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Biotechnology Methods – Review

Basic and applied research on multiple aminoglycoside antibiotic resistance of actinomycetes: an old-timer's recollection

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Abstract: A list of our research achievements on multiple aminoglycoside antibiotic (AG) resistance in AG-producing actinomycetes is outlined. In 1979, the author discovered a novel AG (istamycin)-producing *Streptomyces tenjimariensis* SS-939 by screening actinomycetes with kanamycin (KM)-resistance and plasmid profiles. This discovery directed our biochemical and genetic approaches to multiple AG resistance (AGR) of AG producers. In this article, the following discoveries will be outlined: (1) AGR profiles correlating with the productivity of AGs in AG-producers, (2) Wide distribution of multiple AG resistance in AG-nonproducing actinomycetes, (3) Involvement of ribosomal resistance and AG-acetylating enzymes as underlying AGR factors, (4) Activation by single nucleotide substitution of a silent gene coding for aminoglycoside 3-*N*-acetyltransferase, AAC(3), in *S. griseus*, (5) Discovery of a novel antibiotic indolizomycin through protoplast fusion treatment between *S. tenjimariensis* and *S. griseus* strains with different AGR phenotypes, and (6) Double stage-acting activity of arbekacin (ABK; an anti-MRSA semisynthetic AG) discovered by acetylation of ABK with cloned AACs; that is both ABK and its acetylated derivatives showed remarkable antibiotic activities.

Keywords: Actinomycetes, Multiple aminoglycoside antibiotic resistance, Gene activation, Double stage-acting activity, Plasmid profile screening

Introduction

Antibiotic resistance in actinomycetes has been investigated from various perspectives such as self-resistance (Cundliffe, 1989; Demain, 1974), involvement in antibiotic biosynthesis (Piepersberg & Distler, 1997; Hotta et al., 1995), origin of clinical antibiotic resistance (Benveniste & Davies, 1973), and distribution among actinomycetes (Hotta et al., 1983a). In the 1970s, the author and colleagues started investigations into the self-resistance of AG-producing *Streptomyces* strains (Hotta & Okami, 1976) and the screening of actinomycete isolates for plasmids since plasmid involvement in antibiotic biosynthesis was a leading topic at that time (Okanishi, 1979). Consequently, we discovered *Streptomyces tenjimariensis* SS-939 that produced istamycins (ISMs) as a new astromicin (ASTM) group of AGs (Hotta et al., 1980; Okami et al., 1979) and possessed a novel plasmid profile (Shigyo et al., 1984). However, the production of KM-group AGs could not be detected despite the isolated strain being KM resistant. Thereafter, we examined its resistance to foreign AGs. It turned out that this strain exhibited multiple AGR that could not be explained by the substrate specificity of any of the known AG-inactivating enzymes. Subsequent biochemical characterization revealed that the resistance factor involved ribosomes with novel resistance specificity (Yamamoto et al., 1981a, 1981b). Consequently, we expanded the characterization of other AG producers (Hotta et al., 1981; Yamamoto et al., 1982) and demonstrated the existence of individual AGR profiles correlating with the productivity of their own AGs (Hotta et al., 1983b). The discovered AGR phenotypes were mainly dependent on the functions of self-resistance factors such as AG-modifying enzymes and ribosomes. In some cases, however, additional factors that do not contribute to self-resistance were found to exist (Hotta et al., 1992; Hotta & Okami, 1996). These included AACs of *S. griseus* (Hotta et al., 1988), *S. kasugaensis* (Hotta et al., 1996a, 1996b) and an unidentified actinomycete strain #8 (Zhu et al., 1999) that produced streptomycin (SM), kasugamycin (KSM) but no AG, respectively. These AACs were eventually characterized for substrate specificity as well as gene structure.

The above findings stimulated the idea that it might be possible to obtain new results if we were successful in creating novel AGR by manipulation of AG-producing strains. We challenged an interspecies protoplast fusion using strains of *S. tenjimariensis* and *S. griseus*, taking advantage of their reciprocal resistance to KM and SM. Consequently, this challenge resulted in the emergence of indolizomycin (a novel antibiotic)-producing strain (Gomi et al., 1984; Yamashita et al., 1985a). Protoplast regeneration was then carried out and this resulted in the emergence of KM resistance (Yamashita et al., 1985b) specifically in *S. griseus*.

On the other hand, the above AACs were used for simulative investigations on the possible emergence of AAC-dependent resistance to an anti-MRSA (methicillin-resistant *Staphylococcus aureus*) agent shown to be refractory to AACs of clinical origin arbekacin (ABK) that has been (Kondo & Hotta, 1999). It turned out that ABK was readily acetylated but the acetylated ABK derivatives retained antibiotic activity. This indicated double stage activity of ABK and pointed to the remote possibility of generating of AAC-dependent ABK-resistant MRSA.

In this article, these achievements are outlined.

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Table 1 Profiles and Biochemical Basis of Multiple AG Resistance in Actinomycetes

Strains	Own AGs	AG resistance*								Resistance factor	
		S	K	G	R	B	N	P	I	Self	Nonself
Streptomyces *tenebrarius* ISP5477	TOB	●	●	●	●	●	●	●	●	16SrRNA, AAC(2′), AAC(6′)	APH(6)
Streptomyces kasugaensis MB273	KSM	●			●	○	●	●	●	AAC	AAC(2′)
Streptomyces tenjimariensis SS-939	ISM		●		●	●			●	16SrRNA	
Streptomyces kanamyceticus ISP5500	KM	●	●		●					AAC(6′), 16SrRNA(?)	
Streptomyces fradiae ISP5063	NM		○		●	○	●	●		APH(3′), AAC(3)	
Micromonospora *purpurea* KCC-0074	GM		●	●					●	16SrRNA	
Streptomyces griseus SS-1198	SM	●								APH(6)	
SS-1198PR	–	●	●	●	○		○	○		APH(6)	AAC(3)
Strain #8	?		●	●	●	●	●		●	?	AAC(1), AAC(6′)

*Resistance to 50 μg/ml of AG; ●: resistant, ○: variable, No indication: sensitive. S & SM: streptomycin, K & KM: kanamycin, G & GM: gentamicin, R: ribostamycin, B: butirocin, N & NM: neomycin, P: paromomycin, I & ISM: istamycin, TOB: tobramycin, KSM: kasugamycin, AAC: aminoglycoside *N*-acetyltransferase, APH: aminoglycoside *O*-phosphotransferase.

AG Resistance Profiles: Specificity and Diversity

Table 1 shows AGR profiles of AG producers and other strains. AG producers exhibited specific AGR profiles. All were reproducible in terms of the different strains of AG producers examined (Hotta et al., 1983b). It was conclusively shown that the AG producers demonstrate individual multiple AGR profiles except for *S. griseus* SS-1198. This strain was resistant to only its own AG (SM) and contained a hidden AG resistance factor that was activated by protoplast regeneration treatment (Yamashita et al., 1985b). Interestingly, the strain designated SS-1198PR emerged with additional resistance to KM and GM (gentamicin).

Furthermore, to check the incidence of multiple AGR (Hotta et al., 1983a), we isolated many soil actinomycete strains using an agar medium (ISP No. 4) supplemented with (20 μg/ml) or without one of the following AGs; SM, KM, DKB (dibekacin), GM, RSM (ribostamycin), BT (butirocin), NM (neomycin), PRM (paromomycin), and LVM (lividomycin). It turned out that isolates isolated from AG-containing plates showed wide varieties of multiple AGR, whereas the majority of isolates on AG-free plates showed resistance to none or only a few AGs. As a whole, we confirmed over 150 distinct multiple AGR profiles among about 500 isolates. It was notable that isolates from AG-containing plates provided producers of SM, ISM (istamycin), NM, RSM, GM, PRM, Trehalosamine, and SPCM (spectinomycin), whereas only SM-producing isolates were included among isolates from AG-free plates. It was thereby indicative that in order to obtain novel AG-producing organism, it would be advantageous to screen those with multiple AGR (Takahashi et al., 1986). It was also notable that multiple AGR isolates included rare actinomycetes: for example, *Crossiella cryophile* (Labeda, 2001) that was initially classified as *Nocardiopsis mutabilis* (Takahashi et al., 1986) which produced the novel antibiotic, dopsisamine.

During the characterization of AGR, we were aware that semisynthetic AGs such as ABK inhibited wide varieties of strains with multiple AGR. In this context, strain #8 (isolated as an ABK resistant) was remarkable in that its multiple AGR included NM, but not PRM (as shown in Table 1). Cross resistance between NM and PRM has been generally known in clinically occurring AG-resistant bacteria. Strain #8 was not identified taxonomically but exhibited properties of *Amycolatopsis*.

Biochemical and Genetic Factors Underlying Multiple AGRs of AG Producers

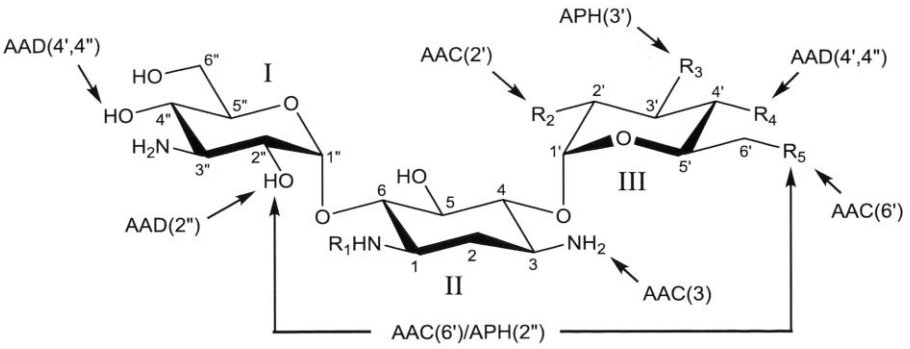
Multiple AGR in AG producers are mainly dependent on self-resistance factors such as ribosomes and/or AG-modifying enzymes as summarized in Table 1. For example, the multiple AGR profile of ISM-producing *S. tenjimariensis* SS-939 is due to ribosomal AGR specificity (Yamamoto et al., 1981a, 1981b). In cases of producers of TOB (tobramycin) and KM, both ribosomes and the substrate specificity of AAC(6′) are involved (Hotta et al., 1981; Yamamoto et al., 1982). In cases of NM and SM producers, AG-inactivating enzymes such as AAC and APH (aminoglycoside-*O*-phosphotransferase) are involved. On the other hand, additional factors such as APH(6) and AAC(2′) turned out to contribute to multiple AGR in the producers of TOB (Yamamoto et al., 1982) and KSM (Hotta et al., 1996a), respectively. Furthermore, additional AGR emerged in *S. griseus* SS-1198PR and turned out to be due to an activated cryptic AAC(3). The factor(s) conferring multiple AGR on strain #8 (Zhu et al., 1999) were categorized as nonessential resistance factor since no AG productivity has (to date) been detected in this strain. However, we cloned an AAC(6′) gene capable of conferring multiple AGR (Zhu et al., 1999) and detected AAC(1) activity (Sunada et al., 1999) using cell free extracts of strain #8. Based on these findings described above, we proposed "primary resistance" for self-resistance and "secondary resistance" for nonself-resistance (Hotta et al., 1992).

Characterization of Nonself-Resistance Factors

We characterized the biochemical basis for the activated AAC(3), AAC(2′), and AAC(6′) of *S. griseus* SS-1198PR, *S. kasugaensis* MB273 and strain #8, respectively. In the biochemical characterization, the author had a strong interest in whether these AACs were capable of acetylating and/or inactivating ABK (Hotta & Kondo, 2017; Kondo & Hotta, 1999) and ASTM (Nara et al., 1977) (refer to Fig. 1). This is because these AACs were believed to be refractory to various known AG-modifying enzymes including AACs. ASTM (= fortimicin) is a natural AG approved in 1985 and ABK is a semisynthetic AG approved in 1990 in Japan (Kondo et al., 1991). In addition, their related AGs such as ISM-B and semisynthetic AGs were also examined.

AAC(3) emerged by activation of a silent gene in S. griseus

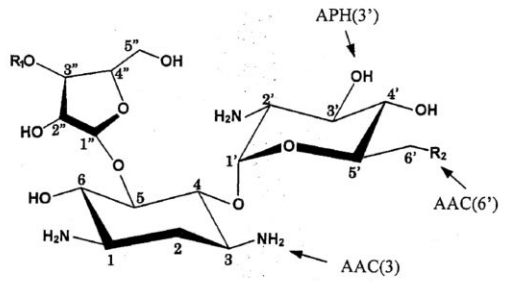
The activated cryptic AAC(3) of *S. griseus* SS-1198PR was found and characterized. In general, SM-producing strains of *S. griseus* are highly resistant to SM but sensitive to KM. In *S. griseus* SS-1198, however, protoplast regeneration treatment (Yamashita et al., 1985a) resulted in the generation of clones with 100- to 200-fold increased KM resistance at a frequency of 10^{-6}. A DNA fragment (1.8 kb *Bgl*II-*Bam*HI) was cloned as the KM resistance gene (designated *kan*) into *S. lividans* TK21 from *S. griseus* SS-1198PR



Kanamycin- and Gentamicin- group AGs

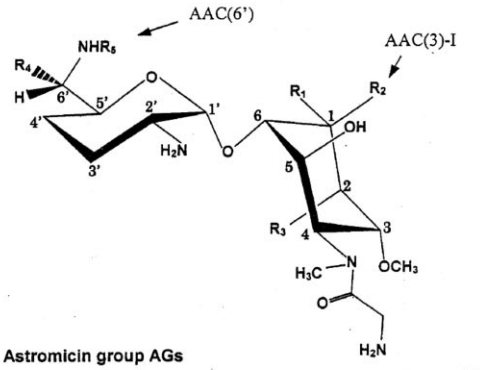
Antibiotic	I	II	III			
		R_1	R_2	R_3	R_4	R_5
Kanamycin (KM)		H	OH	OH	OH	NH_2
Dibekacin (DKB)		H	NH_2	H	H	NH_2
Amikacin (AMK)		AHB*	OH	OH	OH	NH_2
Arbekacin (ABK)		AHB*	NH_2	H	H	NH_2
Gentamicin (GM) -C_1		H				R_6=CH_3 R_7=CH_3
-C_2		H				R_6=CH_3 R_7= H
-C_{1a}		H				R_6= H R_7= H
Micronomicin (MCR)*		H				R_6= H R_7=CH_3
Isepamicin (ISP)		AHP*	OH	OH	OH	NH_2
Sisomicin (SISO)		H				
Netilmicin (NTL)		C_2H_5				

* MCR= GM-C_{2b}, AHB= $COCH(OH)CH_2CH_2NH_2$, AHP= $COCH(OH)CH_2NH_2$



Neomycin group AGs

Antibiotic	R_1	R_2
Neomycin (NM)		NH_2
Paromomycin (PRM)		OH
Ribostamycin (RSM)	H	NH_2



Astromicin group AGs

Antibiotic	R_1	R_2	R_3	R_4	R_5
Astromicin	H	NH_2	OH	CH_3	H
Istamycin B	NH_2	H	H	H	CH_3

Fig. 1. Structures of aminoglycoside antibiotics and enzymatic modification sites

Fig. 1 Structures of aminoglycoside antibiotics and enzymatic modification sites. Adapted from Hotta et al. (2000).

(Hotta et al., 1988). Subsequent biochemical characterization revealed that the KM resistance was due to AAC activity. Structure determination of the acetylation product of KM revealed the enzyme to be an AAC(3). Based on its substrate specificity, the enzyme was putatively designated AAC(3)-VI and later redesignated AAC(3)-Xa (Ishikawa et al., 1988; Shaw et al., 1993).

In order to clarify the underlying mechanism for the emergence of the KM resistance, the wild-type gene designated *kan*^{0} was cloned from strain SS-1198 using the *kan* gene as a probe (Hotta et al., 1992; Ishikawa et al., 1988). Comparison of the size and restriction map of the two 1.8-kb *Bgl*II-*Bam*HI fragments revealed no obvious difference. This indicated that a point mutation is responsible for the emergence of the high level of KM resistance in strain SS-1198PR (Ishikawa et al., 2000). Subsequent analysis of the above 1.8-kb *Bgl*II-*Bam*HI fragments by fragment exchange as well as nucleotide sequencing localized the mutation site to the -10 promoter region (Hotta et al., 1992; Ishikawa et al., 1988); one base substitution of T for C at the first letter of the -10 promoter sequence was found between the *kan* and *kan*^{0} genes (summarized in Table 2). We confirmed the enhanced level of mRNA in strain

Table 2 Correlation of *kan* Gene Activation with One Base Substitution at the -10 Promoter Region

	Streptomyces griseus	
	SS-1198	**SS-1198PR**
Gene	*kan*^{0}	*Kan*
-10 promoter sequence	CATGAT	TATGAT
Promoter activity	Weak	Strong
mRNA level	Low	High
AAC(3) level	Low	High
KM resistance	<5 μg/ml	1000 μg/ml

SS-1198PR as well as the enhanced transcription activity in *kan* gene (Ishikawa et al., 1988, 2000, Ishikawa & Hotta, 1991). It was thus conclusive that these changes resulted in the KM hyperresistance of strain SS-1198PR.

The structure gene of the *kan* was identified as an 855 bp ORF (ATG as the start codon) that coded for a protein consisting of 284 amino acids (Ishikawa et al., 2000). The ORF showed high homology and similarity at the nucleotide and amino acid levels with those of the known AAC(3) enzymes; for example, the AAC(3) gene of a PRM-producer showed 76% and 85% matching identities at the nucleotide and amino acid levels (Ishikawa et al., 2000). In addition, DNA sequences homologous to the AAC(3) gene were found to exist in all strains of *S. griseus* tested (Ishikawa & Hotta, 1991).

Characterization of substrate specificity later revealed that this AAC(3) was capable of acetylating ABK and AMK that had been believed to be refractory to AAC(3) of clinically significant bacteria (Hotta & Kondo, 2017). It should be noted that the acetylation sites were 3′-NH_2 for ABK and AMK (shown in Fig. 1), whereas 3-NH_2 for KM (Hotta et al., 1998).

AAC(2′) of S. kasugaensis

Characterization of the AAC(2′) of *S. kasugaensis* MB273 was carried out (Hotta et al., 1996a, 1996b). As shown in Table 1, this strain exhibited resistance to wide varieties of AGs and especially high levels of resistance (1 mg/ml) to ISM-B (an ASTM group AG) and RSM, but was otherwise sensitive to KM and GM. Modification experiments using cell free extracts revealed AAC dependence on multiple AGR. Since ASTM had been believed to be refractory to known AG-modifying enzymes, we carried out the cloning of the ISM-B resistance gene and followed up by characterizing the AAC encoded by the cloned gene. The ISM-B resistance gene, designated *ist*, was cloned as pANT12 containing a 6.5 kb (*Bam*HI-*Sau*3AI) DNA segment into *S. lividans* TK21. The cell free extract from *S. lividans* TK21/pANT12 was capable of acetylating RSM, ASTM, ISM-A, and ISM-B. The structure determination revealed that RSM, ASTM, and ISM-A were acetylated at 2′-NH_2 indicating the involvement of AAC(2′). In contrast, ISM-B (the 1-NH_2 epimer of ISM-A) was acetylated at 1-NH_2. Since the configuration of 1-NH_2 was axial in ISM-B and equatorial in ISM-A and ASTM (Fig. 1), the acetylation site by the AAC(2′) of the ASTM-group AGs turned out to be dependent on the configuration of 1-NH_2. In addition, the enzyme readily acetylated ABK and DKB (Hotta et al., 1996a) and very slowly acetylated GM and NTL. It was remarkable that the acetylation product of ABK (2′-*N*-acetylABK) retained clear antibiotic activity but not 2′-*N*-acetylDKB as shown in Fig. 2. Under the conditions examined, neither ABK nor ASTM were acetylated by the AAC(2′) of *Providencia* sp. (Kondo & Hotta, 1999). FramePlot analysis (Ishikawa & Hotta, 1999) following nucleotide sequencing revealed an ORF with 924 bp nucleotides (ATG as the start codon) that coded for a protein consisting of 307 amino acids.

AAC(6′) and AAC(1) of an actinomycete strain #8

Strain #8 was isolated from soil as an ABK-resistant actinomycete. AAC activity was detected using cell free extracts of the strain. Subsequently, we cloned a gene segment (0.9 kb) capable of conferring multiple resistance (Zhu et al., 1999) to AGs with 6′-NH_2 (except for NM and ABK). Semisynthetic AGs (ABK, AMK, isepamicin [ISP] and netilmicin [NTL]) and ASTM were acetylated at 6′-NH_2 (refer to Fig. 1). This substrate specificity was not similar to those of known AAC(6′) enzymes, but to that of AAC(6′)-Ie known as the *N*-terminal protein of the bifunctional enzyme AAC(6′)/APH(2′) (Hotta & Kondo, 2017). FramePlot analysis (Ishikawa & Hotta, 1999) following nucleotide sequencing revealed an ORF with 495 bp nucleotides (ATG as the start codon) that coded for the putative AAC(6′) consisting of 164 amino acids.

On the other hand, strain #8 was sensitive to PRM lacking 6′-NH_2 and therefore PRM is free from 6′-*N*-acetylation. However, its cell free extracts unexpectedly acetylated PRM resulting in the formation of 1-*N*-acetylPRM with remarkable antibiotic activity (Sunada et al., 1999), suggesting the existence of AAC(1).

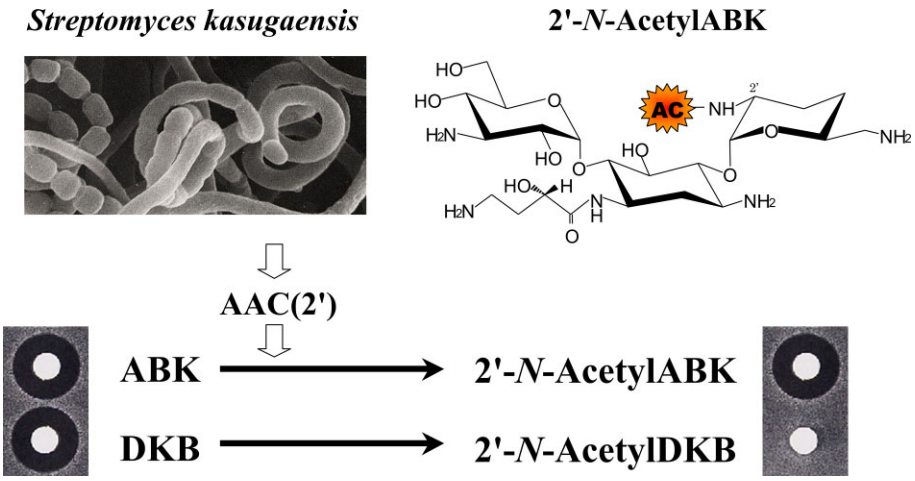


Fig. 2 Difference in antibiotic activity between acetylation products of ABK and DKB by AAC(2') of *S. kasugaensis*. ABK, arbekacin; DKB, dibekacin; AC, acetyl.

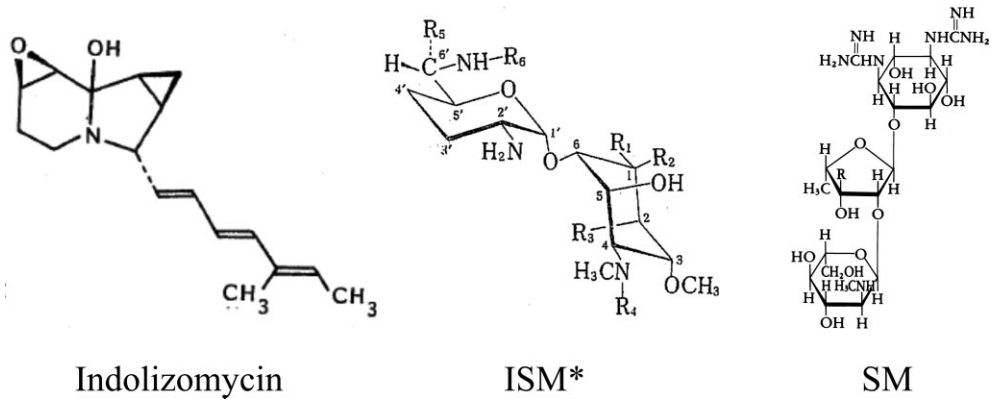


Fig. 3 Structure of indolizomycin discovered by protoplast fusion treatment between *S. tenjimariensis* and *S. griseus*. ISM, istamycin; SM, streptomycin.
*ISM-A: R1 = H, R2 = NH_2, R3 = H, R4 = H, R5 = CH_3, R6 = H
*ISM-B: R1 = NH_2, R2 = H, R3 = H, R4 = H, R5 = H, R6 = CH_3

Applied Researches of AGR

Based on the unique features of AGR obtained, we carried out several application studies. Two representative studies are described.

Indolizomycin discovery by an interspecies protoplast fusion treatment

The fact that different AG producers show different AGR patterns led us to the idea that it might be possible to generate something new if we were able to successfully create novel AGR by manipulation of AG-producing strains. Therefore, we began to challenge protoplast fusion between nonantibiotic-producing mutants of *S. tenjimariensis* SS-939 and *S. griseus* SS-1198. These have reciprocal resistance patterns in terms of resistance to KM and SM (refer to Table 1). Consequently, clones with unique profiles of resistance to AGs including KM, SM, and some other AGs were obtained and examined for antibiotic productivity. As a result, the strain SK2-52 was selected for its unique multiple AGR profile as well as its antibiotic productivity. Structure determination of the antibiotic SK2-52 produced demonstrated a novel antibiotic (Fig. 3) that was named indolizomycin (Gomi et al., 1984; Yamashita et al., 1985b). SK2-52 showed taxonomic properties of *S. griseus* but protoplast regeneration or self-fusion treatment did not provide any clone with the same properties as that of SK2-52.

Double stage-acting activity of ABK as its unique property

AGs have been and still are playing important roles in curing various infectious diseases caused by different varieties of Gram-positive and Gram-negative bacteria. The most serious problem with the activities of AGs is resistant bacteria carrying AG-modifying (or AG-inactivating) enzymes (Hotta & Kondo, 2017; Kondo & Hotta, 1999). In order to overcome this problem, rationally designed semisynthetic AGs such as DKB, ABK, AMK, and ISP (Kondo & Hotta, 1999) have been developed on the basis of the modification mechanisms of AG-inactivating enzymes. Among them, DKB (3′,4′-dideoxy-KM B) was developed and used clinically as the first rationally designed semisynthetic AG refractory to APH(3′) and AAD(4′). Subsequently, AMK, ABK, NTL, and ISP were developed by introducing the following side chains into their 1-*N*-position: 4-ammino-2-hydroxybutyryl (AHB) residue in AMK and ABK (refer to Fig. 1), ethyl residue in NTL and 3-amino-2-hydroxypropionyl (AHP) residue in ISP. These side chains are believed to block the access of a variety of AG-modifying enzymes to their target sites. Hence, these AGs are refractory to various AG-modifying enzymes. However, bacteria clinically resistant to these AGs have emerged. They included MRSA strains carrying APH(3′), AAD(4′), and/or AAC(6′)/APH(2′). In such situations, ABK (1-*N*-AHB-DKB) showed remarkable activity against these MRSA strains as shown in Fig. 4 and was subsequently approved in 1990 as an anti-MRSA agent (Kondo et al., 1991).

Since its approval, ABK has been used clinically and further AGR MRSA strains have emerged at a low incidence. Their ABK resistance level have stayed at low or moderate level (<25 μg/ml) due to AAC(6′)/APH(2′). To the best of my knowledge, no AAC-dependent ABK-resistant MRSA strain has been reported, although the ABK molecule (Fig. 1) possessing possible modification sites at 3-NH_2, 2′-NH_2, and 6′-NH_2 for AAC(3), AAC(2′), and AAC(6′), respectively. Therefore, there remains the possibility of the eventual emergence of AAC-dependent ABK resistance. In order to confirm this possibility, we cloned genes to investigate. ABK was then challenged with the action of the AAC(3), AAC(2′), and AAC(6′). Consequently, it turned out that ABK was readily acetylated by all of these AACs, resulting in the formation of 3′-*N*-acetyl-ABK, 2′-*N*-acetyl-ABK, and 6′-*N*-acetyl-ABK by AAC(3), AAC(2′), and AAC(6′), respectively (Ishikawa &Hotta, 1991; Hotta et al., 1996a, Zhu et al., 1999). It was surprising to see that the former two acetylated ABKs retained remarkably high antibiotic activities (shown in Fig. 4). It was also surprising to see that AAC(3) did not acetylate ABK at 3-NH_2 but at 3′-NH_2. On the other hand, the 3′-*N*-AMK and 2′-*N*-DKB (acetylation products of AMK and DKB, respectively, by the same AAC(2′)) showed only weak activities. Furthermore, the acetylated products by AAC(6′) of AMK, ISP, and NTL were substantially inactive. Therefore, it turned out that ABK was distinctive from the other semisynthetic AGs in terms of the antibiotic activity of their acetylated derivatives. Thus, ABK should be regarded as a "double stage-acting antibiotic" (Hotta et al., 2000) that means the antibiotic capable of retaining antibiotic activity even if enzymatically modified. In addition, it was notable that the acetylation rate by AAC(6′) of ABK was much slower than that of AMK.

Based on the above, it became possible to explain a potential mechanism as to why AAC-dependent ABK-resistant strains of MRSA hardly ever emerge even if they acquired genes of AACs. In this context, *S. lividans* TK21 carrying the cloned AAC(3) or AAC(2′) gene was sensitive to ABK (<2.5 μg/ml), whereas the one carrying the cloned AAC(6′) gene showed a weak resistance to ABK (5 μg/ml).

In spite of these results, we could not rule out the emergence of ABK resistance by acquiring two different AAC genes,

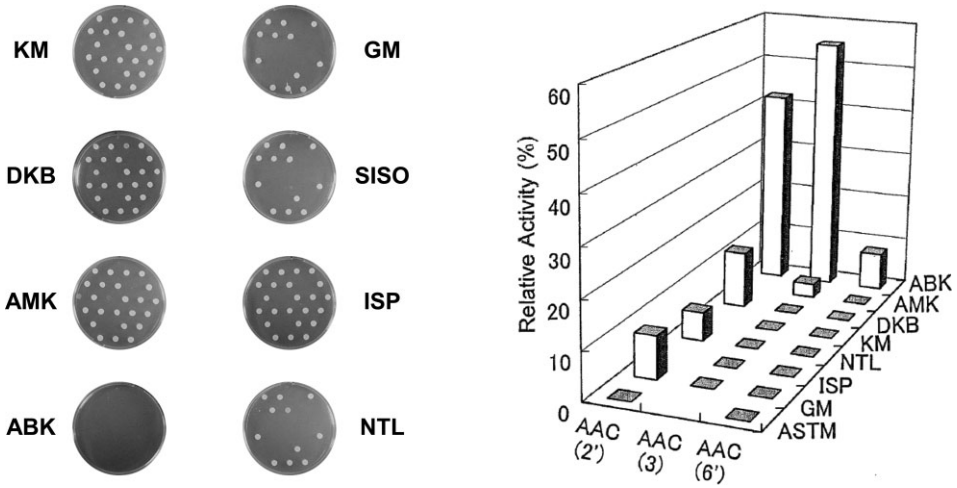


Fig. 4 Colony growth of MRSA (left) on Muller-Hinton agar containing 4 μg/ml of AG and relative activities (right) of the acetylated-AGs isolated from reaction mixtures. KM, kanamycin; GM, gentamicin; DKB, dibekacin; SISO, sisomicin; AMK, amikacin; ISP, isepamicin; ABK, arbekacin; NTL, netilmicin; ASTM, astromicin.

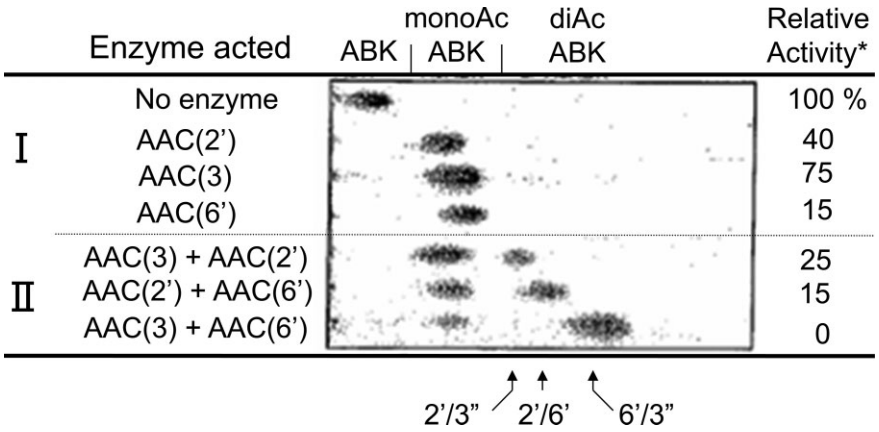


Fig. 5 Conversion of ABK by the action of three different AACs. I, Action of single AAC; II, Action of two mixed AACs.
Acetylation sites: 2′/3′ = 2′- & 3′-NH_2, 2′/6′ = 2′- & 6′-NH_2, 6′/3′ = 6′- & 3′-NH_2
*Relative antibiotic activity of reaction mixtures after enzymatic acetylation.

since we demonstrated the enzymatic formation of diacetylated ABK derivatives (di-*N*-acetylation of NH_2 at 2′/3′, 2′/6′ and 6′/3′ positions) showing no antibiotic activity (Hotta, 2000). Therefore, we constructed *S. lividans* TK21 with two compatible plasmids each containing a different AAC gene (unpublished research). In doing so, we found that the *S. lividans* TK21 containing AAC(6′) plus AAC(3) or AAC(2′) genes showed resistance to ABK at 5∼10 μg/ml whereas the strain containing both AAC(3) and AAC(2′) genes showed no significant ABK resistance. Enzymatic acetylation using cell free extracts revealed that ABK was not readily converted to its diacetylated derivatives but to a mixture of monoacetylated and diacetylated derivatives of ABK (shown in Fig. 5) under conditions allowing for the complete monoacetylation of ABK by each AAC. Based on these findings on the acetylation of ABK by AACs, we speculate that MRSA strains will hardly ever become highly resistant to ABK even if they acquired two different AAC genes.

Closing Remarks

Studies on multiple AGR of actinomycetes led to several new discoveries; (1) wide varieties of multiple AGR in actinomycetes, (2) isolation of a new Genus *Crossiella*, (3) correlation between individual AG resistance profiles with the productivity of intrinsic AGs in AG producers, (4) ribosomes as the self-resistance factor in *S. tenjimariensis* SS-939, (5) additional AG resistance (nonself-resistance) factors, AACs [AAC(3), AAC(2′), and AAC(6′)] with novel substrate specificities, (6) activation of a cryptic AAC(3) gene by one base substitution of the -10 promoter sequence, and (7) double stage-acting activity of ABK.

These findings might allow the author to insist that actinomycetes with novel profiles of multiple AGR will be an eventual treasure chest of "something new" and that "double stage-acting activity" found in ABK will become a new concept for developing new future AGs. Furthermore, our findings may lead to additional future research as shown in the case of the discovery of ribosomal AG resistance that was linked to the sequential molecular basis analysis of AGR of actinomycetes producing ASTM group AGs by other research groups (Ohta et al., 1993; Piendl et al., 1984; Skeggs et al., 1985).

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This article is dedicated to the late Dr. A.L. Demain, a worldwide distinguished microbiologist who passed away on April 3, 2020 of COVID-19. It is dedicated in recognition and appreciation of his friendship, professional activities and contributions to actinomycete researches. There is no doubt that Arny educated, encouraged, and inspired many Japanese actinomycete researchers. The Society of Actinomycetes Japan recognized him an honorary member in 1995 and held a special symposium to celebrate his 80th birthday in 2007. The author was inspired by Professor Demain's review papers, especially ones relating to antibiotic resistance. In this article, therefore, the author has outlined his original research achievements on multiple AG resistance of actinomycetes.

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Conflict of Interest

The author declares no conflict of interest.

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