MULTIPLE RESISTANCE TO AMINOGLYCOSIDE ANTIBIOTICS IN ACTINOMYCETES

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Actinomycetes were characterized in terms of resistance to 11 different aminoglycoside antibiotics (AGs). Strains freshly isolated in AG containing media showed wide varieties of multiple AG resistance, while the majority of ISP (International Streptomyces Project) cultures and the actinomycete strains isolated in an AG free medium were susceptible to all or most of the AGs tested. Marked characteristics were noted in multiple AG resistance of gray and yellow colored actinomycetes and AG-producing strains. In gray colored isolates, multiple resistance to kanamycin A, dibekacin, ribostamycin, butirosin A, istamycin A and neamine was often observed. Yellow colored isolates having multiple AG resistance were mostly resistant to neamine, ribostamycin and streptomycin and, to a lesser extent, istamycin A, dibekacin and butirosin A. Most of the AG producers tested showed unique multiple AG resistance patterns.

We have investigated actinomycetes which produce aminoglycoside antibiotics (AGs) in terms of multiple AG resistance as well as self-resistance, and clarified the mechanisms of multiple AG resistance in strains which produce istamycins[1], kanamycin[2], neomycins[2] and nebramycins[3]. It still remains, however, to clarify whether multiple AG resistance is generally limited to AG producers or whether AG producers have certain specific AG resistance profiles distinct from those of actinomycetes which do not produce AGs. Accordingly, we attempted to survey the distribution of AG resistance in actinomycetes, using fresh soil isolates (mainly streptomycetes), ISP cultures and several known AG producers. This paper describes characteristics of multiple AG resistance observed in actinomycetes isolated in AG containing media and AG producing strains. The AG resistance patterns of the AG producers were so characteristic that it may be likely to predict the AG produced by them.

Materials and Methods

Actinomycete Strains

Actinomycetes were isolated from soils (about 150 samples) collected in Japan (mainly at Tenjin Island, Miura peninsula, Kanagawa prefecture) from June 1979 to April 1980, bimonthly. Soil suspensions in 0.85% NaCl solution were plated on ISP No. 4 medium with or without addition of 20 μg/ml of any one of AGs in Table 1 and 25 μg/ml of both cycloheximide and nystatin and incubated at 27°C.

ISP cultures and known antibiotic producers were those in our laboratory collection.

Examination of Resistance to AGs

Actinomycete strains or isolates growing on ISP No. 4 slants incubated at 27°C for 10~14 days were scraped, transferred to a small volume of 0.85% NaCl solution and then dispersed with a tissue grinder to approximately 10^6 cfu/ml. About 10 μl of the suspension was inoculated on ISP No. 4 agar medium in holes of a micro tissue culture plate (12×8 holes; Nunc, Denmark), each of which contained 50 μg (as free base)/ml of one out of the following 11 AGs; streptomycin sulfate (SM), kanamycin A sulfate (KM), dibekacin (DK), gentamicin C sulfate complex (GM), ribostamycin (RM), butirosin A

Table 1. Effect of AGs on the isolation of actinomycetes resistant to AGs.

AGs used for isolation	Isolates*	Ratio of isolates (%)**			
		Susceptible	Narrow	Middle	Wide
None	77	36.4	46.8	15.6	1.3
SM	74	4.1	40.5	41.9	13.5
NM	61	9.8	24.6	41.0	24.6
NE	54	3.7	31.5	42.6	22.2
KM	51	0	11.8	64.7	23.5
RM	47	4.3	29.8	55.3	10.6
BT	34	8.8	11.8	61.8	17.6
GM	24	0	0	41.7	58.3
LV	9	0	11.1	22.2	66.7
PR	9	0	0	37.5	62.5

* Data were summarized using actinomycete isolates from soils collected at Tenjin island.

** Actinomycete isolates were divided into 4 groups (susceptible, narrow, middle and wide) according to the numbers of AGs to which they showed resistance. Susceptible, narrow, middle and wide referred to resistance to none, 1~3, 4~7 and 8~11 of AGs, respectively.

(BT), neomycin B sulfate (NM), paromomycin (PR), lividomycin A sulfate (LV), neamine (NE) and istamycin A sulfate (IS). The antibiotics were obtained from IMC (Institute of Microbial Chemistry) Collection of Antibiotics. Growth was observed after 7 day incubation.

Results

Effect of AGs on the Isolation of AG-Resistant Actinomycetes

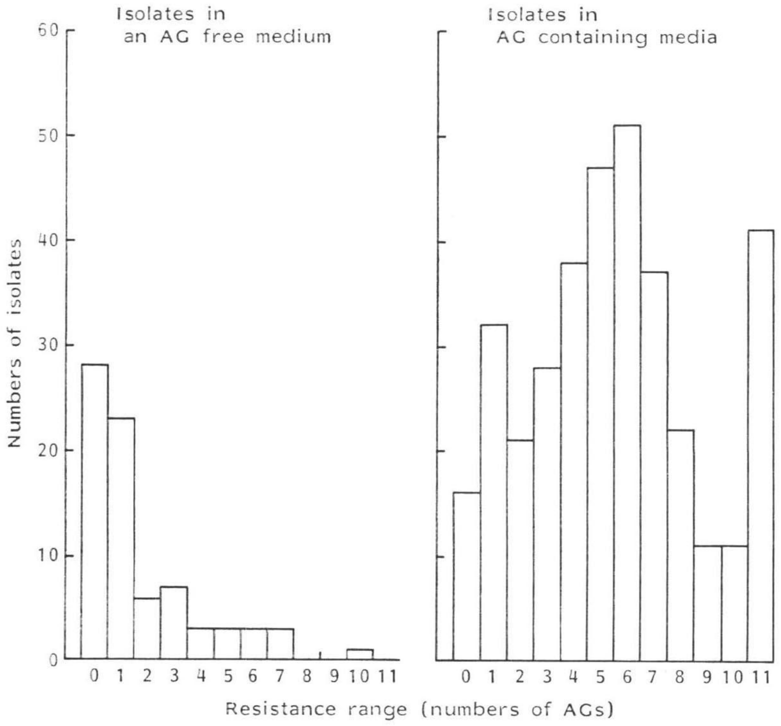
In order to isolate various AG resistant actinomycetes, ISP No. 4 agar medium with or without the addition of various kind of AGs (mainly 9 AGs listed in Table 1) was used. When 20 μg/ml of any one AG was added to the medium, actinomycete colonies appeared at a frequency of about $10^{2\sim3}$ per gram of soil. The numbers of colonies appeared were relatively large in the presence of SM, KM, RM, NM or NE and small in the presence of GM, PR or LV. Colony morphology varied depending on the AG added. On the other hand, in the AG free medium, actinomycete colonies averaged 10^4 per gram of soil.

The actinomycete colonies grown under the above conditions were then picked at random and examined for resistance to 50 μg/ml of 11 AGs. As shown in Fig. 1, actinomycetes obtained from the AG free medium were largely resistant to none (28/77=36.4%) or one (23/77=29.9%) of 11 AGs tested. Actinomycetes resistant to a wide range of AGs were rarely obtained without addition of AG. On the other hand, actinomycete isolates obtained from the AG containing medium largely showed multiple AG resistance.

According to the numbers of AGs to which these actinomycetes showed resistance, they were divided into 3 groups (namely "narrow", "middle" and "wide" which referred to resistance to 1~3, 4~7 and 8~11 AGs, respectively) and the effect of AGs on their isolation was compared (Table 1). The ratio of AG-resistant isolates belonging to the three groups varied depending on the AG added to the isolation medium. The majority of the isolates from the medium containing SM, NM, RM, KM or BT showed the "middle" range of AG resistance. On the other hand, the majority of the isolates from the medium containing GM, PR or LV showed a relatively wide range of AG resistance.

It was rather surprizing that more than 60% of the isolates obtained from the AG free medium were

Fig. 1. Effect of AGs on the isolation of actinomycetes with different resistance range.



AG resistant (they were largely resistant to SM, RM or NM) although the numbers of colonies appearing on AG-containing media were about 10% or less than those appearing on the AG free medium. It seems likely that the larger inoculum size (10^6 cfu/ml) used for the resistance test caused this result. This might also be due to the fact that rapidly growing AG resistant bacteria existing in soil samples often covered the isolation plates before actinomycete colonies grow. This suggested that the wider the variety of AGs used, the wider the variety of AG resistant actinomycetes could be isolated.

Differences in AG Resistance between Soil Actinomycete Isolates and ISP Cultures

AG-resistant strains of both soil actinomycete isolates (435 strains) and known actinomycete strains (91 ISP cultures) were divided into three groups of AG resistance. The soil isolates were found to fall into all three groups whereas the ISP cultures mainly (about 70%) belonged to "narrow" group (Table 2). In order to further illuminate the difference in AG resistance between them, frequency of resistance to each AG was scored (Table 2). In the "narrow" group organisms, relatively large percentages were resistant to NE, SM, RM and IS in case of the soil isolates. The same results except for resistance to IS were obtained in ISP cultures. In the "middle" group, the soil isolates were mainly resistant to RM, NE, IS, DK, KM, BT and SM, while ISP cultures were mainly resistant to RM, NE, SM, BT, PR, LV and DK. Thus a significant difference between the isolates and the ISP cultures was noted in the frequency of isolates resistant to IS in the "narrow" group and in those resistant to IS, KM, PR and LV in the "middle" group. No marked difference was observed in organisms of the "wide" group.

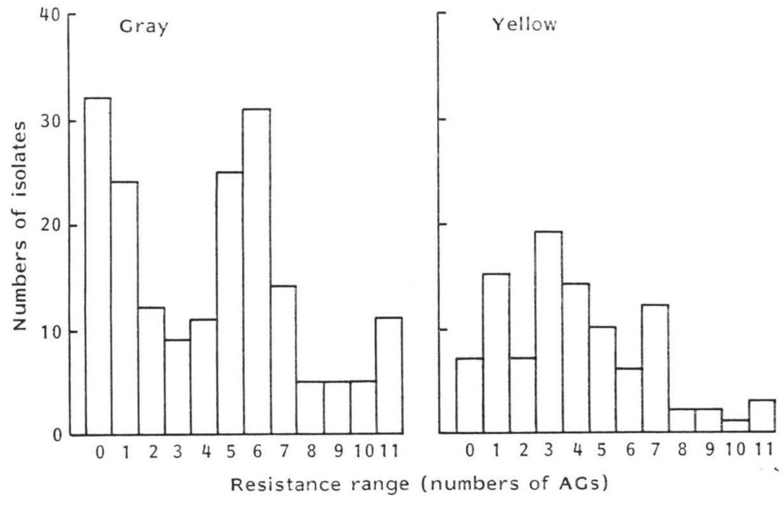
Table 2. Frequency of isolates resistant to each AG in actinomycetes resistant to certain ranges of AGs.

AGs	Frequency of resistants*					
	Isolates (435)			ISP cultures** (91)		
	Narrow (142) (%)	Middle (193) (%)	Wide (100) (%)	Narrow (62) (%)	Middle (14) (%)	Wide (15) (%)
RM	38.7	91.7	98.0	72.6	100.0	100.0
NE	59.9	90.2	98.0	45.2	86.0	100.0
SM	40.8	51.3	88.0	50.0	86.0	93.3
IS	22.5	75.1	96.0	4.8	14.3	100.0
KM	7.0	54.9	87.0	0	7.1	80.0
DK	6.3	60.6	86.0	0	35.7	86.7
BT	4.2	52.3	88.0	1.6	64.3	93.3
GM	4.9	20.2	81.0	1.6	14.3	86.7
NM	1.4	15.0	79.0	0	7.1	100.0
PR	1.4	19.2	92.0	4.8	42.9	100.0
LV	4.9	20.2	88.0	4.8	42.9	80.0

* Percentage of resistant isolates having resistance to each AG. Numbers in the brackets referred to AG resistant strains tested.

** Of 188 strains of ISP cultures examined, 91 strains showed resistance to specific ranges of AGs.

Fig. 2. Gray and yellow colored actinomycetes with different AG resistance range.



Resistance to AGs in Actinomycetes with Different Surface Growth Color

The isolates and ISP cultures were divided into gray, yellow, red and white groups on the basis of the color of their surface growth on ISP No. 4 agar medium in order to examine a possible relationship between color and AG resistance. It was noted that the majority of the gray isolates belonged to the middle group whereas most of yellow isolates belonged to the narrow and middle groups. Red and white colored isolates were distributed almost equally to each of three resistance groups. In ISP cultures, organisms belonging to these color groups were mainly found in the narrow resistance group.

Fig. 2 shows histograms of the total gray and yellow colored isolates as to their resistance range. Two peaks at resistance to none and to 6 AGs were observed in the gray colored isolates. The peak at resistance to 6 AGs was found to reflect the isolates having resistance to the following 6 AGs; KM,

Table 3. Frequency of isolates resistant to each AG in gray and yellow colored actinomycete isolates.

AGs	Frequency of resistants*					
	Gray (152)			Yellow (92)		
	Narrow (45) (%)	Middle (81) (%)	Wide (26) (%)	Narrow (41) (%)	Middle (43) (%)	Wide (8) (%)
NE	60.0	98.8	100.0	63.4	88.4	100.0
RM	37.8	98.8	100.0	43.9	95.3	100.0
SM	37.8	21.0	88.5	56.1	88.4	100.0
KM	8.9	79.0	88.5	4.9	14.0	62.5
DK	6.7	67.9	84.6	7.3	60.5	100.0
BT	4.4	74.1	88.5	2.4	46.5	100.0
IS	13.3	82.7	96.2	29.3	76.7	100.0
GM	4.4	9.9	80.8	0	27.9	75.0
NM	0	7.4	84.6	0	9.3	50.0
PR	2.2	6.2	84.6	0	9.3	87.5
LV	4.4	11.1	88.5	2.4	23.3	87.5

* Percentage of resistant isolates having resistance to each AG. Numbers in the brackets referred to AG resistant strains tested.

DK, BT, IS, NE and RM as shown in Table 3. In the yellow colored isolates, on the other hand, the middle group organisms were resistant mostly to NE, RM and SM and to a lesser extent, IS, DK and BT (Table 3). This indicated that there were considerable numbers of the yellow colored isolates which were resistant to DK but susceptible to KM.

Patterns of AG Resistance in Actinomycetes

Wide varieties of patterns of AG resistance were observed in actinomycetes, though detailed data are not shown here. Distinct patterns in the actinomycete isolates included at least 33 in the narrow group, 85 in the middle group and 30 in the wide range group. Thus the widest variety of patterns of AG resistance were recognized in the middle group isolates. In ISP cultures, on the other hand, 21, 13 and 8 varieties were distinguished in narrow, middle and wide resistance groups, respectively. Thus, soil actinomycetes which were isolated in AG containing media showed AG resistance spectra not found in the ISP cultures tested.

In Table 4, the resistance patterns of known actinomycete strains which produce AGs and other antibiotics are shown. All of the AG producers except SM producing *Streptomyces griseus* were multiply AG resistant. Each spectrum was unique. The same resistance patterns were observed in the actinomycete soil isolates. Most of them were AG producers and will be described in the following paper[4].

Discussion

It became evident in this study that soil actinomycetes isolated in AG containing media showed wide varieties of multiple AG resistance compared to those observed in ISP cultures. Especially marked characteristics were observed in gray and yellow colored actinomycetes having "middle" range AG resistance. Multiple resistance to 6 AGs (KM, DK, RM, BT, IS and NE) was often observed in gray isolates; yellow colored actinomycetes were mostly resistant to NE, RM and SM and to lesser extent, IS, DK and BT. These characteristics may be common to soil actinomycetes in Japan since subsequent surveys using soils collected at other locations have provided similar results. However, it is not known why AG resistance spectra were so different between gray actinomycetes and yellow ones.

Table 4. Antibiotic resistance of antibiotic producing actinomycetes.

Organisms	Antibiotics produced	Resistance* to 50 μg/ml of														
		Aminoglycosides											Others**			
		SM	KM	DK	GM	RM	BT	NM	PR	LV	NE	IS	TC	CP	EM	AC
Streptomyces tenebrarius ISP5477	Nebramycins	●	●	●	●	●	●	●	●	●	●	●	●			
S. spectabilis ISP5512	Spectinomycin	●	○	●	◑	●	●	●	●	●	●	●	●	●	●	●
S. kasugaensis MB273	Kasugamycin	●		◑		●	◑	●	●	●	●	●	NT***	NT	NT	NT
S. tenjimariensis SS-939	Istamycins		●	●		●	●				●	●				
S. kanamyceticus ISP5500	Kanamycins	●	●	●		●					●		◑	●		
S. fradiae ISP5063	Neomycins		○			●	○	●	●		●					
Micromonospora sp. SS-1853	Gentamicins		●	●	●							●				
S. catenulae ISP5258	Paromomycin					●			●		●		○	●		●
S. griseus ISP5236	Streptomycins	●												●	◑	◑
S. aureofaciens ISP5127	Chlortetracycline												●			
S. venezuelae ISP5230	Chloramphenicol												●	●		
S. omiyaensis ISP5552	Chloramphenicol													●		
S. antibioticus ISP5234	Actinomycins															●
S. erythreus ISP5517	Erythromycin														●	
S. griseinus ISP5047	Grisein	●				●					●				●	
S. griseochromogenes ISP5499	Blasticidins	●				●			●	●	●		●	●	●	●

* Solid circle, half solid circle, open circle and no indication refer to good growth, retarded growth, variable growth, and no growth, respectively.
** TC: tetracycline, CP: chloramphenicol, EM: erythromycin, AC: actinomycin D.
*** NT: Not tested.

Differences in AG resistance between actinomycetes and AG resistant bacteria of clinical origin was easily recognized. Many actinomycetes showed AG resistance patterns which were difficult to explain on the basis of the substrate ranges of known inactivating enzymes detected in AG resistant bacteria. Multiple resistance to KM, DK, RM, BT, IS and NE observed in IS producing *S. tenjimariensis* SS-939 and many gray colored actinomycete isolates was a case in point. No acetylating, phosphorylating or nucleotidylating enzymes with such substrate range have been reported in AG resistant bacteria of clinical origin. For instance, if resistance to IS of *S. tenjimariensis* SS-939 were dependent on an aminoglycoside acetyltransferase AAC(3) only which is known to inactivate IS, this strain should be resistant to GM also[5]; however *S. tenjimariensis* is susceptible to GM. Similarly, GM producing strains of *Micromonospora* sp. were found to be resistant to KM and DK but susceptible to NE although NE is a moiety of kanamycin B. Analyses of such resistance has revealed ribosomal resistance mechanisms[1,6] which have not been reported to these AGs in AG resistant bacteria of clinical origin.

With respect to inactivating enzymes, it was of interest that a large number of yellow colored actinomycetes showed multiple resistant to AGs including DK were susceptible to KM. This suggested a wide distribution of an aminoglycoside acetyltransferase, AAC(2′), in yellow colored actinomycetes. It was also notable that neither the isolates nor ISP cultures indicated nucleotidylation of AGs although acetylation and phosphorylation activities were easily detected (data not shown). These results were consistent with those reported by DAVIES and coworkers[7,8] and might be a characteristic of AG resistance in soil actinomycetes distinct from that found in clinically isolated bacteria.

It was noted that most of the known AG producers showed unique multiple AG resistance patterns but most of actinomycetes producing other antibiotics did not. Based on these observations, actinomycete isolates showed multiple resistant to AGs may be likely candidates for production of AGs. Furthermore, the fact that each AG producer showed an individual pattern of AG resistance suggests that specific resistance spectra might correspond to the production of specific AGs (new or predetermined) in actinomycetes. The relationship between AG resistance and AG productivity will be reported in the next paper[4].

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