ZDHY95 contained an ~88 kb plasmid with *bla*<sub>VIM-2</sub>, designated as pZDHY95-VIM-2 (Figure S1). PCR and Sanger sequencing confirmed a transconjugant as *bla*<sub>VIM-2</sub>-encoding PAO1Ri. The comparison of AST of ZDHY95-PAO1Ri and PAO1Ri (Table 1) reveals that ZDHY95-PAO1Ri shows significant drug resistance to ceftazidime, ceftriaxone, cefotaxime, meropenem, imipenem, gentamicin, and ciprofloxacin; intermediate resistance to piperacillin-tazobactam, cefepime, levofloxacin. Although still sensitive to amikacin, its MIC value has increased. Only the MIC of aztreonam remains unchanged. These results indicate that the plasmid pZDHY95-VIM-2 has successfully transferred into recipient PAO1Ri and impacted bacterial resistance.

## Characterization of the Genome of *P. stutzeri* ZDHY95 and In/998

The genome of ZDHY95 consisted of a circular chromosome of 4,500,524 bp and a plasmid of 88,056 bp. The genomic characteristics of *P. stutzeri* ZDHY95 are shown in Table S1. Fourteen drug resistance genes were identified by the Resfinder, of which six were plasmid located; the detailed results are shown in Table 2. Novel class I integron In/998 was identified in the chromosome of *P. stutzeri* ZDHY95 by INTEGRALL. Analysis of the sequence shows that In/998 has a sequenced size of 8183 bp and the gene cassette array includes 5'CS-*aacA3* (aminoglycoside resistance)-*aadA13* (aminoglycoside resistance)-*cmlA8*

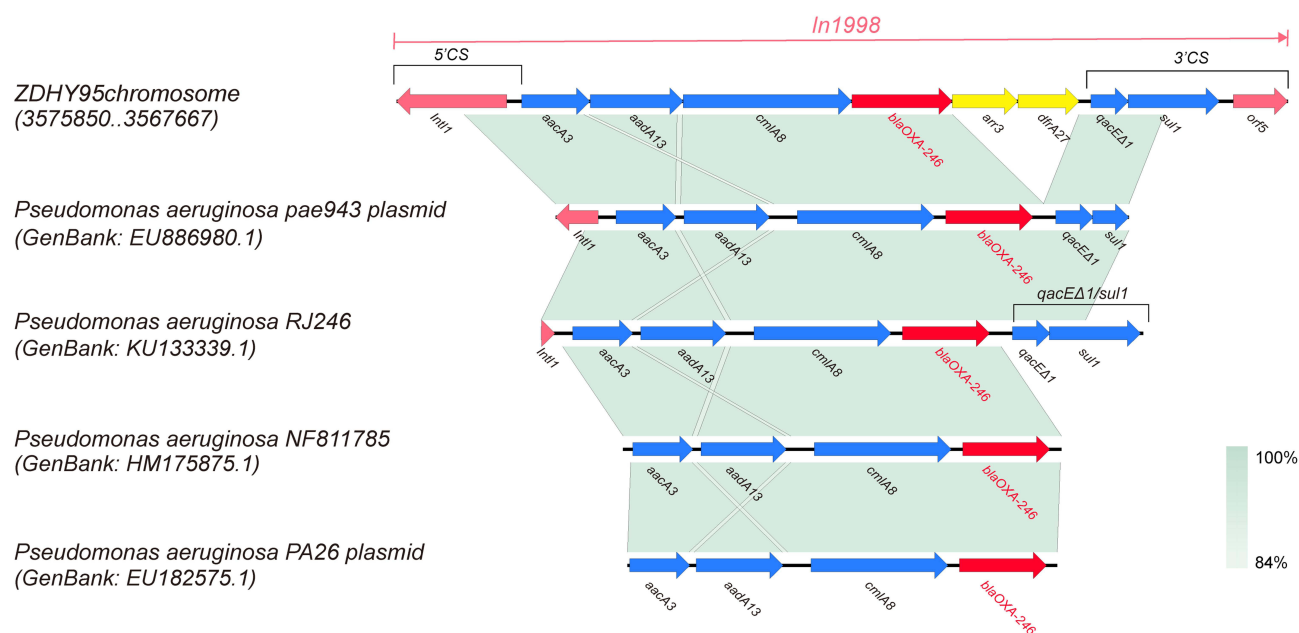
(phenicol resistance)-*bla*<sub>OXA-246</sub> (cephalosporin resistance)-*arr3* (rifamycin resistance)-*dfrA27* (trimethoprim resistance)-3'CS. NCBI BLAST analysis revealed that In/998 shared a high degree of genetic similarity (query coverage over 95% and identity 99%) with *P. aeruginosa* strain pae94323 (EU886980.1), *P. aeruginosa* strain RJ24624 (KU133339.1), *P. aeruginosa* strain NF811785 (HM175875.1), and *P. aeruginosa* strain PA26 (EU182575.1) from different regions of China. The gene cassette array and the genetic map of In/998 based on blastn and sequence analysis are shown in Figure 1.

## Structural Characterization of the Plasmid pZDHY95-VIM-2

The plasmid pZDHY95-VIM-2 has a sequence length of 88,056 bp and contains 91 protein-coding genes with a G +C content of 57% (Table S1). It consists of three central regions: the replication origin site *repA* and putative backbone genes downstream involved in maintaining stability (*parB*, *arc*, *tonB*, and *pill*) and DNA metabolism (*soj*, *dnaB*, *topB*, *rapA*), conjugative transfer region, and multi-drug resistance region. The plasmid could not be categorized into any of the known incompatibility groups by PlasmidFinder. NCBI BLAST analysis revealed that the complete sequence of pZDHY95-VIM-2 shared the highest degree of genetic similarity (query coverage over 17% and identity 99%) with p1160-VIM (MF144194.2) from *P. aeruginosa* 1160.<sup>25</sup> The comparative plasmid circular map shows the genes and their locations (Figure 2A). The conjugative transfer region contains a large number of

**Table 2** Antibiotic Resistance Genes of *P. stutzeri* ZDHY95

Genome	Resistance Gene	Resistance Phenotype	Nucleotide Position	Region Located
Chromosome	<i>sulI</i>	Sulfonamide resistance	3,568,295–3,569,134	In/998
	<i>qacEA1</i>	Quaternary ammonium Compound resistance	3,569,128–3,569,475	
	<i>dfrA27</i>	Trimethoprim resistance	3,569,654–3,570,127	
	<i>arr3</i>	Rifamycin resistance	3,570,260–3,570,712	
	<i>bla</i> <sub>OXA-246</sub>	Cephalosporin resistance	3,570,848–3,571,648	
	<i>cmlA8</i>	Phenicol resistance	3,571,752–3,573,011	
	<i>aadA13</i>	Aminoglycoside resistance	3,573,266–3,574,063	
	<i>aacA3</i>	Aminoglycoside resistance	3,574,121–3,574,675	
pZDHY95-VIM-2	<i>aacA4'-30</i>	Aminoglycoside resistance	60,001–60,639	In/722
	<i>bla</i> <sub>VIM-2</sub>	Carbapenem resistance	60,670–61,470	
	<i>aacA4'</i>	Aminoglycoside resistance	61,549–62,187	
	<i>qnrVC1</i>	Fluoroquinolone resistance	66,658–67,314	
	<i>catB11</i>	Phenicol resistance	67,495–68,127	
	<i>bla</i> <sub>CARB-4</sub>	Beta-lactam resistance	68,277–69,143	



**Figure 1** The gene cassette arrays and the genetic map of In1998 and linear comparison with *P. aeruginosa* strain pae943, *P. aeruginosa* strain RJ246, *P. aeruginosa* strain NF811785, *P. aeruginosa* strain PA26 based on blastn and sequence analysis. Genes are indicated as arrows. Genes, mobile elements, and other features are colored based on function classification.

insertion sequences (ISPpu30, ISPst9, ISAzs36, IS481) and the putative genes associated with the conjugal transfer (*traG*, *traI*) and type IV secretion system (*virB*, *virD4*). The MDR region of plasmid pZDHY95-VIM-2 comprises In1722, the Tn402-like *tmi* module, and transposon Tn5563. In1722 is an unreported integron carrying *bla*<sub>VIM-2</sub> with the gene cassette array of 5'CS-*aacA4'*-30-*bla*<sub>VIM-2</sub>-*aacA4'*-3'CS. Adjoined to In1722 is the Tn402-like *tmi* module, which has insertion mutations compared to the typical Tn402 *tmi* module. The insertion of the gene fragment, including *qnrVCI*, *catB11*, and *bla*<sub>CARB-4</sub>, split *tmiA* into two discontinuous parts, forming the Tn402-like *tmi* module. Transposon Tn5563, with a sequenced length of 6253 bp, has the following structure: *tnpR* (resolvase)–*orf2* (hypothetical protein)–*pliT* (*pilT* domain-containing protein)–*tnpA* (transposase)–*mer* (mercuric resistance gene locus). Further sequence alignment performed between pZDHY95-VIM-2 and p1160-VIM are shown in Figure 2B.

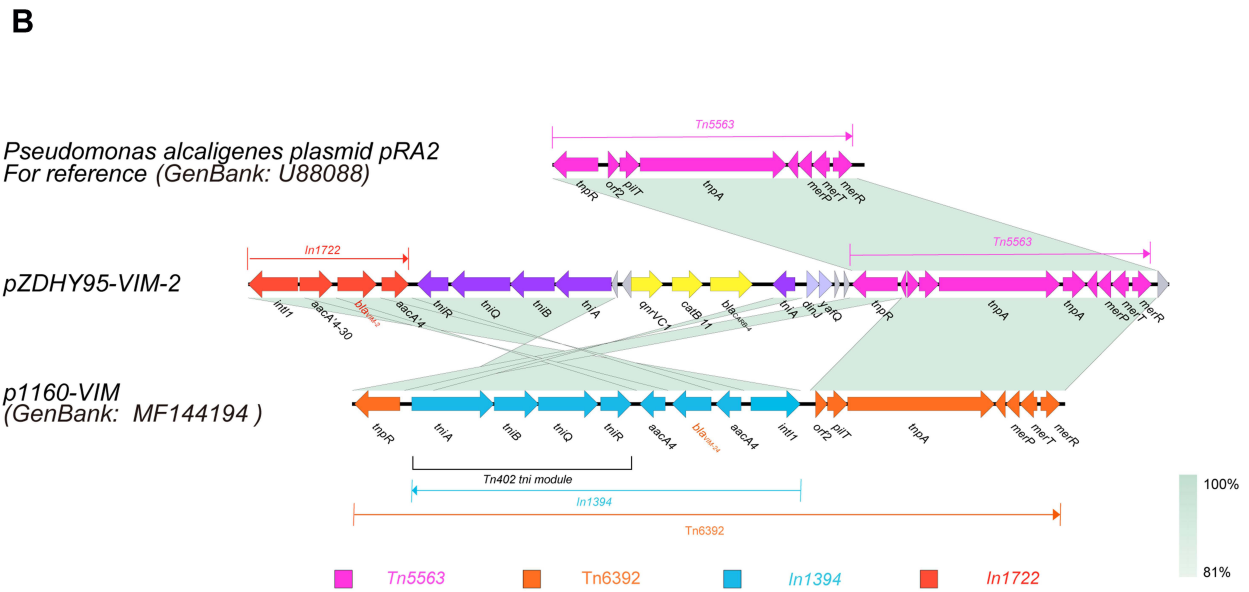
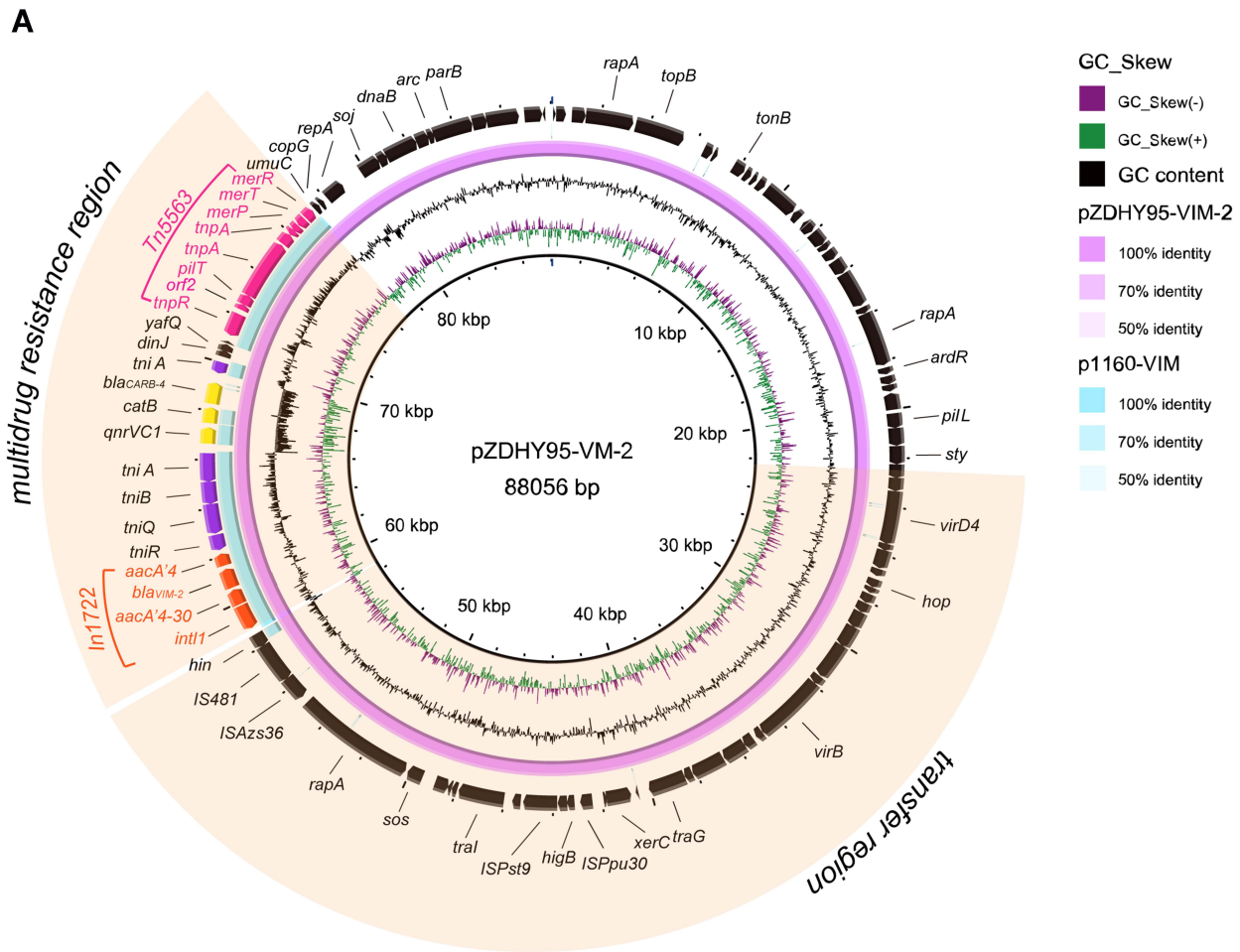
## Phylogenetic Analysis

Twenty-two strains of *P. stutzeri* with drug resistance genes were screened out from a total of 283 in NCBI genome database and used for phylogenetic analysis together with *P. stutzeri* ZDHY95 (Figure 3). The results illustrated that the 23 strains aggregated into two clusters; *P. stutzeri* UBA4963 (GCA\_002487265.1),

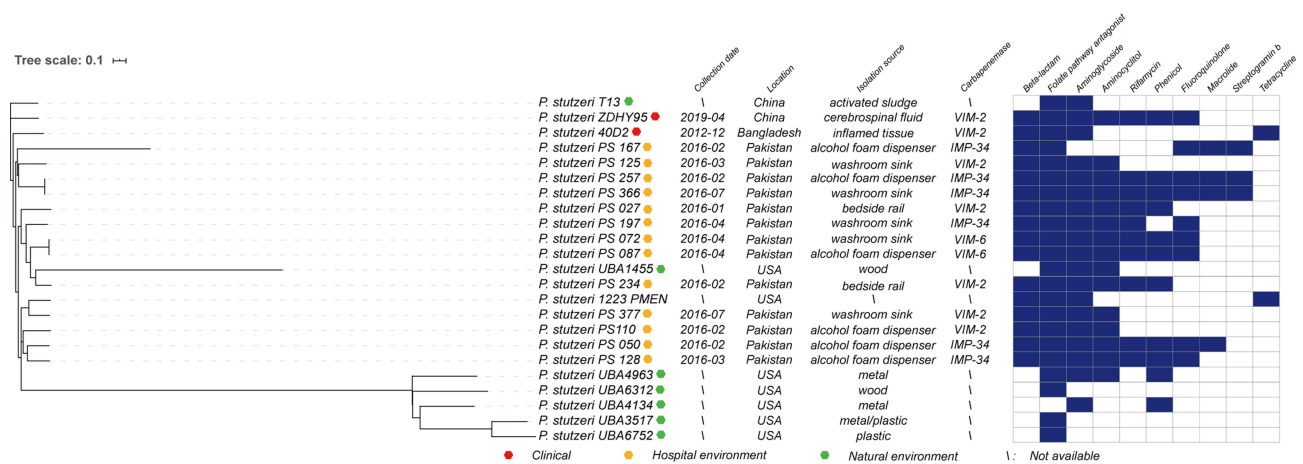
*P. stutzeri* UBA6312 (GCA\_002439185.1), *P. stutzeri* UBA4134 (GCA\_002380885.1), *P. stutzeri* UBA3517 (GCA\_002377205.1), *P. stutzeri* UBA6752 (GCA\_002453575.1) composed a smaller cluster, and the remaining formed the other. *P. stutzeri* strains with drug resistance genes are mainly found in Pakistan and the USA, with a few scattered in China and Bangladesh. The strains isolated in Pakistan are all from accessible surfaces in the hospital ICU, such as bedside rail, wash-room sink, and alcohol foam dispenser. They carry *bla*<sub>VIM-2</sub>, *bla*<sub>VIM-6</sub>, and *bla*<sub>IMP-34</sub>, respectively. The period of strain collection was six months. The strains isolated from the USA constitute the smaller cluster were all collected from natural environments, such as metal, plastic, and wood, and lacked carbapenemases. There are only two isolates from clinical samples, ZDHY95 and 40D2, both carrying *bla*<sub>VIM-2</sub>. *P. stutzeri* ZDHY95 is closely related to *P. stutzeri* T13 (GCA\_000282955.1) isolated from China and *P. stutzeri* 40D2 (GCA\_002027175.1) from Bangladesh.

## Discussion

In recent years, the frequency of isolation of *P. stutzeri* from clinical material has increased remarkably. Nevertheless, these strains rarely exhibit pathogenicity<sup>1</sup> and are considered more likely to represent opportunistic



**Figure 2** Genomic analyses of plasmid pZDHY95-VIM-2. **(A)** The comparative plasmid circular map of pZDHY95-VIM-2 and p1160-VIM, generated using BLAST Ring Image Generator (BRIG), shows the genes and their locations. **(B)** Genetic context of *bla*<sub>VIM-2</sub> on pZDHY95-VIM-2. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification.



**Figure 3** The maximum likelihood core-gene phylogenetic tree of *P. stutzeri* based on the resistance genes, generated by kSNP3.0 using *P. stutzeri* 40D2 as a reference genome. The sources of strains are identified as clinical, hospital, and natural environment and are marked with red, yellow, and green polygons, respectively. The heatmap displays the types of antibiotic resistance genes in each strain. Blue indicates that the isolate carries such genes and colorless means that the genes are not present.

colonization or contamination of patients.<sup>5</sup> However, *P. stutzeri* isolated from sterile sites should be given considerable attention.<sup>1</sup> *Pseudomonas* infection in the central nervous system is generally uncommon, and non-*Pseudomonas aeruginosa* infection is rare. To the best of our knowledge, central nervous system infections related to *P. stutzeri* are sporadic, with fewer than 10 cases reported to date. One case was an infection related to a brain abscess,<sup>26</sup> and the remaining instances caused meningitis.<sup>11,27–31</sup> To our knowledge, this is the first report of multidrug-resistant *P. stutzeri* with a *bla*<sub>VIM-2</sub>-carrying plasmid causing a nosocomially acquired central nervous system infection.

Low virulence and high antibiotic sensitivity are stereotypical characteristics of *P. stutzeri* compared with *P. aeruginosa*.<sup>1,2,32</sup> Indeed, initial studies indicated that isolates of *P. stutzeri* are generally susceptible to third-generation cephalosporins, aminoglycosides, antipseudomonal penicillins, fluoroquinolones, monobactams, carbapenems, and trimethoprim/sulfamethoxazole and are variably vulnerable to ampicillin.<sup>1,5,32,33</sup> However, this has changed since MBLs appeared in *P. stutzeri*. Currently, almost all of the reported *P. stutzeri* carrying carbapenemase are resistant to cefotaxime, ceftazidime, meropenem, and imipenem, and strain ZDHY95 in this study showed resistance to all antibiotics tested except aztreonam. These indicated that *P. stutzeri* is no longer the traditionally appreciated low-virulence, high-sensitivity colonizer or contaminant; instead, it may cause severe infection or mortality.<sup>6,11,34</sup>

Novel class I integron In1998 has three modules the same as classical class I integron: a 5' conserved segment

(5'CS), a variable region with resistance gene cassettes, and a 3' conserved segment (3'CS).<sup>35</sup> Sequence comparison of the strains (Figure 1) mentioned above indicated that *aacA3-aadA13-cmlA8-bla*<sub>OXA-246</sub> might be transferred in bacteria as a resistance gene cassette. The presence of *bla*<sub>OXA-246</sub> embedded in In1998 may result from the horizontal transfer of the resistance gene cassette. In addition to the similar genes shared by all sequences, In1998 carried two other resistance genes, *arr3* and *dfrA27*, which inserted between *bla*<sub>OXA-246</sub> and 3'CS. Although there are no mobile DNA elements in In1998, such as a Tn402 *tni* module, the insertion sequences and transposons upstream and downstream make the horizontal transfer possible (Table S2). To the best of our knowledge, In1998 is the second reported integron carrying *bla*<sub>OXA-246</sub>. OXA-246 is the OXA-10 family class D beta-lactamase, first detected in *P. aeruginosa* in China in 2014.<sup>36</sup> OXA-246 has a nonsynonymous amino acid mutation at Lys141Asn compared with OXA-10 and was considered a non-ESBL  $\beta$ -lactamase for its narrow hydrolysis spectrum of ampicillin, penicillin G, carbenicillin, and ticarcillin.<sup>36</sup> Subsequently, reports of *bla*<sub>OXA-246</sub> only appeared in Vietnam<sup>37</sup> and the Czech Republic,<sup>38</sup> both in *P. aeruginosa*. Our study is the first report of *bla*<sub>OXA-246</sub> in *P. stutzeri* to date.

In plasmid pZDHY95-VIM-2, *bla*<sub>VIM-2</sub> is integrated into the class I integron In1722, designated by the INTEGRALL database, and the gene cassette array was 5'CS-*aacA4'*-30-*bla*<sub>VIM-2</sub>-*aacA4'*-3'CS. Unlike the typical class I integron, the 3'CS of In1722 was replaced by *tniC/R*, a functional transposon in Tn402 (Tn5090), instead of the *qacEA1/sul1*. The same phenomenon in class I integrons carrying *bla*<sub>VIM-2</sub>

has been described in 2007.<sup>39</sup> It was considered an ancestral version of the class I integrons because Tn402 (Tn5090) was initially coupled to the typical 5'CS region of class I integron preceding the *qac/sul* region.<sup>40</sup> In the structure of the MDR region, the Tn402-like *tni* module is connected downstream of In1722. Tn402, also known as Tn5090, is a transposon with a typical transposition module containing four genes: *tniR/tniC*, *tniQ*, *tniB*, and *tniA*.<sup>41</sup> Previous studies predicted that the transposon encodes, in addition to the transposase TniA, two auxiliary proteins, TniB and TniQ, and the serine resolvase TniR/TniC.<sup>40,42</sup> However, in pZDHY95-VIM-2, the *tniA* was truncated by an insertion segment containing *qnrVCI*, *catB11*, and *bla*<sub>CARB-4</sub>, suggesting that the Tn402-like transposon in pZDHY95-VIM-2 may lose the ability to undergo transposition. Unexpectedly, no mobile elements could be found around these three inserted genes. The same phenomenon was observed on the plasmid pVb1978, where resistance genes *qnrVCI* and *catB11* were inserted into *tniA* to cause truncation, but *tniR/tniC* and *tniQ* was deleted within the Tn402*tni* module of pVb1978.<sup>41</sup> It is supposed that the insertion of resistance genes into *tniA* may be related to some particular recognition sequences between gene cassette *intl1* and *tniA*. Tn5563 was initially characterized in *Pseudomonas alcaligenes*, which encoded transposase TnpA, the resolvase TnpR, and the mercuric ion transport proteins MerT, MerP. Previous research confirmed that Tn5563 is a functional Tn3 family transposon with mercury ion resistance.<sup>43</sup> The presence of an MDR region and transfer region in pZDHY95-VIM-2 may cause the prevalence and spread of *bla*<sub>VIM-2</sub>. The ability of plasmid to capture exogenous resistance genes and be transmitted to pathogenic bacteria, such as *P. aeruginosa*, may lead to an epidemic of multidrug-resistant strains in hospitals.

Phylogenetic analysis showed that the isolate collection analyzed in this study was mainly divided into two clusters, the smaller one composed of strains isolated from the Uncultivated Bacteria and Archaea (UBA) project in the US, and the larger cluster mainly isolated from the ICU of a hospital in Pakistan. The continued isolation of *P. stutzeri* carrying MBLs from the ICU of a Pakistani hospital indicates that *P. stutzeri* with MBLs can be widely, continuously, and variably present on surfaces that medical workers and patients routinely touch. Once the patient has experienced long-term wound exposure or intubation, or doctors fail to disinfect relevant surfaces before an interventional operation thoroughly, the *P. stutzeri* hidden in the hospital settings will have the opportunity to enter the wound and cause unexpected infection. In fact, *P. stutzeri* isolated from the

environment and surfaces, including medical equipment and ventilators, have played a role in nosocomial infections on multiple occasions.<sup>6,44,45</sup> Infections caused by *P. stutzeri* vary geographically. It is reported that 62% of all globally reported cases were detected in the Mediterranean Basin.<sup>12</sup> However, no strains from the Mediterranean region are found in Figure 3. Two different explanations are proposed for this phenomenon. One is that the *P. stutzeri* caused the infection has low virulence and high drug sensitivity and lacks drug resistance genes. Another is that because further WGS analysis was not performed, the sequence was unavailable in the public genome database, resulting in missing information.

*P. stutzeri* is ubiquitous in hospital settings, and research indicates that clinical strains come from the same populations as environmental isolates.<sup>2</sup> Moreover, *P. stutzeri* is considered a potential reservoir of antibiotic resistance genes due to its genomic plasticity and capacity to capture genes from the environment.<sup>46</sup> In recent years, populations of the genus *Pseudomonas* present in the hospital environment have frequently caused outbreaks of nosocomial infections and severe postoperative or posttraumatic infections of medical devices,<sup>6–8,22</sup> and the isolated strains are generally multi-drug-resistant and not susceptible to carbapenemase. Studies have revealed that environmental bacteria represent a reservoir for disseminating clinically relevant Metallo- $\beta$ -lactamase genes,<sup>47</sup> which poses a potential threat of outbreak and epidemic of MBLs in hospitals. However, very little is known about chromosomal and transferable MBLs in environmental *Pseudomonas* species, except *Pseudomonas putida*.<sup>48</sup> The comprehensive description of the genetic characterization of *P. stutzeri* with a *bla*<sub>VIM-2</sub>-carrying plasmid and novel integron In1998 provides materials for further related research. Meanwhile, more vigilance and investigation should be provided for *Pseudomonas* in the hospital environment, and strict monitoring and thorough disinfection should be performed to control the spread of the strains.

## Conclusion

In summary, we report the first identification of a clinical *P. stutzeri* isolate, ZDHY95, with a mobile plasmid coproducing VIM-2 and CARB-4 isolated from cerebrospinal fluid. We sequenced its complete genome and detected the novel class I integron In1998 in the chromosome of the strain. We performed a comprehensive phylogenetic analysis of *P. stutzeri*, determined the strain's resistance mechanism, and characterized its genetic environment and plasmid transfer mechanism. Besides, *Pseudomonas* with MBLs in the hospital environment may cause infection in patients with



long-term intubation or after interventional surgery and should be strictly monitored.

## Data Sharing Statement

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

## Ethical Approval

The ethical protocol was approved by the Ethics Committee of First Affiliated Hospital of Zhejiang University (no. 2018-752).

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## Disclosure

The authors report no conflicts of interest in this work.

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