Mechanism of Multiple Aminoglycoside Resistance of Kasugamycin-producing *Streptomyces kasugaensis* MB273: Involvement of Two Types of Acetyltransferases in Resistance to Astromicin Group Antibiotics

KUNIMOTO HOTTA, TETSU OGATA[†], JUN ISHIKAWA, MASANORI OKANISHI and SATOSHI MIZUNO

National Institute of Health,
1-23-1, Toyama, Shinjuku-ku, Tokyo 162, Japan

MOTO MORIOKA[††], HIROSHI NAGANAWA and YOSHIRO OKAMI

Institute of Microbial Chemistry,
3-14-23, Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication February 23, 1996)

The biochemical basis for the multiple resistance to aminoglycoside antibiotics (AGs) of kasugamycin-producing *Streptomyces kasugaensis* MB273 was studied. The strain was resistant to a wide range of deoxystreptamine (DOS)-containing AGs as well as astromicin (ASTM) group antibiotics. These AGs strongly inhibited *in vitro* polyU-directed polyphenylalanine-synthesis using ribosomes from the strain, while they were acetylated and inactivated by the MB273 cell free extract supplemented with acetyl-CoA. It seemed thus likely that the acetyltransferase activity played a critical role for the multiple AG resistance. The acetylation was selective to AGs with 2′-NH_2, suggesting the involvement of aminoglycoside 2′-*N*-acetyltransferase, AAC (2′). Interestingly, the acetylation of istamycin B (ISM-B; an ASTM group AG) resulted in the formation of two different products (1-*N*-acetyl ISM-B and 2″-*N*-acetyl ISM-B) at a similar ratio. In this context, an AAC (2′) gene cloned as an ISM-B resistance gene from the strain MB273 directed the conversion of ISM-B to only 1-*N*-acetyl ISM-B. It seemed likely that two types of AACs [AAC(2′) and a novel one] were involved in the mechanism of resistance to ASTM group AGs.

Antibiotic resistance, mainly self-resistance, of antibiotic producers has been studied in terms of the involvement in antibiotic biosynthesis[1], the possible origin of clinically-occurring antibiotic resistance[2], the resistance mechanism[1,3,4] and the gene evolution[5]. Concerning the origin of antibiotic resistance, actinomycetes which produce aminoglycoside antibiotics (AGs) have been shown to possess AG-inactivating enzymes such as AG-phosphotransferases (APHs) and AG-acetyltransferases (AACs) that modify the same sites of AGs as those modified by APHs and AACs of clinically-occurring resistant bacteria[2]. Comparison of the cloned genes directing the synthesis of these enzymes revealed a high homology at the amino acid level, especially at the *C* terminal region, not only among AG-producers or clinical bacteria but also between both[6~8]. Therefore, characterization of the biochemical and genetic basis of the AG resistance of AG producing-actinomycetes might provide insights into the clinically-occurring AG resistance, especially resistance to new AGs of clinical use. In this context, we have been interested in astromicin (ASTM) group antibiotics and arbekacin (ABK) of which enzymatic inactivation has not been known in actinomycetes but only in clinically-occurring bacteria with AAC(3)-I[9] or AAC(6′)[10] for ASTM and APH (2″)/AAC(6′) for ABK[11].

We have been studying the multiple AG resistance of AG-producing actinomycetes in terms of the resistance mechanism[12~15] as well as the relationship with AG productivity[16,17]. Consequently, we demonstrated that AG producers usually exhibit individual AG resistance patterns correlating with the type of AGs they produce. Biochemical and genetic characterization revealed that these multiple resistances were usually due to AG-modifying enzymes and ribosomal resistance as the self-resistance determinants. In some cases, however, additional AG-modifying enzymes independent of self-resistance were found to contribute to the multiple resistance[18]. Therefore, we proposed that the resistance conferred by non-self-resistance factors should be

Present address: [†] Tokyo Tanabe Co., Ltd.; 2-33-3 Akabanekita, Kita-ku, Tokyo 115, Japan, and [††] Yamanouchi Pharmaceutical Co., Ltd.; 1-1-8, Azusawa, Itabashi-ku, Tokyo 174, Japan.

regarded as secondary resistance in order to distinguish from self-resistance as primary resistance[18,19]. An example falling into this category is a cryptic KM resistance gene in a streptomycin-producing strain of *S. griseus*[20~22]. We demonstrated that the expression of the cryptic gene was activated by one base substitution (C→T) at the first (−12) base of the −10 promoter sequence and resulted in the synthesis of an AAC(3) with a unique substrate specificity[20~22].

Kasugamycin (KSM)-producing *S. kasugaensis* MB273 that had been studied in terms of its plasmids[23,24] and construction of a host vector system[25] shows a multiple resistance to a wide range of AGs including ASTM group antibiotics[16]. The structures of these AGs are different from that of KSM, indicating that the multiple AG resistance of the strain MB273 may fall into the category of secondary resistance. In this paper, we report the correlation between AAC activity [probably AAC(2′)] and the multiple AG resistance. The formation of two distinctive acetylation products from ISM-B (an ASTM group AG) due to AAC(2′) and a novel AAC is also described.

Materials and Methods

Strains

Streptomyces kasugaensis MB273 (←MB273-C4[26]) that produces KSM and aureothricin and *S. lividans* TK21 containing a plasmid pANT10[18] with a cloned ISM-B resistance gene, *ist*, coding for an AAC(2′) were used. These strains have been maintained on Yeast extract-Malt extract agar medium (ISP No. 2; Difco). For the latter strain the medium was supplemented with thiostrepton (10 μg/ml).

Chemicals

Aminoglycoside antibiotics (AGs) were obtained from the antibiotic collection at Institute of Microbial Chemistry. Reagents except for radioactive substances were purchased from Sigma. [Acetyl-^{14}C]-CoA and L-[U-^{14}C]-phenylalanine (25 mCi/mM) were purchased from Amersham.

Antibiotic Resistance

S. kasugaensis MB273 was inoculated on ISP No. 2 medium plates each containing one of the AGs listed in Table 1, as described previously[16]. After incubation at 27°C for 1 week, the growth was scored.

In vitro PolyU-directed Polyphenylalanine-synthesizing System

According to the method described previously[12], S30 and S150 fractions as well as ribosomes were prepared from mycelia of *S. kasugaensis* MB273 grown in Tryptic Soy Broth (Difco) at 27°C for 2 days with shaking. At the final step of preparation, S150 fractions were dialyzed against buffer A at 4°C overnight, and ribosomes were washed twice with buffer D containing 1 M NH_4Cl and then suspended in buffer A so as to give OD_{260} 0.2. All of S30 and S150 fractions as well as ribosomes thus prepared were stored at −80°C until use.

PolyU-directed polyphenylalanine-synthesizing system (50 μl) was established by reconstituting the ribosomes and the S150 fractions according to the method described previously[12]. After incubating at 37°C for 45 minutes, the reaction was arrested by the addition of 10% TCA (0.5 ml) followed by heating (90°C, 10 minutes). The resulting precipitates (TCA insoluble fractions) were transferred onto GFC filters to measure the radioactivity incorporated. Incorporation of L-[U-^{14}C]Phe into the TCA insoluble fraction was regarded as the amount of polyphenylalanine synthesized.

Acetylation of Aminoglycoside Antibiotics

According to the method described previously[14], AGs (100 μg/ml) were incubated at 37°C for 60 minutes in a reaction mixture (100 μl) containing 20 μl of the S150 fractions, 80 mM Tris HCl (pH 7.8), 2 mM magnesium acetate, 2 mM DTT, 0.2 mM acetyl-CoA and 0.1 μCi [acetyl-^{14}C]-CoA. After incubation, 20 μl of the reaction mixture was transferred onto phosphocellulose paper (15 mm × 15 mm) and washed with a large volume of water. The radioactivity remained on the paper was measured as the acetylation activity.

A large scale acetylation was carried out at 37°C for 2 hours in the reaction mixture (100 ml) that contained 20 ml of the 30S fraction, IS-B sulfate (20 mg = about 34 μmoles) and 1 mM acetyl-CoA.

Extraction, Purification and Structure Determination of Acetylated Products of Istamycin B

The reaction mixture after the large scale acetylation was neutralized with HCl and loaded on a column containing 25 ml of Amberlite IRC50 (NH_4^+). After washing the column with 100 ml of H_2O, acetylated products of ISM-B were eluted with 50 ml of 1 N NH_4OH. The eluate was evaporated, dissolved in 100 ml of H_2O, adjusted to pH 7.5, and loaded on a column containing 25 ml of Amberlite CG50(NH_4^+) Type I. The column was washed with H_2O and eluted with 400 ml of a linear gradient (0~0.4 N) of NH_4OH. The eluate was collected as 6 ml fractions and monitored by ninhydrin reaction on silica gel TLC (Merk 5715) after developing with a solvent system of $CHCl_3$: CH_3OH : NH_4OH (20 : 15 : 8). Structures of the purified acetylation products were determined by analyzing the spectra of MS, ^{13}C NMR and ^{1}H NMR.

The antibiotic activity of the acetylated products was examined by the cup assay method using *Bacillus subtilis* PCI 219 as the test organism.

Results

Correlation Between Antibiotic Resistance and Acetyltransferase Activity

As shown in Table 1, *S. kasugaensis* MB273 showed resistance to a wide range of deoxystreptamine (DOS)-containing AGs (ribostamycin, dibekacin, neomycin B and paromomycin) and ASTM-group AGs such as istamycin B (ISM-B), although retarded growth was observed in the presence of kanamycin A, gentamicin C complex, butirosin A or streptomycin. These AGs were then examined for their effect on the *in vitro* polyU-directed polyphenylalanine-synthesizing system. Consequently, polyphenylalanine synthesis was strongly inhibited, indicating that the ribosomes of the strain MB273 were sensitive to the AGs. On the other hand, it seemed unlikely that AG-inactivating enzymes such as APH and AAD (AG-adenylyltransferase) were involved in the AG resistance because the polyphenylalanine-synthesizing system contained a high level of ATP enough for phosphorylation and adenylylation that should result in AG inactivation as well as suppression of the inhibition of polyphenylalanine synthesis. However, we could not rule out the presence of AAC because acetyl-CoA essential for acetylation was not added to the polyphenylalanine-synthesizing system.

Therefore, AGs were subjected to acetylation by S150 fractions supplemented with [acetyl-^{14}C]-CoA. As shown in Table 1, acetylation occurred with AGs possessing 2′-NH_2 but not with KM-A and streptomycin that lack 2′-NH_2, suggesting the involvement of AAC(2′). In a separate acetylation experiment using ISM-B, ASTM, dibekacin and ribostamycin, total inactivation of the antibiotics was observed (data not shown). Thus, there was a good correlation between AG resistance and AAC activity.

Acetylation Products of Istamycin B

We chose the acetylation product of ISM-B (an ASTM group AG) for chemical characterization, since acetylation of ASTM-group AGs had not been known in actinomycetes. When ISM-B (20 mg) was incubated with S30 fractions, approximately 40% was inactivated under the acetylation conditions employed. Ion exchange chromatography yielded 3 ninhydrin positive peak fractions (designated I, II and III on the elution basis). As shown in Fig. 1, peak I provided two distinctive spots designated compounds **A** and **B** at a similar ratio. They showed yellow (**A**) and violet (**B**) colors upon ninhydrin reaction and Rf values of 0.62 (**A**) and 0.58 (**B**) upon silica gel TLC. In order to separate these two substances, peak I fractions were pooled and loaded on Amberlite CG50 (NH_4^+) Type I column and eluted with 400 ml of 0.06 N NH_4OH. Under these conditions, **A** was eluted a little slower than **B**. Fractions giving rise to a single spot on TLC were pooled, while fractions containing both compounds were rechromatographed under the same conditions. After 4 rounds of rechromatography, purified **A** and **B** (2~3 mg each) were obtained, neither of which showed antibiotic activity against *B. subtilis* PCI 219 at 100 μg/ml.

Compound **A** was also obtained as the sole acetylation product by incubating ISM-B with acetylCoA and the cell free extract (S30 fractions) from *S. lividans* TK21/pANT10 containing an AAC(2′) encoding gene (*ist*) from *S. kasugaensis* MB273 (data not shown).

On the other hand, peaks II and III each contained a single compound indistinguishable from ISM-B (II) and ISM-Bo (III), respectively, in terms of Rf value and color (yellow and violet) upon TLC. It seemed thus likely

Table 1. Antibiotic resistance and acetyltransferase activity of *S. kasugaensis* MB273.

Antibiotics	2′-NH_2	Growth[a]	Poly-Phe-Synthesis[b]		Acetylation
None		+++	16,648 dpm	100.0%	67 dpm
Istamycin B	Yes	++	1,152	6.9	12,330
Kanamycin A	No	+	1,046	6.2	70
Dibekacin	Yes	++	1,283	7.7	8,825
Gentamicin C	Yes	+	3,052	18.3	8,614[c]
Ribostamycin	Yes	+++	1,259	7.5	8,910
Butirosin A	Yes	+	nt	—	2,260
Neomycin B	Yes	++	890	5.3	4,780
Paromomycin	Yes	++	1,187	7.1	2,367
Streptomycin	No	+	nt	—	73

[a] Growth relative to the control after 7 day incubation at 27°C on ISP No. 2 medium containing 50 μg/ml of antibiotics. [b] *In vitro* polyU-directed polyphenylalanine synthesis using S150 fraction and ribosomes from the strain MB273. nt: not tested. [c] Gentamicin C complex was replaced with micronomicin as the substrate.

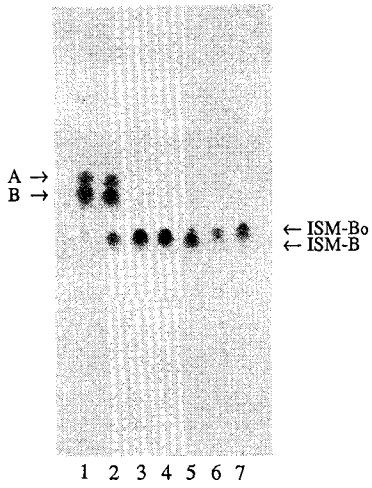
color (yellow and violet) upon TLC. It seemed thus likely that these compounds were not acetylation products but the unconverted substrate (II) and its artificial degradation product (III) due to the use of NH_4OH for elution upon chromatography.

Structure Determination of Compound **A** as 1-*N*-Acetyl ISM-B

The compound **A** provided the signal at *m/z* 431 in FAB-MS, and those at *m/z* 143 and 272 in EI-MS. Since ISM-B (MW 389) consists of one sugar moiety (MW 143) and one aminocyclitol moiety (MW 246) linked together through a glycosidic linkage, it is obvious that *m/z* 431 corresponds to mono-acetylated ISM-B (42+389) and that *m/z* 143 and 272 correspond to the sugar moiety and the mono-acetylated aminocyclitol moiety, respectively.

Table 2 shows ^{13}C NMR (400 MHz) chemical shifts assigned for **A**. Signals at 22.8 and 173.9 ppm that were not found in ISM-B were assigned to methyl and carbonyl carbons, respectively, of 1-NCOMe. The *β*-shift

Fig. 1. Silica gel TLC of acetylation products.



1: Peak I, 2: peak I + ISM-B, 3: peak II, 4: peak II + ISM-B, 5: ISM-B + ISM-Bo, 6: peak III, 7: peak III + ISM-Bo.

Table 2. ^{13}C NMR chemical shifts of compound **A**.

Carbon	ISM-B[a]	Compound **A**[b]
C-1	47.2 ppm	45.5 ppm
C-2	29.3	30.3[c]
C-3	71.9	72.6
3-OMe	56.6	56.3
C-4	56.5	56.8
4-NMe	32.0	31.8
C-5	68.3	69.2
C-6	73.6	74.7[c]
C-1′	93.1	93.1
C-2′	49.6	49.5
C-3′	21.4	21.8
C-4′	26.7	26.4
C-5′	66.5	65.1
C-6′	53.0	52.4
6′-NMe	34.4	34.0
C-1″	168.7	168.6
C-2″	41.3	41.3
1-NCOMe		22.8
1-NCOMe		173.9

[a] ISM-B sulfate. [b] A hydrochloride (pH 5.0). [c] *β*-shifts due to 1-*N*-acetylation.

Table 3. Chemical shifts and coupling constants of ^1H NMR of compound **A**.

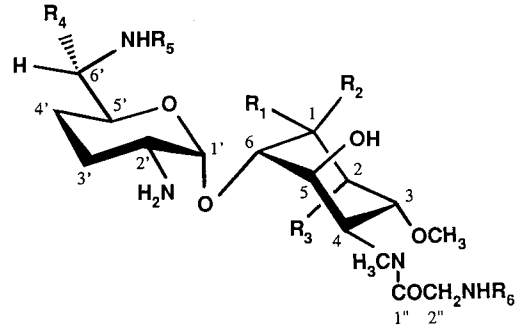
Proton	ISM-B[a]		Compound **A**[b]	
	δ (ppm)	*J* (Hz)	*δ* (ppm)	*J* (Hz)
H-1	3.80	(dt, 3.5, 12)	4.36[c]	(dt, 3.5, 12)
H-2a	1.83	(q, 12)	1.78	(q, 12)
H-2b	2.59	(dt, 4, 12)	2.41	(dt, 4, 12)
H-3	4.04	(dt, 4, 12)	3.99~4.02	?
H-4	4.32~4.46	(dd, 3, 11)	4.41	(dd, 2.5, 10)
H-5	4.32~4.46	(t, 3)	4.27	(t, 3)
H-6	4.00	(t, 3~3.5)	3.99~4.02	?
3-OMe	3.45	(s)	3.45	(s)
4-NMe	3.09	(s)	3.11	(s)
H-1′	5.43	(d, 3.4)	5.32	(d, 3.5)
H-2′	3.61	(m)	3.56	(m)
H-3′	1.42~2.24	(m)	2.05~2.12	(m)
H-4′	1.42~2.24	(m)	1.61~1.99	(m)
H-5′	~4.00	(m)	~4.16	(m)
H-6′a	3.17	(dd, 7.6, 13)	3.18	(dd, 7.5, 13)
H-6′b	3.30	(dd, 3.5, 13)	3.28	(dd, 3.5, 13)
6′-NMe	2.76	(s)	2.75	(s)
H-2″a	4.02	(d, 16)	4.04	(d, 17)
H-2″b	4.12	(d, 16)	4.11	(d, 17)
1-NCOMe			2.06	(s)

[a] ISM-B sulfate, [b] A hydrochloride (pH 5.0), [c] Acyl-shifts by 1-*N*-acetylation.

due to this 1-*N*-acetylation was detected in signals of C-2 and C-6 that appeared in lower field (30.3 and 74.7 ppm) in the **A**, compared to those (29.3 and 73.6 ppm) of ISM-B. The rest of signals showed a reasonably good agreement between ISM-B and **A**. Table 3 shows chemical shifts and coupling constants of **A** upon ^{1}H NMR (COSY; 400 MHz). The signal (3.80 ppm) corresponding to H-1 in ISM-B appeared in lower field (4.36 ppm) in **A**, indicating an acyl-shift by 1-*N*-acetylation. The other δ and J values were in a good agreement between **A** and ISM-B.

Based on these data, we concluded that **A** was 1-*N*-acetyl ISM-B (Fig 2).

Fig. 2. Structures of acetylation products (**A** and **B**) of istamycin B.



	R_1	R_2	R_3	R_4	R_5	R_6
Astromicin	H	NH_2	OH	CH_3	H	H
Istamycin A	H	NH_2	H	H	CH_3	H
Istamycin B	NH_2	H	H	H	CH_3	H
Istamycin B_1	NH_2	H	H	H	CH_3	CHO
A	$NHCOCH_3$	H	H	H	CH_3	H
B	NH_2	H	H	H	CH_3	$COCH_3$

Table 4. ^{13}C NMR chemical shifts of compound **B**.

Position	δ value (ppm)		
	Compound **B**	ISM-B_1[a]	ISM-B[a]
C-1	47.3	47.3	47.2
C-2	29.2	29.3	29.3
C-3	72.0	72.0	71.9
C-4	56.4	56.6	56.5
C-5	68.3	68.3	68.3
C-6	73.6	73.6	73.6
3-OMe	56.6	56.4	56.6
4-NMe	32.1	32.1	32.0
C-1′	92.9	93.0	93.1
C-2′	49.5	49.5	49.6
C-3′	21.4	21.4	21.4
C-4′	26.6	26.6	26.7
C-5′	66.5	66.5	66.5
C-6′	52.9	52.9	53.0
6′-NMe	34.3	34.4	34.4
C-1″	172.2[b]	171.6[b]	168.7
C-2″	42.5	41.2	41.3
2″-*N*-COMe	22.5	—	—
2″-*N*-COMe	175.7	—	—
2″-*N*-CHO	—	165.6	—

[a]Data were available from IKEDA *et al.*[27]. [b]β-shifts due to acetylation (**B**) and formylation (ISM-B_1).

Table 5. ^{1}H NMR chemical shifts and coupling constants for compound **B**.

Position	Compound **B***		ISM-B sulfate*	
	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)
H-1	3.87	$J_{1,2a}=12$, $J_{1,2b}=4$	3.84	$J_{1,2a}=12$, $J_{1,2b}=4$
H-2a	1.84	$J_{2a,2b}=12$, $J_{2a,3}=12$	1.82	$J_{2a,2b}=12$, $J_{2a,3}=12$
H-2b	2.61	$J_{2b,3}=4$	2.61	$J_{2b,3}=4$
H-3	4.05	$J_{3,4}=12$	4.05	$J_{3,4}=12$
H-4	~4.35	m	~4.37	m
H-5	~4.38	$J_{5,6}=3$	~4.39	$J_{5,6}=3$
H-6	4.16	$J_{1,6}=3$	4.19	$J_{1,6}=3$
3-OMe	3.46	s	3.46	s
4-NMe	3.13	s	3.09	s
H-1′	5.45	$J_{1',2'}=3.5$	5.44	$J_{1',2'}=3.5$
H-2′	3.62	m	3.60	m
H-3′	1.6~2.14	m	1.6~2.09	m
H-4′		m		m
H-5′	~4.1	m	4.16	m
H-6′a	3.19	$J_{6'a,6'b}=13$, $J_{5',6'a}=7.5$	3.13	$J_{6'a,6'b}=13$, $J_{5',6'a}=7.6$
H-6′b	3.29	$J_{5',6'b}=3.5$	3.29	$J_{5',6'b}=3.5$
6′-NMe	2.76	s	2.76	s
H-2″a	4.07	$J_{2''a,2''b}=17$	4.05	$J_{2''a,2''b}=16$
H-2″b	4.24		4.16	
2″-*N*-COMe	2.09	s	—	—

* Data of compound **B** and ISM-B sulfate in D_2O (Internal TSP, $\delta=0$) were obtained with 250 MHz and 400 MHz[27] spectra, respectively.

Structure Determination of Compound **B** as 2″-*N*-Acetyl ISM-B

The compound **B** gave rise to the signals at *m/z* 431 and 143 that were assigned to monoacetylated ISM-B and amino-sugar moiety, respectively, in FAB-MS spectra. In addition, **B** provided a signal at *m/z* 300 which could be assigned as the acetylated aminocyclitol resulting from the cleavage of the glycosidic linkage. Thus these results indicated that monoacetylation occurred in the aminocyclitol moiety in **B**.

Table 4 shows ^{13}C NMR data of **B** in comparison with those of ISM-B and ISM-B_1[27]. There was no significant difference in chemical shifts from C-1 to 6′-*N*-Me signals among these 3 compounds. However, signals assigned to C-1″ of **B** (172.2 ppm) and ISM-B_1 (171.6 ppm) appeared at lower fields, compared to that of ISM-B (168.7 ppm). These results indicated β-shifts due to acetylation and formylation at 2″-NH_2 of the glycine residue in **B** and ISM-B_1, respectively. Signals of 2″-*N*-COMe were also observed at 22.5 and 175.7 ppm in **B**. Table 5 shows ^1H NMR data of **A** and ISM-B. Except for those corresponding to 2″-*N*-COMe, both δ and *J* values of ISM-B and **A** agreed well with each other. It was thus obvious that **B** has the same structure as that of ISM-B except for 2″-*N*-COMe.

Based on these results, B was determined to be 2″-*N*-acetyl ISM-B, a novel acetylated compound which has not been known in ASTM group antibiotics (Fig. 2).

Discussion

S. kasugaensis MB273 showed resistance to a variety of DOS-containing AGs and ASTM group AGs. These AGs strongly inhibited *in vitro* polyphenylalanine-synthesis using ribosomes from the strain, while they were acetylated and inactivated by cell free extract from the strain. Therefore, it seemed obvious that the multiple AG resistance was dependent on the acetylating activity. The acetylation showed a good correlation with the presence of 2′-NH_2 in the molecules; *e.g.* RSM and DKB were readily acetylated, while KM lacking 2′-NH_2 was not. Therefore, it seemed likely that an AAC(2′) was involved in the acetylation.

Acetylation of ASTM-group AGs had not previously been reported in actinomycetes, but had been observed in clinically-occurring bacteria with AAC(3)-I[9] or AAC(6′)[10]. In actinomycetes, only ribosomal resistance to ASTM-group antibiotics[18,28,29] had been demonstrated. Therefore, we examined ISM-B, an ASTM group AG that we discovered[30], for acetylation by the cell free extract from the strain MB273 and observed that it was converted to approximately equal amounts of 1-*N*-acetyl ISM-B and 2″-*N*-acetyl ISM-B. The formation of 2′-*N*-acetyl ISM-B was not detected. Based on these results, we first thought that the formation of 1-*N*-acetyl ISM-B was not due to AAC(2′) but to an AAC(3)-I type enzyme that has been shown to acetylate ASTM at 1-NH_2[9]. We also wondered whether the formation of 2″-*N*-acetyl ISM-B was due to a novel AAC or to the one responsible for the formation of 1-*N*-acetyl ISM-B. Therefore, we cloned an ISM-B resistance gene designated *ist* from *S. kasugaensis* MB273 into *S. lividans* TK21 and examined for its ability to direct the conversion of ISM-B. It turned out that the gene coded for an AAC(2′) active upon AGs containing 2′-NH_2[18,31] and the AAC(2′) acetylated the 2′-NH_2 of ASTM or the 1-NH_2 of ISM-B. The acetylation site of ASTM group AGs was dependent on the configuration of 1-NH_2[18] and 2″-*N*-acetyl ISM-B was not formed. Therefore, we concluded that two different AACs were involved in the conversion of ISM-B to 1-*N*-acetyl ISM-B and 2″-*N*-acetyl ISM-B; AAC(2′) due to the *ist* gene product and a novel AAC activity, possibly AAC(2″).

It is therefore interesting that a gene encoding novel AAC activity has recently been isolated from *S. kasugaensis* MB273. This AAC is active against the 2′-NH_2 of KSM but it remains to be established whether it is capable of acting on the 2″-NH_2 of ISM-B. If not, there must be at least 3 AAC activities in *S. kasugaensis* MB273 since the sequence of the gene isolated by HIRASAWA *et al.* (Japan Patent: JP. A05-23187), differs from that of the *ist* gene (unpublished) and KSM is not a substrate for the AAC(2′) activity of the *ist* gene product. That being so, the multiple AG resistance due to the AAC(2′) gene we cloned falls into the category of secondary AG resistance we proposed[18].

AAC(2′) activity has also been shown in clinically-occurring *Providencia*[32,33]. It will be interesting to determine the degree of relatedness between the AAC(2′) genes and proteins of *Providencia*[34] and *S. kasugaensis*.

Acknowledgements

This work was supported in part by a grant from Japan Antibiotics Research Association. We are grateful to Dr. M. HAMADA and Dr. TOMIO TAKEUCHI, Institute of Microbial Chemistry, for *S. kasugaensis* MB273-C4 and encouragement, respectively.

References

1) CUNDLIFFE, E.: Self-protection mechanisms in antibiotic producers. *In* Secondary Metabolites: Their Function and Evolution. *Ed.*, J. C. DEREK & J. WHELAN, pp. 199～214, John Wiley and Sons Ltd., Chichester, England, 1992
2) BENVENISTE, R. & J. DAVIES: Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc. Nat. Acad. Sci. U.S.A. 70: 2276～2280, 1973
3) DEMAIN, A. L.: How do antibiotic-producing micro-organisms avoid suicide ? Ann. New York Acad. Sci. 235: 601～612, 1974

4) CUNDLIFFE, E.: How antibiotic-producing organisms avoid suicide. Annu. Rev. Microbiol., 43: 207~233, 1989
5) PIEPERSBERG, W.; J. DISTLER, P. HEINZEL & J.-A. PEREZ-GONZALEZ: Antibiotic resistance by modification: Many resistance genes could be derived from cellular control genes in actinomycetes—A hypothesis. Actinomycetologica 2: 83~98, 1988
6) HERBERT, C. J.; M. SARWAR, S. S. NER, I. G. GILES & M. AKHTAR: Sequence and interspecies transfer of an aminoglycoside phosphotransferas gene (*APH*) of *Bacillus circulans*. Biochem. J. 233: 383~393, 1986
7) SALAUZE, D.; J. A. PEREZ-GONZALEZ, W. PIEPERSBERG & J. DAVIES: Characterization of aminoglycoside acetyltransferase-encoding genes of neomycin-producing *Micromonospora chalcea* and *Streptomyces fradiae*. Gene 101: 143~148, 1991
8) SHAW, K. J.; P. N. RATHER, R. S. HARE & G. H. MILLER: Molecular genetics of aminoglycoside resistance genes and familial relationship of aminoglycoside-modifying enzymes. Microbiol. Rev. 57: 138~163, 1993
9) SATO, S.; T. IIDA, R. OKACHI, K. SHIRAHATA & T. NARA: Enzymatic acetylation of fortimicin A and seldomycin factor 5 by aminoglycoside 3-acetyltransferase I: [AAC(3)-I] of *E. coli* KY8348. J. Antibiotics 30: 1025~1027, 1977
10) OKUBO, T.; M. INOUE, M. NAGASHIMA, M. OKII, T. IIDA & S. MITSUHASHI: Acetylation of astromicin by the aminoglycoside 6′-*N*-acetyltransferase of a gentamicin-resistant *S. aureus*. J. Antibiotics 38: 122~125, 1985
11) KONDO, S.; A. TAMURA, S. GOMI, Y. IKEDA, T. TAKEUCHI & S. MITSUHASHI: Structures of enzymatically modified products of arbekacin by methicillin-resistant *Staphylococcus aureus*. J. Antibiotics 46: 310~315, 1993
12) YAMAMOTO, H.; K. HOTTA, Y. OKAMI & H. UMEZAWA: Self-resistance of a *Streptomyces* which produces istamycins. J. Antibiotics 34: 824~829, 1981
13) YAMAMOTO, H.; K. HOTTA, Y. OKAMI & H. UMEZAWA: Ribosomal resistance of an istamycin producer, *Streptomyces tenjimariensis*, to aminoglycoside antibiotics. Biochem. Biophys. Res. Commun. 100: 1396~1401, 1981
14) HOTTA, K.; H. YAMAMOTO, Y. OKAMI & H. UMEZAWA: Resistance mechanisms of kanamycin-, neomycin- and streptomycin-producing streptomycetes to aminoglycoside antibiotics. J. Antibiotics 34: 1175~1182, 1981
15) YAMAMOTO, H.; K. HOTTA, Y. OKAMI & H. UMEZAWA: Mechanism of resistance to aminoglycoside antibiotics in nebramycin-producing *Streptomyces tenebrarius*. J. Antibiotics 35: 1020~1025, 1982
16) HOTTA, K.; A. TAKAHASHI, N. SAITO, Y. OKAMI & H. UMEZAWA: Multiple resistance to aminoglycoside antibiotics in actinomycetes. J. Antibiotics 36: 1748~1754, 1983
17) HOTTA, K.; A. TAKAHASHI, Y. OKAMI & H. UMEZAWA: Relationship between antibiotic resistance and antibiotic productivity in actinomycetes which produce aminoglycoside antibiotics. J. Antibiotics 36: 1789~1791, 1983
18) HOTTA, K.; J. ISHIKAWA, T. OGATA & S. MIZUNO: Secondary aminoglycoside resistance in aminoglycoside-producing strains of *Streptomyces*. Gene 115: 113~117, 1992
19) DAVIES, J.: Another look at antibiotic resistance. J. Gen. Microbiol. 138: 1553~1559, 1992
20) HOTTA, K.; J. ISHIKAWA, M. ICHIHARA, H. NAGANAWA & S. MIZUNO: Mechanism of increased kanamycin-resistance generated by protoplast regeneration of *Streptomyces griseus*. I. Cloning of a gene segment directing a high level of an aminoglycoside 3-*N*-acetyltransferase activity. J. Antibiotics 41: 94~103, 1988
21) ISHIKAWA, J.; Y. KOYAMA, S. MIZUNO & K. HOTTA: Mechanism of increased kanamycin-resistance generated by protoplast regeneration of *Streptomyces griseus*. II. Mutational gene alteration and gene amplification. J. Antibiotics 41: 104~112, 1988
22) ISHIKAWA, J. & K. HOTTA: Nucleotide sequence and transcriptional start point of the *kan* gene encoding an aminoglycoside 3-*N*-acetyltransferase from *Streptomyces griseus* SS-1198PR. Gene 108: 127~132, 1991
23) TOYAMA, H.; M. OKANISHI & H. UMEZAWA: Physical characterization of plasmids from *S. kasugaensis* MB273. Plasmid 5: 306~312, 1981
24) AKAGAWA, H.; K. KAWAGUCHI & M. MIZUNO: Plasmids of *Streptomyces kasugaensis* MB273: Their pock formation, their dispensable endonuclease cleavage sites for pock formation, and transformation of *S. kasugaensis* MB273 by them. J. Antibiotics 37: 1016~1025, 1984
25) NABESHIMA, S.; Y. HOTTA & M. OKANISHI: Construction of plasmid vectors from *Streptomyces kasugaensis* plasmids, pSK1 and pSK2. J. Antibiotics 37: 1026~1037, 1984
26) HAMADA, M.; N. KINOSHITA, S. HATTORI, A. YOSHIDA, Y. OKAMI, K. HIGASHIDE, N. SAKATA & M. HORI: *Streptomyces kasugaensis* sp. nov.: A new species of genus *Streptomyces*. Actinomycetologica 9: 27~36, 1995
27) IKEDA, D.; Y. HORIUCHI, M. YOSHIDA, S. KONDO & H. UMEZAWA: Isolation and structures of istamycin components. Carbohydrate Res. 109: 33~45, 1982
28) OHTA, T.; T. DAIRI & M. HASEGAWA: Characterization of two different types of resistance genes among producers of fortimicin-group antibiotics. J. Gen. Microbiol. 139: 591~599, 1993
29) HOLMES, D. J.; DROCOURT, G. TIRABY & E. CUNDLIFFE: Cloning of an aminoglycoside-resistance-encoding gene, *kamC*, from *Saccharopolyspora hirsuta*: comparison with *kamB* from *Streptomyces tenebrarius*. Gene 102: 19~26, 1991
30) OKAMI, Y.; K. HOTTA, M. YOSHIDA, D. IKEDA, S. KONDO & H. UMEZAWA: New aminoglycoside antibiotics, istamycins A & B. J. Antibiotics 32: 964~966, 1979
31) HOTTA, K.; C. B. ZHU, T. OGATA, A. SUNADA, J. ISHIKAWA, S. MIZUNO, Y. IKEDA & S. KONDO: Enzymatic 2′-*N*-acetylation of arbekacin and antibiotic activity of its product. J. Antibiotics 49: 458~464, 1996
32) CHEVEREAU, M.; P. J. L. DANIELS, J. DAVIES & F. LEGOFFIC: Aminoglycoside resistance in bacteria mediated by gentamicin acetyltransferase II, an enzyme modifying the 2′-amino group of aminoglycoside antibiotics. Biochemistry 13: 598~603, 1974
33) YAMAGUCHI, M.; S. MITSUHASHI, F. KOBAYASHI & H. ZENDA: A 2′-*N*-acetylating enzyme of aminoglycosides. J. Antibiotics 27: 507~515, 1974
34) RATHER, P. N.; E. OROSZ, K. J. SHAW, R. HARE & G. MILLER: Characterization and transcriptional regulation of the 2′-*N*-acetyltransferase gene from *Providencia stuartii*. J. Bacteriol. 175: 6492~6498, 1993