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

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(Received August 29, 2007; Accepted September 25, 2007; Published online October 10, 2007)

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, sulphonamide, sulfamethoxazole/trimethoprim, or aminoglycosides has been reported.3–6) 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, b-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.

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 b-lactams, tetracycline, chloramphenicol, sulfamethoxazole/trimethoprim, or aminoglycosides has been reported.3–6) 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)

Key words —— integron, drug resistance, Pseudomonas aeruginosa

MATERIALS AND METHODS

Bacterial Strains —— We isolated 46 multidrug-resistant strains of P. aeruginosa from the spu-
tum samples of different inpatients at Bai Quen 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). 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 GGC GCA AGGT TCTT CGGT-3′) and intI1-D (5′-GAA AGGT CTTG GTCATACATG -3′) were prepared to amplify the 565-bp intI1 gene in the 5′-CS of the class 1 integron. The primers in-F (5′-GGA CATC AAAC AGCAGA-3′) and in-B (5′-AG AGCAG ACTTG A CTGAT-3′) were used for the resistance gene cassettes, while qacED1-F (5′-ATCG CAA TATG TGG CCGA AGT-3′) and sul1-B (5′-GCAAG GCGA AACC CGCC-3′) were used for the 800-bp 3′-CS in the class 1 integron.

The template DNA (1 µl) prepared as described previously was mixed with 5 µl of the PCR buffer (Tris-HCl 100 mM, KCl 500 mM, MgCl2 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. In the case of PCR with qacED1-F and sul1-B, the annealing temperature was changed to 56°C as previously described.

**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 DH5α, 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 Rif² 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.

**Pulsed-field Gel Electrophoresis**—— Pulsed-field gel electrophoresis (PFGE) analysis was performed with the protocol described elsewhere. 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 XbaI for 12 hr at an appropriate temperature. Electrophoresis was carried out by the contour-clamped homogeneous 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 4°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 3'-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 XbaI 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)

<table>
<thead>
<tr>
<th>Strain</th>
<th>AMK</th>
<th>ATM</th>
<th>CAZ</th>
<th>CFP</th>
<th>CHL</th>
<th>CIP</th>
<th>CRO</th>
<th>CTX</th>
<th>FEP</th>
<th>GEN</th>
<th>IPM</th>
<th>PIP</th>
<th>SXT</th>
<th>TOB</th>
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<tbody>
<tr>
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<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>R</td>
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<td>R</td>
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<td>P6</td>
<td>I</td>
<td>I</td>
<td>S</td>
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<td>R</td>
<td>S</td>
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</tr>
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</table>

(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.

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 resistance 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 *intI1* 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 acetyltransferases in multidrug-resistant bacteria including *P. aeruginosa*.⁶ ²⁴) Mazel *et al.*⁸ reported that the *aac(6′)-Ib* gene also contributes 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 Tn1696 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 drugs. 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.

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.

Acknowledgements This work was supported by the Science Foundation of the Ministry of Education of China (70S046), National Natural Science Foundation of China (20436020), and the Scientific and Technological Project of Guangzhou City (2004J1C0161).

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