New plasmid-mediated aminoglycoside 6′-N-acetyltransferase, AAC(6′)-Ian, and ESBL, TLA-3, from a Serratia marcescens clinical isolate

Wanchun Jin, Jun-ichi Wachino*, Kouji Kimura, Keiko Yamada and Yoshichika Arakawa

Department of Bacteriology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan

*Corresponding author. Tel: +81-52-744-2106; Fax: +81-52-744-2107; E-mail: wachino@med.nagoya-u.ac.jp

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Objectives: Enterobacteriaceae clinical isolates showing amikacin resistance (MIC 64 to >256 mg/L) in the absence of 16S rRNA methyltransferase (MTase) genes were found. The aim of this study was to clarify the molecular mechanisms underlying amikacin resistance in Enterobacteriaceae clinical isolates that do not produce 16S rRNA MTases.

Methods: PCR was performed to detect already-known amikacin resistance determinants. Cloning experiments and sequence analyses were performed to characterize unknown amikacin resistance determinants. Transfer of amikacin resistance determinants was performed by conjugation and transformation. The complete nucleotide sequence of the plasmids was determined by next-generation sequencing technology. Amikacin resistance enzymes were purified with a column chromatography system. The enzymatic function of the purified protein was investigated by thin-layer chromatography (TLC) and HPLC.

Results: Among the 14 isolates, 9 were found to carry already-known amikacin resistance determinants such as aac(6′)-Ia and aac(6′)-Ib. Genetic analyses revealed the presence of a new amikacin acetyltransferase gene, named aac(6′)-Ian, located on a 169829 bp transferable plasmid (p11663) of the Serratia marcescens strain NUBL-11663, one of the five strains negative for known aac(6′) genes by PCR. Plasmid p11663 also carried a novel ESBL gene, named blal, HPLC and TLC analyses demonstrated that AAC(6′)-Ian catalysed the transfer of an acetyl group from acetyl coenzyme A onto an amine at the 6′-position of various aminoglycosides.

Conclusions: We identified aac(6′)-Ian as a novel amikacin resistance determinant together with a new ESBL gene, blal, on a transferable plasmid of a S. marcescens clinical isolate.

Keywords: amikacin resistance, S. marcescens, antibiotic resistance genes

Introduction

Aminoglycosides have been widely used for the treatment of bacterial infections caused by Gram-negative and Gram-positive bacteria in combination with β-lactams. However, bacteria are known to acquire various mechanisms of resistance to aminoglycosides. In Enterobacteriaceae, the acquisition of plasmid-mediated 16S rRNA methyltransferase (MTase) genes such as armA, rmtB and rmtC has been reported worldwide and is becoming a major clinical concern because these MTases confer a high level of resistance to clinically important aminoglycosides including amikacin.

We previously reported that 16S rRNA MTase-producing Enterobacteriaceae showing a very high level of amikacin resistance (MIC ≥256 mg/L) in Enterobacteriaceae mostly depends on 16S rRNA MTase production and at the same time raises the question of what resistance determinant is involved in amikacin resistance in Enterobacteriaceae strains without 16S rRNA MTase genes.

Aminoglycoside 6′-N-acetyltransferases, AAC(6′), which acetylate the amino group at the 6′-position of aminoglycosides, and aminoglycoside 3′-O-phosphotransferases, APH(3′), which phosphorylate the hydroxyl group at the 3′-position of aminoglycosides, are known to underlie amikacin resistance by disrupting the ability of aminoglycosides to bind to target 16S rRNA molecules. Several new AAC(6′) enzymes, such as AAc(6′)-Ia, AAc(6′)-Ib, AAc(6′)-Ic, AAc(6′)-Id, AAc(6′)-Ie, AAc(6′)-If, AAc(6′)-Ig, AAc(6′)-Ih, and AAc(6′)-Ii, have been reported exclusively in amikacin-resistant non-fermenting Gram-negative pathogens over the last 10 years in Japan, but it remains unclear whether these AAC(6′) enzymes are involved in amikacin resistance in Enterobacteriaceae. The aim of this study was thus to clarify the molecular mechanism underlying amikacin resistance in Enterobacteriaceae.
resistance in Enterobacteriaceae isolates that do not produce 16S rRNA MTases. Our study shows that Enterobacteriaceae clinical isolates have continuously acquired either amikacin acetyltransferases or 16S rRNA MTases to enable amikacin resistance.

Materials and methods

Bacterial strains

Fourteen amikacin-resistant (MIC >64 mg/L) Enterobacteriaceae clinical isolates (3 Escherichia coli, 4 Klebsiella pneumoniae, 2 Proteus mirabilis and 5 Serratia marcescens) were used in this study.

Susceptibility testing

MICs were determined with the agar dilution method.12 E. coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 strains were used as quality controls.

Detection of amikacin resistance genes by PCR

Detection of aminoglycoside resistance 16S rRNA MTase genes was performed as described previously.13 The presence of aac(6′)-Ia and aac(6′)-Ib, which are prevalent among Enterobacteriaceae as determining factors of amikacin resistance, was determined by PCR using the primers listed in Table S1 (available as Supplementary data at JAC Online).

Conjugation and transformation

E. coli CSH2 (rifampicin′) was used as the recipient for conjugation. The conjugants were selected on LB agar plates containing 2 mg/L amikacin and 100 mg/mL rifampicin.16 Plasmids of the S. marcescens NU1663 strain were extracted and electrophorated into E. coli DH10B. The transformants were selected on LB agar plates supplemented with 2 mg/L amikacin.

Cloning of aac(6′)-Ia

The p11663 plasmid was extracted from the E. coli DH10B transformant using the QIAGEN Plasmid Midi Kit (Qiagen) and partially digested with Sau3AI. The digested fragments were ligated to the pBC-SK+ vector previously digested with BamHI, dephosphorylated and then introduced into E. coli DH5α. The transformants were selected on LB agar plates supplemented with 30 mg/L chloramphenicol and 2 mg/L amikacin.

Complete nucleotide sequence of p11663

Plasmids were extracted from an E. coli DH10B transformant carrying p11663 as described above and subjected to electrophoresis with a CHEF-DR III system (Bio-Rad). The extracted plasmids were digested with S1 nuclease (Takara) and subjected to electrophoresis. The DNA band corresponding to p11663 was extracted using a Wizard SV Gel and PCR Clean-Up System (Takara) and subjected to electrophoresis. The DNA band corresponding to p11663 was extracted using a Wizard SV Gel and PCR Clean-Up System (Takara) and subjected to electrophoresis. The DNA band corresponding to p11663 was extracted using a Wizard SV Gel and PCR Clean-Up System (Takara) and subjected to electrophoresis. The DNA band corresponding to p11663 was extracted using a Wizard SV Gel and PCR Clean-Up System (Takara) and subjected to electrophoresis. The DNA band corresponding to p11663 was extracted using a Wizard SV Gel and PCR Clean-Up System (Takara) and subjected to electrophoresis.

Expression and purification of recombinant AAC(6′)-Ia

The aac(6′)-Ia gene was amplified with the primers listed in Table S1 and cloned into a pET22b(+) vector. The resultant plasmid (pET-AAC) was introduced into E. coli BL21(DE3)pLysS. The cells were cultured in LB broth containing 100 mg/L ampicillin and 30 mg/L chloramphenicol at 37°C. When the OD at 610 nm reached 0.4, IPTG was added and the culture was further continued. The cells were collected, resuspended in PBS (pH 7.4) and disrupted with a French press. After ultracentrifugation, the supernatant was bound with 2 mL of Ni Sepharose™ 6 Fast Flow (GE Healthcare) and eluted with PBS containing 500 mM imidazole. The eluted protein was buffer-exchanged to PBS containing 20 mM imidazole and loaded onto a HiTrap™ HP column (GE Healthcare). The protein was eluted with a linear gradient of 20–500 mM imidazole. The collected protein was dialysed against 20 mM Bis-Tris buffer (pH 6.0) containing 50 mM NaCl, loaded onto a Mono Q column (GE Healthcare) and eluted with a linear gradient of 50–500 mM NaCl. The eluted protein was concentrated, loaded onto a HiLoad 16/60 Superdex 200 pg column (GE Healthcare) and eluted with 20 mM Tris-HCl (pH 7.5) buffer containing 100 mM NaCl. The eluted protein was stored at −80°C until use.

Thin-layer chromatography (TLC) analysis of aminoglycoside acetylation by recombinant AAC(6′)-Ia

Acetylation of aminoglycosides using recombinant AAC(6′)-Ia was performed as described previously.9 Briefly, each aminoglycoside solution (2 mM) was mixed with 2 mM acetyl coenzyme A (acetyl-CoA) and recombinant AAC(6′)-Ia (2.5 μg) in 50 μL of PBS (pH 7.4) and then incubated at 37°C for 16 h. Each mixture was applied to a TLC Silica Gel 60 F254 (Merck) and then developed with 5% phosphate potassium solution. Ninhydrin (Wako) was overlaid on silica gels to detect aminoglycoside derivatives.

HPLC

HPLC was also performed to identify the acetylation of aminoglycosides. AAC(6′)-Iad, the positive control for the AAC(6′) group enzyme, was purified as described previously.7 The purified AAC(6′)-Iad or AAC(6′)-Ia (5 μg) was added to 250 μL reaction mixtures containing 50 mM Tris-HCl buffer (pH 7.6), 15 mM MgCl2, 4 mM acetyl-CoA and 6 mM arbekacin or 10 mM neomycin. The incubation was performed at 37°C for 30 min and equal volumes of 2-propanol and the derivatization reagent were added.13 After incubation for 10 min at 60°C, the sample was loaded onto a Chromobond 5-ODS-H column (4.6×100 mm; Chemco Scientific) equipped with an Agilent 1100 HPLC system (Agilent Technologies). The mobile phase was the same as that described previously.7

Nucleotide sequence accession number

The complete nucleotide sequence of p11663 presented in this study has been deposited in GenBank under accession no. AP014611.

Results and discussion

Detection of AAC(6′) group enzyme gene in amikacin-resistant Enterobacteriaceae

Fourteen amikacin-resistant Enterobacteriaceae without 16S rRNA MTase genes are listed in Table 1. For these isolates, the presence of aac(6′)-Ia and aac(6′)-Ib was first explored by PCR and the results are summarized in Table 1. Nine strains were found to carry aac(6′)-Ia or aac(6′)-Ib, but five strains (one E. coli, three S. marcescens and one P. mirabilis) gave negative results when evaluating resistance genes by PCR and these strains were predicted to have unknown mechanisms underlying amikacin resistance such as a new AAC(6′) group enzyme. S. marcescens
strain NUBL-11663 showed a high level of resistance to amikacin (MIC 128 mg/L). We therefore decided to focus on clarifying the molecular mechanism underlying the amikacin resistance of the *S. marcescens* NUBL-11663 strain.

**Transfer of amikacin resistance and cloning resistance determinants**

The amikacin-resistant phenotype of the *S. marcescens* NUBL-11663 strain was successfully co-transferred with β-lactam resistance to *E. coli* strain CSH2 by conjugation. Introduction of plasmids extracted from the NUBL-11663 strain by electroporation also conferred amikacin resistance to *E. coli* DH10B (Table 2). These results indicate that the amikacin resistance determinant was located on the plasmids. We extracted plasmids from the *E. coli* DH10B transformant and identified one plasmid, named p11663, with a size of ≈170 kb (Figure S1).

Next, we attempted to isolate the amikacin resistance determinant located on p11663 by a cloning experiment and obtained one plasmid (pBC-amk) carrying a 1.2 kb insert by selection using amikacin. This 1.2 kb insert had one 573 bp ORF, encoding a protein consisting of 190 amino acids. A homology database search revealed that this protein has a coenzyme A-binding pocket, assigned to be a putative GCN5-like N-acetyltransferase, and has 97% amino acid identity to amino-glycoside 6′-N-acetyltransferase, AAC(6′), of a whole genome-determined *Acinetobacter baumannii* AYE strain (GenBank accession no. CU459141), although the function of AAC(6′) of *A. baumannii* AYE has not been elucidated yet. Thus, the gene we isolated was predicted to encode an AAC(6′) conferring amikacin resistance and termed as AAC(6′)-Ian. AAC(6′)-Ian has low amino acid identity (25%) to the functionally determined AAC(6′) group enzymes shown in Figure 1.

**Antimicrobial susceptibility testing**

The MICs of various aminoglycosides for the parent *S. marcescens* NUBL-11663 and *E. coli* DH10B transformant strains are shown in Table 1.
Figure 1. Tree view depicting the identity of AAC(6\textsuperscript{-})-Ian with other AAC(6\textsuperscript{-}) enzymes. The tree was constructed using the ClustalWX version 2.1 program (http://clustal.org). GenBank accession numbers: AAC(6\textsuperscript{-})-Ian, this study; AAC(6\textsuperscript{-})-Ia, M18967; AAC(6\textsuperscript{-})-Ib, M23634; AAC(6\textsuperscript{-})-Ic, M94066; AAC(6\textsuperscript{-})-Ih, L29044; AAC(6\textsuperscript{-})-Iq, AF047556; AAC(6\textsuperscript{-})-Iad, AB119105; AAC(6\textsuperscript{-})-Iae, AB104852; AAC(6\textsuperscript{-})-Iaf, AB462903; AAC(6\textsuperscript{-})-Iag, AB472901; and AAC(6\textsuperscript{-})-Iaj, AB709942.

Table 2. A parent S. marcescens NUBL-11663 strain showed resistance to a variety of aminoglycosides, 4,6-disubstituted 2-deoxystreptamines (DOS), 4,5-disubstituted 2-DOS, streptomycin and spectinomycin. The E. coli DH10B transformant showed an aminoglycoside resistance profile very similar to that of the parent strain. In E. coli DH5\textalpha{} carrying aac(6\textsuperscript{-})-Ian, increased MICs were observed for 4,6-disubstituted 2-DOS except for gentamicin and 4,5-disubstituted 2-DOS, but not for the other aminoglycosides. This aminoglycoside resistance phenotype conferred by the production of AAC(6\textsuperscript{-})-Ian was quite similar to that conferred by other aminoglycoside AAC(6\textsuperscript{-}) group enzymes.7–11 The E. coli DH10B transformant harbouring p11663 demonstrated gentamicin and spectinomycin resistance that was not observed in E. coli DH5\textalpha{} carrying aac(6\textsuperscript{-})-Ian, indicating that p11663 carried additional aminoglycoside resistance determinants other than aac(6\textsuperscript{-})-Ian (discussed below). Regardless, these findings clearly show that the production of AAC(6\textsuperscript{-})-Ian was mainly responsible for the amikacin resistance in the S. marcescens NUBL-11663 strain.

Complete nucleotide sequence of p11663 and genetic environment of aac(6\textsuperscript{-})-Ian

The circular map of p11663 is shown in Figure 2(a). Plasmid p11663 had a length of 169829 bp and belonged to incompatibility group A/C2 with a broad host range. The average G + C content of p11663 was 52.0%. Plasmid comparative analysis revealed that the backbone of p11663 exhibited the highest similarity to that of plasmid pP91278 from a Photobacterium damselae subsp. piscicida isolate from the USA (GenBank accession no. AB277724).

The genetic environments of the newly identified aac(6\textsuperscript{-})-Ian are shown in Figure 2(b). The backbone elements upstream of aac(6\textsuperscript{-})-Ian correspond partially to those found in the pHH1107 plasmid (GenBank accession no. FJ012881), which is a low-GC plasmid recovered from soil.18 Heuer et al.18 suggested that the original host of these low-GC antibiotic plasmids may be Acinetobacter species. The IS1106-like element, which was located at the 5’-end of aac(6\textsuperscript{-})-Ian, appeared to be inserted into a genetic region similar to the backbone sequence of the pHH1107 plasmid because a duplicated 8 bp sequence (CTGGCGAA) was found on both sides of the IS1106-like element, which is a hallmark of a previous insertion event. The aac(6\textsuperscript{-})-Ian gene and IS1106-like element were flanked by copies of a unique region (sequence 1) including an ISCR-like element. The genetic region including the aac(6\textsuperscript{-})-Ian gene and its upstream 16 bp and downstream 151 bp had 94% nucleotide identity to those located in the chromosomal DNA of the A. baumannii AYE strain (GenBank accession no. CT025832) (Figure 2b). The aac(6\textsuperscript{-}) gene of A. baumannii AYE was flanked by two copies of ISCR-like elements that were quite different from those surrounding aac(6\textsuperscript{-})-Ian. A similar AAC(6\textsuperscript{-}) enzyme was found in A. baumannii (NCBI Reference Sequence WP_000960976), although its enzymatic function has not been evaluated; thus, aac(6\textsuperscript{-})-Ian mediated by p11663 of the S. marcescens NUBL-11663 strain and aac(6\textsuperscript{-}) found in A. baumannii strains may have evolved from a common ancestor.

Other antibiotic resistance genes in p11663

Another notable antibiotic resistance gene found in p11663 was bla\textsubscript{TLA-3}, a new variant of the TLA-type ESBL gene. TLA-type \(\beta\)-lactamase was identified for the first time in Asian countries including Japan. TLA-3 has 93% and 52% amino acid identity to TLA-1, found in plasmids of Enterobacteriaceae clinical isolates exclusively from Latin America (Figure S2),19–21 and TLA-2, found in the plasmid pRSB101 recovered from a wastewater treatment plant,22 respectively. The introduction of a recombinant plasmid carrying bla\textsubscript{TLA-3} (pBC-TLA-3) conferred resistance to cefazidime, cefotaxime and cefepime, but not to cefmetazole and meropenem (Table 2). The resistance to cefazidime was reduced in the presence of clavulanic acid. These resistant phenotypes, the preferred \(\beta\)-lactam substrate and the high sensitivity to the \(\beta\)-lactamase inhibitor of TLA-3 correspond well to the characteristics of typical ESBLs belonging to class A \(\beta\)-lactamases.23 The surrounding genetic organization of bla\textsubscript{TLA-3} is largely different from that of bla\textsubscript{TLA-1} of pRZA92 (GenBank accession
Figure 2. (a) Circular map of plasmid p11663. Each ORF is shown with an arrow to indicate the direction of transcription. The black inner circle shows the G+C content plotted against the average G+C content of 52.0% and green and purple circles represent GC skew information. The outside grey circle indicates the region genetically similar to pP91278 (GenBank accession no. AB277724). (b) Schematic map of genetic environments of aac(6′)-lan and bla_{TLA-3}. ORFs are shown as arrows indicating the direction of transcription. The aac(6′) and bla_{TLA-3} genes are coloured in red and the other genes involved in antibiotic resistance are coloured in faint orange. The genes related to DNA recombination and transposition are coloured in green. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
no. AF148067) from the E. coli strain R170 (Figure 2b). Only the 1.2 kb sequence containing bla₅LA-3 and its upstream and downstream region showed 91% nucleotide identity to the sequences covering bla₅LA-1 and its surrounding region.

In addition, blaTEM (which encodes a TEM-type β-lactamase with F100L substitution relative to TEM-208), bla₅CO₁ (carbenicillinase gene) and aac(3)-II were present in the p11663 plasmid (Figure 2a). The aac(3)-II gene is a gentamicin resistance determinant in the S. marcescens NUBL-11663 strain.

**Overexpression and purification of histidine-tagged AAC(6')-Ian**

E. coli BL21(DE3)pLysS and the pET22b(+) expression vector were used for overexpression and purification of C-terminus histidine-tagged AAC(6')-Ian. E. coli BL21(DE3)pLysS carrying pET22b(+) was susceptible to amikacin (MIC ≤ 0.25 mg/L), whereas E. coli BL21(DE3)pLysS carrying pET-AAC showed resistance to amikacin (MIC 32 mg/L). The production of histidine-tagged AAC(6')-Ian was functional and responsible for amikacin resistance in E. coli BL21(DE3)pLysS (data not shown).

**Acetylation of aminoglycosides by AAC(6')-Ian**

TLC analysis using a variety of aminoglycosides as a substrate was performed to determine the biochemical characteristics of AAC(6')-Ian. AAC(6')-Ian was predicted to be an acetyltransferase because it contained the acetyl-CoA-binding motif; thus, acetyl-CoA was first used as a cofactor in an *in vitro* reaction. The results of the TLC analysis are shown in Figure 3. AAC(6')-Ian acetylated 4,6- and 4,5-disubstituted 2-DOS aminoglycosides, arbekacin, amikacin, tobramycin, kanamycin, gentamicin, isepamicin, sisomicin, netilmicin, neomycin and ribostamycin, which possess an amino group at the 6'-position, although the modifications of gentamicin were only partial because it was a mixture of derivatives such as gentamicin C1a, C1a and C2. APR, apramycin; ABK, arbekacin; AMK, amikacin; TOB, tobramycin; KAN, kanamycin; GEN, gentamicin; ISP, isepamicin; SIS, sisomicin; NET, netilmicin; NEO, neomycin; RSM, ribostamycin; PRM, paromomycin.

**Table 3. Results of HPLC analysis**

<table>
<thead>
<tr>
<th>Agent(s)</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AAC(6')-Ian</strong></td>
<td>AAC(6')-Ian</td>
</tr>
<tr>
<td>Arbekacin</td>
<td>12.4</td>
</tr>
<tr>
<td>Arbekacin + acetyl-CoA</td>
<td>8.0</td>
</tr>
<tr>
<td>Neomycin</td>
<td>7.5</td>
</tr>
<tr>
<td>Neomycin + acetyl-CoA</td>
<td>3.9</td>
</tr>
</tbody>
</table>

and this peculiar effect against arbekacin was generally attributed to lower *kₘ* and/or higher *kₐₙ* values against arbekacin. AAC(6')-Ian, in addition to AAC(6')-Iad, AAC(6')-Iaj and AAC(6')-Iag, would have high catalytic activity against arbekacin. AAC(6')-group enzymes can commonly modify the 6'-position of aminoglycosides, but their substrate specificity for aminoglycosides may be highly variable considering their large diversity in amino acid constitution (Figure 1).

In addition, it is also possible that the expression of aac(6')-Ian may be enhanced through the original promoter regions or insertion of an upstream IS1106 element, resulting in an increase in the MIC of arbekacin. Further genetic and enzymatic characterization is necessary to explain the elevation of the MIC of arbekacin conferred by aac(6')-Ian.

**Conclusions**

We report here a novel plasmid-mediated amikacin acetyltransferase, AAC(6')-Ian, and ESBL, TLA-3, from a S. marcescens clinical isolate. Our results suggest that Enterobacteriaceae are still acquiring new variants of AAC(6') group enzymes as the mechanisms for resistance to aminoglycosides including amikacin. In Enterobacteriaceae, the prevalence of amikacin resistance is still...
Novel antibiotic resistance genes 

aac(6′)-Ia and 

bla

TLA-3

low (<1%) in Japan (http://www.nih-janis.jp/report/kensa.html), compared with the resistance rate for other classes of antibiotics such as third-generation cephalosporins and fluoroquinolones. Thus, it appears that the potency of amikacin is still sufficient to make it effective for the treatment of infectious diseases caused by Enterobacteriaceae. However, increased use of amikacin would promote the spread of resistance determinants; thus, continuous monitoring is necessary to prevent and control the further spread of resistance determinants such as amikacin acetyltransferases including AAC(6′)-Ia to allow aminoglycosides to continue to be used for treatment of bacterial infectious diseases.

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figures S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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aac(6′)-Ia and 


Pseudomonas aeruginosa 

strain that caused an outbreak in a

neurosurgery ward and its 

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6′-aminoglycoside group 

except 

karabicin 