Characterization of a Conjugative Multidrug Resistance IncP-2 Megaplasmid, pPAG5, from a Clinical Pseudomonas aeruginosa Isolate

Meng Li,a,b Congcong Guan,a,b Gaoyu Song,a,b Xiaoxi Gao,a,b Weina Yang,c Tietao Wang,a,b Yani Zhang,a,b

aKey Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, Northwest University, Xi’an, People’s Republic of China
bProvincial Key Laboratory of Biotechnology of Shaanxi Province, Northwest University, Xi’an, People’s Republic of China
cDepartment of Clinical Laboratory, The Children’s Hospital of Xi’an City, Xi’an, People’s Republic of China

ABSTRACT The spread of resistance genes via horizontal plasmid transfer plays a significant role in the formation of multidrug-resistant (MDR) Pseudomonas aeruginosa strains. Here, we identified a megaplasmid (ca. 513 kb), designated pPAG5, which was recovered from a clinical multidrug-resistant P. aeruginosa PAG5 strain. The pPAG5 plasmid belonged to the IncP-2 incompatibility group. Two large multidrug resistance regions (MDR-1 and MDR-2) and two heavy metal resistance operons (merEDACPTR and terZABCDE) were identified in the pPAG5 plasmid. Genetic analysis demonstrated that the formation of MDR regions was mediated by several homologous recombination events. Further conjugation assays identified that pPAG5 could be transferred to P. aeruginosa but not Escherichia coli. Antimicrobial susceptibility testing on transconjugants demonstrated that pPAG5 was capable of transferring resistance genes to transconjugants and producing a multidrug-resistant phenotype. Comparative analysis revealed that pPAG5 and related plasmids shared an overall similar backbone, including genes essential for replication (repA), partition (par), and conjugational transfer (tra). Further phylogenetic analysis showed that pPAG5 was closely related to plasmids pOZ176 and pJB37, both of which are members of the IncP-2-type plasmid group.

IMPORTANCE The emergence and spread of plasmid-associated multidrug resistance in bacterial pathogens is a key global threat to public health. It is important to understand the mechanisms of the formation and evolution of these plasmids in patients, hospitals, and the environment. In this study, we detailed the genetic characteristics of a multidrug resistance IncP-2 megaplasmid, pPAG5, and investigated the formation of its MDR regions and evolution. To the best of our knowledge, plasmid pPAG5 is the largest multidrug resistance plasmid ever sequenced in the Pseudomonas genus. Our results may provide further insight into the formation of multidrug resistance plasmids in bacteria and the molecular evolution of plasmids.

KEYWORDS Pseudomonas aeruginosa, conjugative plasmid, multidrug resistance, IncP-2 plasmid, plasmid evolution

The presence of multidrug-resistant (MDR) pathogens is one of the most important global public health threats (1). Pseudomonas aeruginosa is an opportunistic pathogen and leading cause of nosocomial infections, which are often difficult to eradicate due to multidrug resistance (2). The mechanisms underlying antimicrobial resistance (AMR) in P. aeruginosa may be intrinsic to the species or acquired through mutation of intrinsic genes or horizontal gene transfer from other bacteria that carry genetic material encoding resistance determinants (3).

Whole-genome sequencing suggests that the spread of resistance genes via horizontal plasmid transfer plays a significant role in determining P. aeruginosa AMR (4). Most of the identified transmissible resistance plasmids in P. aeruginosa belong to the IncP-2 incompatibility group (5). IncP-2 plasmids often possess several typical...
characteristics (5–7). First, they may have a narrow host range, as IncP-2 plasmids have not been transferred to *E. coli* from *P. aeruginosa* by conjugation. Second, they are usually single copy and have a large size (8). Third, IncP-2 plasmids are resistant to metals (tellurium, mercury, and chromate) and possess bacteriophage-inhibiting properties. Recent reports also revealed that IncP-2 plasmids like pJB37, pOZ176, pBT2436, and pBT2101 carry multiple MDR cassettes (2, 5, 6). These characteristics may provide adaptive advantages for their host, especially in hospital settings. However, the step-by-step evolutionary changes in these megaplasmids remain unknown.

Here, we report the largest multidrug resistance plasmid ever sequenced in the *Pseudomonas* genus, pPAG5. In addition, we identify the detailed genetic characteristics of the multidrug resistance IncP-2 megaplasmid pPAG5 and investigate the formation of MDR regions and evolution of pPAG5. Our results may provide further insight into the formation of multidrug resistance plasmids in bacteria and the genomic diversity and molecular evolution of plasmids.

**RESULTS**

**General features of the pPAG5 plasmid.** A previous molecular epidemiologic study used whole-genome sequencing to identify a clinical *P. aeruginosa* PAG5 isolate that contained the megaplasmid pPAG5 (9). However, pPAG5 was not yet characterized. To do this, its genomic backbone was examined in detail. The complete plasmid sequence of pPAG5 was 513,322 bp long and had a guanine-cytosine (GC) content of 56.31%. Plasmid pPAG5 was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and the RAST server. It contained 538 protein-coding sequences (CDS), 57.7% of which encoded hypothetical proteins. The pPAG5 plasmid backbone contains key genes of IncP-2-type plasmids, including replication (*repA*), partitioning (*parA* and *parB*), and transfer (*tra*), *traG*, and *virB4*) genes. Virulence factors were identified in the pPAG5 plasmid, including twitching motility genes (*pilB*, *pilG*, *pilT*, and *pilZ*), a chemotaxis operon (*cheBARZWY*), and virulence regulatory genes (*vfr* and *csrA*). Based on the read coverage versus chromosome sequencing data, pPAG5 was present as a single-copy plasmid.

A BLASTp comparison against the GenBank database using the full plasmid sequence showed that the majority of CDS from the pPAG5 plasmid were similar to those of plasmid pOZ176 in *P. aeruginosa* strain PA96, isolated from a clinical specimen (6). Plasmid pOZ176 was categorized as a member of incompatibility group IncP-2 by phenotypic methods (6). A pairwise comparison of pPAG5 and pOZ176 indicated that these two plasmids encode highly similar RepA replication proteins and ParA and ParB partition proteins (100%, 100%, and 99% amino acid sequence identities, respectively), suggesting that pPAG5 belonged to the IncP-2 group.

**Two MDR regions in plasmid pPAG5.** AMR genes were detected through BLASTn searches against the Comprehensive Antibiotic Resistance Database (CARD) (10). Structure alignments of MDR regions were compared using the Easyfig tool (11). Two concentrated AMR regions were identified in pPAG5 and designated MDR-1 and MDR-2 (25.3 kb and 7.9 kb in length, respectively). Both resistance regions were rich in transposases and integrase. Five different insertion sequences (ISs) were identified as scattered across MDR-1, whereas one IS was identified in MDR-2. The origins of the ISs were diverse and included *Pseudomonas alcaligenes*, *Escherichia coli*, *Aeromonas salmonicida*, and *Proteus vulgaris*. This indicated that ISs play a key role in the acquisition of resistance from different sources.

The MDR-1 region was associated with integron In786, transposon Tn1403-like, Tn1548-like, and Tn6023-like (Fig. 1A). Integron In786, with MDR gene cassette array *aac(6’)-Ib4* -*bla* _SEG45* -*gcu3*- _bla_CAT3*, contributes resistance to aminoglycosides, β-lactams, and chloramphenicol. Upstream from In786, a Tn1403-like transposon containing tnpA and tnpR was also identified and was 99% identical to the amino acid sequence of Tn1403. Downstream from In786, a Tn1548-like transposon carried the resistance module *armA-msr (E)-mph(E)-repA-cfN*, encoding high-level resistance to aminoglycosides and macrolides. Upstream from this module, IS1394 was flanked by ISCR1. ISCR1 elements can transfer adjacent DNA sequences into other bacterial species (12). Downstream from the module, there

Volume 10 Issue 1 e01992-21
was a Tn6023 transposon carrying an \textit{aph(3')-Ia} gene flanked by two IS26 insertion sequences, contributing resistance to aminoglycosides (13). The MDR-1 region demonstrated a high similarity to the MDR region of plasmid pPA1819 in \textit{P. aeruginosa} strain 14.1819 (87% coverage and 99.98% identity) and that of plasmid pSY153-MDR in \textit{Pseudomonas putida} strain SY153 (96% coverage and 99.99% identity) (Fig. 1A).

The MDR-2 region consisted of In1237, \textit{tnpM}-like, and \textit{recA}-like (Fig. 1B). Integron In1237 was flanked upstream by IS6100 and downstream by \textit{tnpM}-like and \textit{recA}-like. In1237 contained the \textit{qnrVC1-gcu165-arr-2-dfrA22e} cassette array, which confers resistance to quinolones, rifampicin, and dihydrofolate. Of note, this cassette was first reported in pPA1819 from \textit{P. aeruginosa} strain 14.1819 (14). The same array also has been observed previously in pSY153-MDR from \textit{P. putida} strain SY153 (15). The \textit{tnpM}-like transposase shared 95% amino acid identity with the \textit{tnpM} transposase of plasmid pNDM-CIT, which belonged to the IncHI1 plasmid type found in the extremely drug-resistant \textit{Citrobacter freundii} strain STE (16). The \textit{recA}-like recombinase displayed 85% amino acid similarity to the \textit{RecA} protein of plasmid pMBU17, which belongs to the
self-transmissible plasmid and IncU plasmid type, carried by an uncultured bacterium isolated from Paradise Creek (17). The MDR-2 region was very similar to the MDR region of plasmid pSY153-MDR from *P. putida* SY153 (94% coverage and 99.98% identity) (Fig. 1B).

**Characterization of heavy metal resistance operons.** Two heavy metal resistance operons, encoding resistance to tellurite and mercury, were observed in pPAG5 (Fig. 2). The tellurite operon consisted of *terZABCDE* genes and two genes encoding
members of the TerD family of proteins. This tellurite operon exhibited 100% amino acid identity with the tellurite operon of plasmid pOZ176 (6). Resistance to tellurite is a key feature of IncP-2 Pseudomonas plasmids; in fact, they all carry resistance to tellurite. The mercury operon contained merEDACPTR genes and was 100% identical to the mercury operon of plasmid p727-IMP (18). Of the 17 pPAG5-related plasmids, only 9 plasmids possess the mercury operon, which can provide resistance to mercury (Fig. 2).

Transmissibility of pPAG5. The annotation of plasmid pPAG5 demonstrated that it contained oriT, virB4, and traG genes required for conjugative transfer (19). Therefore, conjugation experiments were performed to determine whether pPAG5 would undergo intra- and interspecies horizontal transfer. The donor strain PAG5 and recipient strain PAO1-lux were subjected to plasmid transfer experiments. Plasmid pPAG5 encoded high-level resistance to gentamycin (Gm) (Fig. 3), and therefore, the recipient strain PAO1-lux would acquire Gm resistance if pPAG5 was successfully transferred into it. Mating between PAG5 and PAO1-lux in LB for 24 h resulted in the appearance of Gm-resistant bioluminescent transconjugants (Fig. 3). However, it failed to transfer the plasmid to E. coli strain DH5α-lux. This finding showed that pPAG5 is self-transmissible but has a narrow host range.

Multidrug resistance phenotype. The MICs of amikacin, aztreonam, ceftazidime, ciprofloxacin, colistin, cefepime, gentamicin, imipenem, levofloxacin, meropenem, piperacillin, and piperacillin-tazobactam were determined for PAG5, the transconjugant strain PAGST, and PAO1-lux by conducting broth dilution tests. PAG5 showed resistance to all antibiotics except colistin. The transconjugant PAGST exhibited resistance to all antibiotics except aztreonam, colistin, and imipenem. However, PAO1-lux was susceptible to all antibiotics. The transconjugant PAGST and donor PAG5 had similar patterns of MICs to meropenem (MIC > 8 μg/mL), ceftazidime (MIC > 16 μg/mL), piperacillin (MIC > 64 μg/mL), cefepime (MIC > 16 μg/mL), gentamicin (MIC > 8 μg/mL), piperacillin-tazobactam (MIC > 64 μg/mL), and amikacin (MIC > 8 μg/mL) (Table 1). These results indicated that pPAG5 could transfer these associated antibiotic resistance genes from the donor strain PAG5 to the recipient strain PAO1-lux, resulting in transconjugant PAGST exhibiting a resistant phenotype.

Comparative analysis between pPAG5 and related megaplasmids. We carried out homology searches targeting pPAG5 complete sequences using the NCBI non-redundant nucleotide database. The resistance determinants were identified using ResFinder (20) and RGI software from the CARD database (10). Details of the 17 additional megaplasmids identified, including the size range and GC content, are shown in Table 2. Thirteen of 17 megaplasmids were present in strains of P. aeruginosa, and 4 related plasmids were identified in non-aeruginosa Pseudomonas species (Pseudomonas koreensis, Pseudomonas citronellolis, and Pseudomonas putida). Nevertheless, four of the related megaplasmids lacked any AMR genes: p1 (GenBank accession number CP027478.1), pTT512 (GenBank accession number CP009975.1), pRBL16 (GenBank accession number CP015879.1), and plasmid 1-RW109 (GenBank accession number LT969519.1) (Fig. 4). These four megaplasmids were from the environmental isolate strain P. koreensis P19E3, soil strain P. putida S12, sludge strain P. citronellolis SJTE-3, and industrial strain P. aeruginosa RW109, respectively. Other related megaplasmids were sourced from clinical strains, with the majority belonging to P. aeruginosa.

To further investigate and understand the relationships between pPAG5 and related megaplasmids, we constructed a phylogenetic tree based on the concatenated alignment of the high-quality single-nucleotide polymorphisms (SNPs) using the CSI Phylogeny 1.4 software (21). A close phylogenetic relationship was found among pPAG5, pO2176, and pJB37, from the P. aeruginosa PAG5, P. aeruginosa PA96 and P. aeruginosa FFUP_P5_37 clinical isolates, respectively (Fig. 4). All three of these megaplasmids were isolated from clinical P. aeruginosa isolates, and they belong to the same IncP-2 group. Comparative analysis of the 18 complete megaplasmid sequences using BLAST Ring Image Generator (BRIG) revealed that the pPAG5 nucleotide sequence is similar to those of 17 other related megaplasmids (Fig. 2). Theses plasmids shared similar IncP-2 backbones and key traits, but there were several DNA fragment differences between pPAG5 and the 17 megaplasmids (Fig. 2). In particular, the ~13-
kb variable region 1 (VR1; bp 159,544 to 171,526) and the ~41-kb variable region 2 (VR2; bp 11,910 to 51,201) of pPAG5 were absent from any of the other megaplasmids. A BLASTn search of VR1 showed that it was similar (82% coverage and 100% identity) to the IMP-harboring p420352-IMP plasmid (GenBank accession number MN961670.1) isolated from *P. putida* strain 420352. The VR1 fragment was also similar (75% coverage and 96.12% identity) to the AG1 chromosome of a *P. aeruginosa* strain that was recently described in the first report of a *P. aeruginosa* isolate carrying both *bla*VIM-2 and *bla*IMP-18 resistance genes (22). It is noteworthy that VR2 was highly similar to the PAG5 host strain of megaplasmid pPAG5 (94% query coverage and 99.93% nucleotide similarity). Annotation of the unique VR1 and VR2 regions suggested that they had not contributed to any resistance genes. However, the majority of their genes were responsible for encoding type I restriction-modification system endonuclease, which is closely associated with defense against invading foreign DNA and maintaining the integrity of the host genome (23).

**DISCUSSION**

In this study, a hybrid approach combining Nanopore and Illumina sequencing was applied to identify and characterize a megaplasmid in a clinical *P. aeruginosa* isolate. To the best of our knowledge, plasmid pPAG5 is the largest MDR plasmid ever sequenced in the *Pseudomonas* genus. *In silico* analysis of the sequence features showed that pPAG5 was closely related to IncP-2 plasmids. It possesses substantial numbers of diverse mobile elements, such as insertion sequences (ISs), integrons, and transposons. These mobile elements are considered important vehicles for transmitting resistance genes and promote the exchange and rearrangement of genetic information (2). Both ends of the VR1 and VR2 variable regions are ISs in pPAG5; VR1 is flanked by two ISs.

**TABLE 1** Antimicrobial susceptibility patterns of *P. aeruginosa* strain PAG5, its transconjugant strain PAGST, and the recipient strain *P. aeruginosa* PAO1-lux.

| *P. aeruginosa* strain | MIC (mg/L) of: |
|------------------------|----------------|
|                         | IPM  | MEM  | CAZ  | PIP  | FEP  | CIP  | LEV  | GEN  | TZP  | ATM  | AMK  | CST  |
| PAG5                   | >8   | >8   | >16  | >64  | >16  | >2   | >8   | >8   | >64  | >16  | >32  | 1    |
| PAGST                  | 4    | >8   | >16  | >64  | >16  | 2    | 4    | >8   | >64  | 8    | >32  | 1    |
| PAO1-lux               | 2    | ≤1   | 2    | ≤4   | 4    | ≤0.5 | ≤1   | ≤2   | ≤4   | 8    | ≤8   | 1    |

*AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; FEP, cefepime; GEN, gentamicin; IPM, imipenem; MEM, meropenem; LVX, levofloxacin; PIP, piperacillin; TZP, piperacillin-tazobactam.*
### TABLE 2  Megaplasmids identified by BLASTn search with pPAG5

| Plasmid           | % GC content | Score | Query coverage (%) | E value | % identity | Species      | Size (bp) | Yr of isolation | Source | Country      | Genbank accession no. |
|-------------------|--------------|-------|--------------------|---------|------------|--------------|-----------|----------------|--------|--------------|-----------------------|
| pOZ176            | 57.60        | 1.75E+05 | 7.17E+05           | 78      | 0          | P. aeruginosa | 500839   | 2000           | Clinical | China        | KC543497.1            |
| pBM413            | 56.41        | 1.58E+05 | 7.82E+05           | 79      | 0          | P. aeruginosa | 423017   | 2012           | Clinical | China        | CP016215.1            |
| p12939-PER        | 57.30        | 1.58E+05 | 6.47E+05           | 70      | 0          | P. aeruginosa | 496436   | NA*             | Clinical | China        | MF344569.1            |
| p1                | 55.97        | 1.57E+05 | 6.49E+05           | 73      | 0          | P. korensis  | 467568   | 2014           | Environment | Switzerland | NZ_CP027478.1        |
| Unnamed2-AR439    | 56.87        | 1.55E+05 | 6.52E+05           | 73      | 0          | P. aeruginosa | 437392   | NA             | Clinical | NA           | NZ_CP029096.1         |
| pPABL048          | 56.58        | 1.54E+05 | 6.20E+05           | 70      | 0          | P. aeruginosa | 414954   | 2001           | Clinical | USA          | NZ_CP039294.1         |
| Unnamed2-AR_0356  | 57.14        | 1.53E+05 | 6.49E+05           | 73      | 0          | P. aeruginosa | 438531   | NA             | Clinical | USA          | NZ_CP027170.1         |
| p727-IMP          | 56.39        | 1.25E+05 | 7.35E+05           | 75      | 0          | P. aeruginosa | 430173   | NA             | Clinical | China        | MF344568.1            |
| Unnamed3-AR441    | 57.14        | 1.22E+05 | 6.49E+05           | 73      | 0          | P. aeruginosa | 438529   | NA             | Clinical | USA          | NZ_CP029094.1         |
| pJB37             | 57.20        | 1.21E+05 | 6.81E+05           | 74      | 0          | P. aeruginosa | 464804   | 2008           | Clinical | Portugal      | KY494864.1            |
| pBM908            | 56.86        | 1.15E+05 | 7.37E+05           | 74      | 0          | P. aeruginosa | 395774   | 2018           | Clinical | China        | CP040126.1            |
| pRBL16            | 55.57        | 1.09E+05 | 6.31E+05           | 71      | 0          | P. citronellolis | 370338  | 2015           | Sludge | China        | NZ_CP015879.1         |
| pR31014-IMP       | 56.37        | 8.75E+04 | 6.93E+05           | 68      | 0          | P. aeruginosa | 374000   | NA             | Clinical | China        | MF344571.1            |
| pA681-IMP         | 56.35        | 8.15E+04 | 7.17E+05           | 71      | 0          | P. aeruginosa | 397159   | NA             | Clinical | China        | MF344570.1            |
| pSY153-MDR        | 56.56        | 7.71E+04 | 9.09E+05           | 83      | 0          | P. putida    | 468170   | 2012           | Clinical | China        | KY83660.1             |
| pTT12             | 57.85        | 75188   | 6.35E+05           | 71      | 0          | P. putida    | 583900   | 1989           | Soil     | Netherlands  | NZ_CP009975.1          |
| plasmid1-RW109    | 58.09        | 74550   | 6.35E+05           | 71      | 0          | P. aeruginosa | 555265   | NA             | Industrial | NA           | LT969519.1            |

*NA, no data available.*
by recA-like and IS6100, while VR2 is bordered by IS1411 and ISPa1382. The findings revealed that the variable regions were likely captured by homologous recombination.

Two MDR regions in pPAG5 also harbor transposases and integrases, indicating a key role for these mobile genetic elements in the acquisition of resistance and in evolution (12). This suggests that complex rearrangement and homologous recombination events likely occurred during the evolution of pPAG5 (15, 18, 24). Recent analysis showed that members of the megaplasmid family contained diverse resistance elements to form AMR regions via homologous recombination, and some of the resistance genes carried trace back to the late 1970s (2).

Megaplasmids may harbor accessory modules that provide adaptive advantages or broaden the host strain response spectrum. Such advantages include virulence factors, antibiotic resistance, and heavy metal resistance (2). The IncP-2 megaplasmids, such as pPAG5, pOZ176, pJB37, and pBM413, have a common core genetic backbone but different AMR gene profiles, suggesting that the backbone was formed first and then collected diverse cassettes of AMR genes during transfer to different host strains (5, 6, 24). The two larger AMR regions identified in pPAG5 are highly similar to the MDR regions in megaplasmid pSY153 in a _P. putida_ isolate from a clinical environment in China (15).

The MDR-1 genes also have a best match to the MDR region in plasmid pPA1819 in _P. aeruginosa_ strain 14.1819 isolated from a clinical environment in France (14). This difference of the plasmid backbone suggests that the resistance genes have been assembled independently of the backbone and that horizontal gene transfer plays an important role in the dissemination of resistance genes in the clinical setting.

Some members of the IncP-2 megaplasmid family are considered conjugative plasmids (2, 5, 6), but they differ in their transfer genes; for example, pOZ176 carries _traF, traG, virD2_, and _trbBCDEJFGI_ genes, while pBT2436 carries _traGBV, dnaG_, and _type IV_
TABLE 3 Strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics or function | Source |
|-------------------|--------------------------------------|--------|
| **Strains**       |                                      |        |
| *P. aeruginosa* PAG5 | A multidrug-resistant clinical isolate from a urine sample | This study |
| PAO1-lux          | A derivative of *P. aeruginosa* PAO1, carrying luxCDABE genes driven by the *modA* promoter | This study |
| E. coli DH5α-lux  | *E. coli* DH5α containing plasmid pMS402 with a promoterless luxABCDE reporter gene driven by the *modA* promoter | This study |
| **Plasmids**      |                                      |        |
| pMS402            | lux-based promoter reporter plasmid; Km⁰ Tp⁰ | This study |
| pPAG5             | Natural 513-kbp plasmid of *P. aeruginosa* PAG5, carrying diverse resistance genes | This study |

Conjugative IncP-2 Megaplasmid pPAG5

Other members are nonconjugative because transfer genes are absent or disrupted (3, 15, 25). We found that the virB4 gene and *traG* gene are encoded on pPAG5. The VirB-like protein, VirD4, and TraG-like protein interact with the DNA substrate and couple it to the secretion pore via mating pair formation (19, 26). Additionally, the *traI* gene is located upstream from the type IV secretion system (T4SS) cluster. This suggests that the *traI* gene may serve as a relaxase and is involved in initiating DNA transfer (19). Comparative analysis of the transfer region of pPAG5 revealed that it contains a P-type T4SS with all genes required for transfer, which forms a conjugative pilus and mediates mating pair stabilization (27). Importantly, conjugation experiments revealed that pPAG5 can be successfully self-transmissible into recipient strains.

In conclusion, we report a conjugative transferable plasmid, pPAG5, from a *P. aeruginosa* isolate in a hospital environment. It belongs to the IncP-2 group plasmids and carries two large MDR regions. It poses a serious threat, especially for controlling nosocomial infections. To defuse this threat, it is crucial to prevent the rapid transmission of MDR plasmids into our health care systems. A better understanding of the problem of how resistance plasmids evolve in hospitals and the environment is a key to controlling the global threat of antibiotic resistance.

MATERIALS AND METHODS

**Bacterial strains and plasmids used in this study.** The strains and plasmids used in this study are listed in Table 3. *P. aeruginosa* isolate PAG5 exhibited an MDR profile, as previously described (9). *Escherichia coli* strains were maintained in Luria Bertani (LB) broth or LB agar at 37°C. For solid cultivation, 1.5% (wt/vol) agar was added to LB. All *P. aeruginosa* strains were grown in LB or *Pseudomonas* isolation agar at 37°C.

**Antimicrobial susceptibility determination.** The isolates were assessed for MICs of the tested antibiotics by the broth microdilution method according to the 2021 Clinical and Laboratory Standards Institute (CLSI) guidelines (28). The antimicrobial agents tested included amikacin, aztreonam, ceftazidime, ciprofloxacin, colistin, cefepime, gentamicin, imipenem, levofloxacin, meropenem, piperacillin, and piperacillin-tazobactam.

**Construction of recipient strains.** The broad antibiotic resistance conferred by pPAG5 complicates the plasmid transmission assay because selecting, enumerating, and isolating transconjugants usually requires a selective marker carried by recipient strains. Therefore, engineered bioluminescent reporter recipient strains of *P. aeruginosa* PAO1 and *E. coli* DH5α, carrying luxABCDE (driven by the *modA* promoter of PAO1), were constructed. To construct the lux reporter strains, the plasmid pMS402, with a promoterless luxABCDE reporter gene cluster, was used as previously described (29). The *modA* promoter region was amplified by PCR from genomic DNA of PAO1 and cloned into the BamHI-XhoI sites upstream from the *lux* genes in pMS402, resulting in a *modA-lux* fusion plasmid named pKD-modA. The promoter region was confirmed by DNA sequencing. Apart from the plasmid-based lux reporter system, an integration plasmid, CTX 6.1, derived from the plasmid mini-CTX-lux, was used to construct the chromosomal fusion reporter (30). The pMS402 fragment containing the MCS kanamycin resistance marker and *modA-lux* reporter cassette was ligated into integration plasmid CTX 6.1 with the PacI site (31). The new recombinant plasmid was then transformed into PAO1 by electroporation and integrated into the attB chromosome site of PAO1 as previously described (32). This yielded a bioluminescent strain named PAO1-lux (Fig. 3). pKD-modA was transformed into *E. coli* strain DH5α-lux.

**Conjugation experiments.** Conjugation experiments were performed as previously described, with a slight modification (33). The donor strain was the PAG5 clinical isolate. PAO1-lux and *E. coli* DH5α-lux played roles as recipient strains. Overnight donor and recipient bacteria were combined in a 10:1 ratio in 1 mL LB broth and then cocultured using filters on solid LB agar at 37°C without agitation for 12 h. Then, the mixture was resuspended in LB broth and plated onto Mueller-Hinton agar plates supplemented...
with gentamicin (100 μg/mL). After incubation for 24 h at 37°C, growing colonies with biolumi-

cence were selected as transconjugants. Plasmid acquisition in the transconjugants was fur-

ther confirmed by antibiotic susceptibility tests and detection of the gentamicin resistance gene armA of the

plasmid by PCR.

Plasmid analysis. Plasmid pPAG5 was annotated with the NCBI Prokaryotic Genome Annotation

Pipeline (PGAP, version 4.9) (https://www.ncbi.nlm.nih.gov/annotation/annotation_prok/) and the RAST

server (version 2.0) (http://rast.nmpdr.org/). This was followed by manual review through BLASTp

search. Function of genes were annotated via EggNOG mapper (version 4.5.1) (34). The incompatibility type

of the plasmid pPAG5 was determined by using the Plasmid Finder (version 2.1) (https://cge.cbs.dtu.dk/

services/PlasmidFinder/) and Plasmid MLST (https://pubmlst.org/plasmid/) databases. The resistance
determinants were identified using ResFinder (version 3.2) (https://cge.cbs.dtu.dk/services/ResFinder/)

and RGI software from the CARD database (https://card.mcmaster.ca/). Additional software, including

Transposon Registry (35), InSfinder (36), and INTEGRALL (version 1.2) (37), was used to identify mobile

gene elements.

Comparative analysis of pPAG5 and related megaplasmids. Complete pPAG5-related megaplasmid

sequences deposited in GenBank were identified through BLASTn searches against the non-

redundant GenBank database (up to October 2019), using pPAG5 as a query sequence. The maxi-

mum scores reflect complete homologous sequences. Pairwise comparisons of pPAG5 and related

megaplasmids were generated using BLAST Ring Image Generator (BRIG; version 0.95) (38). Easyfig

was used for comparative analysis of MDR regions. The phylogenetic trees were constructed from

single-nucleotide polymorphism (SNP) concatemers using the CSI Phylogeny 1.4 software (21). The

phylogenetic trees and AMR gene contents were visualized and edited with the iTOL tool (version 4.3.3) (39).

Data availability. The complete sequence of plasmid pPAG5 has been submitted to GenBank under

accession number CP045003.

ACKNOWLEDGMENTS

This work was supported by a grant from the Natural Science Foundation of Shaanxi Province of China (grant number 2021JZ-42).

We are grateful to Haihua Liang (Northwest University) provides excellent experimental conditions and Kaichao Chen (City University of Hong Kong) for help in bioinformatics analysis.

We declare that we have no conflicts of interest.

REFERENCES

1. Ullah W, Qasim M, Rahman H, Bari F, Khan S, Rehman ZU, Khan Z, Dworeck T, Muhammad N. 2016. Multi drug resistant Pseudomonas aerugi-

nosa: pathogen burden and associated antibiogram in a tertiary care hospital of Pakistan. Microb Pathog 97:209–212. https://doi.org/10.1016/j.

micpath.2016.06.017.

2. Cazes A, Moore MP, Hall JP, Wright LL, Grimes M, Emond-Rheault JG, Pongchakul P, Santanipard P, Levesque RC, Fothergill JL, Winstanley C. 2020. A megaplasmid family driving dissemination of multidrug resistance in Pseu-

domonas. Nat Commun 11:1370. https://doi.org/10.1038/s41467-020-15081-7.

3. Botelho J, Lood C, Partridge SR, van Noort V, Lavigne R, Grosso F, Peixe L. 2019. Combining sequencing approaches to fully resolve a carbapenemase-encoding

megaplasmid in a Pseudomonas shirazica clinical strain. Emerg Microbes Infect 8:1186–1194. https://doi.org/10.1080/22221751.2019.1648182.

4. Lermimiaux NA, Cameron ADS. 2019. Horizontal transfer of antibiotic resis-

tance genes in clinical environments. Can J Microbiol 65:34–44. https://doi.

org/10.1139/cjm-2018-0275.

5. Botelho J, Grosso F, Quinteira S, Mabrouk A, Peixe L. 2017. The complete

nucleotide sequence of an IncP-2 megaplasmid unveils a mosaic architecture

comprising a putative novel blgA-like harbouising transposon in Pseu-

domonas aeruginosa. J Antimicrob Chemother 72:2225–2229. https://doi.

.org/10.1093/jac/dkx143.

6. Xiong J, Alexander DC, Ma JH, Deraspe M, Low DE, Jamieson FB, Roy PH. 2013. Complete sequence of pOZ176, a 500-kilobase IncP-2 plasmid encod-

ing IMP-9-mediated carbapenem resistance, from outbreak isolate Pseu-

odomonas aeruginosa 96. Antimicrob Agents Chemother 57:3775–3782. https://

doi.org/10.1128/AAC.00423-13.

7. Jacoby GA, Sutton L, Knobel L, Mammrn P. 1983. Properties of IncP-2 plas-

mids of Pseudomonas spp. Antimicrob Agents Chemother 24:168–175.

https://doi.org/10.1128/aaac.24A.2.168.

8. Weiser R, Green AE, Bull MJ, Cunningham-Oakes E, Jolley KA, Maiden MCJ, Hall AJ, Winstanley C, Weightman AJ, Donoghue D, Arnezonita A, Connor TR, Mahenthiralingam E. 2019. Not all Pseudomonas aeruginosa are equal: strains from industrial sources possess uniquely large multireplicon genomes. Microb Genom 5:e000276. https://doi.org/10.1099/mgen.0.000276.

9. Zhang Y, Meng L, Guan C, Zhou Y, Peng J, Liang H. 2020. Genomic charac-
terisation of clinical Pseudomonas aeruginosa isolate PAGS with a multi-

drug-resistant megaplasmid from China. J Glob Antimicrob Resist 21:130–131. https://doi.org/10.1016/j.jgar.2020.03.012.

10. Alcock BP, Raphenya AR, Lau TTY, Tsang KK, Bouchard M, Edalatmand A, Huynh W, Nguyen AV, Cheng AA, Liu S, Min SY, Miroshnichenko A, Tran HK, Werfalli RE, Nasir JA, Oلونi M, Speicher DJ, Florescu A, Singh B, Faltyn M, Hernandez-Koutoucheva A, Sharma AN, Bordeleau E, Pawlowski AC, Zubyk HL, Dooley D, Griffiths E, Maguire F, Winsor GL, Beligo RG, Brinkman FSL, Hsiao WWL, Domelsear GV, McArthur AG. 2020. CARD 2020: antibiotic resistome surveillance with the Comprehensive Antibiotic Resistance Database. Nucleic Acids Res 48:DS17–DS25. https://doi.org/10.1093/nar/gkz395.

11. Rodriguez-Beltran J, Delafuente J, Leon-Sampedro R, MacLean RC, San Millan A. 2021. Beyond horizontal gene transfer: the role of plasmids in bacte-

rial evolution. Nat Rev Microbiol 19:347–359. https://doi.org/10.1038/s41579-

020-00497-1.

12. Partridge SR, Kwong SM, Firth N, Jensen SO. 2018. Mobile genetic ele-

ments associated with antimicrobial resistance. Clin Microbiol Rev 31: e00088-17. https://doi.org/10.1128/CMR.00088-17.

13. Cain AK, Hall RM. 2011. Transposon Tn5392e carrying the aphA1-containing

transposon Tn6023 upstream of strA8 does not confer resistance to streptomycin. Microb Drug Resist 17:389–394. https://doi.org/10.1089/ mdr.2011.0037.

14. Janvier F, Otto MP, Jove T, Mille A, Contargyris C, Meaudre E, Brisou P, Plesiws P, Janneot K. 2017. A case of multiple contamination with methyl-

ase ArmA-producing pathogens. J Antimicrob Chemother 72:618–620. https://doi.org/10.1093/jac/dkx418.

15. Yuan M, Chen H, Zhu X, Feng J, Zhan Z, Zhang D, Chen X, Zhao X, Lu J, Xu J, Zhou D, Li J. 2017. pSY153-MDR, a p12969-DIM-related mega plasmid
Conjugative IncP-2 Megaplasmid pPAG5

carrying bla<sub>IMP-45</sub> and armA, from clinical Pseudomonas putidu. Oncotarget 8:66439–66447. https://doi.org/10.18632/oncotarget.19496.

16. Dolejska M, Villa L, Poirel L, Nordmann P, Carattoli A. 2013. Complete sequencing of an IncHI1 plasmid encoding the carbapenemase NDM-1, the ArmA 165 RNA methylase and a resistance-nodulation-cell division/multidrug efflux pump. J Antimicrob Chemother 68:34–39. https://doi.org/10.1093/jac/dks357.

17. Brown CJ, Sen DY, Yano H, Bauer ML, Rogers LM, Van der Auwera GA, Top EM. 2013. Diverse broad-host-range plasmids from freshwater carry few accessory genes. Appl Environ Microbiol 79:7684–7695. https://doi.org/10.1128/aem.02252-13.

18. Jiang X, Yin Z, Yuan M, Cheng Q, Hu L, Xu Y, Yang W, Yang H, Zhao Y, Zhao X, Gao B, Dai E, Song Y, Zhou D. 2020. Plasmids of novel incompatibility group Inc<sub>mcf-16</sub> from Pseudomonas species. J Antimicrob Chemother 75:2093–2100. https://doi.org/10.1093/jac/dkaa343.

19. Smillie C, Garcillan-Barcia MP, Francia MV, Rocha EP, de la Cruz F. 2010. High quality 3C de novo assembly and annotation of a multidrug resistance plasmid: benchmark of hybrid and non-hybrid assemblers. Sci Rep 10:1392. https://doi.org/10.1038/s41598-019-452.

20. Bortolaia V, Kaas RS, Ruppe E, Roberts MC, Schwarz S, Cattoir V, Philippon A, Allesoe RL, Rebelo AR, Florensa AF, Xavier BB, Malhotra-Kumar S, Westh H, Pinhoit M, Anjum MF, Duggett NA, Kempf I, Nykasenoja S, Oliksola S, Wieczorek K, Amaro A, Clemente L, Mossong J, Losch S, Ragimbeau C, Lund O, Aarestrup FM. 2020. ResFinder 4.0 for predictions of phenotypes from genotypes. J Antimicrob Chemother 75:3491–3500. https://doi.org/10.1093/jac/dkaa345.

21. Kaas RS, Leekitcharoenphon P, Aarestrup FM, Lund O. 2014. Solving the problem of comparing whole bacterial genomes across different sequencing platforms. PLoS One 9:e104984. https://doi.org/10.1371/journal.pone.0104984.

22. Molina-Mora JA, Campos-Sanchez R, Rodriguez C, Shi L, Garcia F. 2020. High quality 3C de novo assembly and annotation of a multidrug resistant ST-111 Pseudomonas aeruginosa genome: benchmark of hybrid and non-hybrid assemblers. Sci Rep 10:1392. https://doi.org/10.1038/s41598-020-58319-6.

23. Bower EKM, Cooper LP, Roberts GA, White JH, Luyten Y, Morgan RD, Dryden A. 2018. A model for the evolution of prokaryotic DNA restriction-modification systems based upon the structural malleability of type I restriction-modification enzymes. Nucleic Acids Res 46:9067–9080. https://doi.org/10.1093/nar/gkz760.

24. Liu J, Yang L, Chen D, Peters BM, Li L, Li B, Xu Z, Shirillf ME. 2018. Complete sequence of pBM413, a novel multidrug resistance megaplasmid carrying qnr<sub>VC6</sub> and bla<sub>IMP</sub>, from Pseudomonas aeruginosa. Int J Antimicrob Agents 51:145–150. https://doi.org/10.1016/j.ijantimicag.2017.09.008.

25. Urbanowicz P, Biter I, Izdebski R, Baraniak A, Litarz E, Hrabak J, Gniałkowski M. 2021. Epidemic territorial spread of IncP-2-type VIM-2 carbapenemase-encoding megaplasmids in nosocomial Pseudomonas aeruginosa populations. Antimicrob Agents Chemother 65:e02122-20. https://doi.org/10.1128/AAC.02122-20.

26. Garcillan-Barcia MP, Alvarado A, de la Cruz F. 2011. Identification of bacterial plasmids based on mobility and plasmid population biology. FEMS Microbiol Rev 35:936–956. https://doi.org/10.1111/j.1574-6976.2011.00291.x.

27. Christie PJ. 2016. The mosaic type IV secretion systems. EcoSal Plus 7. https://doi.org/10.1128/ecosalplus-ESP-0020-2015.

28. Clinical and Laboratory Standards Institute. 2021. Performance standards for antimicrobial susceptibility testing, 31st ed. CLSI M100. Clinical and Laboratory Standards Institute, Wayne, PA.

29. Cao Q, Wang Y, Chen FF, Xia YJ, Zhang X, Yang NN, Sun XX, Zhang Q, Zhuo C, Huang X, Deng X, Yang CG, Ye Y, Zhao J, Wu M, Lan LF. 2014. A novel signal transduction pathway that modulates rhl quorum sensing and bacterial virulence in Pseudomonas aeruginosa. PLoS Pathog 10: e1004340. https://doi.org/10.1371/journal.ppat.1004340.

30. Becher A, Schweizer HP. 2000. Integration-proficient Pseudomonas aeruginosa vectors for isolation of single-copy chromosomal lacZ and lux gene fusions. Biotechniques 29:948–950. https://doi.org/10.2144/00295bm04.

31. Wang T, Du X, Ji L, Han Y, Deng J, Wen J, Wang Y, Pu Q, Wu M, Liang H. 2021. Pseudomonas aeruginosa T6SS-mediated molybdate transport contributes to bacterial competition during anaerobiosis. Cell Rep 35:108957. https://doi.org/10.1016/j.celrep.2021.108957.

32. Hoang TT, Kutchma AJ, Becher A, Schweizer HP. 2000. Integration-proficient plasmids for Pseudomonas aeruginosa: site-specific integration and use for engineering of reporter and expression strains. Plasmid 43:59–72. https://doi.org/10.1016/j.plasmid.1999.1441.

33. Chen K, Dong N, Chan EW, Chen S. 2019. Transmission of ciprofloxacin resistance in Salmonella mediated by a novel type of conjugative helper plasmids. Emerg Microbes Infect 8:857–865. https://doi.org/10.1002/22221751.20191626197.

34. Huerta-Cepas J, Forslund K, Coelho LP, Szklarczyk D, Jensen LJ, von Mering C, Bork P. 2017. Fast genome-wide functional annotation through orthology assignment by eggNOG-Mapper. Mol Biol Evol 34:2115–2122. https://doi.org/10.1093/molbev/msx148.

35. Tanssinchaiya S, Rahman MA, Roberts AP. 2019. The Transposon Registry. Mob DNA 10:40. https://doi.org/10.1186/s13100-019-0182-3.

36. Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M. 2006. iSfinder: the reference centre for bacterial insertion sequences. Nucleic Acids Res 34:D32-D36. https://doi.org/10.1093/nar/gkj014.

37. Moura A, Soares M, Pereira C, Leitao N, Henriques I, Correia A. 2009. INTEGRALL: a database and search engine for integrons, integrase and gene cassette. Bioinformatics 25:1096–1098. https://doi.org/10.1093/bioinformatics/btp105.

38. Alikhani NF, Petty NK, Ben Zakour NL, Beatson SA. 2011. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics 12:402. https://doi.org/10.1186/1471-2164-12-402.

39. Letunic I, Bork P. 2019. Interactive Tree Of Life (ITOL) v4: recent updates and new developments. Nucleic Acids Res 47:W256–W259. https://doi.org/10.1093/nar/gkz239.