Journal of Industrial Microbiology (1996) 17, 352–358
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Diversity in aminoglycoside antibiotic resistance of actinomycetes and its exploitation in the search for novel antibiotics

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Wide varieties of multiple aminoglycoside antibiotic (AG) resistance that are not found in known *Streptomyces* cultures were found among actinomycetes isolated as AG-resistant. Screening of about 170 AG-resistant isolates demonstrated their high probability (63%) of antibiotic production including the production of eight different AGs. We found specific AG-resistance patterns correlating to the productivity of specific AGs and established an AG-targeted assay system using AG producers as test organisms. Consequently, AG-directed screening totally on the basis of multiple AG-resistance was established. Necessity of biochemical and genetic studies on primary metabolism in order to find a new basis to search for novel metabolites is also discussed.

Keywords: actinomycetes; aminoglycoside resistance; aminoglycoside-directed screening

Introduction

Actinomycetes have been, and still are, regarded as the richest source of bioactive secondary metabolites such as antibiotics. They have provided about two thirds (more than 5000) of the naturally occurring antibiotics discovered [45]. However, there are many known antibiotics and the probability of discovering new antibiotics is declining. Various efforts have therefore been made in order to enhance the probability of discovering novel compounds. Such efforts include rare actinomycetes, targeted screening and the use of novel fermentation conditions [34]. In this context, we developed screening strategies on the basis of multiple antibiotic resistance of actinomycetes.

Antibiotic production by actinomycetes is strain-specific and conditional. It has long been known that there are actinomycete strains belonging to the same species that produce antibiotics different from one another, and also that there are strains belonging to different species that produce the same antibiotics. Antibiotic production is, therefore, not species-specific, but strain-specific. There should be specific genotypes conferring strain-specific antibiotic production. Gene clusters for antibiotic biosynthesis may be regarded as representing such strain-specific genotypes. The gene clusters contain self-resistance (resistance to the cell's own antibiotics) genes [5]. Therefore, if specific phenotypes due to the expression of the self-resistance genes are identified and can be easily detected, they must be of great help in antibiotic screening. Usually, one may regard the resistance to own antibiotics as the specific phenotype conferred by the resistance gene. However, the resistance to specific antibiotics is not specific to strains which produce them, because there are many strains with multiple antibiotic resistance; for example, there are many streptomycin (SM)-resistant actinomycetes other than SM-producers. In this context, we have been studying actinomycete strains in terms of the relationship between aminoglycoside antibiotic (AG) resistance and AG productivity. Consequently, we discovered that AG-producers show multiple AG-resistance the patterns of which are individual and specific to the producer. This finding led us to develop a novel screening strategy based on multiple AG-resistance. In addition, we were aware that unique AG-resistance patterns could be useful for genetic manipulation as selective markers. In this short review, diversity in AG-resistance of actinomycetes and our approaches to develop novel strategies in search for new antibiotics are described and discussed.

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Received 11 March 1996; accepted 24 June 1996

Diversity in AG-resistance of actinomycetes

Ranges and profiles of AG-resistance

We accidentally learned that AG-producers generally exhibit multiple AG-resistance the profiles of which are distinct. When we discovered istamycins (ISMs) as new AG antibiotics belonging to the astromicin (ASTM) group, the producer *Streptomyces tenjimariensis* SS-939 (ATCC 31603) was isolated as a kanamycin (KM)-resistant strain [12]. This led us to question why this strain did not produce antibiotics in the KM group but in the ASTM group. We then examined the strain together with other AG-producers for their resistance to a variety of AGs. It turned out that they were resistant not only to their own antibiotics but also to some other AGs; namely they showed multiple AG-resistance. It was noted that their multiple AG-resistance patterns were individual so that identification of the type of AGs they produce might be possible on the basis of their AG-resistance patterns.

In order to check this point as well as to survey the diversity of their AG-resistance, we isolated actinomycete strains from soils by the use of agar plates with or without addition

of AGs (20 μg ml^{-1}), and examined their AG-resistance to 11 different AGs at 50 μg ml^{-1} [13]. As shown in Table 1, the incidence of multiple AG-resistance was high in strains isolated on the AG-added plates, but low in strains isolated on the AG-free plates. For comparison, numbers of ISP cultures (international reference strains of *Streptomyces*) were also examined and consequently a low incidence of multiple AG-resistance was revealed. As a whole, wide varieties of multiple AG-resistance were found in the 440 soil isolates tested. They included strains with resistance to AGs ranging from 1 to 11 AGs in which about 150 distinct resistance patterns were distinguished. When the resistance ranges were arbitrarily divided into three groups (narrow, middle and wide referring to the resistance to 1–3, 4–7 and 8–11 AGs, respectively), the narrow, middle and wide groups included at least 33, 85 and 30 distinctive resistance patterns, respectively. The middle group included the widest varieties of resistance patterns and most of the AG-producers. On the other hand, 21, 13 and 8 varieties were distinguished in the narrow, middle and wide groups, respectively, in the 188 ISP cultures including the producers of SM, KM, neomycin (NM), paromomycin (PRM), spectinomycin (SPCM) and tobramycin (TOB). Thus, the soil isolates showed numbers of AG-resistance patterns which were not found in the ISP cultures. It was also notable that AG-resistance patterns of strains with indistinguishable morphology (surface growth including color) showed varieties. Taken together, it seemed likely that the number and variety of multiple AG-resistant strains which had been screened in the conventional approaches were rather limited, and therefore there remain unexplored ones with productivity of novel antibiotics. It was thus confirmed that there existed AG-producer specific resistance patterns [14], and indeed, the use of a variety of AGs was advantageous for isolation of wide varieties of multiple AG-resistant actinomycetes.

Emergence of additional AG-resistance due to the activation of a cryptic gene

In an SM-producing strain, *S. griseus* SS-1198, we discovered a silent or cryptic AG-resistance gene which, when activated, resulted in the emergence of additional AG-resistance [15,23,49,50]. This was first observed when we attempted a protoplast fusion between the strain and the ISM-producing *S. tenjimariensis* SS-939 [49]. Then, protoplast regeneration of *S. griseus* SS-1198 resulted in the emergence of the additional AG-resistance [50].

Resistance mechanism

As shown in Table 2, AG-modifying (or inactivating) enzymes and ribosome (target of AG)-resistance have been characterized as two major biochemical bases for AG-resistance [1,4,5]. Various AG-resistance genes have been cloned from AG-producers and characterized at the molecular level [5,38,39]. We characterized the biochemical basis of AG-resistance of AG-producers. First, we examined ISM-producing *S. tenjimariensis* SS-939 and demonstrated its ribosome-dependent AG-resistance [46,47]. Subsequently, ribosomal AG-resistance was reported in the producers of gentamicin (GM) [35,36], TOB [41,42,48] and ASTM group [10,32], and demonstrated to be due to methylation of 16S rRNA [10,32,36,41,42]. On the other hand, AG-modifying enzymes such as aminoglycoside phosphotransferase (APH) and aminoglycoside acetyltransferase (AAC) have also been shown to contribute to multiple AG-resistance in AG-producers; both APH(3′) and AAC(3) in the producers of ribostamycin (RSM), NM and PRM, APH(6) and APH(3″) in the SM-producer, AAC(2′) in the producers of SPCM, TOB and NM, and AAC(6′) in the KM-producer (Table 2). It should be noted that no AAD (aminoglycoside adenylyltransferase) has been reported in actinomycetes. The lack of AAD may set actinomycetes apart from clinically occurring AG-resistant bacteria in which various types of AAD have been reported [39].

The multiple AG-resistance of AG-producers is usually due to the substrate specificity of AG-modifying enzymes and/or the resistance specificity of ribosomes as the self-resistance determinants. All the strains with the same antibiotic productivity and morphology turned out to show the same pattern and biochemical basis of AG-resistance. In SM-producing *S. griseus* [30] and ASTM (= fortimicin A)-producing *Micromonospora olivasterospora* [6,8], self-resistance genes are located in the gene clusters for the biosynthesis of SM and ASTM, respectively.

In some AG-producers, there exist additional resistance determinants which do not contribute to the resistance to their own antibiotics. We demonstrated that *S. griseus* SS-1198, *S. kasugaensis* MB273 and *S. tenebrarius* ISP5477, which produce SM, kasugamycin (KSM) and TOB, respectively, contained a cryptic AAC(3) [15,21,23,24], a novel AAC(2′) [15,19] and an APH(6) [48], respectively. The cryptic gene designated *kan* was activated by one base substitution (C → T) at the first (-12) letter of the -10 promoter sequence, resulting in the synthesis of an AAC(3) with a unique substrate specificity. Consequently, *S. griseus* SS-

Table 1 Resistance range of fresh soil isolates and ISP cultures of actinomycetes

	Isolation medium[a]	Strains tested	AG-resistance group[b]			
			Sensitive	Narrow	Middle	Wide
Soil isolates	AG	363	4.4%	24.0%	47.9%	23.7%
	Non-AG	77	36.4	46.8	15.6	1.3
ISP cultures	?	188	51.6	33.0	7.4	8.0

[a]Soil suspensions were spread on inorganic salts–starch agar (ISP No. 4) medium with or without addition of AGs (20 μg ml^{-1}).
[b]Cultures were incubated at 27°C for 7 days on ISP No. 4 medium added with one of the following AGs (50 μg ml^{-1}): SM, KM, DKB, GM, RSM, butirosin, NM, PRM, lividomycin, neamine and ISM-A. Sensitive, narrow, middle and wide refer to resistance to 0, 1–3, 4–7 and 8–11 AGs, respectively.

Table 2 Biochemical basis of resistance to aminoglycoside-producers

Organisms	Primary resistance				Secondary resistance		
	Own AG	APH	AAC	Ribosome^R	Foreign AG	APH	AAC
Streptomyces griseus	Streptomycin	APH(6) APH(3″)	–	+	Kanamycin	–	AAC(3)
S. fradiae	Neomycin	APH(3′)	AAC(3) AAC(2′)	–			
S. ribosidificus	Ribostamycin	APH(3′)	AAC(3)	–			
S. rimosus forma *paromomycinus*	Paromomycin	APH(3′)	AAC(3)	–			
S. spectabilis	Spectinomycin	+	AAC(2′)				
S. kasugaensis	Kasugamycin	–	+	–	Astromicin	–	AAC(2′)
S. kanamyceticus	Kanamycin	–	AAC(6′)	+			
S. tenebrarius	Tobramycin	–	AAC(2′) AAC(6′)	16S rRNA	Streptomycin	APH(6)	–
Micromonospora purpurea	Gentamicin	–	–	16S rRNA			
M. olivasterospora	Astromicin	–	–	16S rRNA			
S. tenjimariensis	Istamycin	–	–	16S rRNA			
S. sannanensis	Sannamycin	–	–	16S rRNA			
Saccharopolyspora hirsuta	Sporaricin	–	–	16S rRNA			
Dactylosporangium matsuzakiense	Dactimicin	–	–	16S rRNA			

Adapted from Hotta *et al* [15]. Information on ribosomal resistance was obtainable from Piendl *et al* [36], Holmes *et al* [10], Ohta *et al* [31,32] and Nakano *et al* [29].

1198 which is originally resistant only to SM became resistant to KM, DKB, GM, PRM and NM in addition to SM [15]. The *S. kasugaensis* AAC(2′) plays a critical role for the resistance to RSM, ASTM and some other AGs having 2′-NH_2 such as ISM, DKB, NM and PRM [15,19]. *S. kasugaensis* MB273 contains two other types of AAC, one of which is specifically responsible for acetylating the 2′-NH_2 of its own antibiotic, KSM (Hirasawa *et al*; JP (Japan Patent) A0523187). This AAC gene has no significant homology with the ASTM-acetylating AAC(2′) gene (unpublished).

Based on these findings, Hotta *et al* [15] proposed the concept of secondary AG-resistance which depends on non-self-resistance determinants to distinguish it from primary resistance which depends on self-resistance determinants.

Distribution of phenotypes and genes of AG-resistance

Among AACs and APHs, the APH(6) and AAC(3) genes of SM-producing strains of *S. griseus* were investigated for their distribution in *Streptomyces* strains [20]. DNA sequences homologous to the APH(6) gene were present specifically in the SM-producing strains of *S. griseus*. On the other hand, the AAC(3) gene (*kan*) showed distinct homology with all the DNAs from all the *S. griseus* strains tested, but not with DNAs from other species. Thus, it seemed likely that the SM gene cluster containing the APH(6) gene is specific to the SM-producing strains and the AAC(3) gene is specific to the species of *S. griseus*. There is an exception concerning the AAC(3) gene; the AAC(3) gene of a PRM-producer shows 76% and 85% identities at the nucleotide and amino acid levels with the *S. griseus* AAC(3) [24].

Ribosomal resistance to AGs is widely spread among *Micromonospora* spp which produce AGs such as GM and ASTM [26]. Producers of ASTM-group AGs include *S. tenjimariensis* ATCC 31603 (ISMs), *S. sannanensis* IFO 14239 (sannamycin), *M. olivasterospora* ATCC 21819 (ASTM), *Micromonospora* sp ATCC 31580 (SF2052), *Saccharopolyspora hirsuta* ATCC 20501 (sporaricin) and *Dactylosporangium matsuzakiense* ATCC 31570 (dactimicins) as shown in Table 2. M Hasegawa and his coworkers cloned and characterized the gene cluster for ASTM biosynthesis from *M. olivasterospora* as well as ASTM resistance genes (*fmr* genes) from all the above ASTM group producers [6,8,31,32]. They demonstrated two types of *fmr* genes in the producers. The ones cloned from the producers of ISM, sannamycin and sporaricin were homologous with one another, but not homologous with the *fmr* genes from the producers of ASTM, dactimicin and SF-2052, although the organization of their biosynthetic gene clusters are similar. Phenotypically, the two types of *fmr* genes are similar in conferring resistance to ASTM and KM, but different in conferring resistance to GM and NM (the former *fmr* genes conferred resistance to NM but not to GM, and the latter *fmr* genes vice versa).

It should be noted that multiple AG-resistance is spread not only among AG-producers but also among AG-nonproducers. For example, strains with the same AG-resistance pattern due to ribosomal resistance as that of *S. tenjimariensis* were found among the soil actinomycete isolates, although they did not show antibiotic productivity under the conditions we used (unpublished). In addition, multiple AG-resistant strains include rare actinomycetes; for example, *Saccharothrix cryophilis* [25] (initially classified as *Nocardiopsis mutabilis* subsp *cryophilis* [43]).

Exploitation of multiple AG-resistance in searching for novel antibiotics

Antibiotic productivity of multiple AG-resistant strains

Approximately 200 strains were selected at random from soil actinomycete isolates with or without multiple AG-resistance and examined for their antibiotic productivity by incubating them in a starch–soy bean meal medium [14]. AG-resistant strains (about 170 strains) showed as high as 63% probability of antibiotic production; especially the middle and wide resistance groups showed a very high probability (>70%) of antibiotic production. All the AGs except SM were found in the middle and wide groups (Table 3). The middle group provided the widest varieties of AGs at a very high hit rate of about 15% which we had never experienced in our conventional screening. On the other hand, the sensitive and narrow groups showed high rates (40 and 50%, respectively) of antibiotic production. However, SM was the only AG found in the narrow group and no AG was found in the sensitive group. The majority of known AG-producers (stock cultures) fell into the middle and wide groups (Table 3). The soil isolates identified as AG producers were indistinguishable in their AG-resistance pattern and morphology from those of the known AG-producers. It was thus concluded that there exist specific AG-resistance patterns correlating with the productivity of specific AGs (Table 4 illustrates some of them). In a search for AGs, it is undoubtedly advantageous to screen multiple AG-resistant strains and new AGs will be found in strain(s) with novel AG-resistance patterns.

Antibiotics other than AGs found in the 200 isolates screened were, for example, amicetin, negamycin, ristocetin, streptothricins and viomycin, which are water soluble, basic compounds like AGs, and mycinamicin (macrolide).

AG-directed screening

Cross gradient AG plate for efficient isolation of AG-resistant strains: The use of varieties of AGs as the selective pressure is advantageous for isolation of wide varieties of multiple AG-resistant actinomycetes [13]. However, we are often met with the following problem; the population of multiple AG-resistant actinomycetes is rather sparse, whereas there are numbers of AG-resistant bacteria that outgrow AG-resistant actinomycetes. To solve this problem, Hotta *et al* devised the cross gradient plate method (Figure 1) in which two kinds of selective pressure substances (two AGs in this case) form cross density gradients [17]. On this plate, one can expect the effect of each one of the antibiotics at both ends of the plate and their combination effect at the center part of the plate. Figure 1 shows typical colony formation on the plate; dense-populated colonies at both ends and restricted numbers of colonies at the center part. This isolation method allowed us to isolate strains with a variety of multiple AG-resistance from soil suspensions with dense microbial populations.

Elimination of strains with known AG-resistance patterns: To enhance the chance of finding novel antibiotics, it is important to avoid repetitive screening of strains that produce known antibiotics as well as to distinguish unexplored strains that may have a higher possibility of novel antibiotic production. In this regard, determination of AG-resistance patterns of strains is useful. Based on our findings, we preferentially selected and screened hundreds of strains (including bacteria) with novel AG-resistance patterns for their ability to produce antibiotics. As a result, the selected strains were found to produce siso-

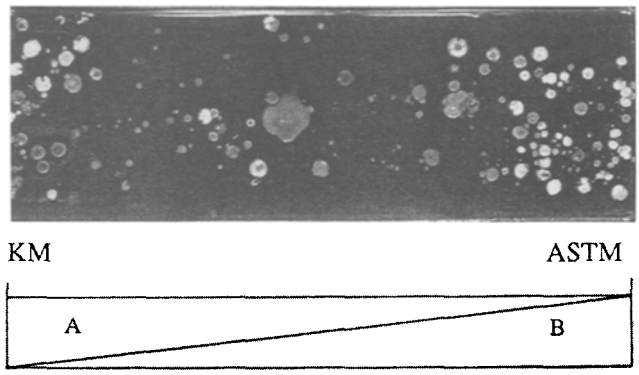

Figure 1 Cross aminoglycoside density gradient plate. Kanamycin (KM) and astromicin (ASTM) were added at 25 μg ml^{-1} into A and B sides of the plate, respectively.

Table 3 Relationship between antibiotic production and aminoglycoside resistance range

Resistance group[a]	Soil isolates			Stock cultures	
	Strains tested	Antibiotic producers	Aminoglycoside antibiotics found	Aminoglycoside antibiotic producers	
Wide	34	76.5%	Spectinomycin	Spectinomycin	*S. spectabilis* ISP5512
				Tobramycin	*S. tenebrarius* ISP5477
Middle	66	72.2%	Gentamicins	Gentamicins	*M. purpurea* KCC-0074
			Istamycins	Istamycins	*S. tenjimariensis* SS-939
			Neomycins	Neomycins	*S. fradiae* ISP5063
			Ribostamycin		
			2-Aminotrehalose		
			4-Aminotrehalose		
Narrow	69	49.3%	Streptomycin	Paromomycins	*S. catenulae* ISP5258
Sensitive	33	42.4%		Streptomycin	*S. griseus* ISP5236

[a]Sensitive, narrow, middle and wide refers to resistance to 0, 1–3, 4–7 and 8–11 AGs, respectively.

micin and 6‴-deamino-6‴-hydroxyparomomycin not previously isolated in our laboratory and two novel antibiotics, dopsisamine [43] and bagougeramine (a bacterial product) [44].

AG-targeted assay system using AG-producers as test organisms: Bacterial strains with specific resistance and/or sensitivity to the target antibiotics are often used as test organisms for speculation of specific classes of antibiotics. In this regard, AG producers are individually resistant or sensitive to certain types of AGs as shown in Table 4. They can grow quite fast on nutritionally rich media such as malt extract–yeast extract agar (ISP No. 2) so that their growth can be distinctive after 1 day incubation at 27°C. Based on these, we established an AG-targeted assay system using AG-producers as the test organisms (Table 4), which allowed us to identify AGs formed in fermentation broths. For example, antibiotics in a fermentation produced by strain X was speculated as follows. The culture broth filtrate of strain X showed the same antibiotic spectrum as that of PRM, and strain X itself showed the same AG-resistance pattern as that of a PRM producer, *S. catenulae* ISP 5258 (Table 4). These results indicated the formation of PRM or its derivative, and structure determination revealed that X substance was 6‴-deamino-6‴-hydroxyparamomycins I and II. Thus, this AG-targeted assay system using AG-producers as test organisms verified its usefulness.

The other success was the use of another assay system using two strains of actinomycetes, *S. griseus* SS-1198 and an unidentified strain SS-1878, as test organisms. The former is resistant to wide varieties of antibiotics, but sensitive to AGs except for SM, and the latter showed a very wide antibiotic resistance, but specifically sensitive to ASTM group AGs (unpublished). Antibiotics active upon both strains were only ASTM-group antibiotics. Therefore, we expected that if any strain showed activity to both test organisms, its active substance would be in the ASTM-group or a novel one. A soil isolate, TS-1980, in spite of a weak activity upon cup assay, was thus selected from multiple AG-resistant strains. Physicochemical characterization of its fermentation product revealed a novel polyamine-type antibiotic named dopsisamine [44]. Taxonomic characterization identified the strain as a new species of a rare actinomycete, *Saccharothrix cryophilis* [25,43].

Our approach to discovering novel AGs can be regarded as a total AG-directed screening system summarized as follows: 1) isolation of AG-resistant actinomycetes using the cross AG-gradient plate; 2) diagnosis of AG-resistance patterns of the isolates; 3) selection of strains with novel AG-resistance patterns; 4) determination of antibiotic spectrum by an AG-identification system using AG-resistant actinomycete strains as test organisms; and finally 5) physicochemical characterization of the products.

Manipulation of multiple AG-resistant strains for novel antibiotic production

Interspecific protoplast fusion: As described so far, strains with multiple AG-resistance showed a very high probability of antibiotic production. In addition, strains which produce different antibiotics differed in their AG-resistance pattern from one another. Therefore, we expected that if mutant or recombinant strains which express novel AG-resistance patterns are obtained by genetic manipulation, they may have a high possibility of novel antibiotic production. Accordingly, we attempted an interspecific protoplast fusion between non antibiotic-producing mutants of *S. griseus* SS-1198 and *S. tenjimariensis* SS-939 (producers of SM and ISM, respectively). These strains were chosen because their patterns and biochemical mechanisms of AG-resistance had already been clarified and their resistance patterns to SM and KM are reciprocal to each other. Therefore, clones exhibiting resistance to both KM and SM were targeted after the fusion. Consequently, KM^rSM^r clones with antibiotic productivity emerged. Among them, *Streptomyces* strain SK2-52 was selected as the one showing the most stable productivity as well as a unique AG-resistance pattern and analyzed for its antibiotic. It turned out that the strain produced a novel antibiotic named indolyzomycin the structure of which was unrelated to the antibiotics of the parent strains [48]. The follow-up investigation revealed that *Streptomyces* SK2-52 exhibited taxonomic properties falling into those of *S. griseus* and its acquired AG-resistance was not derived from *S. tenjimariensis* but due to the activation of an AAC gene of *S. griseus* SS-1198 [18].

Table 4 Aminoglycoside targeted assay system using aminoglycoside-producers

Organisms	Antibiotics produced	Antibiotic resistance[a]									
		SM	KM	GM	RM	BT	NM	PR	LV	IS	X
S. tenjimariensis SS-939	Istamycins		⊕		⊕	⊕				⊕	
S. kanamyceticus ISP5500	Kanamycins	⊕	⊕		⊕						
S. fradiae ISP5063	Neomycins		⊕		⊕	⊕	⊕	⊕			⊕
Micromonospora sp SS-1853	Gentamicins		⊕	⊕						⊕	
S. catenulae ISP5258	Paromomycins				⊕			⊕			⊕
S. griseus ISP5236	Streptomycin	⊕									
Strain X					⊕			⊕			⊕

[a]Resistance to 50 μg ml^{-1} of the following AGs; SM (streptomycin), KM (kanamycin), GM (gentamicin), RM (ribostamycin), BT (butirosin), NM (neomycin), PR (paromomycin), LV (lividomycin), IS (istamycin B). ⊕ and no indication refer to resistant and sensitive, respectively.

Conversion of known AG antibiotics: Novel antibiotics have been obtained by direct biosynthesis [7], mutasynthesis [7,37] or gene technology-based hybrid biosynthesis [11]. The underlying basis for these achievements can be regarded as the low substrate specificity of biosynthetic enzymes. In this context, conversion of known antibiotics to novel molecules with the use of known AG-producers has been carried out; combimicins from KMs A and B by a GM-producing strain of *Micromonospora* [33], and amikacin from KM-A by a butirosin-producing strain of *Bacillus circulans* [2]. We also carried out the conversion of fortimicin B (an intermediate of ASTM = fortimicin A) by *S. tenjimariensis* SS-939 with expectation of formation of 1-epidactimicin as a novel antibiotic, since *S. tenjimariensis* SS-939 has a biosynthetic pathway similar to that of the ASTM-producer *M. olivasterospora* and exhibits epimerization activity of 1-NH_2 which the ASTM-producer lacks [16,28]. As expected, fortimicin B was converted to 1-epidactimicin (dactimicin is 2″-*N*-formimidoyl ASTM).

Perspective

According to a recent survey [27], over 70% of novel bioactive substances of microbial origin in the decade 1984–1993 were derived from actinomycetes. We believe that actinomycetes will continue to play a major role in providing novel bioactive substances in the next decade. However, considering that the probability of finding novel metabolites is increasingly declining as the known metabolites increase, innovative methods will be required in order to enhance the probability. Accordingly, a new basis will need to be established for isolating and distinguishing strains with potential productivity of novel bioactive metabolites.

Isolation and selection of actinomycetes have been, and still are, dependent on taxonomists who have special diagnostic eyes or experiences in terms of the cultural property and morphology of actinomycetes. Over the last decade, they have shown increasing interests in rare actinomycetes as a potential source of bioactive secondary metabolites. They developed methods to isolate wide varieties of rare actinomycetes [9,40] and were successful in finding numbers of novel bioactive substances. Taxonomists will keep playing important roles for screening of actinomycetes in the next decade. However, a taxonomic approach contains the following limitation: taxonomic characterization remains at the species level, although the production of antibiotics and other secondary metabolites is strain-specific. Therefore, a new basis will be required to distinguish isolates at the strain level in terms of potential productivity of novel compounds. In this context, isolation and characterization of actinomycetes on the basis of multiple AG-resistance can be regarded as a new basis that meets the requirement.

For finding other new bases, primary metabolism should be considered as a key. It is obvious that supply of primary metabolites as precursors is necessary for biosynthesis of secondary metabolites. Therefore, it seems likely that the primary metabolism of antibiotic producers is adequately regulated so as to supply precursors for biosynthesis of their specific secondary metabolites. In the case of AG-producers, their primary metabolisms should be regulated so as to supply sugar precursors for AG biosynthesis. Similarly, producers of amino acid-related antibiotics and polyketides such as macrolides will have specifically regulated primary metabolism to supply precursors for their own products. Therefore, if phenotypes relating to characteristic primary metabolism are found, they will form a good basis for screening novel secondary metabolites.

In this regard, Hotta *et al* [22] investigated resistance to amino acid analogues (AAA) of actinomycetes, based on the following consideration; in bacteria it has been known that mutants with increased productivity of specific amino acids are obtainable by selecting mutants with specific AAA resistance [3]. In such mutants, increased accumulation of a specific amino acid occurs as the result of deregulation of feedback inhibition. Based on this, Hotta *et al* examined producers of amino acid-related antibiotics for their AAA resistance and found that the producers showed multiple AAA resistance. Subsequently, hundreds of AAA-resistant actinomycetes were isolated and screened for novel metabolites, and consequently several novel bioactive metabolites including a diketopiperadine, a depsipeptide and a cyclic peptide with a fatty acid side chain were found [22]. Their structures suggested that they were biosynthesized via amino acid pathways.

Therefore, it seems likely that primary metabolism is an emerging key for establishing a new basis for searching for novel metabolites. In actinomycetes, however, primary metabolism has been studied rather poorly, compared to secondary metabolism. Therefore, biochemical and genetic studies on primary metabolism will be necessary for finding a new basis for detecting novel metabolite producers.

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