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# Characterisation of a class 1 integron associated with the formation of quadruple $bla_{GES-5}$ cassettes from an IncP-1$\beta$ group plasmid in *Pseudomonas aeruginosa*

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ABSTRACT

Integrons are genetic platforms responsible for the dissemination of antimicrobial resistance genes among Gram-negative bacteria, primarily due to their association with transposable elements and conjugative plasmids. In this study, a cassette array containing four identical $bla_{GES-5}$ genes embedded in a class 1 integron located on an IncP-1$\beta$ group plasmid from a clinical *Pseudomonas aeruginosa* strain was identified. Comparative genome analysis and conjugation assay showed that the plasmid pICP-4GES lacked the *trbN, trbO* and *trbP* genes but was conjugable. Antimicrobial susceptibility testing revealed that compared with single-copy $bla_{GES-5}$ complementary strains, both the cloned and chromosome-targeted expression of four copies of $bla_{GES-5}$ increased the minimum inhibitory concentration (MIC) by one to two dilutions for most of the selected antimicrobials. Quantitative real-time reverse transcription PCR (RT-qPCR) showed that the four consecutive cassettes increased $bla_{GES-5}$ expression by approximately two-fold compared with the single-copy $bla_{GES-5}$ strain, suggesting that the level of gene expression was not directly proportional to copy number. In addition, the gene cassette capture assay showed that the global $bla_{GES-5}$ transfer frequency reached $5.38 \times 10^{-4}$.



## 1. Introduction

Nosocomial infections caused by carbapenem-resistant *Pseudomonas aeruginosa* are one of the most common challenges for antimicrobial therapy. Resistance to carbapenems in *P. aeruginosa* can be attributed to a multitude of intrinsic mechanisms, including various efflux pumps, loss of the porin OprD, and increased AmpC $\beta$-lactamase expression [1,2]. In addition to intrinsic mechanisms, acquired mechanisms of resistance, such as metallo-$\beta$-lactamase and extended-spectrum $\beta$-lactamase production, have facilitated the emergence of carbapenem-resistant isolates, which has resulted in limited treatment options during recent decades [3]. Minimum inhibitory concentration (MIC) breakpoints of carbapenems, such as imipenem and meropenem, in *P. aeruginosa* have been established, and values $\geq$16 mg/L are defined as clinical resistance [3]. $bla_{GES}$ is a class A carbapenemase-encoding gene, formerly identified as $bla_{IBC-1}$ [4], and has recently been demonstrated to be widely distributed throughout Europe, South Africa and the Far East in at least five bacterial species, residing as gene cassettes mainly on class 1 integrons [3–6]. In addition, many $bla_{GES}$ cassette-harbouring integrons are located on conjugative plasmids [4–11], laying the molecular basis for rapid spread of a carbapenem-resistant phenotype through horizontal gene transfer.

Class 1 integrons are one of the five classes of mobile integrons that are notorious for the dissemination of antimicrobial resistance genes and function as a genetic platform for gene cassette capture and resolution [12–16]. At least 200 different gene cassettes have been identified from class 1 integrons and the mechanisms involved in integrase-mediated gene cassette fixation and excision have been well elucidated [17–23]. Although as many as eight gene cassettes have been found in a single class 1 integron [24], few studies have reported that the same multiple gene cassettes simultaneously appeared in a single integron. In 2016, Chen et al.

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identified two consecutively identical gene cassettes associated with fosfomycin resistance in a naturally occurring class 1 integron located on a non-conjugative plasmid (pGES-GZ) [25]. Furthermore, different gene cassettes have a distinct transfer frequency, which can be reflected by biased emergence of cassettes in naturally occurring class 1 integrons. In this study, a class 1 integron harbouring four identical $bla_{GES-5}$ gene cassettes was identified. The resistance activity of this cassette array was characterised both in multi-copy and single-copy states. The gene expression level both in single and quadruple $bla_{GES-5}$-harboring strains was also evaluated. Finally, a cassette capture assay was carried out to characterise the transfer efficiency of the $bla_{GES-5}$ gene cassette.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmid elimination and conjugation assay

*Pseudomonas aeruginosa* PA1280 wild-type strain was isolated from a 96-year-old male patient with an upper respiratory tract infection at the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) in 2012. The patient was treated with meropenem prior to isolation of the bacterial strain. The plasmid of *P. aeruginosa* PA1280 wild-type strain was eliminated (ΔPA1280) using a high-voltage (25 μFD, 200 Ω and 2.5 kV; Bio-Rad, Richmond, CA) electroporation method [26]. The plasmid conjugal transfer experiment was performed with rifampicin-resistant and *recA*-inactivated *Escherichia coli* EC600 as the recipient and *P. aeruginosa* PA1280 as the donor. Overnight cultures (3 mL) from the donor and recipient were mixed and were then harvested and re-suspended in 80 μL of brain–heart infusion (BHI) medium. The mixture was spotted onto a 1 $cm^2$ filter membrane, which was subsequently incubated on a BHI agar plate at 37 °C for 16 h. Bacteria were washed from the membrane, which was further spotted on a Muller–Hinton agar plate containing 1000 μg/mL rifampicin and 100 μg/mL ampicillin to select for pICP-4GES-positive transconjugants. The transconjugants were thus named EC600[pICP-4GES]. 16S rRNA and $bla_{GES-5}$ gene amplification and sequencing were used to confirm the positive transconjugants. Multilocus sequence typing (MLST) of *P. aeruginosa* PA1280 wild-type strain was performed using seven housekeeping genes as described previously [27].

### 2.2. Plasmid sequencing and analysis

The plasmid of PA1280 was extracted using an alkaline lysis method as described elsewhere [28]. A 20-kb library was generated using a SMRTbell™ Template Prep Kit (Pacific Biosciences, Menlo Park, CA) according to the PacBio standard protocol and was sequenced on a PacBio RS II instrument (Pacific Biosciences). In addition, an Illumina library with 300-bp insert sizes was also constructed and was sequenced from both ends using a HiSeq 2500 platform (Illumina Inc., San Diego, CA). The PacBio long reads were initially assembled using Canu software [29]. The Illumina reads were subjected to adaptor trimming and quality filtering and were further mapped onto the primary assembly to identify the low-quality bases and false 'insertions and deletions' generated by the primary assembly using Burrows–Wheeler Aligner (BWA) and the Genome Analysis Toolkit [30,31]. A custom-derived script was used to extract the consensus sequence. Potential open reading frames (ORFs) were predicted using Glimmer software [32] and were annotated against the non-redundant (Nr) protein database using the BLASTX program. Comparative genome analysis of pICP-4GES with other IncP-1$\beta$ group plasmids was performed by BLASTN program and custom-derived Biopython scripts. The complete nucleotide sequence of pICP-4GES has been submitted to NCBI under accession no. **MH053445**.

### 2.3. Molecular cloning and gene knock-in assay

Single and four $bla_{GES-5}$ complete ORFs alone with the integron Pc promoter were amplified using *P. aeruginosa* PA1280 DNA as a template. The PCR primer pairs used are listed in Supplementary Text S1. PCR products containing one and four $bla_{GES-5}$ genes were digested with *Eco*RI–*Bam*HI and *Eco*RI–*Xba*I, respectively, and were ligated into the same sites on a pUCP24 vector (gentamicin-resistant), resulting in pUCP24::$bla_{GES-5}$ and pUCP24::4$bla_{GES-5}$. Both constructed vectors were transformed into the plasmid-eliminated *P. aeruginosa* PA1280 (abbreviated as ΔPA1280) and *ampG*-deleted *P. aeruginosa* PAO1 (abbreviated as ΔPAO1) strains [33], respectively, forming the transformants named ΔPA1280[pUCP24::$bla_{GES-5}$], ΔPA1280[pUCP24::4$bla_{GES-5}$], ΔPAO1[pUCP24::$bla_{GES-5}$] and ΔPAO1[pUCP24::4$bla_{GES-5}$]. Single or quadruple $bla_{GES-5}$ was cloned into mini-Tn*7* using a one-step cloning method. The recombinant mini-Tn*7* vector and the helper plasmid pTNS were co-transformed into ΔPAO1 according to the protocol reported by Choi and Schweizer [34]. Bacterial strains with chromosome-targeted $bla_{GES-5}$ and 4$bla_{GES-5}$ were validated by PCR and Sanger sequencing.

### 2.4. Antimicrobial susceptibility testing

In total, 12 bacterial strains were selected for susceptibility testing against 15 antimicrobial agents, including carbapenems, using a standard agar dilution method as follows: ampicillin; piperacillin; piperacillin/tazobactam; cefoxitin; ceftriaxone; cefotaxime; ceftazidime; cefepime; cefoperazone; cefoperazone/sulbactam; aztreonam; imipenem; meropenem; kanamycin; and gentamicin. The results were interpreted according to Clinical and Laboratory Standard Institute (CLSI) 2015 recommendations. *Pseudomonas aeruginosa* ATCC 27853 was used as the reference strain for quality control.

### 2.5. Cassette stability test

A purified clone of transconjugant EC600[pICP-4GES] was inoculated in 5 mL of lysogeny broth (LB) medium without any antibiotics at 37 °C and shaking at 240 rpm overnight. Then, 10-μL cultures were transferred to 5 mL of fresh LB medium for a second round of cultivation. After 20 similar rounds of culture, the bacterial cultures were diluted and were spread on LB agar. A total of 100 clones were randomly picked and were used as templates for PCR amplification to test the stability of the cassettes.

### 2.6. Gene expression analysis

Overnight bacterial cultures were subcultured in LB medium and were further grown to an optical density at 600 nm ($OD_{600}$) of 1.0. Total RNA from each strain was extracted using an RNAprep Pure Cell/Bacteria Kit with on-column DNase I digestion (TIANGEN Biotech, Beijing, China). RNA was further treated by DNase I and was purified using an RNAclean Kit (TIANGEN Biotech). RNA concentration and purity were evaluated with a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA integrity was verified by denaturing agarose gel electrophoresis. cDNA was synthesised using a Super RT Reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Quantitative real-time reverse transcription PCR (RT-qPCR) was carried out using AceQ® qPCR SYBR® Green Master Mix (Vazyme Biotech, Nanjing, China). The 30S rRNA *rpsL* gene was used as an internal control to normalise gene expression quantities between samples. Primer sequences are listed in Supplementary Text S1.

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