Molecular Cloning and Nucleotide Sequencing of a Novel Aminoglycoside 6'-N-Acetyltransferase Gene from an R-Plasmid of *Salmonella typhimurium* S24 Isolated in Taiwan

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Abstract A conjugative aminoglycoside resistance plasmid pST2 has been isolated from *Escherichia coli* K-12 14R525, which was mated with a clinical isolate of *Salmonella typhimurium* S24. A novel resistance gene of aminoglycoside 6'-N-acetyltransferase[AAC(6')] was cloned from plasmid pST2 on a 1,393 kilobase (kb) of *Sph*I-*Sal*I fragment into vector pACYC184 and pUC18. This novel AAC(6') gene in plasmid pST2 acetylated kanamycin, amikacin, dibekacin, tobramycin, gentamicin, netilmicin, and sisomicin. The complete nucleotide sequence of the novel AAC(6') gene and its neighboring sequences were also determined. Minicell experiments detected only one protein of 24.7 kilodaltons (kDa) translated from an open reading frame of the 618 base pairs (bp) gene.

Resistance to aminoglycoside antibiotics in clinical isolates has become increasingly apparent with the extensive use of these drugs in hospitals (19, 22). Aminoglycoside-modifying enzymes (AMEs) are found to be the major resistance mechanisms to inactivate aminoglycosides (6, 27). According to the modification on target sites of aminoglycosides, they are classified as aminoglycoside O-phospho-transferases (APHs), aminoglycoside O-nucleotidytransferases (ANTs), and aminoglycoside N-acetyltransferases (AACs). In particular, AACs have a diverse group of AAC(1'), AAC(2'), AAC(3'), and AAC(6') (20). These AMEs are often encoded by resistance genes on chromosomes (8, 37), conjugative plasmid (33), nonconjugative plasmid (3), and transposon (24) in both gram-positive (15) and gram-negative bacteria (17).

During 1986 and 1987, a total of 136 multidrug resistant strains of *Salmonella typhimurium* was isolated from an outbreak of salmonellosis in southern Taiwan. *S. typhimurium* S24, one of these multiresistant isolates, was selected for further study. *S. typhimurium* S24 harbors two conjugative R-plasmids of 130-kilobase (kb) IncFI pST1 and 56-kb IncN pST2. Plasmid pST1 conferred high-level resistance to chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, and β-lactam antibiotics. However, plasmid pST2 encoded resistance to β-lactam and amino-
glycoside antibiotics. In this report, we describe the aminoglycoside resistance phenotypes encoded by plasmid pST2 contained in S. typhimurium S24. We have cloned the aminoglycoside resistance gene from plasmid pST2. The nucleotide sequence of the aminoglycoside resistance gene in plasmid pST2 has been determined. The mechanism of resistance to aminoglycoside presented in plasmid pST2 is also described.

MATERIALS AND METHODS

Bacterial strains, bacteriophage and plasmids. S. typhimurium S24 was isolated in September 1986 at Kaohsiung Medical College Hospital, Kaohsiung, Taiwan, from a patient suffering from gastroenteritis during an outbreak of salmonellosis. E. coli K-12 strains, bacteriophage and plasmids used in this study are shown in Table 1. E. coli JM101 was used as the recipient strain for M13 bacteriophage vectors M13mp18 and M13mp19 and was grown in 2×YT broth (23).

DNA enzymes and chemicals. Restriction endonucleases, T4 DNA ligase and E. coli alkaline phosphatase were purchased from Bethesda Research Laboratories (Life Technologies, Inc., Gaithersburg, Md., U.S.A.) or Boehringer Mannheim Biochemicals (Mannheim, Germany) and were used according to the instruction of the manufacturers. Reagents for nick translation were purchased as a commercial kit from Boehringer Mannheim Biochemicals. All biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). All radiolabeled compounds were obtained from Du Pont Co., NEN Research Products (Wilmington, Del., U.S.A.).

Susceptibility testing. The antibiotic resistance phenotypes were determined by disk susceptibility testing (Difco Laboratories, Detroit, Mich., U.S.A.). The minimum inhibitory concentrations (MICs) of various antibiotics were determined on Mueller-Hinton agar (BBL, Microbiology Systems, Cockeysville, Md., U.S.A.) by using two-fold agar dilution method of Steers et al (26) with an inoculum of 10^5 CFU per spot.

Genetic techniques of plasmid DNA. Plasmid DNA was prepared by the alkaline-SDS method (Birnboim and Doly) and purified using ethidium bromide—cesium chloride density gradients (26). Restriction endonuclease analysis and agarose gel electrophoresis of plasmid DNA were performed as described before (26). Conjugation was done as previously described (26). Transformation with plasmid DNA was performed by the method of Chung et al (10). Transconjugants and transformants were isolated from selective media containing suitable antibiotics.

Identification of aminoglycoside-modifying enzyme. Crude cell-free enzyme extracts were prepared as described by Haas et al (13). The phosphocellulose paper (Whatman P81) binding assay was used to demonstrate the presence of aminoglycoside-modifying enzymes. Radioisotopes including [γ-32P]ATP(25 μCi/ml), [U-14C]ATP(50 μCi/ml), [1-14C]acetylcoenzyme A(5 μCi/ml) were used as cofactors in enzyme reactions. The aminoglycoside-modifying enzymes were classified on the basis of the modified substrate profiles at eight concentrations (10^-4, 5×10^-4,
10^{-3}, 3 \times 10^{-3}, 10^{-2}, 3 \times 10^{-2}, 10^{-1}, \text{and } 3 \times 10^{-1} \text{mM} ) \text{ as described by Bongaerts et al. (5).}

**Analysis of gene products in minicells.** *E. coli* P678-54 was used to produce minicells (1). The purification of minicells, labeling of plasmid-encoded proteins with 45 μCi of L-[^35]S]methionine, and autoradiography were done as described by Gaastra and Klemm (12). Plasmid-encoded proteins were resolved by electrophoresis in 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS-PAGE).

**DNA sequence determination.** DNA nucleotide sequence was determined with[^35]S]dATP labeling, by the dideoxy chain termination method of Sanger et al (29) using the restriction DNA fragment cloned into M13mp18 RF and M13mp19 RF (38). Sequencing was performed with T7 DNA polymerase Deaza sequencing system (Promega Co., Madison, Wis., U.S.A.) by following the reaction protocol supplied by the manufacturer. The universal 20 mer M13/pUC forward primer was used to sequence the fragment cloned in M13mp18 and M13mp19. Three oligonucleotides (20 mer) were also synthesized (Clontech Laboratory, Inc., Palo Alto, Calif., U.S.A.) and were used as internal primers. Nucleotide sequence and amino acid sequence were compared and analyzed with GenBank DNA sequence library (IntelliGenetics, Inc., Mountain View, Calif., U.S.A.) (4).

**RESULTS**

**Aminoglycoside Resistance Phenotypes Encoded by Plasmid pST2 in S. typhimurium S24**

The aminoglycoside resistance phenotypes in *S. typhimurium* S24 were encoded by the conjugative multiresistance plasmid pST2. Plasmid pST2 could be transferred from parental strain to recipient strain *E. coli* 14R525. Transconjugant of *E. coli* C24, carrying plasmid pST2, was selected for further genetic study. *E. coli* C24 also expresses the aminoglycoside resistance to kanamycin, amikacin, dibekacin, tobramycin, gentamicin, netilmicin, and sisomicin, but is susceptible to neomycin and streptomycin. Furthermore, plasmid pST2 could re-transfer from *E. coli* C24 to a plasmid-free, sensitive strain of *S. typhimurium* S1103. This transconjugant (designated *S. typhimurium* SC24), acquiring plasmid pST2, exhibited the same resistance phenotypes as *E. coli* C24. The MICs for the different aminoglycoside resistance phenotypes of donor cells, recipient cells, and transconjugants are shown in Table 2. It was noted that *E. coli* 14R525, harboring plasmid pST2, showed lower MIC values than *S. typhimurium* S24 for aminoglycoside antibiotics resistance.

**Molecular Cloning of the Aminoglycoside Resistance Gene from Plasmid pST2**

The aminoglycoside resistance determinant was cloned into vector pACYC184 by ligating a 12 kb *Sau3A* fragment of plasmid pST2 into *BamHI*-cut pACYC184 DNA and transforming into *E. coli* K-12 RR1 ΔM15. The resulting recombinant clone, designated pAG1, had the same aminoglycoside resistance phenotype as the original plasmid pST2. In order to locate precisely the aminoglycoside resistance gene, a series of deletion derivatives of plasmid pAG1 was constructed (Fig. 1).
Table 1. Bacterial strains, plasmids and bacteriophages

<table>
<thead>
<tr>
<th>Strain, plasmid and bacteriophage</th>
<th>Relevant characteristics(^a)</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>S. typhimurium</strong></td>
<td></td>
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<tr>
<td>S24</td>
<td>Clinical isolate</td>
<td>This study</td>
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<tr>
<td>S1103</td>
<td>Clinical isolate, sensitive strain</td>
<td>This study</td>
</tr>
<tr>
<td>SC24</td>
<td>Transconjugant of <em>S. typhimurium</em> S1103</td>
<td>This study</td>
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<td></td>
<td>Carrying plasmid pST1 and pST2</td>
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<td><strong>E. coli K-12 strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RRI dJM15</td>
<td>(F^+) lacIqZ \&amp; M15&amp;6+</td>
<td></td>
</tr>
<tr>
<td>P678-54</td>
<td>(F^-, \text{thr}^-, \text{leu}^-, \text{thr}^-, \text{supE, lacY, gal}^-, \text{mal}^-, \text{xyl}^-, \text{ara}^-, \text{mlz}^-, \text{min}^-)</td>
<td>28</td>
</tr>
<tr>
<td>14R525</td>
<td>(\text{Nal}^R)</td>
<td>2</td>
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<tr>
<td>C24</td>
<td>BLA&amp; AGR</td>
<td>This study</td>
</tr>
<tr>
<td>JM101</td>
<td>(F^{proAB} \text{ lacIq Z } &amp; M15 &amp; tra D36)</td>
<td>38</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pACYC184</td>
<td>(\text{Cm}^R \text{ Tc}^R) vector</td>
<td>9</td>
</tr>
<tr>
<td>pUC18</td>
<td>(\text{Ap}^R \text{ lacZ}') vector</td>
<td>38</td>
</tr>
<tr>
<td>pST1</td>
<td>(\text{BLA}^R \text{ Cm}^R \text{ Tc}^R \text{ SXT}^R)</td>
<td>This study</td>
</tr>
<tr>
<td>pST2</td>
<td>(\text{BLA}^R \text{ AGR})</td>
<td>This study</td>
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<tr>
<td>pAGR5</td>
<td>(\text{Cm-AGR}) ((1.3\text{ kb } &amp;&amp;hI-SalI\text{ of pST2 cloned into pACYC184}))</td>
<td>This study</td>
</tr>
<tr>
<td>pUCAGR5</td>
<td>(\text{Ap-AGR}) ((1.3\text{ kb } &amp;&amp;hI-SalI\text{ of pST2 cloned into pUC18}))</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Bacteriophages</strong></td>
<td></td>
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<tr>
<td>M13 mp18</td>
<td></td>
<td>38</td>
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<tr>
<td>M13 mp19</td>
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<td>38</td>
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</table>

\(^a\) \(\text{NaI}^R, \text{Cm}^R, \text{Tc}^R, \text{Ap}^R, \text{AGR}, \text{BLA}^R,\) and \(\text{SXT}^R\) indicate that resistance is specified to nalidixic acid, chloramphenicol, tetracycline, ampicillin, aminoglycosides, \(\beta\)-lactams, and trimethoprim-sulfamethoxazole, respectively.

Table 2. Antibiotic susceptibility of *S. typhimurium* S24, *S. typhimurium* S1103, *E. coli* 14R525, and their transconjugants

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC ((\text{ug/ml})) against strain:</th>
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<tr>
<td></td>
<td><strong>S. typhimurium</strong></td>
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<tr>
<td></td>
<td>S24</td>
</tr>
<tr>
<td>Amikacin</td>
<td>128</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>128</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>&gt;1,024</td>
</tr>
<tr>
<td>Dibekacin</td>
<td>512</td>
</tr>
<tr>
<td>Netilmicin</td>
<td>512</td>
</tr>
<tr>
<td>Neomycin</td>
<td>8</td>
</tr>
<tr>
<td>Sisomicin</td>
<td>512</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>8</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>512</td>
</tr>
</tbody>
</table>
Fig. 1. Restriction endonuclease maps of plasmid pAGR1 and their subclones. Letters below horizontal lines indicate restriction enzyme site: B, BamHI; H, HindIII; S, SalI; SP, SphI. Symbols: \( \square \), region of plasmid pACYC184; ——, cloned DNA from plasmid pST2; \( \square \), location of the aminoglycoside resistance gene.

Fig. 2. Substrate profiles of the novel AAC(6') enzyme produced by E. coli 14R525 harboring plasmid pAGR5. Abbreviations: ■, kanamycin; +, amikacin; □, dibekacin; ×, tobramycin; ○, gentamicin; △, netilmicin; ▽, sisomicin; ●, neomycin; •, streptomycin. cpm: counts per minute. The enzymatic activity is expressed as cpm of [14C]acetylated aminoglycoside in 25 µl after incubation for 30 min at 37 C.
Recombinant plasmid pAGR2 was constructed by inserting an 8.2 kb *SalI* fragment of plasmid pAGR1 into the *SalI* site of vector pACYC184. The resulting plasmid pAGR2 also had the aminoglycoside resistance phenotype. Finally, a 1.3 kb *SphI*-*SalI* fragment from recombinant plasmid pAGR4 was ligated to the same site of vector pACYC184. This recombinant plasmid, pAGR5, showed the aminoglycoside resistance phenotype when transformed into *E. coli* 14R525. Furthermore, the 1.3 kb *SphI*-*SalI* fragment was ligated into the same site of plasmid pUC18, this recombinant plasmid (designated plasmid pUCAGR5) was also showing the same resistance phenotype as that of plasmid pAGR5.

**Character of Aminoglycoside-Modifying Enzyme**

Cell lysates of *E. coli* 14R525, harboring plasmid pAGR5, were used to examine the activities of aminoglycoside-modifying enzymes of ANT, AAC, and APH. On the basis of the radiolabeled cosubstrate profiles, the cell lysates had the activity of AAC(6') enzyme. However, no ANT and APH activity was detected. This AAC(6') enzyme modified kanamycin, amikacin, dibekacin, tobramycin, gentamicin, netilmicin, sisomicin, but not neomycin and streptomycin. In addition, the remarkable substrate inhibition with all modified aminoglycosides at concentrations greater than $3 \times 10^{-2}$ mm was also demonstrated (Fig. 2). The modifying

Fig. 3. Plasmid-encoded proteins labeled with $[^{35}S]$methionine expressed in minicell-producing strain *E. coli* p678-54. Minicells were prepared, resolved by 12% SDS-PAGE and examined by autoradiography. Lane A, *E. coli* p678-54; lane B, *E. coli* p678-54 transformed with plasmid pAGR5; lane C, *E. coli* p678-54 transformed with plasmid pACYC184; lane D, *E. coli* p678-54 transformed with plasmid pUCAGR5; lane E, *E. coli* p678-54 transformed with plasmid pUC18. Numbers at the right represent the standard molecular weight of the methylated $^{14}$C-labeled proteins (kilodaltons); bovine serum albumin (69 kDa); ovalbumin (46 kDa); carbonic anhydrase (30 kDa); lactoglobulin A (18.4 kDa); cytochrome C (12.3 kDa). Abbreviations: BLA, $\beta$-lactamase; CAT, chloramphenicol acetyltransferase; AAC, aminoglycoside 6'-N-acetyltransferase.
enzyme encoded by plasmid pAGR5 also acetylated butirosin and ribostamycin but not paromomycin and lividomycin A. These data suggested that this modifying enzyme was designated as a novel AAC(6') enzyme.

Expression of Plasmid-Encoded Protein in Minicells

To identify the gene product encoded by the aminoglycoside resistance gene, recombinant plasmid pAGR5 and pUCAGR5, and vector pACYC184 and pUC18 were transformed into minicell-producing strain *E. coli* p678-54. Minicells were isolated and their protein contents labeled with [35S]methionine, resolved by SDS-PAGE and examined by autoradiography. In comparison with the proteins encoded by control plasmid of pACYC184 and pUC18, recombinant plasmid of pAGR5 and pUCAGR5 expressed one additional protein of approximately 24.5 kDa (Fig. 3). This protein is probably a product of the aminoglycoside resistance gene of the plasmid pAGR5 and pUCAGR5.

Nucleotide Sequences of the Novel AAC(6') Gene and Its Surroundings

The sequence of 1,393 bp *Sph*I-*Sal*I fragment from plasmid pAGR5, which includes the novel AAC(6') gene, is shown in Fig. 4. An open reading frame of 618 nucleotides, that codes a protein of 206 amino acid residues, is located between the start codon ATG at nucleotides 575 to 577 and the termination TAA codon at nucleotides 1,193 to 1,195. The ATG codon is separated by 33 bp from the sequence AGGAA, which is a putative ribosome-binding site. Further upstream sequences which could be the $-10$ region (TACAGT, nucleotides 464 to 469) and the $-35$ region (TTGTAA, nucleotides 444 to 449) are of a potential promoter site. The calculated molecular size of the derived protein of the novel AAC(6') gene is 24.7 kDa. To confirm the region of the novel AAC(6') gene in plasmid pAGR5, two fragments of *Sph*I-*Cla*I (794 bp) and *Cla*I-*Sal*I (599 bp) from plasmid pAGR5 were cloned into vector pACYC184, respectively. These two recombinant plasmids no longer confer the resistance phenotype to *E. coli* 14R525 transformants (data not shown). From the results we conclude that the locus of novel AAC(6') gene is located at the *Sph*I-*Sal*I fragment of plasmid pAGR5.

DISCUSSION

In Taiwan, *Salmonella* spp. are still an important common pathogen in gastroenteritis (36). Among the clinical isolates of *Salmonella*, *S. typhimurium* is the most common serotype for human salmonellosis and shows a high percentage of multiple resistance to commonly used antibiotics. These multiresistant isolates of *S. typhimurium* cause serious clinical problems in the treatment of salmonellosis. In this study, *S. typhimurium* S24 was found to carry two different types of group IncFI plasmid pST1 and group IncN plasmid pST2 that together mediated resistance to multiple antibiotics. Plasmid pST2 is an aminoglycoside resistance plasmid encoding resistance to seven clinically important aminoglycoside antibiotics and other related agents.
Fig. 4. Nucleotide sequence of the novel AAC(6') gene and its surroundings of the cloned 1,393 bp SphI-SalI fragment in plasmid pST2 from S. typhimurium S24. Only the 5'→3' strand is shown. The proposed –35 region and the potential –10 region are underlined. SD (Shine-Dalgarno sequence), the putative ribosome-binding site. The start codon at position 575 and the stop codon at position 1,193 are indicated by asterisks. The region of homology (position 1 to 489) with other resistance genes is boxed.
The aminoglycoside resistance gene, encoding the novel AAC(6') enzyme, has been cloned from plasmid pST2. The recombinant clones of plasmid pAGR5 and pUCAGR5 showed a unique acetylating activity on, in addition to amikacin, also on gentamicin and other related aminoglycosides. Minicell experiments demonstrated that the cloned determinant specified a single protein with a molecular weight of 24.5 kDa. Nucleotide sequence analysis of the cloned SphI-SalI fragment revealed an open reading frame of 618 bp specifying a protein of 206 amino acid residues and with a molecular weight of 24.7 kDa. Therefore the novel AAC(6') gene was identified in agreement with the analysis of the protein expressed in minicells.

Several AAC(6') genes (Ia, Ib, Ic, II, III, and IV) have been cloned and sequenced from gram-negative bacteria (8, 24, 31, 33, 35). Accordingly, AAC(6')-Ia, Ib, Ic, and IV genes mediated resistance to amikacin, whereas AAC(6')-II and III genes specified resistance to gentamicin. However, a novel AAC(6') gene in plasmid pAGR5 acetylated both amikacin and gentamicin. This novel AAC(6') enzyme activity resembles that of typical AAC(6')-II depending on their remarkable strong substrate inhibition profiles and no activity on neomycin (5). We gave to the new enzyme a provisional AAC(6')-V.

The 1,393 bp DNA sequence of the cloned SphI-SalI fragment in plasmid pST2 isolated from S. typhimurium S24 was compared with other resistance genes. It was noted that the 5' flanking region of nucleotides 1 to 489 is nearly identical with the sequence upstream previously reported from plasmid genes of aacAl (33), aacCI (34), AAC(6')-II (31), aadA (16), aadB (7), dhfrII (11), dhfrV (32), OXA-1 (25), and OXA-2 (14). Whether the nucleotide sequence of homology is the common promoter region for the resistance determinants or not, will be further investigated. However, the occurrence of the branching point of the sequence GTTA three times in the promoter region (positions 446–449, 489–492, and 525–528) was considered as the common points of divergence for the novel AAC(6') V gene (32). Furthermore, it can be seen that potential stem and loop structures are located between ATTC sequence (position 1,242 to 1,245) and the fourth GTTA sequence (positions 1,326 to 1,329) (32). The recombinational hot spot of the sequence CCAAGTTA (positions 485 to 492) associated with Tn21-related element previously reported (31) is also observed in the region upstream of the potential Shine-Dalgarno sequence. Therefore the 1,393 bp SphI-SalI fragment of pAGR5 contained in plasmid pST2 is an intact resistance gene of aminoglycoside antibiotics.

As described in previous reports (21, 24, 30, 33, 37), several aminoglycoside resistance genes, together with ampicillin resistance gene, are included in transposable elements that characterized a part of bacterial R-plasmids. Its resistance to ampicillin was usually mediated by a TEM-1 β-lactamase. We have demonstrated that plasmid pST2 showed a transposition event indicated by cointegrate formation (data not shown). The TEM-1 β-lactamase gene, specified by plasmid pST2, conferred high resistance to amoxicillin, carbenicillin, pipercillin, sulbenicillin, ticarcillin in addition to ampicillin. The dissemination of resistance gene for
bacterial phenotypic resistance was evaluated to show higher clinical significance than that of a bacterial strain or a plasmid epidemic (18). Therefore, the resistance genes presented in this transposon plasmid pST2 may play an important role in emergence of bacterial resistance to antibiotics.

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