Nucleotide Sequence of Aminoglycoside 6'-N-Acetyltransferase [AAC(6')] Determinant from Serrata sp. 45

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Gene for aminoglycoside 6'-N-acetyltransferase [AAC(6')] from Serrata sp. 45 was cloned into E. coli. The enzyme produced in E. coli carrying the recombinant plasmid was compared to the Serrata enzyme. Both enzymes acetylated the 6'-C position of amikacin, dibekacin, tobramycin, sisomicin, gentamicin C1, and kanamycin but lacked this activity towards gentamicin C2, gentamicin C3, and micromycin minimally. No significant difference in optimal pH, isoelectric point or molecular weight was detected. The nucleotide sequence of the gene was determined. Initiating with a GTG codon for methionine, it was composed of 552 base pair coding for 184 amino acids. The molecular weight of the enzyme was about 20418. Comparison of the amino acid sequence of this AAC(6') with the amino acid sequence of aacA4 gene from Serratia marcescens (G. Tran Van Nhieu and E. Collatz, J. Bacteriol., 169, 5708 (1987)) showed 98.3% homology.

Keywords 6' aminoglycoside acetyltransferase; Serrata sp.; cloning; nucleotide sequence

Introduction

Enzymatic inactivation of aminoglycoside antibiotics is one of the main mechanisms of resistance of both gram-positive and gram-negative clinical pathogens. The major aminoglycoside modification mechanisms include acetylation (AAC) of the amino group and phosphorylation (APH) and adenylation (AAD) of the hydroxy groups of the antibiotics. Aminoglycoside 6'-N-acetyltransferase [AAC(6')] has been classified further into 4 subgroups, AAC (6')-I, -II, -III and -IV, and is based on its substrate profiles and the susceptibility of organisms to the drugs. The enzyme AAC(6')-I acetylates kanamycin-A (KM-A), kanamycin-B (KM-B) and neomycin (NM). The enzyme AAC(6')-II acetylates KM-A, KM-B, NM, gentamicin-C14, gentamicin-C2 (GMC2) and dibekacin (DKB). The enzyme AAC(6')-III acetylates KM-A, KM-B, NM, GMC14, GMC2, DKB and amikacin (AMK). Recently new types of AAC(6') were found among clinical isolates of Staphylococcus aureus, Serratia spp. and Klebsiella pneumoniae. The enzyme produced by S. aureus acetylates GMC14, GMC2, sisomicin (SISO), netilmicin (NTM), KM-A, KM-B, DKB, tobramycin (TOB), AMK, ribostamycin (RSN), butirosin-A (BUT-A), neomycin-C (NM-C) and fortimicin (FTM) but not GMC1, kanamycin-C (KM-C), paromomycin (PRM) or lividomycin (LV). The enzyme produced by Serratia spp. acetylates KM-A, KM-B, NM, GMC14, DKB and AMK but GMC2 and 1-N-[O-(3-amino-2-hydroxypropionyl)]-gentamicin B (HAP-A) minimally. These two AAC(6')s are entirely different in their substrate profiles. We describe the cloning of the AAC(6') gene from Serratia sp. 45, a wild strain producing this new type AAC(6'), in E. coli and compared the enzymatic properties of the recombinant plasmid with the donor. We present the nucleotide sequence of this new AAC(6') gene from Serratia sp. 45 and additionally compared its nucleotide sequence to other acetyltransferases.

Materials and Methods

Bacterial Strain and Plasmid  Serratia sp. 45 was isolated from clinical sources and maintained as a stock culture in this laboratory. E. coli C600 was used as the plasmids' host cell. Plasmids used and their derivation are listed in Table I.

Medium Heart infusion medium (HI; Difco), Heart infusion agar (HIA; Difco) and Mueller Hinton agar (MHA; Difco) were used for cultivation and the determination of bacterial drug resistance, respectively.

Antibiotics Susceptibility Test Minimum inhibitory concentrations (MICs) were determined by the agar dilution method with an inoculum of approximately 5 x 10^5 cells per spot.

Antibiotic and Chemicals KM, DKB, streptomycin (SM), TOB, AMK and ampicillin (AP) were obtained from Sigma Chemical Co., Ltd., HAPA-B, micromycin (MCR), SS, and FTM were provided by the laboratories of Toyo Jozo Co., Ltd., Kyowa Hakko Kogyo Co., Ltd., Essex Nippon Co., Ltd., and Kyowa Hakko Kogyo Co., Ltd., respectively. GMC14, GMC2 and GMC3 were prepared in our laboratory. Adenosine 5'-triphosphate (ATP), and acetylCoA were purchased from Sigma Chemical Co., Ltd. [8-3^C]ATP (56mCi/mmol), [r-3^P]ATP (5000 mCi/mmol) and [1-14C]acetylCoA (54 mCi/mmol) were purchased from the Radiochemical Center, Amersham. Japan. Restriction endonuclease, deoxyribonuclease (DNA) polymerase and alkaline phosphatase were purchased from Takara Syuzo Co., Ltd.

Conjugal Transfer of Resistance Plasmid transfers were performed by the following method. One tenth milliliter of overnight cultures of the recipient (E. coli X1037: ryf) and donor (Serratia sp. 45) each were inoculated in 10 ml of fresh HI and shaken at 37°C for 3 h. One part of the donor culture was mixed with four parts of recipient culture, and the mixture was passed through a millipore membrane filter (0.45 μm, Millipore Co., Ltd.). The filter was placed on drug free HIA. After incubation at 37°C for 2 h, the millipore membrane filter was placed into 5 ml of phosphate buffered saline (PBS) and blended by a vortex mixer. One tenth milliliter of the mixed culture was placed on selective plates containing rifampicin (200 μg/ml) and aminoglycoside antibiotics (AMK, DKB, TOB and KM).

Assay of Aminoglycoside-Modifying Enzymes Substrate specificity was determined by a cellulosic phosphate binding assay. For acetylation, the assay mixture contained 30 μl of a 10500 x g supernatant (S10 fraction), 10 μl of 1 mM antibiotic solution, 10 μl of 20 mM MgCl2 solution and 10 μl of [1-14C]acetylCoA (10 μCi/mmol) containing 1 mm acetylCoA solution, 60 μl in total. The reaction mixture was incubated at 37°C for 15 min, and then heated at 100°C for 3 min. Twenty microliters of supernatant, obtained by centrifugation at 15000 rpm for 3 min, was placed onto phosphocellulose paper (0.49 cm²; Whatman p81), washed with a large volume of distilled water, dried and counted in a scintillator counter. For phosphorylation or adenyllylation, the same reaction mixture was used, except that it contained [r-3^P]ATP (10 μCi/ml) or [8-3^C]ATP (2 μCi/ml) instead of acetylCoA solution, and was incubated for 60 min. The bioassay method was performed as described previously. Protein concentration was measured by the method of Lowry et al. using bovine serum albumin as the standard.

Optimum pH Tris-maleic acid buffer (0.5 M; ranging from pH 6.0 to 7.5) and Tris HCl buffer (0.2 M; ranging from pH 8.0 to 8.5) were used to determine the optimal pH value. Enzyme activity was measured at each pH value by the bioassay method using AMK as the substrate.

Determination of the Isoelectric Point (pI) of the Enzyme Isoelectric focusing of enzyme was performed according to the method with a 110 ml capacity electrophoresis column using carrier ampholytes having a pH range of 4.0 to 12.0 in solution. After electrophoresis for 48 h, the content of the column was collected in 2 ml fractions, and the enzyme activity and pH of the fractions were determined. Enzyme activity was
measured by bioassay.

**Determination of Enzyme Molecular Weight**

The molecular weights (M.W.) of the enzymes were determined by gel filtration using Sephadex G75. Bovine serum albumin, ovalbumin, chymotrypsinogen A and ribonuclease A (Pharmacia Fine Chemicals) having M. Ws. of 67000, 43000, 25000, and 13700 were used as a standard.

**Isolation of Inactivated AMK**

AMK was inactivated in a reaction mixture containing 70 ml of S-105 fraction, 200 mg acetylCoA, 200 mg of AMK and 0.2 ml Tris HCl buffer (pH 8.0) containing 20 mm MgCl2. After incubation for 18 h at 37 °C, the reaction was stopped by heating in boiling water for 5 min. The supernatant collected by centrifugation at 15000 rpm for 15 min. The reaction mixture (about 200 ml) containing inactivated AMK was passed through a column of IRC 50 (NH₄⁺ form, 15 by 700 mm). After washing with 500 ml of distilled water, the inactivated AMK was eluted with 500 ml of 1 M NaOH. The fraction which had a positive ninhydrin reaction was collected and neutralized with 1 M HCl. Inactivated AMK was subjected to CM-Sephadex C-25 (NH₄⁺ form, 50 ml) column chromatography for purification. After washing with 1000 ml of distilled water, elution was carried out with a linear gradient of NH₄OH (0-0.1 M). Ninhydrin-positive fractions were collected and lyophilized to yield 30 mg of product. The chemical structure of the inactivated AMK was confirmed by mass spectroscopy and proton nuclear magnetic resonance (¹H-NMR) and carbon-13 nuclear magnetic resonance (¹³C-NMR) spectroscopy.

**DNA Procedures**

Plasmid and chromosomal DNA were prepared by either the rapid alkaline extraction or CsCl–ethidium bromide equilibrium density gradient centrifugation as described previously. Digestion and ligation of DNA

Endonuclease digestion and ligation reactions were performed according to the instructions of the manufacturer (Takara Shuzo Co., Ltd.). Restriction endonuclease were used with buffers prepared according to the recommendation of the supplier. The ligation reaction was carried out with excess T4 DNA ligase for 34 h at 4°C. Restriction analysis and ligation with T4 ligase were performed using the enzymes recommended by the supplier.

**DNA Nucleotide Sequence Determination**

The nucleotide sequence was determined by the dideoxy termination method using restriction fragments cloned into M13 derivative mp18 and mp19. The nucleotide sequence data and search for sequence homologies were analyzed by computer using the program of GENIAS system (Research Institute, Mitsu Knowledge Industry Co., Ltd.).

**Results**

**Construction of Plasmid**

We examined conjugal transferability of aminoglycoside-resistance in *Serratia* sp. 45 using *E. coli* X1037 (rif) as the recipient. We were unable to acquire the transconjugant when the selection was made for AMK, DKB, TOB and KM. Additionally, we were unable to find plasmid DNA either by the rapid alkaline extraction method or the CsCl–ethidium bromide equilibrium density gradient centrifugation method. Therefore, chromosomal DNA from *Serratia* sp. 45 (5.04 µg) was digested by the restriction endonuclease EcoRI and ligated with an EcoRI digest from pACYC184 (3.55 µg). For transformation, 10 to 20 µl of plasmid DNA in ligation mixture was added to 300 µl of competent cells and kept on ice for at least 40 min. After incubation at 42 °C for 3 min, the mixture was added to 5 ml of H1 and cultured at 37°C for 60 min. The transformants were selected by H1 containing KM (25 µg/ml). Five colonies were found to be resistant to KM and tetracycline (TC) but sensitive to chloramphenicol (CM). All transformants carried the same plasmid, approximately an 8-kb fragment. Accordingly, we chose one DNA plasmid (pMT2) for further study. One recognition site for BamHI was found on the inserted DNA fragment. pMT2 was cleaved by both EcoRI and BamHI and ligated with pBR322. After transformation of the ligated DNA into *E. coli* C600, both KM and AP resistant transformants were selected. One of them, plasmic pMT2 carried a 2.5-kb insert. We tried further subcloning and obtained plasmid pMT2 carrying *BbeI* and *HincII* to digest pMT2 and pUC13 as the vector plasmid. Finally, a 1.2-kb of *BbeI*–*HincII* fragment was cloned into pUC13 (Fig. 1).

**Resistance Patterns of Aminoglycoside Antibiotics**

Table I shows the MIC of *Serratia* sp. 45, *E. coli* C600 carrying pMT22 or pMT222, *Serratia marcescens* TL-1 and plasmid-free *E. coli* C600 against 12 different aminoglycoside antibiotics. *Serratia* sp. 45, wild strain, was resistant to AMK, DKB, TOB, SISO, GMC₁, and KM but susceptible to GMC₂, GMC₃, HAPA-B, SM and FTM. The sensitivity to GMC₁, GMC₃, and MCR of the transformants (pMT22, pMT222) was 8 to 64 times higher than the wild strain. However, they showed similar resistance levels as the wild-type strain to AMK, DKB, KM, TOB and HAPA-B.

**Identification of Acetyltransferase by *Serratia* sp. 45,** pMT22 and pMT222

Each S-105 fraction from *Serratia* sp. 45 and transformants pMT22 and pMT222 was examined for activity of the three amino-glycoside-modification enzymes (AAC, APH and AAD) to most amino-glycosides. Strains harboring plasmid and wild type strain were found to contain AAC but no AAD nor APH (AAD, APH data not shown). Consequently, it was concluded that aminoglycosides were inactivated only be acetylation in these three strains. The substrate profiles of the AAC extracted from strains *E. coli* C (pMT22, pMT222) and *Serratia* sp. 45 were very similar (Table II). The transformants had roughly 3- to 9-fold higher AAC activity than the wild strain, and the transformant carrying pMT22 possessed almost the same AAC activity as the transformant carrying pMT222.

**Some Properties of Each Enzyme**

The relationship
between AAC activity and pH was determined by varying the pH of the reaction mixture using 0.5 M Tris–maleic acid buffer (pH 6.0, 7.0 and 7.5) and 0.2 M Tris HCl buffer (pH 8.0, 8.5). The optimal pH for AMK inactivation was about 8.0 (data not shown). The inactivation decreased at pH values below 7.0 or over 8.5. No marked differences were observed between the two species. S-105 fractions were used as samples to determine the pl. AAC(6')s from the wild strain and transformant pMT22 both had pIs of 4.6 (data not shown). The M. W. of different Aacs was determined by gel filtration. The M. W. of AAC from the wild strain was about 20000 and the AAC from transformant pMT22 also was about 20000.

**Identification of Inactivated AMK** AMK was completely inactivated by the reaction described in Material and Methods. On thin layer chromatography (TLC) with the solvent of 10% CH3COONa–CH3OH (1:1), Rf values of the inactivated AMK and AMK were 0.26 and 0.04, respectively. The (1H-NMR) spectrum of the inactivated AMK suggested the existence of a formed N-acetyl-group (2.20 ppm singlet) and in the 13C-NMR spectrum of the same product, two other signals consistent with the presence of an acetate group were observed (data not shown). In view of the above experimental finding and the molecular ion peak in mass spectrum, the chemical structure of the inactivated AMK was 6'-N-acetyl-AMK. Both enzymes (wild strain and pMT22) acetylated the 6'-amino position.

**Nucleotide Sequence of the AAC (6') Gene** The strategy for the nucleotide sequencing of the AAC(6') gene region of pMT222 is shown in Fig. 2. The nucleotide sequence of the 1169-bp, HincII–Bbel fragment was determined by the Sanger's dideoxy chain termination method. Possible open reading frame (ORF) on both strands of the DNA were examined by the GENIAs computer program (Matsui

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**Table II. Resistance Patterns of Serratia sp. 45, E. coliC600 Carrying Recombinant Plasmids, E. coliC600 and S. marcescensTL-1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (mg/ml)</th>
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<tr>
<td></td>
<td>Serratia sp. 45</td>
</tr>
<tr>
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<td>3.13</td>
</tr>
<tr>
<td>AMK</td>
<td>25</td>
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<td>MCR</td>
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</tr>
<tr>
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<td>FTM</td>
<td>1.56</td>
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<tr>
<td>SM</td>
<td>0.78</td>
</tr>
</tbody>
</table>

\(^{a}\) These strains were transferred from recombinant plasmids into E. coliC600 as a recipient.

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Fig. 2. **Nucleotide Sequence of AAC (6') Gene of pMT222**

The nucleotide sequence is numbered from the first base of the initiation codon GTG. Amino acid sequence is shown under the nucleotide sequence. A probable Shine–Dalgarno sequence (GAGG, −14 to −11) and three promoters (−35 region and Praesnov box) are underlined. The alternative amino acid were those identified by G. Tran Van Nhieu et al.\(^{19}\)

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\[\text{HincII} \]

\[\text{Bbel} \]

\[\text{RF}\]
Knowledge Industry, Japan). ORF was found between the start codon GTG at positions +1 to +3 and the termination codon TAA at positions 553 to 555. This structure gene was composed of 552 base pair and 184 amino acid residues. Ten bases upstream from the GTG codon, there was a 4-base sequence, GAGG (−14 to −11), which may serve as the ribosome binding site (Shine–Dalgarno sequence\(^1\)) for the translation of AAC (6')\(^2\). This ORF codes for protein with a M. W. of 20418.

Discussion

The most common mechanism of aminoglycoside resistance in bacteria is enzymatic inactivation. Aminoglycoside antibiotics are inactivated by phosphorylation, adenyllylation and acetylation. AMK has been an effective antimicrobial agent against bacteria resistant to DKB, GM, TOB and KM, but recently, several authors have reported strains of Serratia marcescens resistant to AMK.\(^3\)\(^-\)\(^6\) Mitsuhashi classified AAC (6') into four groups [AAC(6')-1, -2, -3 and -4] on the basis of substrate profiles and drug susceptibility (1975).\(^1\) AMK resistance is believed to be due primarily to AAC (6')-4. Recently, a new enzyme has been found in a few clinical isolates of Serratia spp. Which is resistant to AMK, TOB, DKB and KM, but susceptible to GMC\(_2\) and HAPA-B. This enzyme has been named AAC (6').\(^3\) We cloned the AAC (6') gene from Serratia sp. 45 using vector plasmids (pACYC184, pBR322 and pUC13) and gene expression was confirmed in the recipient E. coli C 600. Recombinant plasmids pMT22 and pMTR22 carried the natural AAC (6') gene, having the 2.5-kb EcoRI–BamHI and 1.2-kb Bbel–HincII inserts, respectively. The enzyme produced from pMT22 was identical to the wild strain in terms of optimal pH, pl and M. W. Transformants carrying plasmids pMT22 and pMTR22 produced levels of AAC (6') activity 6-13-fold higher than the wild strain. This is believed to be due to the high copy numbers produced by plasmids pBR322 and pUC13. The nucleotide sequence of the AAC (6') gene region revealed only one ORF encoding a protein composed of 184 amino acid residues. This ORF was preceded by a putative Shine–Dalgarno sequence\(^1\) and by a potential promoter sequence.\(^1\)\(^-\)\(^2\) Three putative promoters (−35 region and Pribnow box) are shown in Fig. 2; these are TTGATGATACCTGTCGCGTAAACT (−577 to −549), TTGATGAGGCTGATGACGTTGATATAA (−152 to −127) and TTGATGAGGCGAATCGTTGATATA (−125 to −99). It has been reported that GTG is the initiation codon for KM nucleotide transferase encoded by plasmids pUB110 and pTB 913 in gram-positive bacteria.\(^1\) The initiation codon of the AAC (6') gene was GTG in our study. The AAC(6') gene from Serratia sp. 45 was compared to other gram-positive and gram-negative bacteria. The sequence of another 6'-N-acetyltransferase, aac44 gene from S. marcescens has been determined.\(^9\) It was confirmed that the whole nucleotide sequence was identical between both determinants of aac44 and pMTR22 except for four bases. Cytosine at position +305, thymine (+546), guanine (+547) and adenine (+548) in structural gene in pMT22 were replaced by thymine, guanine, thymine, thymine in aac44 gene, respectively. The amino acid sequences of the two acetyltransferases differed by only three amino acids. This protein of 184 amino acids is seventeen N-terminal amino acids shorter than the coding potential for the aac44 gene. The homology with the amino acid sequence of this protein showed 98.3% with the aac44 gene. Moreover, this protein has been compared with AAC(6')-II from Pseudomonas aeruginosa.\(^7\) The number of amino acids in both the pMT22 and the AAC(6')-II gene was the same (184 amino acids), but amino acid homologies were 77.7%. The amino acid sequences of the AAC(6') gene from Serratia sp. 45 were compared to the amino acid sequences of AAC(6')-APH(2') from S. faecalis;\(^1\) but the two sequences were not homologous. Whether the nucleotide sequence of each of the AAC(6') subclasses is the same or not and whether there are different isoenzymes of AAC(6') are interesting problems which require further study.

Acknowledgments

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References

1) S. Mitsuhashi, Czechoslovak Medical Press, Prague, Avicennum, 1975

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<tr>
<th>Substrate</th>
<th>Incorporation of ([^{14}C])Acetate into Various Aminoglycoside Antibiotics (dpm)</th>
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<tr>
<td>Serratia sp. 45</td>
<td>pMT22</td>
</tr>
<tr>
<td>HAPA-B</td>
<td>3541</td>
</tr>
<tr>
<td>AMK</td>
<td>6311</td>
</tr>
<tr>
<td>GMC(_2)</td>
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<tr>
<td>GMC(_{1a})</td>
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<tr>
<td>GMC(_2)</td>
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<tr>
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