AadA36, a novel chromosomal aminoglycoside nucleotidyltransferase from a clinical isolate of Providencia stuartii

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In this study, we characterized a novel chromosome-encoded aminoglycoside nucleotidyltransferase (ANT), AadA36, from the Providencia stuartii strain P14 isolated from the sputum specimen of a burn patient at a hospital in Wenzhou, China. Among the functionally characterized ANTs, AadA36 shared the highest amino acid sequence identity of 51.91% with AadA14. The whole genome of P. stuartii P14 consisted of one chromosome and two plasmids (designated pP14-166 and pP14-114). A total of 19 genes with ≥80% similarity with functionally characterized antimicrobial resistance genes (ARGs) were identified in the whole genome, including aminoglycosides [aac(2′)-Ia, aph(6′)-Ia, aph(3′)-Ib, aac(6′)-Ib, ant(3′)-Ia, aph(3′)-Ia], β-lactams (blaCMY-2 and blaOXA-10) and so on. Antimicrobial susceptibility testing showed that the aadA36 gene conferred specific resistance to spectinomycin and streptomycin, and the minimum inhibitory concentration (MIC) of these antimicrobials increased 128- and 64-fold compared with the control strain. The kinetic parameters of AadA36 were consistent with the MIC data of spectinomycin and streptomycin, with $k_{cat}/K_m$ ratios of $(1.07\pm 2.23) \times 10^5 M^{-1} s^{-1}$ and $(8.96\pm 1.01) \times 10^5 M^{-1} s^{-1}$, respectively. The identification of a novel aminoglycoside resistance gene will help us further understand the complexity of the resistance mechanisms and provide deep insights into the dissemination of resistance genes in the microbial population.

KEYWORDS
AadA36, Providencia stuartii, aminoglycoside nucleotidyltransferase, novel aminoglycoside resistance gene, kinetic analysis
Introduction

Aminoglycoside antimicrobials are broad-spectrum agents with strong antibacterial effects that are used for the treatment of bacterial infections, especially those caused by Gram-negative bacilli, including Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter cloacae, Enterobacter aerogenes, Providencia spp., Proteus spp., Morganella spp., and Serratia spp. (Krause et al., 2016). This class of antimicrobials exerts bactericidal effects mainly by interfering with bacterial protein synthesis. Moreover, these antimicrobials can also be combined with other classes of antimicrobials to treat severe infections. In recent years, the resistance of Gram-negative bacilli to aminoglycosides has become increasingly severe. In the clinical setting, the most common mechanism of resistance to the aminoglycoside (AG) antimicrobials is the enzymatic modification (Wright, 1999; Ramirez and Tolmasky, 2010; Labby and Garneau-Tsodikova, 2013). According to their modification ability, the aminoglycoside-modifying enzymes (AMEs) are divided into three main groups: aminoglycoside acetyltransferases (AACs), aminoglycoside phosphotransferases (APHs), and aminoglycoside nucleotidyltransferases or adenyltransferases (ANTs or AADs; Ramirez and Tolmasky, 2010).

The ANT group can be further divided into five subtypes based on the specific position adenylated by the enzymes on aminoglycosides, including ANT(6), ANT(9), ANT(4'), ANT(3*), and ANT(2*). To date, more than 30 types of ANT(3*) enzymes have been described in the Comprehensive Antibiotic Resistance Database (CARD), designated AadA1 to AadA31 with some numbers missing (Ramirez and Tolmasky, 2010). The ANT (3*) enzymes are the most common ANTs and include two subclasses [ANT (3*)-I, ANT (3*)-II] that confer specific resistance to streptomycin and spectinomycin based on the mechanism of adenylation on the 3'- and 9-hydroxyl groups of streptomycin and spectinomycin, respectively (Shaw et al., 1993). Moreover, the genes encoding ANT (3*)-Ia proteins are most commonly namedaadA (Hollingshead and Vapnek, 1985). Correspondingly, the proteins encoded by aadA genes are designated AadA.

Bacteria of the genus Providencia in the family Morganellaceae are Gram-negative opportunistic pathogens. The Providencia group originated from the paracolon bacterial strain 29911, which was discovered in 1943 (Ewing et al., 1954; Hawkey, 1984). This genus experienced considerable taxonomic instability from 1952 to 1962 because the Providencia strains appeared to be an intermediate group between Proteus morganii and Proteus rettgeri. Providencia stuartii was named in 1962 and was further verified based on DNA–DNA hybridization in 1978 (O’Hara et al., 2000). P. stuartii is one of the most common pathogens of Providencia spp. (Yuan et al., 2020) and occurs naturally in soil, water and sewage (Clifford et al., 2012). As a common clinical isolate, P. stuartii can cause urinary tract infections (UTIs) and other nosocomial infections in humans, such as pneumonia, meningitis, endocarditis, and wound and bloodstream infections (Keane et al., 1975; O’Hara et al., 2000; Armbruster et al., 2014; Kurmasheva et al., 2018). Additionally, the role of P. stuartii as a nosocomial pathogen in the dissemination of plasmid-mediated resistance has been confirmed (Franceschini et al., 1998; O’Hara et al., 2000). Currently, 15 Providencia species are recognized: Candidatus P. Siddallii, P. Alcalifaciens, P. Burhodogranariea, P. Entomophila, P. Heimbachae, P. Huaxiensis, P. Rettgeri, P. Rustigianii, P. Sneathia, P. Stuartii, P. Thailandensis, P. Vericola, P. Friedericiana, P. Manganoxydans and P. Wenzhouensis.2

In this work, we report a novel chromosome-encoded ANT gene, designated aadA36, in the strain P. stuartii P14, which was isolated from a sputum specimen from a burn patient. Whole-genome sequencing, genetic context analysis and kinetic parameter analyses were performed to characterize the molecular features of the aadA36 gene and its related sequences.

Materials and methods

Bacterial strains and plasmids

A total of 25 Providencia isolates were collected from a hospital in Wenzhou, China, of which P. stuartii P14 was obtained from the sputum specimen of a burn patient. Species identification of these isolates was conducted by the VITEK 2 Compact instrument (bioMerieux, Inc., Craponne, France), 16S rRNA gene homology comparison and average nucleotide identity (ANI) analyses. The strains and plasmids used in this work are listed in Table 1.

Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) was determined using the agar dilution method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI), and the susceptibility pattern was interpreted as described by the CLSI M100 (31st Edition, 2021) and the European Committee on Antimicrobial Susceptibility Testing (version 11.0, 2021). Escherichia coli ATCC 25922 was used as a reference strain for quality control. pUCP20/DH5α was used as the control strain for investigating the activity of the aadA36 gene. The MIC experiment was performed on Mueller-Hinton (MH) agar plates with 2-fold serial dilutions of the antimicrobials, incubating the plates at 37°C for 20 h. The resistance breakpoint for florfenicol was determined according to a previous publication for E. coli (Wasyli et al., 2013), and the values for spectinomycin and streptomycin were interpreted according to the criteria proposed by the US FDA and the publications by Jouybari MA

1 https://card.mcmaster.ca/ontology/36357
2 https://lpsn.dsmz.de/search?word=Providencia
TABLE 1 Bacteria and plasmids used in this work.

| Strain or plasmid | Relevant characteristic(s) | Reference or source |
|-------------------|----------------------------|---------------------|
| Strain            |                            |                     |
| P14               | The wild-type strain of *Providencia stuartii* P14 | This study |
| DH5α              | *Escherichia coli* DH5α was used as a host for cloning the *aadA36* gene | Our laboratory collection |
| BL21              | *Escherichia coli* BL21 was used as a host for expression of the *aadA36* gene | Our laboratory collection |
| ATCC 25922        | *Escherichia coli* ATCC 25922 was used as quality control for antimicrobial susceptibility testing | Our laboratory collection |
| pUCP20-∗aadA36/   | DH5α carrying the recombinant plasmid pUCP20-∗aadA36 | This study |
| DH5α              | bl21 carrying the recombinant plasmid pCold I-∗aadA36 | This study |
| Plasmid           | Cloning vector for the PCR products of the *aadA36* gene with its upstream promoter region, AMP<sup>r</sup> | Our laboratory collection |
| pCold I           | Expression vector for the PCR products of the ORF of the *aadA36* gene, AMP<sup>r</sup> | Our laboratory collection |

<sup>r</sup>, Resistance; AMP, Ampicillin.

(Hu et al., 2017; Jouybari et al., 2021), respectively. All the tests were performed in triplicates.

Whole-genome sequencing and functional analysis

Bacterial genomic DNA was extracted using the Generay Genomic DNA Miniprep Kit (Shanghai Generay Biotech Co., Ltd., Shanghai, China). Whole-genome sequencing was achieved using the Illumina NovaSeq (for all isolates) and PacBio RS II (only for an isolate to obtain a complete genome) platforms by Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The short reads from Illumina sequencing of each isolate were assembled by MEGAHIT v1.2.9 (Li et al., 2016). To obtain a complete genome sequence for a certain isolate, the PacBio long reads were initially assembled using Trycycler v0.5.1 (Wick et al., 2021) and Flye v2.9-b1768 (Lin et al., 2016), and the quality of the draft genome assembly was further corrected with the short reads from Illumina sequencing by Pilon v1.24 (Walker et al., 2014). The open reading frames (ORFs) were then predicted using Prokka v1.14.6 (Seemann, 2014) and annotated by DIAMOND v2.0.11 (Buchfink et al., 2021) against the NCBI non-redundant protein database. The promoter region was characterized based on genome sequence information using BPROM. The resistance genes were annotated by Resistance Gene Identifier v5.2.0 (RGI)<sup>4</sup> based on CARD (McArthur et al., 2013). ANI was computed using FastANI v1.33 (Jain et al., 2018). Multiple sequence alignment and neighbor-joining phylogenetic tree construction were performed using MAFFT v7.487 (Katoh and Standley, 2013) and IQ-TREE v 2.0.7 (Tamura et al., 2021), respectively. Plasmid prediction was performed by PlasmidFinder 2.0.<sup>5</sup> Linear representation and visualization of the gene maps were achieved through genoPlotR v0.8.9 (Guy et al., 2010) and GView Server (Petkau et al., 2010), respectively.

Molecular cloning of the resistance gene

The ORF of the predicted resistance gene with its promoter region (∼155 bp) was amplified by PCR and then ligated into the pUCP20 vector with a T4 DNA ligase cloning kit (Takara Bio, Inc., Dalian, China). The recombinant plasmid was transformed into *E. coli* DH5α by the calcium chloride method, and then the transformants were cultured on Luria-Bertani (LB) agar plates supplemented with 100 μg/ml ampicillin. The inserted sequence in the recombinant was verified by Sanger sequencing. The primers used in this work are listed in Table 2.

Expression and purification of recombinant AadA36

To obtain the AadA36, the ORF of the *aadA36* gene was amplified by PCR and then inserted into the pCold I vector between the cleavage sites of the restriction enzymes BamHI and HindIII (Qing et al., 2004). The resultant recombinant plasmid pCold I-∗aadA36 was introduced into *E. coli* BL21 competent cells. The transformants (pCold I-∗aadA36/BL21) were selected on LB agar plates supplemented with 100 μg/ml ampicillin. The presence of the *aadA36* gene in the recombinant strain was confirmed by PCR and Sanger sequencing of the PCR product (Shanghai Sunny Biotechnology Co., Ltd., Shanghai, China).

The overnight culture of the recombinant strain (pCold I-∗aadA36/BL21) was added to LB broth supplemented with ampicillin (at a final concentration of 100 μg/ml) at a ratio of 1:100 for further incubation at 37°C in a shaker at 250 rpm for 3 hours. Then, the recombinant protein was purified using the Ni-NTA high-binding resin (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. After washing with buffer A, the protein of interest was eluted from the resin using buffer B (100 mM imidazole, 250 mM NaCl, 50 mM Tris-HCl, pH 8.0). The eluted fractions were further purified by size exclusion chromatography using a Superdex 200 gel filtration column (GE Healthcare, Buckinghamshire, UK) equilibrated in buffer B. The collected fractions were dialyzed against buffer B and stored at -80°C until use.

3. [http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb](http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb)
4. [https://github.com/arpcard/rgi](https://github.com/arpcard/rgi)
5. [https://cge.food.dtu.dk/services/PlasmidFinder-2.0/](https://cge.food.dtu.dk/services/PlasmidFinder-2.0/)
250 rpm. To induce the expression of AadA36, sterile isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the broth at a final concentration of 1 mM when the OD_{600} of the culture reached 0.6, as detected by ultraviolet–visible spectrophotometry, and then continue cultivating for 24 h at 15°C. Cells were harvested by centrifugation (8,000 × g, 10 min) at 4°C, resuspended in 3 ml of non-denaturing lysis buffer and disrupted by sonication for 5 min. The recombinant protein was purified using the BeyoGold His-tag Purification Resin and subsequently eluted with the nondenaturing eluent (50 mM NaH_{2}PO_{4}, 300 mM NaCl, 50 mM imidazole) from the His-tag Protein Purification Kit (Beyotime, Shanghai, China) according to the manufacturer’s instructions. The His-tag was removed from the samples using thrombin for 24 h at 37°C. Confirmation of the presence of AadA36 was obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent staining with Coomassie Brilliant Blue. The protein concentration was determined spectrophotometrically using a BCA protein assay kit (Beyotime, Shanghai, China).

### Enzyme kinetic studies of AadA36

The kinetic parameters of AadA36 were determined as reported previously with slight modifications (Kim et al., 2006). AadA36 activity was measured by coupling the enzymatic reaction to the UDP-glucose pyrophosphorylase, phosphoglucomutase and glucose-6-phosphate dehydrogenase reactions. The ANT(3′) catalytic activity was assayed by monitoring the accumulation of NADPH at 340 nm with a SpectraMax 190 (Molecular Devices, CA). The reaction mixtures contained 50 mM HEPES (pH 7.5), 10 mM MgCl_{2}, 0.2 mM UDP-glucose, 0.2 mM glucose 1,6-bisphosphate, 0.2 mM NADP, 0.2 mM dithiothreitol (DTT), 2 units/ml UDP-glucose pyrophosphorylase, 20 units/ml phosphoglucomutase, 20 units/ml glucose-6-phosphate dehydrogenase, 1 mM ATP, 8.8 × 10^{-4} μM purified AadA36, and variable concentrations of an aminoglycoside (5–150 μM) in a total volume of 0.2 ml. Reactions were initiated by the addition of the purified AadA36 enzyme. The steady-state kinetic parameters (K_{m} and V_{max}) were determined by nonlinear regression of the initial reaction rates with the Michaelis–Menten equation in Prism (v9.4.0) software (GraphPad Software, CA, United States; Chen et al., 2019).

### Nucleotide sequence accession numbers

The nucleotide sequences of the chromosome, two plasmids (pP14-166 and pP14-114) of _P. stuartii_ P14 and the _aadA36_ gene have been deposited in GenBank under accession numbers NZ_CP097380, NZ_CP097381.1, NZ_CP097382.1 and ON520657, respectively.

### Results

#### Identification of candidate novel resistance genes

Based on the annotation of the antimicrobial resistance genes (ARGs) in the genome sequences of the 25 isolates sequenced in this work, we screened the potential novel resistance genes that shared identities of <80% with the functionally characterized resistance genes, of which 9 hypothetical aminoglycoside resistance-related genes were chosen for further functional analysis. These genes included the _aac(6′)-Iy-, ant(9)-Ia-, aadA10-, aac(6′)-If-, aac(6′)-Iz-, ant(3′)-Ib-, aac(6′)-Iaa-, aac(6′)-Ib- and aac(6′)-Ia-like genes_. Through preliminary _in vitro_ antimicrobial susceptibility testing, among the recombinants with these cloned genes, we found that the _aadA10_-like gene (with the highest identity of 48.61% with the functionally characterized resistance genes, finally designated _aadA36_ in this work) was functional. Of the 25 isolates sequenced in this work, the gene was found to be encoded in the P14 genome alone, and its molecular characteristics were subsequently analyzed.

#### Characteristics and general features of _Providencia stuartii_ P14 genome

The isolate P14 shared the closest relationship (97.14% coverage and 99.73% identity) with _P. stuartii_ ATCC 29914...
(NR_024848) based on 16S rRNA gene sequence analysis. Further analysis of the ANI revealed that it shared the highest identity (99.24%) with *P. stuartii* ATCC 33672 (NZ_CP008920.1). This was consistent with the clinical laboratory identification of the VITEK 2 Compact instrument. Therefore, this isolate was finally named *P. stuartii* P14.

The whole genome of *P. stuartii* P14 consisted of one chromosome that was 4,375,942 bp in length, encoding 3,967 coding sequences (CDSs) with an average GC content of 41.3% (Table 3). It contained two plasmids that were 166,313 bp and 114,876 bp in length, designated pP14-166 and pP14-114, respectively. A total of 19 resistance genes with ≥80% similarities with the functionally characterized ARGs were identified in the whole genome. These genes conferred resistance to 8 classes of antimicrobials: aminoglycosides [*aac(2′)-Ia, aph(6)-Ia, aph(3′)-Ib, aac(6′)-Ib, ant(3′)-IIa, aph(3′)-Ia, β-lactams (bla*TEM*, and bla*OXA*), fluoroquinolones (*rsmA* and *qnrVC*4), phenicols (*catIII, floR* and *cmlA*5), tetracyclines [*tet(B)* and *tet(A)*], sulphonamides (*sul2*), quinolones (*aacL*) and macrolides (*dfrA4* and *mepA*). Among them, *rsmA*, *tet(B)*, *catIII* and *aac(3′)-Ia* were located on the chromosome, and the other 15 resistance genes were on the plasmid pP14-166. Furthermore, the *in vitro* antimicrobial susceptibility test showed that the isolate P14 was resistant to 16 of the 25 tested antimicrobials, including aminoglycosides (spectinomycin, streptomycin, neomycin, ribostamycin, tobramycin, gentamicin, kanamycin and paromomycin), β-lactams (ampicillin, cefazolin, cefotaxime and ceftazidime), tetracycline, chloramphenicol and florfenicol, and nalidixic acid. In addition, the strain was susceptible to some other antimicrobials, such as amikacin, meropenem, and aztreonam. The MICs of the 25 antimicrobials and their corresponding resistance genes are shown in Table 4.

**aadA36** confers resistance to spectinomycin and streptomycin

The *aadA36* gene is 843 bp in length and encodes a 280 amino acid protein with a molecular mass of 31.74 kDa and a pI value of 5.03. The antimicrobial susceptibility results of the recombinant strain (pUCP20-*aadA36*/DH5α) showed that it conferred resistance to spectinomycin and streptomycin, with the MIC levels of both antimicrobials increasing 128-fold and 64-fold, respectively, compared with that toward the control strain (pUCP20/DH5α; Table 4). The kinetic parameters of *AadA36* were consistent with the MIC data. The enzyme specifically adenylates spectinomycin and streptomycin with *kcatKm* ratios of (1.07 ± 2.23) × 10^4 M^−1 s^−1 and (8.96 ± 1.01) × 10^3 M^−1 s^−1, respectively. As expected, no adenosine transfer was detected with tobramycin. The steady-state kinetic parameters for *AadA36*-catalyzed reactions are summarized in Table 5.
| Antimicrobial class | Chromosome-encoded related resistance gene | Plasmid-encoded related resistance gene | Antibiotics | ATCC 25922 | DH5α | pUCP20/ DH5α | pUCP20-paadA36/DH5α | Providencia stuartii P14 |
|---------------------|------------------------------------------|----------------------------------------|-------------|------------|------|-------------|-----------------------|--------------------------|
| Aminoglycosides     | aac(2')-Ia                             | ant(3')-Ia, aph(6)-Ia, aph(3')-IIb,  | Spectinomycin | 8          | 8    | 8           | 1,024                | 512                      |
|                     |                                          | aph(3')-Ia, aac(6')-Ib                | Streptomycin | 4          | 2    | 2           | 128                  | 64                       |
|                     |                                          |                                        | Neomycin     | 1          | 1    | 1           | 1                    | 32                       |
|                     |                                          |                                        | Sisomicin    | 0.25       | 0.25 | 0.25        | 0.25                 | 2                        |
|                     |                                          |                                        | Ribostamycin | 2          | 2    | 2           | 2                    | 512                      |
|                     |                                          |                                        | Tobramycin   | 0.25       | 0.25 | 0.25        | 0.25                 | 32                       |
|                     |                                          |                                        | Gentamicin   | 0.25       | 0.25 | 0.5         | 0.25                 | 16                       |
|                     |                                          |                                        | Amikacin     | 1          | 1    | 1           | 1                    | 0.5                      |
| β-Lactams           | bla <sub>Ceph</sub> ·bla<sub>Cep</sub>  |                                        | Ampicillin   | 4          | 2    | /           | /                    | 512                      |
|                     |                                          |                                        | Cefoxitin    | 4          | 4    | /           | /                    | 16                       |
|                     |                                          |                                        | Cefazolin    | 1          | 1    | /           | /                    | 128                      |
|                     |                                          |                                        | Cefotaxime   | 0.125      | 0.125 | /           | /                    | 16                       |
|                     |                                          |                                        | Cefazidime   | 0.25       | 0.125 | /           | /                    | 16                       |
|                     |                                          |                                        | Meropenem    | 0.03       | 0.03 | /           | /                    | 0.06                     |
|                     |                                          |                                        | Aztreonam    | 0.125      | 0.03 | /           | /                    | 2                        |
| Quinolones          | rsmA                                    | qacL, qnrVC4                           | Nalidixic acid | 4          | 4    | /           | /                    | 32                       |
|                     |                                          |                                        | Levofloxacin | 0.03       | 0.03 | /           | /                    | 1                        |
| Tetracyclines       | tet(B)                                  | tet(A)                                 | Tetracycline | 2          | 2    | /           | /                    | 128                      |
|                     |                                          |                                        | Tigecycline  | 0.25       | 0.5  | /           | /                    | 8                        |
| Phosphonic acid derivatives | catIII                              | floR, cmlA5                             | Fosfomycin   | 2          | 2    | /           | /                    | 2                        |
| Chloramphenicol     |                                          |                                        | Chloramphenicol | 4          | 4    | /           | /                    | 128                      |
| Trimethoprim and sulfonamides* | sul2                            |                                        | Florfenicol  | 4          | 8    | /           | /                    | 512                      |
| Macrolides*         |                                          |                                        | MphA, dfrA14 | /          | /    | /           | /                    | /                        |

*The antimicrobials with corresponding genes carried by the chromosome and plasmid pP14-166 but the susceptibility test was not performed.
region (approximately 21 kb in length) including the ORF of \(aadA36\) along with the approximately 10 kb upstream and downstream sequences was used as a query to search the NCBI non-redundant nucleotide database. A comparative genomic analysis of the \(aadA36\) encoding region with those of homologous sequences in three other \(P. stuartii\) strains and one \(Providencia\) sp. strain showed that the IS200-nema-aadA36-mnmH-encoding fragment in the \(P. stuartii\) P14 chromosome exhibited high similarity with its relatives, and the fragment was flanked by two pairs of 9-bp imperfect IRs (Figure 3). IS200 in this work was an imperfect insert sequence encoding an 85-amino-acid transposase that shared 97.6% amino acid sequence identity with a transposase (AIN64626.1) of the IS200-like family. The finding indicates that this novel resistance gene might be transferable. To determine the origin of the novel resistance gene, more genomes of bacteria from different sources should be sequenced.

**Discussion**

In this work, we identified a novel chromosome-encoded ANT gene designated \(aadA36\) from a clinical \(P. stuartii\) isolate P14. Of the 11 aminoglycoside antimicrobial agents tested, the recombinant strain (pUCP20-\(aadA36\)/DH5α) showed resistance only to streptomycin and spectinomycin. Phylogenetic analysis revealed that AadA36 is distantly related to the other AadA proteins. The closest relatives among the functionally characterized resistance genes were the genes encoding AadA14 and AadA31 of the ANT (3')-Ia family, which shared amino acid sequence identities of less than 55%.
The MIC values of *aadA36* to spectinomycin (1,024 μg/ml) and streptomycin (128 μg/ml) was roughly consistent with the genes of the *ant(3″)-Ia* family, such as *aadA14* (≥512 and 256 μg/ml), *aadA31* (>512 and 256 μg/ml), *aadA7* (>64 and >64 μg/ml) and *aadA25* (≥512 and ≥64 μg/ml; Ahmed et al., 2004; Kehrenberg et al., 2005; Michael et al., 2012; Cameron et al., 2018).

Different ANTs have different aminoglycoside substrates. ANT(3″) adenylates streptomycin and spectinomycin (but not tobramycin; Kehrenberg et al., 2005), while ANT(6) confers resistance to streptomycin (Abril et al., 2010), and ANT(9) mediates resistance to spectinomycin (Kanchugal and Selmer, 2020). ANT(4′) and ANT(2″), however, confer resistance to multiple antimicrobials, including tobramycin (Wiedemann and Husing, 1985; Semper et al., 2020). Therefore, AadA36 could be distinguished from the other four subclasses of the ANT family and was assigned as a novel lineage of the ANT(3″)-Ia family.

The structural mechanism of AadA (Q8ZPX9) has been verified that the determinants for adenylation activity on streptomycin were amino acid residues W173 and D178, and on spectinomycin were E87, W112, D182, and 185H/N. Besides, the last four residues were conserved in all ANT (3″)(9) and ANT(9) enzymes (Stern et al., 2018). Multiple sequence alignments of AadA36 with the other AadA enzymes revealed that the six amino acid residues were conserved in them, except AadA9 (D203E) and AadA27 (W165I). Moreover, it has been reported that the structure of AadA31 was consistent with AadA122, and the residues implicated in ligand binding and catalysis was conserved within the active site (Cameron et al., 2018). It indicates that the mechanism of action of *aadA36* on streptomycin and spectinomycin may be related to these six amino acid residues.

The novel aminoglycoside resistance gene *aadA36* was related to a transposon-like sequence. The presence of the transposase gene upstream of the *aadA36* gene and three pairs of imperfect IRs flanking the *aadA36* encoding fragment demonstrated that the novel resistance gene carrying transposon-like sequence might be transferable. Furthermore, based on the amino acid sequence similarity analysis between AadA36 and other proteins retrieved from the database, we found that 21 proteins with identities >98% were all from the genus *Providencia* (including 18 from *P. stuartii*), and all the other proteins showed identities of less than 74%. The results suggested that at present, the *aadA36* gene is conserved in species of the genus *Providencia*. The source and transmission mechanism of this novel resistance gene remain to be further studied.

The transposon-like structure related to *aadA36* of this work was located in the chromosome, and the *ant(3″)-Ia* genes discovered so far have been found to be encoded on either plasmids or chromosomes, with many of them carried by the mobile genetic elements such as the class 1 integrons (Sandvang, 1999; Partridge et al., 2002; Michael et al., 2005). Overuse of antimicrobials exerts strong selective pressure on bacteria and facilitates the evolution and spread of drug-resistant strains. To some extent, strains carrying genes like *aadA36* can cope with
the selection pressure of spectinomycin and streptomycin. On the other hand, the evolution of antimicrobial resistance may carry a fitness cost, in terms of reduced competitive ability when bacteria encounter an antimicrobial-free environment (Paulander et al., 2009). It has been found that chromosomal resistance mutations carry a larger cost than acquiring resistance via a plasmid (Vogwill and MacLean, 2015). However, due to its uncertain origin and the instability of compensatory evolution, the survival of strains carrying \textit{aadA36} in the absence of antimicrobial selection pressure cannot be determined, which requires more efforts to verify.

**Conclusion**

In this study, based on whole-genome sequencing, we characterized a novel chromosome-encoded ANT gene, \textit{aadA36}, in a clinical \textit{P. stuartii} isolate P14, which showed resistance to spectinomycin and streptomycin. Besides the chromosomal resistance genes, \textit{P. stuartii} P14 also harbored a plasmid (pP14-166) encoding multidrug-resistance genes that conferred resistance to various antimicrobials, including aminoglycosides, β-lactams, tetracycline, and chloramphenicol. Sequence analysis revealed that the \textit{aadA36} gene is related to a transposon-like sequence, which might indicate the possibility of transmission of this novel resistance gene between bacteria of different species. Identification of a novel resistance gene and characterization of its molecular characteristics will help us further elucidate the resistance mechanisms of clinical opportunistic pathogens and better cope with the corresponding infections.

**Data availability statement**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

**Ethics statement**

Individual patient data was not involved, and only anonymous clinical residual samples during routine hospital laboratory procedures were used in this study. It was approved by the ethics committee of the Second Affiliated Hospital and Yuying Children’s Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China.

**Author contributions**

JL, QB, and HZ conceived and designed the experiments. MG, YJ, WS, LZ, SL, AL, XZ, and QL performed the experiments. MG, CF, JL and QB data analysis and interpretation. MG, CF, QB, and HZ drafting of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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