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Role of Aminoglycoside 6′-Acetyltransferase in a Novel Multiple Aminoglycoside Resistance of an Actinomycete Strain #8: Inactivation of Aminoglycosides with 6′-Amino Group Except Arbekacin and Neomycin

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From a rare actinomycete strain #8 isolated from soil as arbekacin (ABK) resistant, we cloned a gene segment (0.9 kb) conferring multiple resistance to aminoglycoside (AG) antibiotics with 6′-NH_2 including semisynthetic ones except ABK and neomycin (NM). Enzymatic modification using cell free extracts from *Streptomyces lividans* TK21/pANT-S2 carrying the cloned gene revealed that the gene coded for an AG 6′-acetyltransferase [AAC(6′)] capable of acetylating all of the tested AGs with 6′-NH_2 including semisynthetic ones and astromicin. The substrate specificty of the enzyme was thus similar to that of AAC(6′)-Ie of *Enterococcus faecalis*. Antibiotic assay revealed a weak but clear antibiotic activity of 6′-*N*-acetylABK (8% of ABK activity) in contrast with substantial inactivation by the AAC(6′) of the other AGs including amikacin and isepamicin. The NM acetylation by the AAC(6′) also did not result in NM inactivation. It seems thus likely that AAC(6′)-dependent resistance to ABK and NM, if it emerges, will remain at low level.

Aminoglycoside (AG) antibiotics have been and still are playing important roles in curing various infectious diseases caused by Gram-positive as well as Gram-negative bacteria. Especially, semisynthetic AGs such as amikacin (AMK) and isepamicin (ISP) have been most widely used in the last decade[1,2]. Furthermore, arbekacin (ABK)[3], an anti-MRSA (methicillin-resistant *Staphylococcus aureus*) agent in Japan, has also been used widely since its approval in 1990. The most serious problem to the activty of these AGs is resistant bacteria carrying AG-modifying or inactivating enzymes[1~7]; *i.e.* AG phosphotransferases (APHs), adenylyltransferases (AADs), acetyltransferases (AACs) and a bifuntional enzyme AAC(6′)/APH(2″). According to recent reports[8~11], AAC(6′) and AAC(6′)/APH(2″) have been increasing their importance in clinically-occurring AG resistant bacteria such as *Pseudomonas aeruginosa*, MRSA and *Enterococcus faecalis*.

As to ABK resistance in MRSA, AAC(6′)/APH(2″)-dependent resistance[1,12] has been exclusively reported although modification sites for AAC(3), AAC(2′) and AAC(6′) exist in ABK molecule. Therefore, HOTTA *et al.* have been interested in the possible emergence of AAC-dependent ABK resistance and to check this possibility they employed AACs of actinomycete origin. Actually AAC(3) and AAC(2′) of *Streptomyces* origin were examined for capability of modification as well as inactivation of ABK[13,14]. Consequently, it turned out that ABK was relatively readily converted to 3″-*N*-acetylABK and 2′-*N*-acetylABK by the AAC(3) and AAC(2′),

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respectively. However, these two acetylated ABK products were found to retain substantial antibiotic activity so that the cloned AAC(3) or AAC(2′) did not confer ABK resistance.

HOTTA *et al.* have been studying the phenotypic diversity in multiple AG resistance of actinomycetes and the underlying biochemical and genetic basis[15,16]. Recently they isolated a soil actinomycete strain designated #8 with resistance to ABK to which actinomycetes are generally sensitive. Since the strain showed a novel AG resistance profile, they started to characterize the underlying genetic and biochemical basis for the resistance. This paper deals with the modification and inactivation of AGs by an AAC(6′) cloned from the strain #8. We believe this is the first characterization report on the AAC(6′) of actinomycete origin.

Materials and Methods

Strains

Strain #8 is an actinomycete that was isolated as an ABK resistant from a soil collected at a water fall (Johren-no-taki) in Izu peninsula in Japan. *Streptomyces lividans* TK21 was used as the host for gene manipulation.

Chemicals

AG antibiotics were available from the antibiotic collection at National Institute of Infectious Diseases. The other chemicals used for enzymatic modification and DNA manipulation were commercially available.

Taxonomic Characterization

According to the methods[17] used regularly for the characterization of actinomycetes, strain #8 was characterized in terms of morphology and physiology. Diaminopimelic acid type of cell wall and the G+C content of DNA were also analyzed according to the methods[18,19] reported, respectively.

Cloning of AG Resistance Gene

Preparation of total DNA and protoplasts, and transformation were carried out according to a genetic manipulation method of *Streptomyces*[20]. Total DNA prepared from mycelia grown in Tryptic Soy Broth (Difco) was cut partially with *Sau* 3AI and run on an agarose gel to separate DNA fragments. The resulting fragments ranging 4~10 kb were extracted with GeneClean II kit (BIO101) and ligated with plasmid vector pIJ702 cut with *Bgl* II. Subsequently, a protoplast suspension (40 μl) prepared from *S. lividans* TK21 was mixed with the ligation mixture (12 μl) in order to get transformed. Then the protoplasts were spread and incubated at 27°C for 2 weeks on soft R2YE medium supplemented with thiostrepton (10 μg/ml). The resulting colonies were replica plated on ISP No. 2 agar medium containing 10 μg/ml of an AG.

Enzymatic Modification of AGs

Acetylation reaction was carried out under the following conditions; 250 μg/ml AG, 0.1 M phosphate buffer (pH 7.0), 10% (v/v) cell free extract (S30) and 4 mM acetylCoA in a 50 μl reaction mixture. After incubation at 37°C, the acetylation of AGs and the remaining antibiotic activity of the reaction mixtures were monitored by TLC and paper disk assay, respectively. TLC was carried out on a silica gel plate (E. Merck Art. 5712) by developing with 5% KH_2PO_4. AGs and their acetylation products were detected by spraying ninhydrin reagent.

Antibiotic Resistance

Aerial mycelium of strain #8 grown on ISP No. 4 agar medium (Difco) for 2 weeks at 27°C was streaked on ISP No. 2 agar medium plates supplemented with AGs. The growth was scored after 7 day incubation at 27°C.

Isolation and Structure Determination of Acetylated Products

AGs [ABK (20 mg; 36.2 μmol), AMK (20 mg; 34.2 μmol) and ASTM (10 mg; 24.7 μmol)] were incubated with 5 mM acetylCoA and 3~10% (v/v) cell free extracts (72.6 mg protein/ml) in 20 ml (10 ml for ASTM) of the reaction mixture containing 0.1 M phosphate buffer (pH 7.0). After incubated at 37°C for 6~8 hours, the reaction mixtures were loaded on columns of Amberlite CG50 (NH_4^+, 20 ml). Subsequently, the columns were washed with 40 ml of water, and eluted with aqueous ammonia (1%, 0.4% and 0.2% for acetylated products of ABK, AMK and ASTM, respectively). The eluates were collected as approximately 2 ml fractions and the fractions with positive reactions to ninhydrin and Rydon-Smith reagents were pooled and concentrated to yield a colorless solids. As results, 15.1 mg, 14.4 mg and 7.9 mg of the purified acetylation products of ABK, AMK and ASTM, respectively, were obtained.

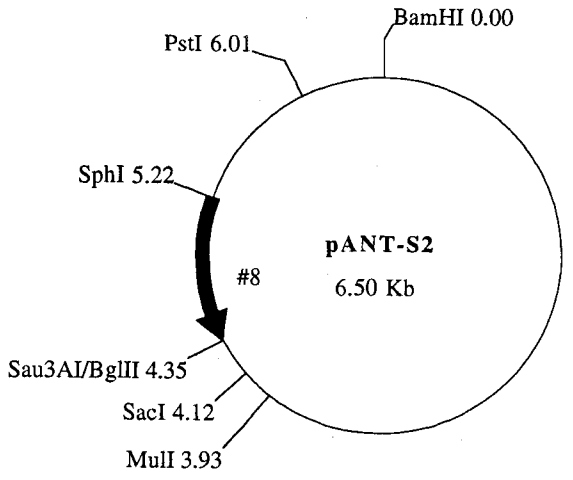
Results

Taxonomic Properties of Strain #8

Taxonomically, strain #8 showed the following

properties. On ISP media (Nos. 2, 3, 4 and 5), the strain showed good growth with white surface color and developed flexuous aerial mycelia and spores with smooth surface. The strain is capable of utilizing the following sugars; *i.e.* glucose, arabinose, xylose, galactose, sucrose, mannitol, inositol, rhamnose and raffinose as sole carbon sources for growth. The following physiological tests gave negative results; melanine production, gelatin liquifaction, milk peptonization, milk coagulation and starch hydrolysis. Nitrate reduction was positive. The G+C content of DNA was estimated at 73.2%, provided that 71.7% was obtained from the nucleotide sequence of the cloned DNA segment containing an AAC(6′) gene (unpublished). Cell wall analysis using whole mycelia indicated that cell wall contained *meso*-diaminopimelic acid (data not shown) so that the strain was regarded to be categorized into so called rare actinomycetes. No antibiotic productivity has been detected so far.

Fig. 1. Plasmid pANT-S2.



Cloning of AG Resistance Gene

The transformation treatment of *S. lividans* TK21 protoplasts with the ligation mixture of *Bgl* II-cut pIJ702 (a high copy number vector plasmid) and *Sau* 3AI-cut DNA fragments from strain #8 resulted in the formation of a number of colonies on ISP No. 2 medium containing ribostamycin (RSM). One of the colonies was then subcultured and designated as strain #8-2. This RSM-resistant strain was confirmed to contain a recombinant plasmid designated pANT8-2 with a 6.9 kb *Sau* 3AI fragment derived from the strain #8. Subsequent subcloning provided pANT-S2 (Fig. 1) containing a 0.9 kb *Sph* I-*Sau* 3AI fragment derived from the strain #8.

AG Resistance

As shown in Table 1, strain #8 showed multiply resistance (25 μg/ml or higher) to kanamycin (KM)-, gentamicin (GM)-, astromicin (ASTM)- and neomycin (NM)-group AGs, but specific sensitivity (<2.5 μg/ml) to paromomycin (PRM) and GM when examined on ISP No. 2 agar medium supplemented with AG. The AGs to which the strain #8 was resistant turned out to possess 6′- NH_2 commonly.

On the other hand, *S. lividans* TK21/pANT-S2 showed clear resistance ranging 10~200 μg/ml to RSM and other AGs including semisynthetic AGs such as AMK, dibekacin (DKB), ISP and netilmicin (NTL). These AGs commonly possess 6′-NH_2, suggesting pANT-S2 contains an AAC(6′) gene. However, the strain did not show clear resistance to ABK and NM both of which also possess 6′-NH_2, although the resistance levels to these two AGs were a little bit higher than those of *S. lividans* TK21.

AG Acetylation by the Cell Free Extract from *S. lividans* TK21/pANTS-2

As shown in Fig. 2, all of the examined AGs but PRM were converted in the presence of acetylCoA. The acetylated AGs commonly possess 6′-NH_2 including ones (ABK and NM) to which *S. lividans* TK21/pANT-S2 did not show clear resistance. Thus all of the semisynthetic AGs such as ABK[1] and ISP[5] known to be refractory to AAC(6′) of clinical origin were acetylated by the AAC(6′) of strain #8. The acetylation rate was relatively fast with AMK and ISP and relatively slow with ABK and DKB. Exceptionally, no PRM acetylation and incomplete gentamicin (GM) acetylation were observed. These results should be due to that PRM lacks 6′-NH_2 and GM contains a component (GM-C_1 with 6′-C- as well as 6′-*N*-methyl groups) known to be refractory to AAC(6′)[5].

When the reaction mixtures with complete substrate conversion were examined for antibiotic activity, those of ABK and NM showed a clear antibiotic activity. By contrast, the other reaction mixtures showed no significant antibiotic activity.

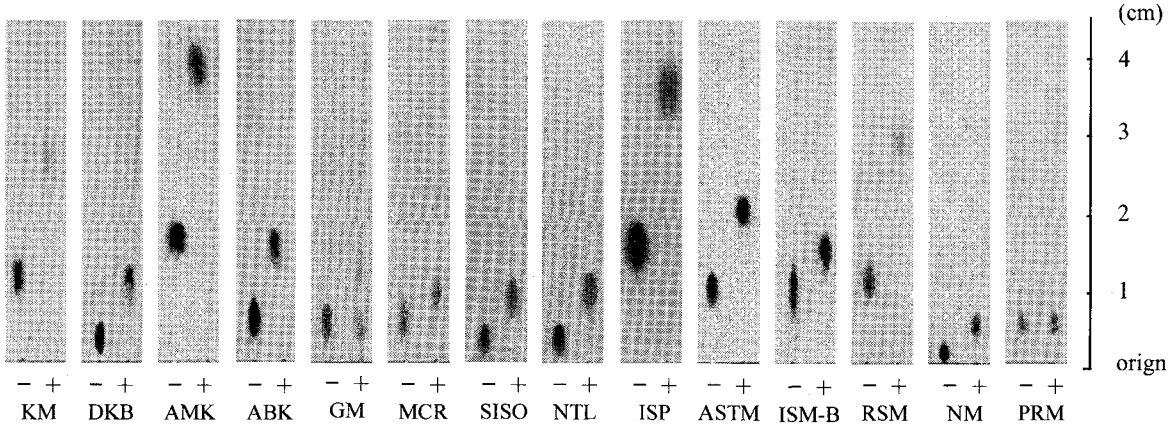
On the other hand, neither phosphotransferase activity nor adenylyltransferase activity was detectable (data not shown).

Table 1. Resistance to and acetylation of aminoglycoside antibiotics due to the cloned gene.

Group	Antibiotics	Resistance [a] (μg/ml)			TK21/pANT-S2	
		#8	TK21	TK21/pANT-S2	Acetylation [b]	Activity [c] (%)
Kanamycin	KM	100	<2.5	50	++	<1
	DKB [d]	100	<2.5	50	++	<1
	AMK [d]	25	<2.5	10	++	<1
	ABK [d]	25	<2.5	5	++	30
Gentamicin	GM	≦2.5	<2.5	<2.5	+	nt
	SISO	25	<2.5	<50	++	<1
	MCR	25	<2.5	10	++	<1
	ISP [d]	100	<2.5	25	++	<1
	NTL [d]	10	<2.5	50	++	<1
Astromicin	ASTM	50	<2.5	50	++	<1
	ISM-B	100	<2.5	100	++	<1
Neomycin-Paromomycin	RSM	200	<2.5	50	++	<1
	NM	25	<2.5	≦2.5	++	50
	PRM	<2.5	<2.5	<2.5	−	100

a: Strains were streaked on ISP No. 2 agar plates containing AGs (2.5-200 μg/ml).
b: Complete (++), incomplete(+) and no(-) acetylations were observed upon TLC.
c: Antibiotic activity of the incubated reaction mixtures with acetylCoA relative to that of the mixtures without acetylCoA. Paper disk assay using ISP No. 2 agar plate seeded with *B. subtilis* ATCC6633 was carryed out.
d: Semisynthetic AGs.
Abbreviation: SISO: sisomicin, MCR: micromomicin, ISM-B: istamycin-B.

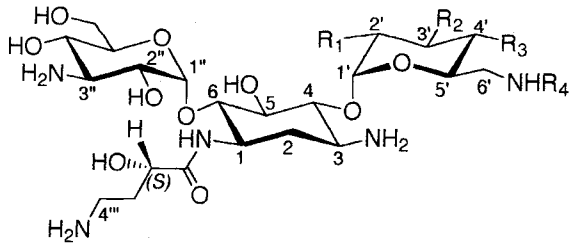
Fig. 2. TLC of the reaction mixtures of aminoglycosides incubated with the cell free extract from *S. lividans* TK21/pANT-S2 in the presence (+) or absence (−) of acetylCoA.



Determination of Acetylation

Structures of the acetylated products of ABK, AMK and ASTM were determined by spectral analyses of FAB-MS (Jeol JMX-SX102) and ^{1}H and ^{13}C NMR in D_2O at pD 1.7~1.9 (Jeol JNX-EX400). These products turned out to be identical with the known compounds; 6′-*N*-acetylABK (Fig. 3)[12], 6′-*N*-acetylAMK (Fig. 3)[22] and 6′-*N*-

Fig. 3. Structures of the enzymatically acetylated derivatives of ABK and AMK.



Arbekacin (ABK) $R_1 = NH_2$ $R_2 = H$ $R_3 = H$ $R_4 = H$
6'-*N*-AcetylABK $R_1 = NH_2$ $R_2 = H$ $R_3 = H$ $R_4 = Ac$
Amikacin (AMK) $R_1 = OH$ $R_2 = OH$ $R_3 = OH$ $R_4 = H$
6'-*N*-AcetylAMK $R_1 = OH$ $R_2 = OH$ $R_3 = OH$ $R_4 = Ac$

acetylASTM[23], respectively.

6'-*N*-acetylABK: FAB-MS (positive) *m/z* 595 $(M+H)^+$; The 6'-H signals (δ 3.32 and 3.43) shifted to lower field than those of ABK, and β-carbon shift of C-5' (δ 69.6) was also observed.

6'-*N*-acetylAMK: FAB-MS (positive) *m/z* 628 $(M+H)^+$; The 6'-H signals (δ 3.51 and 3.59) and C-5' (δ 72.0) signal shifted due to the 6'-*N*-acetylation.

6'-*N*-acetylASTM: FAB-MS (positive) *m/z* 448 $(M+H)^+$; the 6'-H (δ 3.99) and the β-carbon shifts (C-5' at δ 71.9 and 6'-CCH_3 at δ 16.1) were observed.

6'-*N*-acetylABK showed 8% activity of ABK, whereas neither 6'-*N*-acetylAMK nor 6'-*N*-acetylASTM showed significant antibiotic activities (0.3% and 0.2% activities of AMK and ASTM, respectively).

Discussion

The acetyltransferase encoded by the cloned gene turned out to be AAC(6') responsible for the 6'-*N*-acetylation of AGs as well as the multiple resistance to AGs with 6'-NH_2 except for ABK and NM. Therefore, it was concluded that the AAC(6') substantially contributed to the multiple AG resistance of the strain #8. However, since the strain #8 is resistant to both ABK and NM, the strain #8 should possess some additional resistance determinant(s) distinct from AAC(6'). Actually, we demonstrated that the strain #8 contained AAC(1) and perhaps other AACs in addition to AAC(6')[21].

It was noted that the AAC(6') of strain #8 was capable of acetylating all of the examined AGs with 6'-NH_2 including semisynthetic ones such as ABK and ISP that have been known to be rather refractory to the action of AAC(6')s of clinical origin. Since ASTM was also acetylated at 6'-NH_2, the AAC(6') of strain #8 was regarded to have similarity in substrate specificity to AAC(6')-Ie[5]. In fact, the deduced amino acid sequence of the former showed similarity to that of the latter (unpublished). Furthermore, it was remarked that acetylation by AAC(6') of ABK and NM did not result in inactivation, whereas that of the other AGs resulted in inactivation. The antibiotic activity of 6'-*N*-acetylNM[24] as well as the NM sensitivity of *E. faecalis* with AAC(6')-Ii[25] have been known, but the weak antibiotic activity of 6'-*N*-acetylABK has never been reported. In this context, we reported that the acetylation derivatives by AAC(3) and AAC(2') of ABK retain substantial antibiotic activity[13,14]. By contrast, NM was inactivated by AAC(3) although data was not shown. Thus, ABK is distinct from the other AGs in terms of the antibiotic activity of acetylation products and therefore we may call ABK the double stage active antibiotic.

ABK is an anti-MRSA agent with a broad antimicrobial spectrum. Although it has been widely used since its approval in 1990, the emergence of ABK-resistant MRSA has remained low and all of the emerged ABK-resistant MRSA strains have so far been dependent on AAC(6')/APH(2")[1,26,27]. Based on our findings, AAC-dependent ABK-resistant MRSA may hardly emerge although AAC(6') as well as AAC(6')/APH(2") have been increasing their importance in clinical AG resistance[8~11], provided that ABK-resistant MRSA with two different AACs may emerge.

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