# A Plasmidic Class 1 Integron from Five *Pseudomonas aeruginosa* Clinical Strains Harbored *aacA4* and Nonsense-mutated *cmlA1* Gene Cassettes

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**Five strains of multidrug-resistant *Pseudomonas aeruginosa* (*P. aeruginosa*) were isolated from inpatients at a local hospital in China. The most frequent resistance was to cefoperazone, ciprofloxacin, ceftriaxone, cefotaxime, gentamicin, piperacillin, trimethoprim sulfamethoxazole, and tobramycin. These strains were found to contain the class 1 integron, in which the 2360-bp gene cassettes were flanked by 5′- and 3′-conserved segments. Sequence analysis revealed that the gene cassettes contained *aacA4* and *cmlA1* genes; however, the latter gene had a nonsense mutation resulting in the production of a truncated protein. To the best of our knowledge, this is the first report of a nonsense mutation in the *cmlA1* gene. Moreover, the *P. aeruginosa* strains showed identical profiles in pulsed-field gel electrophoresis, suggesting that they were derived from the same clone. These results emphasize the importance of controlling the spread of multidrug-resistant pathogens in hospitals.**

**Key words** —— integron, drug resistance, *Pseudomonas aeruginosa*

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## INTRODUCTION

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a ubiquitous, gram-negative, opportunistic human pathogen implicated particularly in infections of immunocompromised hosts,[1,2] and this species is known to be inherently resistance to a wide variety of antimicrobials.[2,3] For example, the resistance to $\beta$-lactams, tetracycline, chloramphenicol, sulfamethoxazole/trimethoprim, or aminoglycosides has been reported.[4–6] Multidrug resistance is frequently caused by the enzymatic inactivation of antibiotics; however, enzyme-independent resistance such as accumulation of or defect in uptake of an antimicrobial agent is also common.[6] Transfer of the antibiotic-resistance genes is very likely because the genes are often located on plasmids, transposons, or integrons.[7] Integrons potentially play a major role in the dissemination of multidrug-resistance genes in gram-negative bacteria, especially in enteric bacteria and *Pseudomonas* sp.[8,9]

To date, based on the sequences of the integrase genes, integrons are divided into nine classes.[10,11] However, class 1 integrons have been found to be the most prevalent.[12] The class 1 integron usually consists of two conserved segments (CS) flanking the antibiotic-resistance gene cassette(s).[13] The 5′-CS contains the *intI1* gene, an *attI* recombination site, and a strong promoter. The 3′-CS normally carries the antiseptic-resistance gene *qacED1* and the sulfonamide-resistance gene *sul*.[13] On the other hand, the gene cassette is a small, mobile genetic element consisting of a single gene and a recombination site called the 59-base element (59-be).[14–16] More than 75 different gene cassettes conferring resistance to aminoglycosides, $\beta$-lactams, chloramphenicol, trimethoprim, or rifampin have been identified, although those for resistance to aminoglycoside or trimethoprim are the most common.[8,17] The 59-be found in the gene cassettes varies in length from 55- to 141-bp, but all contain consensus regions at boundaries.[8]

In the present study, we detected and characterized the class 1 integrons from five clinical strains of *P. aeruginosa*, which might have derived from a single clone.

## MATERIALS AND METHODS

**Bacterial Strains** —— We isolated 46 multidrug-resistant strains of *P. aeruginosa* from the spu-

tum samples of different inpatients at Bai Qiuen Peace Hospital in Hebei, China. Among them, 5 strains (10.9%) were used in this study because they showed highly similarity in their antibiotic-resistance profiles. *Vibrio cholerae* O1 strain SK-10 and *Escherichia coli* (*E. coli*) strain C600 were used as a positive and negative control for the *intI1* gene, respectively.

**Antibiotic Resistance** —— Susceptibility to antimicrobial agents was tested using the disk agar diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI).[18] The antimicrobial agents used were amikacin (30 μg), aztreonam (30 μg), cefepime (30 μg), cefoperazone (75 μg), cefotaxime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), piperacillin (100 μg), sulfamethoxazole/trimethoprim (23.75 μg/1.25 μg), and tobramycin (10 μg).

**Polymerase Chain Reaction (PCR)** —— In this study, the PCR primers intI1-U (5′-ACGA GCGCAAGGTTTCGGT-3′) and intI1-D (5′-GAAAGGTCTGGTCATACATG-3′) were prepared to amplify the 565-bp *intI1* gene in the 5′-CS of the class 1 integron. The primers in-F (5′-GGCATCCAAGCAGCAAGC-3′) and in-B (5′-AAGCAGACTTGACCTGAT-3′) were used for the resistance gene cassettes,[19] while qacED1-F (5′-ATCGCAATAGTTGGCGA AGT-3′) and sul1-B (5′-GCAAGGCGGAAACCC GCGCC-3′) were used for the 800-bp 3′-CS in the class 1 integron.[19]

The template DNA (1 μl) prepared as described previously[19] was mixed with 5 μl of the PCR buffer (Tris-HCl 100 mM, KCl 500 mM, $MgCl_2$ 15 mM, pH 8.3), 3 μl of each of the primers (10 pmol/μl), 4 μl of dNTPs mixture (2.5 mM), 0.25 μl of *Taq* DNA polymerase (5 U/μl), and 33.75 μl of sterilized distilled water. The conditions for amplification of the *intI1* gene with primers intI1-U and intI1-D were: initial denaturation for 5 min at 94°C and 30 amplification cycles consisting of denaturation for 30 sec at 94°C; annealing for 30 sec at 52°C; extension for 1 min at 72°C; and a final extension for 7 min at 72°C in a thermal cycler (GeneAmp PCR 24 System 2400, Applied Biosystems, Foster City, CA, U.S.A.). For PCR using the primers in-F and in-B, the annealing was carried out for 2 min at 52°C.[19] In the case of PCR with qacED1-F and sul1-B, the annealing temperature was changed to 56°C as previously described.[19]

**Sequencing of the Gene Cassette Region** —— The gene cassette region amplified by PCR was purified with a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and inserted into the pMD18-T easy vector (TaKaRa-Bio, Ohtsu, Japan). The hybrid plasmid thus constructed was transformed into *E. coli* strain DH5$\alpha$, and the transformant was selected on Luria-Bertani (LB) agar (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) containing ampicillin (100 μg/ml). The nucleotide sequence of the gene cassette cloned was determined with a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) and ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

**Plasmid Conjugation** —— *E. coli* strain RG488 $Rif^r$ was used as a recipient strain in the conjugation experiment. A single colony of each *P. aeruginosa* strain (donor strain) and the recipient were inoculated into LB broth and cultivated for 12 hr at 37°C. The culture was transferred to a Mueller-Hinton agar (Merck KGaA, Darmstadt, Germany) plate containing rifampin (50 μg/ml) and kanamycin (30 μg/ml), and the plate was cultivated overnight at 37°C. Thereafter, the transconjugants were selected and verified in PCR using two primer pairs, intI1-U/intI1-D and in-F/in-B.

**Southern Hybridization of the *intI1* Gene** —— The plasmid DNA was isolated from each of the *P. aeruginosa* strains, and Southern hybridization to detect *intI1* gene was performed with the DNA probe prepared as reported.[19]

**Pulsed-field Gel Electrophoresis** —— Pulsed-field gel electrophoresis (PFGE) analysis was performed with the protocol described elsewhere.[20, 21] Briefly, the genomic DNA isolated from each *P. aeruginosa* strain was fixed into the agarose plugs, and the plugs were equilibrated with the restriction enzyme buffer (pH 7.5) for 1 hr at room temperature. Thereafter, the DNA was digested with 50 U of *Xba*I for 12 hr at an appropriate temperature. Electrophoresis was carried out by the contour-clamped homogenous electric field method on a CHEF Mapper system (Bio-Rad, Hercules, CA, U.S.A.). A DNA size standard ladder (Bio-Rad) was used as molecular size markers, and a model 1000 Mini-Chiller (Bio-Rad) was used to maintain the temperature at 14°C. The run condition was set up using the autoalgorithm mode of the system with a size range of 20 to 300-kb.

## RESULTS

### Antibiotic Susceptibility

The susceptibilities of five *P. aeruginosa* isolates to various antibiotics were tested according to the CLSI recommendation. As shown in Table 1, all strains were demonstrated to be resistant to cefoperazone, ciprofloxacin, ceftriaxone, cefotaxime, gentamicin, piperacillin, trimethoprim-sulfamethoxazole, and tobramycin. However, they showed susceptibility to ceftazidime, chloramphenicol, and imipenem.

### Detection of Class 1 Integrons

PCR using the primer pair intI1-U/intI1-D revealed common amplification of a 565-bp fragment, suggesting that all isolates might be positive for the class 1 integron containing the *intI1* gene in the 5′-CS (data not shown). To detect the 3′-CS of the integron, the strains were further investigated using PCR with the primers qacED1-F and sul1-B. All of the five isolates also produced an 800-bp amplicon (data not shown), and thus the presence of the class 1 integron was confirmed.

### Characterization of Class 1 Integrons

Through PCR amplification with the primer set in-F and in-B, all *P. aeruginosa* isolates were found to have 2360-bp variable regions in the integrons, suggesting the presence of the same genes contributing to antibiotic resistance. Analysis of the nucleotide sequences revealed that each of the 2360-bp fragments contained two gene cassettes, *aacA4* and *cmlA1*. The *aacA4* gene showed complete identity with the *aacA4* gene [*aac*(*6′*)-*Ib* gene] of the class 1 integron reported previously (GenBank accession number DQ089809), which encodes an aminoglycoside 6′-*N*-acetyltransferase and confers resistance to kanamycin and tobramycin. On the other hand, the *cmlA1* gene encodes a chloramphenicol exporter belonging to the major facilitator (MF) family. However, the *cmlA1* gene in the *P. aeruginosa* strains tested was found to have a nonsense mutation at the 267th nucleotide (Fig. 1). Therefore a functional protein is probably not produced from the gene. The nucleotide sequence of the 2360-bp fragment was deposited in GenBank under the accession number AB214531.

### Plasmidic Localization of Class 1 Integrons

In the conjugation experiment, the gene cassettes were transferred from the *P. aeruginosa* strain to the recipient *E. coli* strain so that the PCR amplicon of the cassettes could be detected from the *E. coli* transconjugants (data not shown).

In agarose gel electrophoresis, the plasmid preparations from five *P. aeruginosa* strains showed the same profile, and the plasmid with a molecular size of about 20-kb was detected in all preparations (Fig. 2A). Additionally, Southern hybridization with the *intI1* probe confirmed that the *P. aeruginosa* strains carried the class 1 integron on the plasmid (Fig. 2B).

### PFGE

When the genomic DNA was digested with *Xba*I and its fragments were compared using PFGE, the five *P. aeruginosa* strains showed identical profiles (data not shown). This indicates that these strains may be derived from the same clone.

## DISCUSSION

Certain species of gram-negative bacteria are important causative agents of nosocomial or community-acquired infections. *P. aeruginosa* is a species in this category and is characterized by re-

**Table 1.** Antibiotic Susceptibility Results of Five *P. aeruginosa* Clinical Isolates[a)]

| Strain | Antimicrobial agent[b)] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AMK | ATM | CAZ | CFP | CHL | CIP | CRO | CTX | FEP | GEN | IPM | PIP | SXT | TOB |
| P5 | S | I | S | R | S | R | R | R | S | R | S | R | R | R |
| P6 | S | I | S | R | S | R | R | R | S | R | S | R | R | R |
| P10 | I | I | S | R | S | R | R | R | I | R | S | R | R | R |
| P14 | I | I | S | R | S | R | R | R | I | R | S | R | R | R |
| P26 | I | I | S | R | S | R | R | R | I | R | S | R | R | R |

[a)]S, susceptible; I, intermediate; R, resistance. [b)]AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CFP, cefoperazone; CHL, chloramphenicol; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; FEP, cefepime; GEN, gentamicin; IPM, imipenem; PIP, piperacillin; SXT, sulfamethoxazole/trimethoprim; TOB, tobramycin.

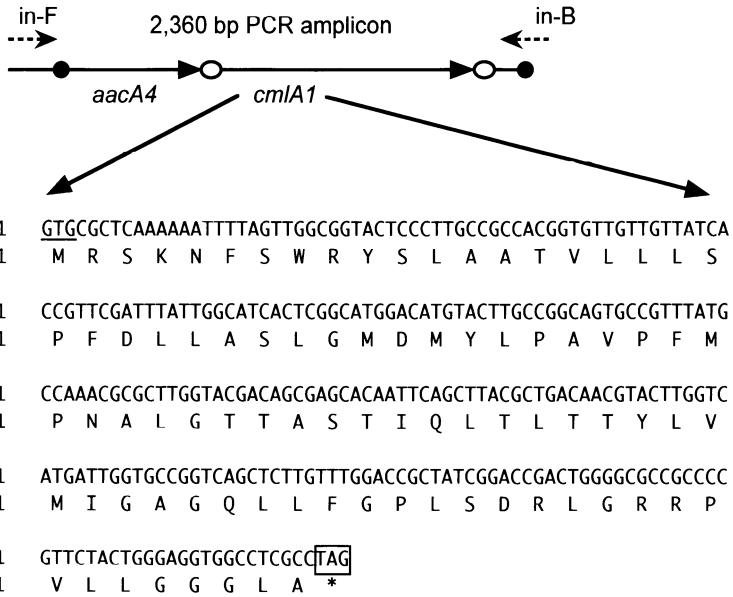


**Fig. 1.** Schematic Representation of the 2360-bp Fragment of Five *P. aeruginosa* isolates, Part of the Nucleotide Sequence of the *cmlA1* Gene, and Deduced Amino Acid Sequence of Its Product

The 59-base elements (59-be) are represented by ellipses, the start codon with underlining, and the nonsense mutation point is boxed.

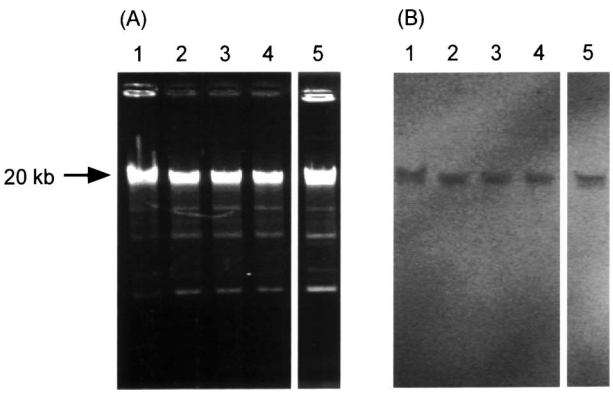


**Fig. 2.** (A) Profiles of Agarose Gel Electrophoresis of Plasmids Isolated from *P. aeruginosa* Strains. (B) Southern Hybridization of the Plasmids with the *intI1* Probe. Lane 1, strain P5; lane 2, strain P6; lane 3, strain P10; lane 4, strain P14; lane 5, strain P26.

sistance to a wide variety of antimicrobials.[2,3] The integrons play an important role in dissemination of multidrug resistance.[8,9] We isolated 11 strains of multidrug-resistant *P. aeruginosa* from inpatients at a local hospital and found that they were positive for the *intl1* gene, suggesting the presence of the class 1 integron causing resistance to chloramphenicol. However, among them, only 5 strains showed susceptibility to that antibiotic. In the present study, we found that, although the class 1 integron in the *P. aeruginosa* strains contained the *aacA4* and *cmlA1* genes, the latter gene had a nonsense mutation. This mutation may cause chloramphenicol sensitivity.

The susceptibility test using various antimicrobial agents indicated clearly that the *P. aeruginosa* strains were resistant to two aminoglycosides, gentamicin and tobramycin. *P. aeruginosa* is known to be resistant to aminoglycosides owing to the enzymatic *N* acetylation.[6,22,23] Our study showed that the *P. aeruginosa* strains tested have the *aacA4* gene cassette and that the gene in this cassette was 100% identical to the *aac*(6′)-*Ib* gene encoding an aminoglycoside 6′-*N*-acetyltransferase. The enzymes that modify the 6′ position (6′-*N* acetyltransferase [AAC (6′)]) are the most common acetyltranferases in multidrug-resistant bacteria including *P. aeruginosa*.[6,24] Mazel *et al.*[8] reported that the *aac*(6′)-*Ib* gene also contributs to amikacin resistance. However, the present study demonstrated that strains P5 and P6 were sensitive to amikacin and that other strains showed intermediate sensitivity. More detailed investigations are needed to interpret this inconsistency. Strain P5 and P6 were also more susceptible to cefepime than the others. This finding suggests that they may have a mutation causing reduction of the natural resistance to several antibiotics.

It should be mentioned that all of the *P. aeruginosa* strains had the nonsense-mutated *cmlA1* gene in the class 1 integron. The *cmlA1* gene is not re-

lated to enzyme-dependent resistance but encodes an exporter in the MF family. About 25 years ago, nonenzymatic resistance to chloramphenicol was first reported in a *P. aeruginosa* strain carrying a transposon Tn*1696* encoding the CmlA protein.[25] In the present study, the *cmlA1* gene was detected in the *P. aeruginosa* clinical strains; however, the gene had the nonsense mutation at codon 267. This finding suggests that the strains may not be resistant to chloramphenicol. This concept was supported by the results of the antimicrobial susceptibility test. Nevertheless, global comparative studies with other strains and/or expression experiments with the exporter are required to confirm that the nonsense mutation in the *cmlA1* gene is crucial for sensitivity to chloramphenicol. Although it is unclear why the clinical isolates of *P. aeruginosa* have the nonsense mutation in the *cmlA1* gene, the presence of the nonsense-mutated gene may be related to the prohibition against the clinical use of chloramphenicol.

All strains tested were resistant to multiple antibiotics, although the presence of the class 1 integron might account for resistance to only a few drugs. In *P. aeruginosa*, many mechanisms conferring multidrug resistance have been reported. The formation of biofilm, for example, always shows considerable resistance to the bactericidal effects of antibiotics.[26] The results from the conjugation and Southern hybridization experiments revealed that the class 1 integron was carried in the 20-kb plasmid. More studies are needed to clarify whether other determinants of antibiotic resistance reside on the plasmid.

In the present study, five *P. aeruginosa* strains isolated from patients at one hospital showed indistinguishable profiles in PFGE analysis. These strains were also found to have the same size of plasmid and the class 1 integron of which the structures are identical. Therefore it appears that, although the antibiotic resistance patterns were not exactly identical, they have a common origin. *P. aeruginosa* is an opportunistic human pathogen causing nosocomial infections, particularly in immunocompromised patients. Outbreaks of *P. aeruginosa* infections at hospitals are frequently reported. Because the class 1 integron in plasmids may contribute to the horizontal dissemination of antibiotic-resistance gene cassettes, studies of integrons and their gene cassettes can provide important information on the mechanism of acquisition of multidrug-resistance genes in the clinical isolates.

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