A SCREENING METHOD TO IDENTIFY ANTIBIOTICS OF THE AMINOGLYCOSIDE FAMILY

GILLES ETIENNE, ELISE ARMAU†, MONIQUE DASSAIN and GÉRARD TIRABY*

Laboratoire de Microbiologie et de Génétique appliquées du CNRS,
CRBGC, Université Paul Sabatier,
118 route de Narbonne, 31062 Toulouse Cédex, France
†Laboratoire de Recherches CAYLA,
Avenue de Larrieu, 31094 Toulouse Cédex, France

(Received for publication February 27, 1991)

A simple and selective screening method was developed for detecting new aminoglycoside (AMG) antibiotics from actinomycetes strains propagated on solid culture media. The first phase of the screening program is designed to isolate AMG-type activities using: 1) two *Serratia marcescens* strains, one susceptible and one resistant to AMGs, 2) the high tolerance of AMGs to heat in acidic solutions and 3) the specific resistance of a streptothricin producing strain of *Streptomyces lavendulae* to streptothricin antibiotics. The second phase of the screening program identifies already known AMG antibiotics through the characteristic spectrum of action which each AMG shows toward a group of bacterial strains synthesizing various AMG-inactivating enzymes.

Aminoglycoside (AMG) antibiotics constitute a major class of therapeutic agents whose discovery dates from that of streptomycin in 1944; there are presently more than 200 natural compounds in the group[1]. All these antibiotics contain a cyclitol-type aglycone and glycosidically linked amino sugar, the AMG family being subdivided according to the structure and the mode of substitution in the cyclitol moiety. Actually, antibiotics belonging to all the different sub-groups of the AMG family have found a large range of applications and thus it seems likely that the probability of finding an application is higher for a new member of the AMG family than for all other families of antibiotics. Despite their relative toxicity in mammals[2], AMGs are widely used in human and veterinary therapeutics and in agriculture. Due to the broad spectrum of action of AMGs, no other antibiotic type, provides such comprehensive coverage of pathogenic microorganisms[1].

The recent development of new AMGs, isolated either from nature (*e.g.*, inosamycin[3] or boholmycin[4]) or by chemical modification of existing compounds (*e.g.*, arbekacin (habekacin[5]) or trospectomycin[6]) indicates continued interest in this important family of antibiotics. The development of new members of this group is necessitated by the appearance of pathogenic bacteria which have become resistant to AMGs through the acquisition of inactivating enzymes[7]. Alternatively, the discovery of a new AMG unrelated structurally to the already known AMGs will lead to the development of a new promising sub-group in this family, as with the fortimicin-type compounds[8].

In a program designed to screen for actinomycete strains synthesizing new AMG antibiotics, it is of prime importance, first to isolate AMG-producing strains, and then to identify as early as possible those strains producing already known AMGs. AMGs are water-soluble basic compounds very closely related to each other in their physico-chemical properties for which chromatographic system of identification have been developed. TLC[9] or HPLC[10] could be used to detect already known compounds. However, these analytical methods require previous knowledge of the nature of antibiotics under characterization, are

limited in the number of strains that can be handled and thus are not very useful in the early steps of the screening program. In contrast, microbiological methods, allow the analysis of a greater number of strains and are more attractive to carry out these first steps. Methods using bacterial strains showing differential resistance to AMGs have been previously described, as for example one using an AMG-hypersensitive mutant of *Klebsiella pneumoniae*[11]. An alternative methodology can exploit the relationship between multiple AMG-resistance and AMG-productivity of actinomycete strains to be able to predict their potential for production of new AMGs[12]. In addition, some assays to characterize AMGs can be inferred from the literature, using AMG-resistant transport mutants of *Pseudomonas aeruginosa*[13] or bacteria synthesizing AMG-inactivating enzymes which show a characteristic pattern of resistance to some AMGs[7]. Other methods to characterize AMGs can exploit some physico-chemical properties common to all the compounds belonging to this family. Salts[14] or acidic pH[15] have an antagonistic effect on the biological activities of AMGs. Another remarkable property of AMG is their high resistance to thermal inactivation[16].

We have combined these different properties to elaborate a screening procedure selective for AMG antibiotics. The first phase of the screen, *i.e.* isolation of strains producing AMG-type activity, use two *Serratia marcescens* strains, one a wild-type AMG-susceptible strain and the other a mutant strain specifically AMG-resistant. Streptothricin (STH) antibiotics, water soluble basic drugs which are frequently produced by *Streptomyces* strains[17] are differenciated from AMGs by using a STH-producing *Streptomyces lavendulae* strain specifically resistant to its own antibiotics. Characterization of AMG-type compounds can be achieved by determining the effect of heat treatment upon their biological activities. The second phase of the screen, *i.e.* identification of already known AMGs, is based on the fact that each AMG shows a characteristic spectrum of action on a group of bacterial strains synthesizing various AMG-inactivating enzymes.

Materials and Methods

Antibiotics

Xylostatin (Takeda Chemical Industries, Japan), seldomycin, micronomicin (sagamicin) and astromicin (fortimicin A, Kyowa Hakko Kogyo Co., Japan), verdamicin (Schering Corporation, U.S.A.), saccharocin (Eli Lilly Co., U.S.A.), streptothricin AY23484 (Laboratoires Ayerst, Canada) and streptothricins D and F (Rhône-Poulenc, France) were gifts from the corresponding company. Other antibiotics were obtained from commercial sources. The standard antibiotic solutions have the following concentrations. AMGs: 0.1 mg/ml H_2O, except for spectinomycin which was 1 mg/ml; STHs, β-lactams, tetracyclines, anthracyclines, quinolones, bleomycins, chloramphenicol, novobiocin, bacitracin, vancomycin and fosfomycin: 0.1 mg/ml H_2O; colistin and polymyxin B: 1 mg/ml H_2O; macrolides, ansamycins: 0.1 mg/ml 10% MeOH; polyethers: 0.1 mg/ml of MeOH; pristinamycins: 0.1 mg/ml 10% DMSO.

Bacterial Strains

Indicator bacterial strains used to identify AMGs, and actinomycetes strains producing AMGs and STHs are listed, respectively in Tables 1 and 4.

Culture Conditions and Media

The media for growth of the indicator bacteria were as follows: *Escherichia coli*, antibiotic No. 2 medium (BioMérieux, France), pH 8.5; *S. marcescens*, half-strength antibiotic No. 2 (no2/2) medium, pH 9.0; *Streptomyces tenebrarius*, *Streptomyces kanamyceticus* and *S. lavendulae*, Trypcase-Soy Agar (BioMérieux, France), pH 8.

Susceptibility Disk Assay Method

Just before use, 150 ml of appropriate medium were inoculated with 0.1 ml of an indicator bacterial

Table 1. Indicator strains for AMG identification.

Strain designation	AMG inactivating enzyme or phenotype	Source or ref
Serratia marcescens		
Sma 62	Wild-type strain	Soil isolate
St101	Streptomycin-resistant mutant	This paper
Sp11	Spectinomycin-resistant mutant	This paper
AG4410	AMG-resistant mutant	This paper
SM1065	AAC (6′)	KABINS and NATHAN[24]
Z677	Multi-resistant strain	Clinical isolates (Toulouse, France)
P585	Multi-resistant strain	Clinical isolates (Toulouse, France)
P657	Multi-resistant strain	Clinical isolates (Toulouse, France)
Escherichia coli		
C1a	Wild-type strain	P. COURVALIN (Paris, France)
K-12 J5/R112	APH (3′)-1	P. COURVALIN (Paris, France)
K-12 J5/R148	APH (3′)-2	P. COURVALIN (Paris, France)
C1a/pAT3	APH (3′)-3	P. COURVALIN (Paris, France)
K-12 J5/R135	AAC (3)-1	P. COURVALIN (Paris, France)
K-12 J5/R176	AAC (3)-2	P. COURVALIN (Paris, France)
W677/pJR225	AAC (3)-4, HPH	P. COURVALIN (Paris, France)
JR66/W677	APH	PERLIN and LERNER[25]
MP1	Derivative of JR66/W677	PERLIN and LERNER[25]
Bacillus subtilis		ATCC 6633

suspension prepared to give dense but not confluent growth. The medium was then gently mixed and poured into sterile plates (Nunc Inter-Med, 245 × 245 mm dishes for diffusion tests). 50 μl of the standard solution of each antibiotic were adsorbed onto 9 mm paper disks (SCHLEICHER and SCHÜLL, RFA) and duplicate disks were placed on the surface of the bacteria-seeded agar plates. The plates were held for 2 hours at 4°C in order to facilitate diffusion of the antibiotics and then were incubated overnight at either 27°C (*S. marcescens* and *Streptomyces*) or 37°C (*E. coli*). Plates were treated in the same way to determine the antibacterial activities of actinomycete strains by the agar piece method or the well method (see below).

Antibiotic Thermostability Determination

Thermostability of known antibiotics and that of unidentified antibiotics which were excreted into solid medium by producing organisms was determined by the same method. For known compounds, GAPY medium (glucose 1%, soluble starch 2%, yeast extract 0.5%, soya peptone 0.5%, $CaCO_3$ 0.1%, agar 1.5%, pH 7.2[18]) containing antibiotic at the standard concentrations were prepared, aliquoted into glass tubes and melted for 5 minutes in boiling water, before being adjusted with HCl to pH 3. The media were autoclaved 60 minutes at 120°C and then rapidly neutralized. Because the agar was hydrolyzed by heating under acidic conditions, the remaining antibacterial activity must be determined by the well method. A 5 mm diameter well was cut into the medium seeded with an indicator strain, and 50 μl of an antibiotic solution was deposited. An antibiotic solution (standard concentration) made up in GAPY broth served as control. All the tests were performed in duplicate.

Detection of Antimicrobial Activities from Actinomycetes Strains

Actinomycete strains were propagated on GAPY solid medium[18] and incubated at 27°C for 7 to 10 days. Antimicrobial activities were tested by the agar piece method[19].

Results

Characterization of AMGs

Indicator organism used to screen for AMG-producing bacteria must be highly susceptible to these antibiotics; moreover if this organisms is also resistant to a large variety of non-AMG antibiotics the

Table 2. Thermostability of AMG antibiotics in acidic solutions.

Antibiotics	Inhibition zone diameter (mm)		Antibiotics	Inhibition zone diameter (mm)	
	Untreated	Treated pH 3 60 minutes at 120°C		Untreated	Treated pH 3 60 minutes at 120°C
AMGs			AMGs		
4,5-Disubstituted 2-DOS:			4,6-Disubstituted 2-DOS:		
NEO	25.5	27.5	GEN	30	31
PAR	19.5	25	VER	28	29.5
LIV	27	29.5	SIS	27.5	30.5
BUT	29	31.5	NET	28	31.5
RIB	25	28.5	Monosubstituted 2-DOS:		
XYL	23.5	26.5	APR	22.5	24.5
4,6-Disubstituted 2-DOS:			HYG	14	19
KAN	30	30	Non-2-DOS diaminocyclitol:		
TOB	28.5	31	FOR	18	23.5
DIB	29	32	STR	28.5	30.5
AMI	31	32.5	SPE	30	29.5
STH AY 23484	20	15	Bleomycin	27	—

—: No inhibition zone around the disk.

Each antibiotic at the standard concentration was tested on *Bacillus subtilis* ATCC 6633 (antibiotic No. 2 medium, pH 9) by the well method (see Materials and Methods).

Abbreviations used: NEO, neomycin; PAR, paromomycin; LIV, lividomycin; BUT, butirosin; RIB, ribostamycin; XYL, xylostatin; KAN, kanamycin; TOB, tobramycin; DIB, dibekacin; AMI, amikacin; GEN, gentamicin; VER, verdamicin; SIS, sisomicin; NET, netilmicin; APR, apramycin; HYG, hygromycin B; FOR, fortimicin A; STR, streptomycin; SPE, spectinomycin; 2-DOS, 2-deoxystreptamine.

efficacy of the AMG-detection step of the screening program could be considerably improved. The strain *S. marcescens Sma* 62, isolated from a soil sample collected in Toulouse, France, and identified by the API20 Enterobacteriaceae micromethod (API system, France) meets these criteria: *Sma* 62 is a Gram-negative bacterium, naturally resistant to the anti-Gram-positive antibiotics.

MICs of AMGs toward *Sma* 62 range from 0.1 to 0.3 μg/ml with the exception of streptomycin (1 μg/ml) and spectinomycin and ribostamycin (3 μg/ml). STH (MIC: 1 μg/ml) and bleomycin (MIC: 0.1 μg/ml) act like AMGs towards this strain. Of 27 other non-AMG antibiotics, only mitomycin and rifampicin inhibit the growth of *Sma* 62, as determined by the susceptibility disk assay method. In particular this strain is completely insensitive to tetracyclines and β-lactams, two of the major families of broad-spectrum antibiotics.

A mutant strain of *Sma* 62, not susceptible to the AMGs, has been isolated after three rounds of mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and selection for cells resistant to increasing concentrations of AMG antibiotics. In order to obtain a wide range of cross-resistance to AMGs, different AMGs were used in each selection step. The mutant strain, AG4410, was found to be at least 100 times more resistant than wild-type strain *Sma* 62 to all 22 AMGs tested except for spectinomycin. The determination of the susceptibility of this strain to 29 non-AMG antibiotics showed no or only a slight decrease of MICs with the exception of the three STHs to which strain AG4410 was resistant. Therefore, use of both strains *Sma* 62 and AG4410 is not completely adequate to selectively characterize the AMGs since they cannot be differentiated from STHs.

We have examined the thermostability of AMGs to complete the characterization of these antibiotics. When heated, AMGs are particularly thermostable, especially in acidic conditions (Table 2). They retain

all of their biological activity against bacteria after autoclaving for 1 hour at 120°C, pH 3. STHs are only partially inactivated under these conditions while bleomycins completely loose their biological activity. Thus, AMG antibiotics can be discerned from the other water-soluble antibiotics due to their remarkable thermostability. Among other non-AMG antibiotics, only anthracyclines, novobiocin and ionophorous polyethers maintain their biological activities after heating in acidic solutions (data not shown), but they can be easily differentiated from AMGs as they act similarly on the strains *Sma* 62 and AG4410.

Discrimination of AMGs from STHs

Antibiotic-producing *Streptomyces* strains are resistant to the antibiotics they produce; for example, the nourseothricin-producer, *Streptomyces noursei*, is highly resistant to STHs and shows no cross-resistance to AMG antibiotics other than spectinomycin[20]. Comparatively, the STH producer, *S. lavendulae* ATCC 8664, is resistant to three STH antibiotics and susceptible to 22 AMG antibiotics tested. However, this strain is susceptible to a large variety of other antibiotics (*e.g.* tetracyclines, macrolides, ansamycins, bleomycins, novobiocin), which could prevent characterization of STHs if co-produced with these other antibiotics.

To palliate this objection, we decided to pair *S. lavendulae* with *S. tenebrarius* ATCC 17920 which is resistant to all AMGs[21]. This latter strain displays a susceptibility pattern similar to that of *S. lavendulae* for 29 non-AMG-antibiotics tested with the exception of bleomycins and novobiocin, to which *S. tenebrarius* is resistant. *S. tenebrarius* is particularly resistant to STHs.

Thus, an AMG-like compound can be characterized by its ability to inhibit the growth of *S. lavendulae* without or with less inhibition of *S. tenebrarius*, if co-produced with another antibiotics. AMGs are thus easily discriminated from STHs which act similarly on both *Streptomyces* strains. Bleomycins and novobiocin cannot be confused with AMGs, due to the susceptibility of *Sma* 62 and AG4410 to these two antibiotics.

Finally, a typical pattern of activity for AMG can be defined as an antibiotic active, after heating in acidic conditions, on the strains *Sma* 62 and *S. levendulae* and inactive on the strains AG4410 and *S. tenebrarius*.

Identification of the Different AMG Antibiotics

In a screening program for AMGs, it is necessary to identify new molecules as early as possible. Elimination of already known AMG can be achieved by identifying these antibiotics by their characteristic spectra of action displayed towards a group of bacterial strains. These strains are mainly multi-resistant isolates from hospitals but have also been obtained by selection of AMG-resistant mutants from strains such as *Sma* 62 (Table 1).

The spectra of action of 22 AMGs and three STHs has been determined with regard to 19 bacterial strains (Table 3). For example, streptomycin and spectinomycin are easily identified with both specifically resistant *S. marcescens* strains, Sp11 and St101. Likewise, the apramycin sub-group displays a characteristic activity towards *S. kanamyceticus* ATCC 21252. Of the 22 AMGs only lividomycin could be confused with apramycin but lacks activity on *S. marcescens* Z677 and P657 strains.

AMGs of the 4,5-disubstituted 2-DOS groups except for butirosin are typically active on SM1065 (with the exception of ribostamycin and xylostatin) and P585 strains, but inactive both in *S. marcescens* Z677 and P657. These compounds can be distinguished from each other with the APH (3′) producing *E. coli* strains K-12 J5/R112, K-12 J5/R148 and C1a/pAT3, and with W677/pJR225 which is, in addition, highly susceptible to the fortimicin sub-group (data not shown).

Table 3. Keys for AMG and STH identification (second phase of the screening procedure).

Strains	4,5-Disubstituted 2-DOS				4,6-Disubstituted 2-DOS								Mono-substituted 2-DOS		Non-2-DOS diaminocyclitol			STHs
	NEO, PAR	LIV	BUT	RIB, XYL	KAN	TOB	SEL	AMI	DIB	GEN, SIS, VER	SAG	NET	APR, SAC	HYG	FOR	STR	SPE	
Serratia marcescens																		
Sma 62	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
St101	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
Sp11	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S
SM1065	S	S	S	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S
P585	S	S	S	S	R	S	S	S	R	S	S	S	S	S	S	S	S	S
Z677	R	R	S	R	R	S	S	S	S	S	R	S	S	S	R	R	R	S
P657	R	R	S	R	R	S	S	S	S	S	R	S	S	S	R	S	R	S
Escherichia coli																		
C1a	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
K-12 J5/R112	R	R	S	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
K-12 J5/R148	R	S	R	R	R	S	S	S	S	S	S	S	S	S	S	R	S	S
C1a/pAT3	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
K-12 J5/R176	S	S	S	S	R	R	R	S	R	R	R	R	R	R	R	R	S	R
K-12 J5/R135	S	S	S	S	S	S	R	S	S	R	R	S	R	S	R	R	R	S
JR66/W677	R	S	R	R	R	S	R	S	R	S	S	S	S	S	S	R	S	S
MP1	R	S	R	R	R	R	R	S	R	R	R	S	R	R	R	R	S	R
W677/pJR225	R	R	S	R	R	R	R	S	R	R	R	R	R	R	S	S	S	S
Streptomyces tenebrarius ATCC 17920	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
S. kanamyceticus ATCC 21252	R	S	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R
S. lavendulae ATCC 8664	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R

Frames indicate the characteristic spectrum fraction of the corresponding antibiotic.
Inhibition zone size: S, susceptible; R, resistant.
Abbreviations used: SEL, seldomycin; SAG, sagamicin; SAC, saccharocin; and at footnote in Table 2.

Kanamycin is the only AMG which is inactive on the four *S. marcescens* strains of clinical origin. Tobramycin and hygromycin B have an identical and unique spectrum of action and so they cannot be differenciated from each other. In the same way, the antibiotics of the gentamicin sub-group show a typical spectrum of action but are not separable from each other.

AMG-Screening Evaluation

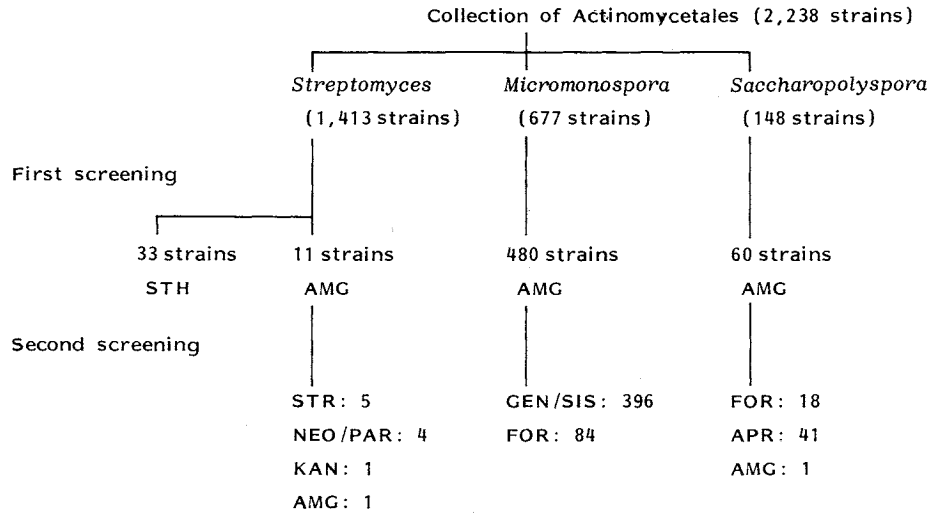
Sensitivity and selectivity of the screening procedure was evaluated using actinomycete strains which produce AMG (24 strains) or STH (3 strains) antibiotics. Except for *Streptomyces kasugaensis* and *Streptoverticillium flavopersicum*, all the control strains produced an AMG/STH-like activity which was detected in the first phase of the screening procedure. From other 25 strains, only the antibiotic activity of the *Streptomyces hygroscopicus* strain failed to be characterized as AMG or STH. Particularly, the products of the three STH-producing strains were not unambiguously identified.

Supernatants of collection strains were heated for 60 minutes at 120°C, pH 3 and subjected to the second phase of the screening procedure. The heating step not only inactivates potential non-AMG antibiotics co-produced by the strains, but also increases the AMG sensitivity of the test. Indeed, we have noticed for most of the collection strains that the yields of the AMG-like antibacterial activities from

Table 4. Performance of AMG bioassay on collection strains producing AMG and STH antibiotics.

Strains	Antibiotic produced	Source	Result of AMG screening system
Primary phase positive strains			
AMG-producing strains			
Streptomyces fradiae	Neomycins	ATCC 10745	NEO/PAR
S. catenulae	Catenulin	ATCC 12476	NEO/PAR
S. ribosidificus	Ribostamycin	ATCC 21294	RIB/XYL
S. rimosus f. paromomycinus	Paromomycins	ATCC 14827	NEO/PAR/LIV
S. lividus	Lividomycins	ATCC 21178	NEO/PAR/LIV
S. kanamyceticus	Kanamycins	ATCC 21252	KAN
S. griseus	Streptomycins	ATCC 12475	STM
Micromonospora purpurea	Gentamicins	ATCC 15835	GEN/SIS
M. inyoensis	Sisomicin	NRLL 3292	GEN/SIS
M. grisea	Verdamicin	NRLL 3800	GEN/SIS
M. olivoasterospora	Fortimicins	ATCC 21819	FOR
M. echinospora	X 14847	NRLL B-12180	FOR
Saccharopolyspora hirsuta	Sporaricins	ATCC 20501	FOR
S. hirsuta	Saccharocin	ATCC 27875	APR/SAC
Streptomyces tenebrarius	Apramycin	NRLL 3816	APR/SAC
S. tenebrarius	Nebramycins	ATCC 17920	AMG
S. hofuensis	Seldomycins	ATCC 21970	AMG
S. rimofaciens	Destomycins	ATCC 21066	AMG
S. spectabilis	Spectinomycins	ATCC 25465	AMG
Streptoalloteichus hindustanus	Nebramycins	ATCC 31158	AMG
Micromonospora pilospora	Lysinomycin	NRRL 11415	FOR
STH-producing strains			
Streptomyces lavendulae	STHs	ATCC 8664	STH
S. xantophaeus	Geomycin	ATCC 19819	STH
S. luridus	Luridin	ATCC 19782	STH
Primary phase negative strains			
AMG-producing strains			
Streptomyces hygroscopicus	Hygromycins	ATCC 27438	AMG/STH
S. kasugaensis	Kasugamycin	ATCC 15714	No activity
Streptoverticillium flavopersicum	Spectinomycin	ATCC 19756	Non-AMG/STH

Fig. 1. Procedure and results of screening.



Abbreviations used: STR, streptomycin; NEO, neomycin; PAR, paromomycin; KAN, kanamycin, GEN, gentamicin; SIS, sisomicin; FOR, fortimicin A; APR, apramycin.

autoclaved culture media were enhanced, presumably by release of a portion of the AMGs from the mycelium. In a few cases (*e.g. Micromonospora purpurea*) identification of the AMG produced was only possible after the heat treatment.

The AMGs produced by 15 of the 21 strains screened in the second phase of the procedure have been correctly identified at the level of the sub-group (Table 4). A more precise identification was only achieved for the products of *S. kanamyceticus* and *Streptomyces griseus* (kanamycin and streptomycin, respectively). One other strain, *Streptoalloteichus hindustanus*, which synthesizes the nebramycins complex, can inhibit all *S. marcescens* and *E. coli* indicator strains, and the AMG it produces remains unindentified. The five last strains, *Micromonospora pilospora*, *Streptomyces hofuensis*, *Streptomyces rimofaciens*, *Streptomyces tenebrarius* ATCC 17920 and *Streptomyces spectabilis* display no or only weak activities on the indicator strains, and therefore the AMG antibiotics they produce cannot be identified.

We have screened 2,238 Actinomycetales strains (*Streptomyces*, 63%, *Micromonospora*, 30% and *Saccharopolyspora*, 7%) from the laboratory collection (Fig. 1) for the production of AMG antibiotics. From 1,413 *Streptomyces* strains isolated at random from soil, 11 strains (0.8%) produced AMG-like activities and 33 strains (2.3%) STH-like activities. AMG antibiotics have been identified in the second phase of the screening procedure as streptomycin (5 strains), neomycin or paromomycin (4 strains) and kanamycin (1 strain). Antibiotic activity of the remaining *Streptomyces* strain inhibits all indicators in the second screen; that strain can be presumed to synthesize a complex of AMG-type compounds.

The *Micromonospora* and *Saccharopolyspora* strains were isolated from soil with selective media containing gentamicin. 480 (70.9%) of the 677 *Micromonospora* strains produced an AMG-type activity: tested in the second phase of the screening program, 396 strains (58.5%) and 84 strains (12.4%) were shown to synthesize AMG antibiotics from the gentamicin or fortimicin sub-groups, respectively. In the same way, 60 *Saccharopolyspora* strains (40.5%) synthesized AMG, identified as apramycin (41 strains, 27.7%) and fortimicin sub-group (18 strains, 12.2%). An attempt to identify the product of the remaining

strain of *Saccharopolyspora* is in progress.

Discussion

Different strategies can be employed to develop a productive program to screen for actinomycete strains which produce new AMG antibiotics. But in each case, the first phase of the screen should quickly distinguish the AMG-producing strains from the other isolates. This paper is not concerned with how to isolate AMG producing strains. However, by specifically isolating AMG-resistant organisms (HOTTA *et al.*[22]) one can greatly enrich for AMG-producing strains and therefore enhance the efficiency of the screening program. A convenient way to characterize AMG-producing strains would be to use two indicator strains: one, AMG-susceptible and the other AMG-resistant (ref 11 and this paper). However, water-soluble basic antibiotics which inhibit protein synthesis and promote misreading as AMGs do (*e.g.* STHs or negamycin[23]), could not be distinguished by this method. This was demonstrated with the STH-resistance of the *Sma* 62 mutant, AG4410. STH antibiotics can be identified using the STH-producing strain *S. lavendulae*, but behavior in this test of STH-like compounds and *a fortiori* of other water-soluble basic antibiotics as negamycin remains unknown. On the other hand, the high thermo-stability of AMGs in acidic solutions is one of the more characteristic properties of these antibiotics, eventhough some other compounds demonstrate similar stability. Finally, only a combination of the results of antimicrobial activities of actinomycetes strains against *S. marcescens*, *S. lavendulae* and *S. tenebrarius* strains after thermal treatment, can lead to an adequate isolation of AMG producing strains. In fact 21 of 24 AMG producing collection strains were positive in the first phase of the screen.

The second phase of this program leads to the identification of some AMG antibiotics. It seems likely that an actinomycete strain producing a new AMG would have a unique spectrum of activity against the group of AMG-multiresistant bacterial strains. However, only an AMG antibiotic structurally unrelated to known compounds might be predicted to behave in this way. For instance, boholmycin exhibits a broad-spectrum of activity against bacteria including some AMG-resistant strains[4] and would probably have been characterized as a new compound in the second phase of our screen. On another hand, inosamycin[3] which is closely related to the neomycin group, should have a pattern of activity indiscernible from that of neomycin or paromomycin based on analysis of their chemical structures. Thus, the inosamycin-producing strain would probably have been identified in the screen as a neomycin-producing strain, resulting in the loss of a strain synthesizing a promising new AMG antibiotic, which, in fact, has a lower acute human toxicity than neomycin. Applied to 21 actinomycete strains characterized as AMG producers in the first phase, the second phase has led to the identification of the precise AMG for two strains and of the correct sub-group of AMG for 14 strains. The product of 5 other strains has not been identified as the level of the bactericidal activity of these strains is too weak. The limitation of our screening program demonstrated by these results could be attributed to a loss of antibiotic productivity by the collection strains. Effectively, such a problem has not been encountered with the actinomycete strains we have recently isolated from soil. Thus, the secondary phase will be useful in two ways: 1) a unique spectrum of activity would indicate a new structurally unrelated AMG; 2) an indexed spectrum of activity should indicate an already known AMG or more generally a sub-group in the AMG family of antibiotics. Such results will considerably improve the efficiency of physico-chemical analysis which follows the screening program. Indeed, knowledge of the precise antimicrobial spectrum of the antibiotic results in a preliminary identification of the AMG. Coupled with methods (as described by INCHAUSPE *et al.*[10]) which permit an early physico-chemical characterization of AMGs, determination of their biological properties will lead to an accurate identification of the new AMG antibiotics.

Acknowledgments

We are particularly grateful to all the managers of private companies who have kindly supply us with antibiotics samples used in this work. We thank M. PERLIN and S. A. LERNER, S. A. KABINS, N. MOATTI and P. COURVALIN for providing *E. coli* and *S. marcescens* strains, and D. STASSI and F. DROCOURT for help in preparing the manuscript.

References

1) BERDY, J.; A. ASZALOS, M. BOSTIAN & K. L. MCNITT: Handbook of Antibiotic Compounds. I. Carbohydrate

antiboitics. CRC Press, Inc., 1980
2) KOEDA, T.; K. UMEMURA & M. YOKOTA: Toxicology and pharmacology of aminoglycoside antibiotics. *In* Aminoglycoside Antibiotics. *Eds.*, H. UMEZAWA & I. R. HOOPER, pp. 293~356, Springer-Verlag, 1982
3) TSUNAKAWA, M.; M. HANADA, H. TSUKIURA, K. TOMITA, K. TOMATSU, T. HOSHIYA, T. MIYAKI, M. KONISHI & H. KAWAGUCHI: Inosamycin, a complex of new aminoglycoside antibiotics. I. Production, isolation and properties. J. Antibiotics 38: 1302~1312, 1985
4) SAITOH, K.; M. TSUNAKAWA, K. TOMITA, T. MIYAKI, M. KONISHI & H. KAWAGUCHI: Boholmycin, a new aminoglycoside antibiotic. I. Production, isolation and properties. J. Antibiotics 41: 855~861, 1988
5) UMEZAWA, H.; S. KONDO & I. KITASATO: Development of new semisynthetic aminoglycoside antibiotics. Drugs Exptl. Clin. Res. 10: 631~636, 1984
6) ZURENKO, G. E.; B. H. YAGI, J. J. VAVRA & B. B. WENTWORTH: *In vitro* antibacterial activity of trospectomycin (U-63366F), a novel spectinomycin analog. Antimicrob. Agents Chemother. 32: 216~223, 1988
7) DAVIES, J. & D. I. SMITH: Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. 32: 469~518, 1978
8) HOOPER, I. R.: The naturally occuring aminoglycoside antibiotics. *In* Aminoglycoside Antibiotics. *Eds.*, H. UMEZAWA & I. R. HOOPER, pp. 1~35, Springer-Verlag, 1982
9) CLAES, P. J. & H. VANDERHAEGHE: Thin-layer chromatographic identification of aminoglycoside antibiotics. J. Chromatogr. 248: 483~487, 1982
10) INCHAUSPE, G.; C. DESHAYES & D. SAMAIN: Use of perfluorinated counter ions for the combination of ion pair HPLC and field desorption mass spectrometry. Application to the early characterization of aminoglycoside antibiotics. J. Antibiotics 38: 1526~1535, 1985
11) NUMATA, K.; H. YAMAMOTO, M. HATORI, T. MIYAKI & H. KAWAGUCHI: Isolation of an aminoglycoside hypersensitive mutant and its application in screening. J. Antibiotics 39: 994~1003, 1986
12) 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
13) BRYAN, L. E. & S. KWAN: Aminoglycoside-resistant mutants of *Pseudomonas aeruginosa* deficient in cytochrome *d*, nitrite reductase, and aerobic transport. Antimicrob. Agents Chemother. 19: 958~964, 1981
14) BEGGS, W. H.: Suppressive actions of inorganic ions on the bioactivity of aminoglycoside antibiotics. Life Sci. 24: 663~668, 1979
15) URI, J. V.; P. ACTOR & J. A. WEISBACH: Presumptive identification of aminoglycoside antibiotics by the pH susceptibility disc agar-diffusion method. Experientia 35: 1034~1035, 1979
16) HUGHES, D. W.; A. VILIM & W. L. WILSON: Chemical and physical analysis of antibiotics. Part III. Aminoglycosides. Can. J. Pharm. Sci. 13: 21~30, 1978
17) BETINA, V.: The chemistry and biology of antibiotics. *In* Pharmacochemistry Library. Vol. 5. *Eds*, W. T. NAUTA & R. F. REKKER, pp. 59~114, Elsevier Scientif. Publishing Co., 1983
18) DANIEL, D. & G. TIRABY: A survey of plasmids among natural isolates of *Streptomyces*. J. Antibiotics 36: 181~183, 1983
19) ICHIKAWA, T.; M. DATE, T. ISHIKURA & A. OZAKI: Improvement of kasugamycin-producing strain by the agar piece method and the prototroph method. Folia Microbiol. 16: 218~224, 1971
20) HAUPT, I.; H. THRUM & D. NOACK: Self-resistance of the nourseothricin-producing strain *Streptomyces noursei*. J. Basic Microbiol. 26: 323~328, 1986
21) 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
22) HOTTA, K.; A. TAKAHASHI, N. SAITO, Y. OKAMI & H. UMEZAWA: Multiple resistance to aminoglycoside antibiotics in actinomycetes. J. Antibiotics 36: 1748~1754, 1983
23) UEHARA, Y.; S. KONDO, H. UMEZAWA, K. SUZUKAKE & M. HORI: Negamycin, a miscoding antibiotic with a unique structure. J. Antibiotics 25: 685~688, 1972
24) KABINS, S. A. & C. NATHAN: *In vitro* activity of 5-episisomicin in bacteria resistant to other aminoglycoside antibiotics. Antimicrob. Agents Chemother. 14: 391~397, 1978
25) PERLIN, M. H. & S. A. LERNER: Amikacin resistance associated with a plasmid-borne aminoglycoside phosphotransferase in *Escherichia coli*. Antimicrob. Agents Chemother. 16: 598~604, 1979