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Overexpression and Characterization of an Aminoglycoside 6′-*N*-Acetyltransferase with Broad Specificity from an ε-Poly-L-lysine Producer, *Streptomyces albulus* IFO14147

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Streptomyces albulus **IFO14147 produces ε-poly-L-lysine, which exhibits antimicrobial activity. In the MIC studies with antibiotics, *S. albulus* IFO14147 was shown to be resistant to kanamycin and amikacin, which are aminoglycoside (AG) antibiotics. We report here the isolation of the AG-resistance gene from *S. albulus* IFO14147 and the substrate specificity of the gene product, AAC(6′)-Isa, which catalyzes *N*-acetylation at the 6′ position of AGs, thereby inactivating them. Kinetic studies revealed that this enzyme has remarkably wide substrate specificity. The V_{max}/K_m values determined for AGs vary by a factor of up to 6,300, a much wider range than those observed for the AAC(6′)s from *Enterococcus faecium* [AAC(6′)-Ii] and *Salmonella enteritidis* [AAC(6′)-Iy]. In addition, AAC(6′)-Isa was able to acetylate lividomycin A, which has a hydroxy group at the 6′ position. Enzymatically acetylated lividomycin A was found to be highly susceptible to mild base hydrolysis, suggesting that the enzyme also catalyzes *O*-acetyltransfer.**

Key words: aminoglycoside, aminoglycosides 6′-*N*-acetyltransferase, antibiotic resistant gene, ε-poly-L-lysine, *Streptomyces albulus*.

Abbreviations: AG, aminoglycoside; AAC, aminoglycoside acetyltransferase; ANT, aminoglycoside nucleotidyltransferase; APH, aminoglycoside phosphotransferase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); IPTG, isopropyl-β-D-thiogalactoside; LB, Luria-Bertani; MIC, minimal inhibitory concentration; TNB, 5-thio-2-nitrobenzoate; ε-PL, ε-poly-L-lysine; ORF, open reading frame.

ε-Poly-L-lysine (ε-PL) is a microbiostatic L-lysine homopolymer that consists of 25–30 residues, with a linkage between the α-carboxyl group and ε-amino group, produced by the actinomycete *Streptomyces albulus* IFO14147 (*1*–*3*). ε-PL exhibits antimicrobial activity towards a wide spectrum of microbes, including Gram-positive and -negative bacteria (*4*), as well as antiphage activity (*5*). Due to its safety and biodegradability, ε-PL has entered the commercial market as a food preservative in Japan, being produced commercially by a mutant of *S. albulus* (*6*). However, the biosynthetic mechanisms of ε-PL remain unclear, although Kawai *et al*. have reported the cell-free synthesis of ε-PL (*7*). An effective genetic system such as a host-vector system for *S. albulus* would be a powerful tool for understanding the biosynthetic mechanisms.

We recently found a novel plasmid, pNO33, in *S. albulus* IFO14147 (*8*). This high-molecular-size plasmid (37-kbp) is a cryptic plasmid, as none of its functions is yet known. It would be suitable for use as a cloning vector provided that a selectable marker gene coding for resistance to an antibiotic were introduced into it. Because many actinomycetes are naturally resistant to several antibiotics, only a limited number of antibiotic resistance markers are currently used for selection in these genera (*9*). Among them, aminoglycoside (AG) resistance genes are frequently used as selective markers in recombinant DNA work. AGs are natural or semisynthetic broad spectrum bactericidal compounds consisting of a central six-membered amino cyclitol ring linked to two or more deoxy-amino sugars by glycosidic bonds (Fig. 1). Their primary mechanism of action is the inhibition of bacterial protein synthesis by binding to the small ribosomal subunit (*10*).

Bacterial resistance to AGs can result from three causes (*11*): decreased antibiotic uptake and accumulation, modification of 16S RNA or ribosomal protein, and enzymatic modification of AGs, which is the most common resistance mechanism. There are three types of AG-modifying enzymes (*12*), each of which transfers a functional group to the AG structure, thereby inactivating the antibiotics: AG nucleotidyltransferases (ANTs) transfer a nucleotide triphosphate (*O*-nucleotidylation); AG phosphotransferases (APHs) transfer the phosphoryl group from ATP (*O*-phosphorylation); and AG acetyltransferases (AACs) transfer the acetyl group from acetyl-CoA (*N*-acetylation). AACs are further subclassified into four classes, AAC(1), AAC(2′), AAC(3), and AAC(6′), on the basis of the site of the regioselective acetylation of the AGs (Fig. 1). Among them, *N*-acetylation at the 6′ position of AGs by AAC(6′) is one of the most prevalent forms of modification (*13*). AAC(6′)-I confers resistance to amikacin, tobramycin, kanamycin A, dibekacin, and sisomicin. Genes for at least 24 AAC(6′)-I enzymes have been identified in both Gram-negative and -positive microorganisms (*11*). The only two known AAC(6′)-II

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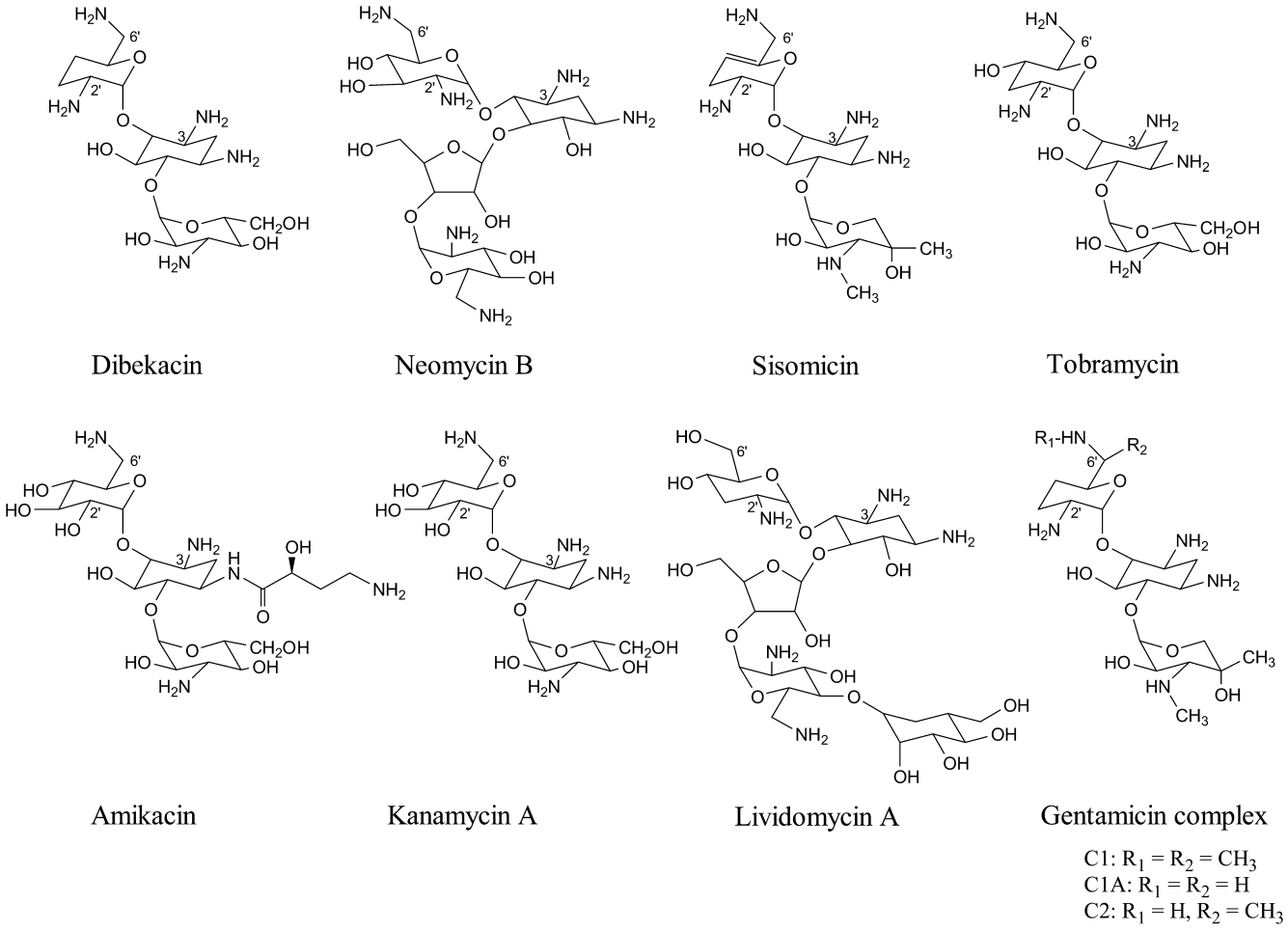

Fig. 1. **Chemical structures of aminoglycosides used in this study.**

enzymes confer resistance to gentamicin, tobramycin, kanamycin A, dibekacin, and sisomicin, but not to amikacin. Table 1 summarizes the resistance profiles of AAC(1), AAC(2′), and AAC(3), as well as AAC(6′).

In order to employ an appropriate AG-resistance gene as a selective marker, an AG resistance or susceptibility profile was investigated in *S. albulus* IFO14147. MIC studies showed that this strain is able to grow on agar plates containing the AG kanamycin (64 μg/ml), thereby negating the possibility of using the kanamycin-resistance gene as a selective marker in this strain (Hoshino, Y. and Takagi, H., unpublished observations). In addition, it was expected that the intrinsic kanamycin-resistance gene of *S. albulus* IFO14147 could confer resistance to different AGs because of cross-resistance between the AGs. Therefore, the mechanism of natural resistance to kanamycin was investigated for the following stage of development of a genetic system for this strain.

Herein we report the cloning and characterization of the *S. albulus* IFO14147 *aac(6′)-I* gene, which was discovered in studies on AGs resistance profiles. *In vitro* analysis revealed that the gene product has remarkably wide substrate specificity as an AAC(6′)-I-type enzyme. In particular, we describe its substrate specificity for various AGs and CoA thioester analogs.

Table 1. **Resistance profiles of aminoglycoside acetyltransferases.**

Acetyltransferase	Resistance to[a]:
AAC(6′)	
I (at least 24 different enzymes)	Amk, Dbk, KamA, Sis, Tob
II	Dbk, Gen, KamA, Sis, Tob
AAC(3)	
Ia, Ib	Gen, Sis
IIa, IIb, IIc	Dbk, Gen, Sis, Tob
IIIa, IIIb, IIIc	Dbk, Gen, KamA, LivA, NeoB, Sis, Tob
IV	Dbk, Gen, Sis, Tob
VII	Gen
AAC(1)	LivA
AAC(2′)-Ia	Dbk, Gen, NeoB, Tob

Amk, amikacin; Dbk, dibekacin; Gen, gentamicin; KamA, kanamycin A; LivA, lividomycin; NeoB, neomycin; Sis, sisomicin; Tob, tobramycin. [a]Aminoglycosides used in this study are listed.

MATERIALS AND METHODS

Chemicals—The aminoglycosides (AGs) were obtained from Sigma. The other chemicals were all analytical grade.

Bacterial Strains and Plasmids—*S. albulus* IFO14147 was used as the DNA source for cloning of *aac(6′)-Isa*. The media and growth conditions for *S. albulus* IFO14147 have been previously described (*8*). *E. coli* XL1-Blue MRF′ (*recA1 thi endA1 supE44 gyrA46 relA1 hsdR17 lac*/F′ [*proAB lacI*^{q} *lacZ*ΔM15::Tn10{Tet^{r}}]) (Toyobo, Osaka, Japan) and the plasmids pKF3 and pUC119 were used for the subcloning experiments and sequencing analysis. The plasmid pQE30 (Qiagen, CA, USA) was used for overexpression of the recombinant protein.

DNA Isolation and Manipulation—Plasmids from *E. coli* were prepared using a Qiagen Plasmid Kit (Qiagen). All restriction enzymes, T4 ligase, and calf intestinal alkaline phosphatase were obtained from Toyobo and used according to the manufacturer's protocols. The transformation of *E. coli* with plasmid DNA by electroporation was performed under standard conditions using a BTX ECM 600 electroporation system (Biotechnologies and Experimental Research, CA, USA). Other general procedures were performed as described by Sambrook *et al.* (*14*).

Cloning of the Kanamycin Resistance Gene—The genomic DNA of *S. albulus* IFO14147 was partially digested with *Sau*3AI. *Sau*3AI fragments larger than 3.0-kbp were ligated into the *Bam*HI site of plasmid pKF3, carrying the chloramphenicol resistance gene (Takara Shuzo, Kyoto, Japan). *E. coli* XL1-Blue MRF′ was transformed with the ligated DNA, and over 5,000 independent chloramphenicol-resistant colonies were then replica-plated onto Luria-Bertani (LB) agar plates (*14*) containing kanamycin (25 μg/ml) and chloramphenicol. Two kanamycin-resistant colonies were isolated. The kanamycin resistance was of the plasmid-dependent phenotype. Two plasmids, pKF3-kat3.7 and pKF3-kat5.1, had 3.7-kbp and 5.1-kbp fragments, respectively, which overlapped 3.7-kbp inserts. The complete nucleotide sequence of the 5.1-kbp fragment was determined using a model PRISM 3100 sequencer (Applied Biosystems, CA, USA) by means of the dideoxy chain termination method. Moreover, the 705-bp *Pvu*II–*Ehe*I fragment containing the coding region and its putative promoter and terminator regions of ORF1 was ligated into the *Sma*I site of pKF3 to give pKF3-orf1.

The nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number AB116646.

Measurement of Acetyltransferase Activity—*E. coli* XL1-Blue MRF′ harboring pKF3-orf1 was grown for 16 h at 37°C in LB medium. Cells were then collected and suspended in a lysis buffer comprising 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM 2-mercaptoethanol. The cells were disrupted by ultrasonic oscillation at 4°C for 5 min with a model UD-200 ultrasonic oscillator (Tomy, Tokyo, Japan). The cell-free extract obtained after centrifugation was used for the enzyme assay. In the measurement of the AAC activity of *S. albulus* IFO14147, cell-free extract was prepared from cells cultivated for 24 h at 30°C in LB medium in the presence or absence of kanamycin (20 μg/ml). The AAC activity was determined spectrophotometrically by measuring the increase in A_{412} due to the formation of 5-thio-2-nitrobenzoate (TNB, 15,570 M^{-1} cm^{-1}) resulting from the reaction between the sulfhydryl group of the product of the acetyltransfer reaction, CoA-SH, and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). The reaction mixture (1 ml) contained 50 mM Tris-HCl (pH 8.0), 1 mM DTNB, 0.1 mM acetyl-CoA, and cell-free extract, and the reaction was initiated by the addition of 1 mM kanamycin. One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 μmol TNB per min at 30°C. The protein concentrations were determined using a Bio-Rad Protein Assay Kit (Bio-Rad, CA, USA). Bovine serum albumin was used as the standard protein.

Overproduction of the ORF1 Gene Product in E. coli—The following set of primers was designed and used to amplify the ORF1: 5′-GGG*GGATCC*GAGCTGCGCGGGGACGACGTC-3′ (forward) and 5′-ACC*AAGCTT***TC-A**GGTCAGTTCGTCGGCGAG-3′ (reverse). Restriction enzyme sites (italic letters) and a stop codon (bold letters) were introduced for the in-frame expression of recombinant proteins in the pQE30 expression vector. The PCR was carried out under standard conditions. After sequence confirmation, a 0.5-kbp *Bam*HI–*Hin*dIII fragment was inserted into the same site of pQE30. Plasmid pQE30-orf1, in which recombinant protein was expressed as *N*-terminal 6× His-tagged fusion proteins under the control of the T5 promoter, was selected. *E. coli* XL1-Blue MRF′ harboring pQE30-orf1 was grown at 37°C in LB medium with ampicillin (100 μg/ml). The expression of the recombinant protein was induced by adding 1 mM isopropyl-β-D-thiogalactoside (IPTG) when the optical density at 600 nm reached about 0.8. The cultivation was continued for an additional 5 h at 37°C.

Purification of His-tagged AAC(6′)-Isa—His-tagged AAC(6′)-Isa was purified according to the manufacturer's protocol (Qiagen). The purified protein was analyzed by SDS-PAGE on 15% gel. The native molecular mass of the purified protein was estimated by gel filtration using a COSMOSIL 5Diol-300 (7.8 mm × 600 mm) column (Nacalai tesque, Kyoto, Japan), which had been equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 100 mM Na_2SO_4.

Kinetic Studies of AAC(6′)-Isa—Kinetic assays were performed under the same conditions as described above, except that the enzyme concentration (0.1 μg/ml), acetyl-CoA concentration (100 μM), and reaction time (2 min) were scaled down to fit the measurement of the steady-state kinetic parameters. Reactions were initiated by the addition of AGs and carried out at 30°C. The assay was linear with respect to protein concentration up to 0.1 μg/ml for 5 min of incubation, and all assays were carried out under these linear conditions. Control reactions without AGs were also performed at the various substrate concentrations used in the kinetic assay to evaluate the actual enzyme activities. For the kinetic constants of the acyl-CoA compounds, reactions were carried out by using sisomicin (25 μM). Triplicate sets of enzyme assays were performed at each substrate concentration, and a Lineweaver-Burk plot was used for the estimation of the kinetic constants.

TLC Analysis of Acetylated AGs Produced by AAC(6′)-Isa and Base Hydrolysis of Acetylated AGs—The enzyme assays were basically the same as those described above, except for the concentrations of the substrates (AGs and acetyl-CoA, 1 mM) and the enzyme (500 μg/ml) in a final volume of 20 μl. After 12 h of reaction, 2 μl of 1 N NaOH

Table 2. **Aminoglycoside resistance profiles of *S. albulus* and *E. coli*.**

	MIC[a] (μg/ml) of:							
	Amk	Dbk	Gen	KamA	LivA	NeoB	Sis	Tob
S. albulus IFO14147	32	8	4	64	16	8	<1	16
E. coli XL1-blue MRF′								
+pKF3 (no insert)	4	4	2	2	16	8	2	4
+ pKF3-orf1	25	25	2	25	16	8	2	25
+ pQE30 (no insert)	4	4	2	2	16	8	2	4
+ pQE30-orf1	32	64	2	64	16	8	8	32

[a]MICs were determined with LB plates containing ampicillin (100 μg/ml), IPTG (0.1 mM), and AGs. Plates were incubated for 20 h at 37°C.

was added and the reaction mixture was incubated at 80°C (final pH 11–12). Samples were neutralized by addition of 2 μl of 1 N HCl. Aliquots of 5 μl of each reaction mixture were spotted on silica gel TLC (Merck, 60F254) and developed with 5:2 methanol:ammonium hydroxide. The AGs and their acetylation products were detected by spraying ninhydrin reagent (*15*, *16*).

RESULTS AND DISCUSSION

Kanamycin Acetyltransferase Activity in S. albulus IFO14147—By MIC determination (Table 2), *S. albulus* IFO14147 was revealed to be resistant to kanamycin A and amikacin but not gentamicin and neomycin B. We therefore expected that *S. albulus* IFO14147 might possibly produce a modification enzyme of the AAC(6′)-I-type. To test this possibility, AAC activities with kanamycin were measured by using cell-free extracts of *S. albulus* IFO14147. Also, because the expression of some antibiotic resistance genes in actinomycetes is known to be induced by the antibiotic in question, cell-free extracts of *S. albulus* IFO14147 cultivated in LB medium in the presence or absence of kanamycin (20 μg/ml) were prepared and used for the AAC assay. A high level of activity was detected in the cells when kanamycin was added to the medium (46 mU/mg). However, low activity was observed in the absence of kanamycin (14 mU/mg), presumably due to repression of the enzyme synthesis. These results suggested that *S. albulus* IFO14147 produces an AAC(6′)-I enzyme, and that its production is induced by kanamycin in a manner similar to the β-lactamase produced by this strain (*17*).

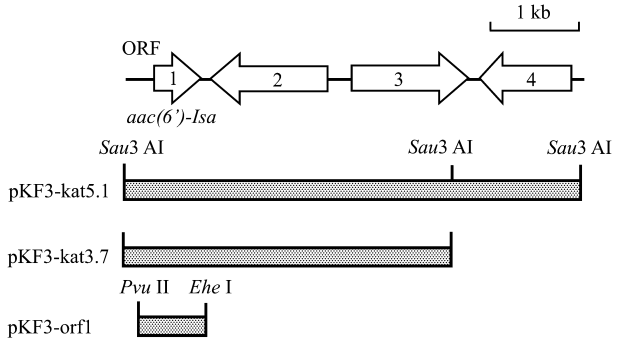

Fig. 2. **Schematic organization of the cloned 5.1-kbp DNA fragment.** Plasmids pKF3-kat5.1 and pKF3-kat3.7 had an overlapping 3.7-kbp *Sau*3AI insert. Plasmid pKF3-orf1 contained only ORF1 as an insert fragment. The hatched boxes represent the cloned fragments in vector pKF3.

Cloning and Sequencing Analysis of the Kanamycin Resistance Gene from S. albulus IFO14147—To clone the putative *aac(6′)-I* gene from *S. albulus* IFO14147, here designated the *aac(6′)-Isa* gene, a genomic DNA library of this strain was constructed using a high-copy-number plasmid, pKF3. From two independent kanamycin-resistant transformants, two plasmids (pKF3-kat3.7 and pKF3-kat5.1) were obtained and found to have an overlapping 3.7-kbp fragment by sequencing analysis (Fig. 2). Computer analysis of the DNA sequence by Frame Analysis (*18*) showed four ORFs on the 5.1-kbp fragment. ORFs 1, 2, 3, and 4 encoded a protein of 157 (molecular mass, 17,533 Da), 375 (40,055 Da), 374 (39,513 Da), and 274 (30,747 Da) amino acids, respectively. A database search with BLAST (*19*) showed that the deduced amino acid sequence of ORF1 has a significant similarity with that of AAC(6′) from *S. kanamyceticus* (61% identity), and has weak similarity with those of AAC(6′)-Ib from *Klebsiella pneumoniae* (21%), AAC(6′)-IIa from *Pseudomonas aeruginosa* (21%), and AAC(6′)-IIb from *Pseudomonas fluorescens* (23%). In addition, the putative acetyl-CoA binding motif, which is highly conserved among AAC(6′)s, was also conserved in ORF1 from *S. albulus* IFO14147. Figure 3 shows the alignment of the deduced amino acid sequences of these proteins generated by using the CLUSTAL W program (*20*). Because the results of the homology search also suggested that the ORF1 gene encodes an AAC(6′)-I-type modification enzyme, thereby conferring the kanamycin resistance, we subsequently constructed a plasmid, pKF3-orf1, which carries a 705-bp *Pvu*II–*Ehe*I fragment containing the coding region and the putative promoter and terminator regions of the putative *aac(6′)-Isa* gene. In fact, it was demonstrated that *E. coli* transformed by pKF3-orf1 was capable of growing on kanamycin A (25 μg/ml)-containing plates (data not shown), and that the putative *aac(6′)-Isa* gene is operative in *E. coli* under its own promoter.

The deduced amino acid sequences of the three other ORFs, ORF 2, ORF 3, and ORF 4, exhibited 83%, 44%, and 52% identity to a putative oxidoreductase from *S. coelicolor* A3(2) (accession no. NP_628469), glycosyltransferase from *Magnetospirillum magnetotacticum* (ZP_00049877), and a putative DNA-binding protein from *S. avermitilis* MA-4680 (BAC72361), respectively.

Functional Analysis of the aac(6′)-Isa Gene Product—AACs are divided into four classes of enzymes: AAC(1), AAC(2′), AAC(3), and AAC(6′). They utilize acetyl-CoA as the donor of the acetyl group in modifying AGs at positions 1 and 3 of the 2-deoxystreptamine ring and posi-

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AAC(6')-Isa     1  ------------------MELRGDDVVLRPVADGEGEVLDRIVREPEVAAWWSPP---- 37
AAC(6')-SK      1  MRAHRSCCIRRRGLGHNAGVELNGEKVLLRPVLDSDVKKLDKIVREPEVAAWWSTP---- 56
AAC(6')-Ib      1  --MSIQHFQTKLGITKYSIVTNSNDSVTLRLMTEHDLAMLYEWLNRSHIVEWWGGEEARP 58
AAC(6')-IIa     1  ------------------MSASTPPITLRLMTRRDLPMLHDWLNRPHIVEWWGDEERP 41
AAC(6')-IIb     1  --------------------MHPGVVTLRPMTEDDIGMLHEWLNRPHIVEWWGGE--RP 37

AAC(6')-Isa    37  ----ED---FAGMLA--------IVFEGEVVGAIQ-----------FYEETDPEFHHAGI 71
AAC(6')-SK     56  ----DD---YEEMLA--------ITLDGEVIGAVQ-----------YEEEEDPEFRHAGI 90
AAC(6')-Ib     59  TLADVQEQYLPSVLAQESVTPVIAMLNGEPIGYAQSYVALGSGDGWWEETDPGVR--GI 116
AAC(6')-IIa    42  TLDEVLEHYLPRAMAEESVTPYIAMLGEEPIGYAQSYVALGSGDGWWEDETDPGVR--GI 99
AAC(6')-IIb    38  SLEEVKEDYRPSALAEEGVTPYIGLLDGTPFAFAQSYVALGSCGGWWEESTDPGVR--GI 95

                          ********
AAC(6')-Isa    72  DVFLTARHQ-GKGLGTDAVRTLARWLVAERGHHRLTIDPAAANTAAIRSYRKVGFRPVGI 130
AAC(6')-SK     91  DIFLTASRH-GLGLGTDTVRTVARWLIDERGHHRITIDPAVANAGAIRSYSKVGFKPVGV 149
AAC(6')-Ib    117  DQLLANASQLGKGLGTKLVRALVELLFNDPEVTKIQTDPSPSNLRAIRCYEKAGFERQGT 176
AAC(6')-IIa   100  DQSLADPTQLNKGLGTRLVRALVELLFSDPTVTKIQTDPTPNNHRAIRCYEKAGFVREKI 159
AAC(6')-IIb    96  DQSIADSGLLGRGYGTRLVQALVDLLFADPQVSKVQTDPSPNNMRAIRCYEKAGFRKVKV 155

AAC(6')-Isa   131  MRAYGRDHRTGRWQDALLMDLLADELT 157
AAC(6')-SK    150  MRSYARDHTSGVWQDALLMDLLAEELV 176
AAC(6')-Ib    177  VTTPDGPAVYMVQTRQAFERTRSVA-- 201
AAC(6')-IIa   160  ITTPDGPAVYMVQTRQAFERKRGVA-- 184
AAC(6')-IIb   156  VSTPDGPAMYMLHERPLVNGLRSAA-- 180
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Fig. 3. **Alignment of the deduced amino acid sequence of AAC(6′)-Isa with those of homologous proteins.** Identical and similar amino acid residues are shown by dark and light shaded boxes, respectively. The numbers on the left and right are the residue numbers for each protein. The amino acid residues involved in the binding of acetyl-CoA are indicated by asterisks. The alignments were derived with the Clustal W program (*20*). Abbreviations and accession numbers: *Streptomyces kanamyceticus* [AAC(6′)-SK, GenBank accession no. BAD11815], *Klebsiella pneumoniae* [AAC(6′)-Ib, AAA98404], *Pseudomonas aeruginosa* [AAC(6′)-IIa, AAA25688], *Pseudomonas fluorescens* [AAC(6′)-IIb, AAA25680].

tions 2′ and 6′ of the 6-aminohexose ring. The cloned gene could encode an AAC(6′)-type enzyme because of its primary structure and MIC profiles for *S. albulus* IFO14147. To verify the acetylation of kanamycin A and other AGs, MIC studies (Table 2) and AAC enzyme assays were carried out with *E. coli* XL1 blue MRF′ transformed by pKF3-orf1. The transformant showed clear resistance to kanamycin A and dibekacin, but it grew slowly on agar plates containing amikacin or tobramycin (25 μg/ml), and showed no growth in the presence of gentamicin, lividomycin A, neomycin B, or sisomicin (data not shown). In AAC activity, a weak activity (2.8 mU/mg protein) was detected only when the cell-free extract was incubated with kanamycin, while no activity with kanamycin was detected in the cell-free extract of the transformant harboring only the vector (data not shown). Thus, we unfortunately could not verify the putative *aac(6′)-Isa* gene product by means of the resistance profile and substrate specificity of AGs, presumably due to its low level of expression in *E. coli*. Therefore, the putative *aac(6′)-Isa* gene product was subsequently overexpressed in *E. coli* in order to perform further functional analysis.

We constructed a recombinant plasmid (pQE30-orf1) to express the enzyme as an *N*-terminal 6× His-tagged fusion protein. A soluble protein extract from *E. coli* harboring pQE30-orf1 or pQE30 (no insert) was analyzed by SDS-PAGE (Fig. 4). After induction with IPTG, the His-tagged ORF1 (18 kDa), which was in good agreement with the values calculated from the deduced amino acid sequence of the enzyme, was expressed at a high level. We first carried out MIC studies with AGs for *E. coli* transformant harboring either pQE30 or the recombinant plasmid pQE30-orf1. The MICs obtained (Table 2) demonstrated that under these experimental conditions, where the *aac(6′)-Isa* gene was placed under the control of the strong T5 promoter, the putative *aac(6′)-Isa* gene product was able to confer to *E. coli* XL1-blue MRF' apparent resistance to amikacin, dibekacin, kanamycin A, sisomicin, and tobramycin, but did not affect the effect of gentamicin, lividomycin A, and neomycin B. This resistance profile was characteristic of the AAC(6′)-I-type modification shown in Table 1, confirming that the cloned gene encodes AAC(6′)-I. Moreover, the addition of a 6× His-tag at the N terminus of the protein did not abolish the ability of the protein to confer AG resistance.

Substrate Specificity of AAC(6′)-Isa—So far, genes for at least 24 AAC(6′)-I enzymes have been identified in both Gram-negative and -positive microorganisms (*11*), and a limited number of kinetic studies on AAC(6′)s have been done by several groups. The earliest studies were conducted in the early 1980s by Radika and Northrop, who focused on kanamycin acetyltransferase AAC(6′)-Ib [also known as AAC(6′)-IV] (*21*, *22*). More recently, two independent groups conducted kinetic studies on AAC(6′)-Ii from *Enterococcus faecium* (*23–25*) and AAC(6′)-Iy from *Salmonella enteritidis* (*26*, *27*). These enzymes commonly showed the broad substrate specificity for AGs. Therefore, we further carried out the kinetic studies on AAC(6′)-Isa, because the deduced amino acid sequence of this enzyme has a low degree of similarity with those of the kinetically characterized AAC(6′)s [AAC(6′)-Ib (21% identity), AAC(6′)-Ii (11% identity), and AAC(6′)-Iy (12% identity)]. We purified the 6× His-tagged AAC(6′)-Isa from a crude cell extract in one step using Ni-nitrilotriacetic acid agarose (Fig. 4), and used it for

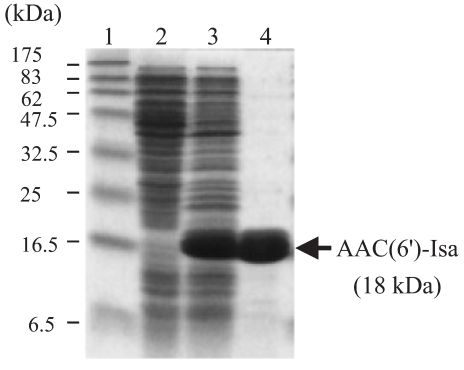

Fig. 4. **Electrophoresis of overproduced and purified AAC(6′)-Isa.** Molecular mass standard (lane 1), total soluble proteins from the *E. coli* harboring pQE30 (lane 2), pQE30-orf1 (lane 3), purified His-tagged AAC(6′)-Isa (lane 4) were analyzed by SDS-PAGE (15%). Proteins were stained with Coomassie Brilliant Blue R-250.

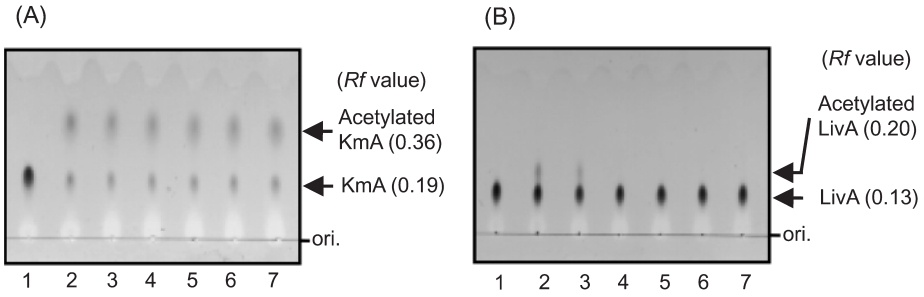

Fig. 5. **Conversion of aminoglycosides by AAC(6′)-Isa and base hydrolysis of acetylated aminoglycosides.** Kanamycin (panel A) and lividomycin A (panel B) were incubated with AAC(6′)-Isa (lane 2) or heat denatured AAC(6′)-Isa (lane 1). The reaction mixtures were subsequently treated with alkali for 0, 5, 10, 15, or 20 min. (lane 3 to 7). Aliquots of 5 μl of each reaction mixture were analyzed by silica gel TLC.

kinetic studies. The native molecular mass of the His-tagged ORF1 was estimated to be 38 kDa by gel filtration (data not shown), suggesting that AAC(6′)-Isa existed as a homodimer. Kinetic studies were carried out with the various AGs listed in Fig. 1. Gentamicin was not used for the kinetic studies because it is a mixture of different forms. The kinetic constants for AGs, determined at a saturating concentration of acetyl-CoA (50 μM), are presented in Table 3. All AGs tested in this research were substrates. In particular, AGs with a 6′ free amino group were effectively acetylated. The V_{max}/K_m values determined for AGs varied by a factor of up to 6,300, a much wider range than those observed for the AAC(6′)s from *E. faecium* [AAC(6′)-Ii] (*23*) and *S. enteritidis* [AAC(6′)-Iy] (*26*). Using these kinetic constants, AG substrates can be divided into three classes. The first class includes dibekacin and neomycin B, which displayed a relatively high steady-state affinity for the enzyme (K_m values range between 0.38 and 0.52 μM). The second class includes sisomicin, tobramycin, amikacin, and kanamycin A, which exhibited a lower steady-state affinity for the enzyme (K_m values range between 3.24 and 7.4 μM). Although the carbon skeletons of dibekacin, sisomicin, tobramycin, amikacin, and kanamycin A are basically the same, dibekacin, a 3′,4′-dideoxy analog of kanamycin A, was found to be the best of these substrates on the basis of the relative V_{max}/K_m values. These results indicated that the OH group(s) at position 3′ and/or 4′ affect the binding to AAC(6′)-Isa. Amikacin, which differs from kanamycin A in the modification of N-1 of the 2-deoxystreptamine ring with a 2-hydroxybutyrylamine group, showed specificity comparable to that of kanamycin A. This result suggested that only substitutions of the 6-aminohexose ring module do not affect the binding to AAC(6′). In fact, neomycin B was also effectively acetylated, although it possesses 3′,4′-dihydroxyl groups.

Table 3. **Kinetic parameters for aminoglycosides.**[a]

Substrate	K_m (μM)	V_{max} (U[b]/mg)	V_{max}/K_m (U[b]/mg/μM)
Dbk	0.38 ± 0.04	24.46 ± 0.93	63.82
NeoB	0.52 ± 0.04	10.62 ± 0.48	20.5
Sis	3.24 ± 0.26	41.45 ± 1.5	12.78
Tob	3.53 ± 0.05	36.92 ± 0.42	10.45
Amk	4.34 ± 0.05	34.14 ± 0.36	7.86
KamA	7.4 ± 0.46	34.81 ± 2.25	4.7
LivA	41.88 ± 3.21	0.22 ± 0.01	0.01

[a]Kinetic parameters were determined with 50 μM acetyl-CoA as an acyl donor. [b]U, μmol production/min. Each value is the mean ± SD of three experiments.

Furthermore, we found acetylated neomycin B to retain substantial antimicrobial activity. Our findings are in good agreement with the results reported by two independent groups (*15*, *23*).

Surprisingly, lividomycin A, which has a hydroxyl group at the 6′ position, was also a substrate for AAC(6′)-Isa, although an extremely low amount of AAC activity was detected. Blanchard and co-workers reported that AAC(6′)-Iy exhibited very low rates of AG-independent hydrolysis of acetyl-CoA (*26*). Therefore, AAC(6′)-Isa was suspected of having the same hydrolysis activity. However, enzyme assays with or without lividomycin A showed that the weak acetyltransferase activity was lividomycin A-dependent, and that there is no AG-independent hydrolysis activity of acetyl-CoA in AAC(6′)-Isa. These results suggested either acetylation of an alternate amino group on the molecule or *O*-acetyltransfer to position 6′. Because AAC(6′)s commonly have a very stringent regiospecificity of acetyl transfer, *O*-acetylation at position 6′ was though to be a possible reaction. The sole example of *O*-acetylation by AAC(6′) was shown in AAC(6′)-APH(2″) from enterococci (*28*). This enzyme is unique in that it is bifunctional, possessing activities of two types of AG-modifying enzymes: an N-terminal AAC [AAC(6′)-Ie] and C-terminal APH [APH(2″)-Ia]. To confirm the acetylation of lividomycin, the enzyme assay mixtures after reaction were analyzed by silica gel TLC. A small spot which seemed to be acetylated lividomycin A was detected by spraying ninhydrin reagent (Fig. 5B). Moreover, enzymatically acetylated lividomycin A was found to be highly susceptible to mild base hydrolysis, supporting the *O*-acetyltransfer hypothesis. Thus, AAC(6′)-Isa is the first example of a monofunctional (not fused) AAC to harbor *O*-acetyltransfer activity. Acetylated kanamycin (Fig. 5 A) and five other AGs possessing an amino group at the 6′ position (data not shown) were clearly detected on the TLC plate. *N*-acetylated AGs were tolerant to this base condition.

It has been demonstrated that the V_{max}/K_m value is positively correlated with the MIC for the AG-modifying enzymes AAC(6′)-IV (*29*), APH(3′)-IIIa (*30*), and ANT(2″) (*31*, *32*). However, it appears that, like AAC(6′)-Ii (*23*), AAC(6′)-Isa does not exhibit this relationship. As for AAC(6′)-Ii, Wright proposed that this enzyme may have an alternate physiological function other than AG detoxification, because of the low specificity constants and the lack of correlation between the MIC and kinetic parameters (*23*). In fact, Blanchard *et al*. have reported that the AAC(6′)-Iy dimer is most structurally similar to the *Saccharomyces cerevisiae HPA*2-encoded histone acetyltransferase, and that AAC(6′)-Iy catalyzes both acetyl-

Table 4. **Kinetic parameters for acyl-CoA.**[a]

Substrate	K_m (μM)	V_{max} (U[b]/mg)	V_{max}/K_m (U[b]/mg/μM)
acetyl-CoA	18.7 ± 3.1	42.76 ± 3.35	2.28
n-propionyl-CoA	16.1 ± 1.2	5.14 ± 0.19	0.32
malonyl-CoA	580.1 ± 130.7	26.27 ± 3.57	0.05
n-butyryl-CoA	68.6 ± 22.6	0.04 ± 0.01	0.001

[a]Kinetic parameters were determined with 25 μM sisomicin. [b]U, μmol production/min. Each value is the mean ± SD of three experiments.

CoA-dependent self-α-*N*-acetylation and the acetylation of eukaryotic histone proteins and the human histone H3 N-terminal peptide (*33*). Thus, some AAC(6′)s that exhibit a wide substrate specificity and have no correlation between the MIC and kinetic parameters seem to have an alternate physiological function. Because AAC(6′)-Isa also exhibits such enzymatic and biological characteristics, it may also have an alternate physiological function. More studies such as an inactivation of *aac(6′)-Isa* gene and crystal structure analysis of the recombinant AAC(6′)-Isa will be required to confirm our assumption.

The steady-state kinetic parameters for acetyl-CoA and various CoA derivatives at a saturating sisomicin concentration (25 μM) are summarized in Table 4. On the basis of the relative V_{max}/K_m values, acetyl-CoA was the most effective substrate as an acyl-donor. The decrease of V_{max}/K_m values from the addition of one or two methyl groups on the substrate molecule compared to acetyl-CoA suggests a very sterically restricted acyl donor binding site. The highly conserved amino acid sequence of the acetyl-CoA binding motif of AAC(6′)-Isa also supports this rigid substrate specificity. Thus, in contrast to its broad AG specificity, this enzyme has a very narrow specificity for CoA derivatives.

In conclusion, we found a unique AAC(6′)-I-type enzyme, AAC(6′)-Isa, from ε-PL-producing *S. albulus* IFO14147 in studies of AG resistance profiles to find appropriate selective makers. By functional analysis and kinetic studies *in vitro*, it was shown that AAC(6′)-Isa can acetylate not only AGs having a free amino group at the 6′ position (*N*-acetylation), but also an AG having a hydroxy group at the 6′ position, namely, lividomycin A (*O*-acetylation), and that the V_{max}/K_m values determined for AGs vary by a factor of up to 6300. AAC(6′)-Isa thus has a much wider substrate specificity than other AAC(6′)s known to date.

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