INTERSPECIFIC RECOMBINATION AMONG AMINOGLYCOSIDE PRODUCING STREPTOMYCETES

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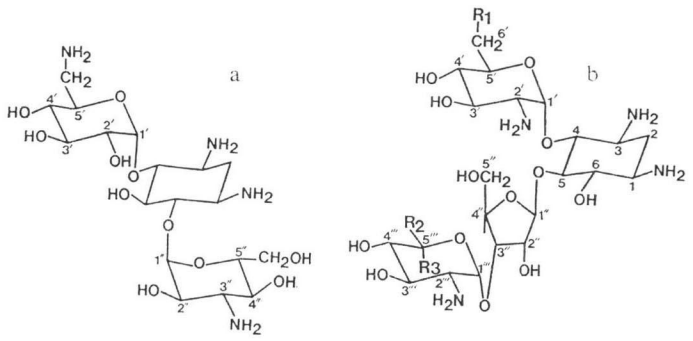
From auxotrophic double mutants of *Streptomyces rimosus* forma *paromomycinus* and *Streptomyces kanamyceticus* producing little or no antibiotic, stable prototrophic recombinants were obtained with low frequencies. Most of the recombinants differed from the parents in morphology and antibiotic production. The most frequent classes of recombinants behaved as streptomycetes of the "red" series and produced a wide range of neomycin yields, in contrast to the parents which produced paromomycin and a small proportion of neomycin, or kanamycin, respectively. Hypotheses on the nature of the genetic material exchanged are discussed.

Intraspecific recombination in antibiotic producing streptomycetes is well known[1] and is particularly suitable for strain improvement[2]. In contrast, interspecific recombination, which is limited by incomplete homology between genomes[1], is far less well documented, even though it could lead to new products[3]. Only a few examples of true interspecific recombinants have been reported[3~6].

In order to test the possibility that hybrid antibiotic molecules could be obtained by the cooperation of segments of the genomes of strains producing different antibiotics, we explored interspecific recombination. The aminoglycoside antibiotics seemed favourable material since most compounds of this family are synthesized by similar pathways in which small numbers of building blocks are assembled and modified by a limited number of enzymes. Moreover, these enzymes, as has been shown by mutasynthesis experiments, accept considerable modifications in their normal substrates[7].

Crossing experiments were performed between pairs of strains from a group producing apramycin, destomycin, kanamycin, neomycin, paromomycin (Fig. 1) or ribostamycin, each bearing at least two

Fig. 1. Structures of kanamycin, paromomycins and neomycins.



Kanamycin A

	R_1	R_2	R_3
Paromomycin II	OH	CH_2NH_2	H
Paromomycin I	OH	H	CH_2NH_2
Neomycin C	NH_2	CH_2NH_2	H
Neomycin B	NH_2	H	CH_2NH_2

mutations as genetic markers. Matings were carried out by the conventional technique involving mixed growing cultures.

In this report we describe stable recombinants producing neomycin isolated from crosses between *Streptomyces rimosus* forma *paromomycinus* and *Streptomyces kanamyceticus*.

Materials and Methods

Parent Strains

Streptomyces rimosus forma *paromomycinus* UC 57 and *Streptomyces kanamyceticus* UC 137 were from the Roussel Uclaf industrial collection and are, respectively, paromomycin and kanamycin producers.

Media

Minimum medium[8] was used for detection of auxotrophs. Complete medium A had the following composition: potatoes 10 g, NZ-Amine B (Sheffield Chemical) 1 g, agar 2.5 g, distilled water 100 ml. Medium B contained: potato starch (Roquette frères, Lille) 0.5 g, potassium phosphate dibasic 0.015 g, magnesium carbonate 0.05 g, sodium chloride 0.025 g, ammonium nitrate 0.01 g, distilled water 100 ml. ATCC No. 343 medium was used as an additional complete medium.

Paromomycin production on agar was detected on medium C of the following composition: corn protein (Roquette frères, Lille) 1.2 g, soya bean flour (Société Industrielle des oléagineux, Paris) 1 g, ammonium chloride 0.4 g, calcium carbonate 1.5 g, magnesium sulfate 0.02 g, glucose 2.5 g, agar 2.5 g, distilled water 100 ml.

Kanamycin and neomycin production on agar were detected, respectively, on medium D: dextrin 2.65 g, soya bean flour 3 g, sodium chloride 0.125 g, agar 2.5 g, distilled water 100 ml; and medium F: corn steep liquor (Roquette Frères, Lille) 0.4 g, dextrin 2 g, magnesium sulfate 0.1 g, calcium carbonate 0.1 g, agar 2.5 g, distilled water 100 ml.

Paromomycin production in shaken flasks was carried out according to AUTISSIER *et al.*[9] and neomycin according to BAUD *et al.*[10]. Kanamycin production was carried out in a liquid medium with the same nutrients as medium C at double concentration.

Mutagenesis

Suspensions of mycelium fragments as main colony forming units were prepared from cultures of the parent strains incubated for 7 days at 30°C on medium A. The general procedure of mutagenesis was treatment by N-methyl-N′-nitro-N-nitrosoguanidine (NTG) of these suspensions in liquid minimum medium. Two successive mutagenic treatments were carried out to obtain the markers, designated as A for an auxotroph and as M for a low or a non antibiotic producer.

For paromomycin mutants the first step was realized by 0.5 mg/ml of NTG for 7 hours, and the second by 0.1 mg/ml for 5 hours. For kanamycin mutants the respective concentrations and times were 0.1 mg/ml for 15 hours and 0.5 mg/ml for one hour. In each case approximatively 99% of the population was killed.

Isolation and Description of Mutants

Mutations in antibiotic production were detected by the agar piece method[11]. Auxotrophic mutants were isolated and characterized according to HOPWOOD and SERMONTI[12]. Four double mutants were constructed from *S. rimosus* f. *paromomycinus* UC 57 and two from *S. kanamyceticus* UC 137 (Table 1).

Cross Procedure

Normal crossings were carried out according to HOPWOOD[8], the mixed suspensions of complementary strains being incubated on medium A for seven days at 30°C. Then the mycelial mat was scraped into 10 ml of a solution of sucrose at 8.5 g per 100 ml, homogenized by agitation for 15 minutes on a rotary shaker in the presence of glass beads and diluted before plating on minimum medium for recombinant recovery, and on appropriately supplemented media and on the complete medium A for viable counts. Colonies appearing on minimal medium were streaked on the same selective medium and single

Table 1. Origin and characters of *Streptomyces* strains used in this investigation.

Strain		Nutrition	Antibiotic production[6]	Morphology[1]	Pigmentation[1]
S. rimosus f. *paromomycinus*	UC 57	Prototrophic	Paromomycin ###[4]	W. t_1.	No pigmentation
S. kanamyceticus	UC 137	Prototrophic	Kanamycin ###	W. t_2.	Greyish-green
Parent strain	Mutants				
UC 57	$A_{67}M_{12}$	Auxotrophic[2]	Paromomycin ±[3]	W. t_1. but reduced growth	No pigmentation
	$A_{67}M_{15}$	Thr B	6'''-deamino-paromomycin ±	W. t_1.	Yellow
	$A_{67}M_{17}$	Thr B	Paromomycin ±	W. t_1.	No pigmentation
	A_3M_7	Trp, His[5]	Paromomycin ±	W. t_1.	No pigmentation
UC 137	M_1A_1	Met B	Non-producer	W. t_2.	Greyish-green
	M_7A_3	Ile A	Non-producer	W. t_2.	No pigmentation

[1] Morphology and pigmentation on agar medium A:
Wild type 1 (W. t_1) = mycelium production limited to dense growth of substrate mycelium forming wide bacillary fragments,
Wild type 2 (W. t_2) = thin non fragmented substrate mycelium forming sparse aerial mycelium with light sporulation.

[2] Multiple nutritional requirements (not determined).

[3] 0.5 % of the wild strain production.

[4] In addition to its normal paromomycin production, this strain gives, on some media, about 1 % neomycin, compared to paromomycin.

[5] The two markers were obtained in one step of mutation.

[6] Identification of antibiotics: see Table 2.

colonies were purified by reisolation. The resulting strains were tested for antibiotic production by the agar piece method; those showing an antibacterial activity were considered as probable recombinants and carefully examined for antibiotic production in liquid medium.

Identification of Antibiotics (Table 2)

Antibiotics were isolated, purified and identified according to AUTISSIER *et al.*[9].

Results

Antibiotic Production by Double Mutants

Mutants unable to produce any measurable antibiotic activity, *i.e.*, less than 5 μg/ml were easily obtained from *S. kanamyceticus*, but all the *S. rimosus* f. *paromomycinus* mutants (ten out of 3,000 mutagenized colonies) showed a small inhibition zone with *Sarcina lutea* ATCC 15957 as the test microorganism. The same was true for eight double mutants isolated from 6,500 mutagenized colonies.

Table 2. Identification of the antibiotics produced in liquid cultures of parent strains and of prototrophic recombinants[a].

	R_B[b]	
	Paper chromatography	Thin-layer chromatography (solvent 1)
Neomycin B	1.00	1.00
Neomycin C	0.60	1.25
Paromomycin	1.07	1.60
Mono-N-acetylneomycin B	1.10	1.60
6'''-Deaminoparomomycin	0.69	2.40
Kanamycin	0.84	1.90

[a] Chromatographic comparison of compounds were performed according to Ref. 9.

[b] In all cases mobilities are relative to neomycin B (R_B). The antibiotic produced in liquid cultures of the strains under experiment gave the same mobilities as the standards of the related antibiotics used as control.

Table 3. Frequency of prototrophs in crosses of auxotrophic non producing mutants of *S. rimosus* f. *paromomycinus* and *S. kanamyceticus*.

Strains mixed Mutants from UC 57×UC 137	Spontaneous reversion to prototrophy		Prototroph frequency from cross	Prototrophic antibiotic producers Number studied
	UC 57 mutants	UC 137 mutants		
$A_{67}M_{12}\times M_1A_1$	$0/2\times10^{10}$	$0/5\times10^{10}$	$2\times10^4/2\times10^{10}$	1*
$A_{67}M_{15}\times M_1A_1$	$0/2\times10^{11}$	$0/5\times10^{10}$	$11/10^{10}$	6**
$A_{67}M_{17}\times M_1A_1$	$0/5\times10^{10}$	$0/5\times10^{10}$	$30/4\times10^{10}$	12
$A_3M_7\times M_1A_1$	$0/3\times10^{10}$	$0/5\times10^{10}$	$0/5\times10^9$	0
$A_3M_7\times M_7A_3$	$0/3\times10^{10}$	$0/1.5\times10^{11}$	$1/1\times10^9$	1

* About a hundred other clones were non producers.
** 3 other clones were non producers.

Table 4. Antibiotic production and morphology of prototrophic recombinants.

Cross parents strains	Recombinant strains	Antibiotic production[5]	Morphology[1]	Pigmentation[1]
$A_{67}M_{12}\times M_1A_1$	39	Paromomycin +[2] Neomycin ±	Mainly fragmented substrate mycelium with sparse patches of white aerial mycelium	Orange
$A_{67}M_{15}\times M_1A_1$	89	6'''-Deamino-paromomycin +[3]	W. t_1.	No pigmentation
$A_{67}M_{17}\times M_1A_1$	40	Neomycin ⅲ[4]	W. t_1.	Orange
	57	Neomycin ⅲ[4] N-Acetylneomycin ⅱ	W. t_1. but less fragmented	No pigmentation
	60	Neomycin ⅲ[4]	W. t_1.	No pigmentation
	64	Neomycin ⅲ[4]	W. t_1.	No pigmentation
	66	Neomycin ⅲ[4]	as strain 39	Orange
	68	Neomycin ⅲ[4]	W. t_1.	Yellow
$A_3M_7\times M_7A_3$	42	Paromomycin +[2] Neomycin +	W. t_1. with reduced growth	No pigmentation

[1] Morphology and pigmentation on agar medium A.
[2] Paromomycin production: 15% of the wild type, so about 30 times the parent strains $A_{67}M_{12}$ and A_3M_7.
[3] 6'''-Deaminoparomomycin: 2~3 times higher than the production of the parent strain $A_{67}M_{15}$.
[4] See Table 5.
[5] Identification of antibiotics: see Table 2.

Identification of antibiotics synthesized by the four mutants used in recombination experiments indicated that strains $A_{67}M_{12}$, $A_{67}M_{17}$ and A_3M_7 produce paromomycin and neomycin in about the same ratio as the starting UC 57 *S. rimosus* f. *paromomycinus* strain (99: 1), but the quantity produced was only about 0.5 per cent of the parent. Strain $A_{67}M_{15}$ gives a small amount of a new antibiotic, 6'''-deaminoparomomycin[9].

Crosses

Conventional crosses from mixed cultures inoculated with mixtures of parents in the ratios 10^7 to 10^8, 10^8 to 10^8 and 10^8 to 10^7 and grown on medium A were plated on minimum medium. Prototrophic colonies were detected only in crosses where colony forming units of the paromomycin mutant were ten times higher than those of the other parent. Based on the recovery of prototrophic clones the frequency

Table 5. Neomycin production relative to the *S. fradiae* control strain.

Clone No.	Activity*	Proportion of isomers (by weight)	
		Neomycin B	Neomycin C
40	30	60	40
57	60	92	8
60	85	72	28
64	95	65	35
66	95	84	16
68	115	88	12
Control strain	100**	80	20

* The whole antibacterial activity of the broth was measured on *Klebsiella pneumoniae* ATCC 9997 and corrected according to the respective activity of isomers B and C.

** The ponderal productivity of the grandparent strains, in their optimal liquid media, related to the neomycin production of the *S. fradiae* control strain, was respectively for *S. rimosus* f. *paromomycinus* 35% and for *S. kanamyceticus* 25%.

of recombination was very low, in the range of $1\times10^{-9}\sim1\times10^{-6}$ for the four so-called fertile crosses (Table 3). These frequencies, although significantly higher than the spontaneous reversion to prototrophy, are not high enough for a conclusive proof of recombination. However, a small number of supposed recombinants were purified, and since they did not segregate back to parental phenotypes, they were examined for phenotypic characters other than prototrophy, particularly antibiotic production and morphology.

Antibiotic production obtained under the normal conditions for paromomycin biosynthesis is reported in Table 4. Recombinant strains 39 and 42 gave the same antibiotics as the mutant and the wild type strains of the *S. rimosus* f. *paromomycinus*. However, quantitative paromomycin production was lower than in the wild-type grandparent and far higher than in the A_{67}-M_{12} and A_3M_7 parents. Strain 89 also showed a higher synthesis of 6'''-deaminoparomomycin than the $A_{67}M_{15}$ parent. Strain 57 was peculiar in giving under kanamycin production conditions N-acetylneomycin in the range of 30% of the total antibiotic production.

Strains 40, 57, 60, 64, 66 and 68, which showed in the preliminary shake flask test a very high antibiotic activity identified mainly as neomycin, were studied more carefully, this time by a procedure close to that described by BAUD, *et al*[10], in comparison with the industrial neomycin producing strain of *S. fradiae* (Table 5). This small set of recombinant strains displayed a fairly wide spectrum of antibiotic productivity, 30~115% of a very high producing strain. The composition of the mixtures of the two isomers, neomycins B and C, was also quite variable.

The high neomycin producing strains 40, 57, 60, 64, 66 and 68 showed distinctive features on agar medium B. The kanamycin parent M_1A_1 did not grow on this medium, and the paromomycin parent $A_{67}M_{17}$ produced a sparse white aerial mycelium. However, all the recombinant strains grew better than the paromomycin parent, giving an abundant white aerial mycelium and at the end of the incubation period reddish spores, more or less abundant depending on the strain.

The same kind of features were observed on medium ATCC 343, the difference being that the kanamycin mutant M_1A_1 grew on this medium with the wild type morphology.

The recombinant strain 68 was particularly attractive because of its high production of neomycin. It behaved as a strain of the red series[13] and more specifically as a *S. fradiae* strain with red spores and no melanoid pigment. Therefore we compared this strain to the industrial *S. fradiae* strain.

Although the susceptibility of both strains to neomycin was about the same (approximately 60 μg/ml) the production of neomycin and the morphology of colonies were different in the agar piece process on medium F: the reference strain produced only substrate mycelium and a small amount of neomycin, whereas strain 68 gave an abundant pink sporulation and twice the titre of antibiotic.

The spores of the reference strain were in chains in the form of hooks on medium ATCC 343 and medium B, whereas strain 68 did not show hooks under these conditions. The new strain produced hooks only on minimum medium. Susceptibility of the two strains to several inhibitors was quite different: for instance the reference strain grew equally well in the presence and absence of 4 mg per ml of α-aminobutyric acid, while strain 68 was largely inhibited by this concentration of α-aminobutyric acid.

In liquid culture, the reference strain was not inhibited at all by up to 8 mg/ml of glycine but strain 68 failed to grow in the presence of 5 mg/ml. Strain 68 was almost resistant to the lethal action of NTG: for the reference strain, 0.002% of spores shaken in minimal medium for 15 hours at 30°C in the presence of 1 mg/ml remained viable, whereas for strain 68 the proportion was 5%.

Discussion

This report describes the isolation of new strains producing antibiotics of the aminoglycoside family by interspecific crosses. Through conventional mating procedures the frequency of recombination was low. However, because of the number of different phenotypic characters shown by the parent and recombinant strains, involving morphology, sporulation capacity, pigmentation as well as antibiotic production, these experiments demonstrate that recombination is possible between at least some strains of paromomycin and kanamycin producing streptomycetes.

The small number of recombinant strains carefully examined produced more antibiotic than the parents involved in the crosses. For some of them the main antibiotic produced was the minor one in the parent and grandparent. Thus, the amount of neomycin produced was an order of magnitude higher than the initial productivity of the grandparent strains, which produced mainly paromomycin and kanamycin.

We can only speculate on the nature of the genetic material exchanged by the strains: structural gene for introduction of the 6′-amino group from the kanamycin parent to the paromomycin recipient; a regulatory function provoking a better expression of the very weak endogenous 6′-amino gene of the paromomycin strain; or information involving the primary metabolism inducing an indirect effect on antibiotic synthesis. The fact that a group of strains was obtained, producing neomycin in variable amount, shows that this recombination process, if not frequent, is not exceptional, and confirms that quantitative characters of secondary metabolites, depending on a large number of genes, are indeed able to vary continuously.

These results, therefore, show that interspecific matings, like intraspecific crosses are suitable for strain improvement, and also probably for production of new hybrid antibiotic molecules, although the latter has not been definitely proved.

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