Genet. Res., Camb. (1985), **45**, *pp.* 341–344
Printed in Great Britain

SHORT PAPER

Genetic experiments on sisomicin production by *Micromonospora rosea*

By ISTVÁN GADÓ, ANTÓNIA BOKÁNY and GYÖRGY SZVOBODA

Institute for Drug Research, Budapest, Hungary

(*Received 27 November 1984 and in revised form 18 January 1985*)

SUMMARY

Chromosomal localization of a gene involved in sisomicin production of *Micromonospora rosea* n.sp. was suggested by the results of matings between antibiotic producer and non-producer auxotrophic mutants. The frequency of producers among prototrophic recombinants varied with the chromosomal markers used for selection. The *sis* allele of the parent which was able to overgrow its partner in mixed culture predominated amongst the recombinants.

1. INTRODUCTION

A fundamental question in the genetics of antibiotic production is whether the structural or regulatory genes of antibiotic biosynthesis of particular actinomyces are on the chromosome or on plasmids. This problem has been clarified only for a few of the many thousands of known antibiotics. (Hopwood, 1978, 1979*b*, 1983; Chater & Hopwood, 1983; Okanishi, 1979). There are no data at all in respect of antibiotics produced by the genus *Micromonospora* (gentamicin, sisomicin, sagamicin, etc.).

On the genetics of *Micromonospora* strains only few studies have been published. Conjugal recombination was reported by Beretta, Betti & Polsinelli (1971) and fusion experiments were carried out between *Micromonospora* protoplasts (Szvoboda *et al.* 1979), but genetic maps have not been made. Appearance of a plasmid could be induced by UV irradiation in *Micromonospora* inyoensis (Parag & Goedeke, 1984); its role in antibiotic production was not revealed.

To localize the genetic determinants of sisomicin production in a strain of *Micromonospora rosea* n.sp., a variety of techniques was applied. These techniques were: plasmid elimination with ethidium bromide and by means of protoplast formation and regeneration on the basis of method of Shaw & Piwowarski (1977) and that of Chater *et al.* (1982), respectively; rapid detection of plasmid DNA with methods of Birnboim & Doly (1979) and Kado & Liu (1981); plasmid isolation with methods of Bibb, Freeman & Hopwood (1977) and Dobritsa, Dobritsa & Tanyshin (1978). All of these experiments yielded negative results: non-producer variants did not appear in significant quantity (1 out of 500 after ethidium bromide treatment; 0 out of 200 among regenerated protoplasts), nor did we detect plasmid DNA. Similarly, the infectious transfer of productivity in mixed culture of producer and non-producer strains could not be observed (0 out of 500 of the non-producer parent became producers).

These negative results, however, do not exclude the plasmid-localization of sisomicin production. To obtain positive evidence of its chromosomal localization, in the present work *in vivo* recombination experiments were made between producer and non-producer auxotrophic mutants starting from the premise, that if the *sis* gene in question was on

a plasmid, the frequency of the *sis*$^-$ genotype among the prototrophic recombinants should be independent of the selective markers (Hopwood, 1983).

2. METHODS

(i) *Organisms*

Micromonospora rosea n.sp. strain S1 was isolated from Hungarian water sample. It was deposited at the National Collection of Micro-organisms in the State Institute of Hygiene, Budapest, Hungary (No. 00182). Leu$^-$Ade$^-$ and non-producer (*sis*$^-$) mutants were made with MNNG treatment. After crossing these mutants with each other by mating, Leu$^-$*sis*$^-$, Leu$^-$*sis*$^+$, Ade$^-$*sis*$^-$ and Ade$^-$*sis*$^+$ recombinants were isolated.

(ii) *Media*

Minimal agar (MM): 1 g KNO_3, 0·5 g K_2HPO_4, 0·5 g $MgSO_4 . 7H_2O$, 0·01 g $FeSO_4 . 7H_2O$, 20 g water soluble starch, 15 g Bacto agar (Difco) in 1 litre distilled water, pH = 7·0.

Complete agar (CM): 3 g meat extract, 5 g tryptone (Oxoid), 1 g glucose, 24 g water soluble starch, 5 g yeast extract (Oxoid), 2 g $CaCO_3$, 2 g $MgSO_4 . 7H_2O$, 0·2 g $FeSO_4 . 7H_2O$, 0·002 g $Co/NO_3/_2$, 15 g Bacto agar (Difco) in 1 litre tap water, pH = 7·0.

Production medium: 40 g soymeal, 35 g corn starch, 10 g peptone, 5 g glucose, 5 g $CaCO_3$, 3 g $MgSO_4 . 7H_2O$, 0·2 g $FeSO_4 . H_2O$, 0·008 g $Co/NO_3/_2$ in 1 litre tap water, pH = 7·5.

(iii) *Mating*

Mixed cultures on CM slants were inoculated with vegetative mycelium of producer and non-producer auxotrophic strains. After incubation for 4 days the mycelia were harvested and plated on CM and MM agar without filtration. The relative frequencies of the two parents were determined among the colonies obtained on CM. From MM plates well-grown colonies appearing on a very weak background growth were isolated and transferred 3 times to slants of the same medium. These cultures were inoculated into flasks containing production medium. After shaking for 7 days, platings were made from each flask on CM (to check parental segregants) and on MM agar: 20–20 colonies from the latter were isolated, and their productivity was determined in shake cultures made as above. The cultures on agar media were incubated at 37 °C. Flasks were shaken on a horizontal shaker with 250 rev/min at 28 °C.

(iv) *Assay of antibiotic production*

An agar diffusion method was applied using *Staphylococcus epidermidis* ATCC 22228 as test organism and sisomicin standards between 0·5 and 2 μg/ml. Sisomicin was produced as the main antibiotic component; the proportion of antibiotically active minor components was 5–15% for all the producer strains as determined by thin layer chromatography and bio-autography. The total antibiotic activity of all the producer strains was equivalent, with 150–350 μg/ml sisomicin. Non-producer strains did not exhibit any antibiotic activity.

3. RESULTS AND DISCUSSION

Two matings were carried out with the *sis*$^-$ and *sis*$^+$ alleles situated in opposite polarity beside otherwise identical auxotrophic markers (Table 1). As can be seen, there was a very great difference in the distribution of *sis* alleles among prototrophic recombinants depending on the markers applied.

The mating experiment reported here was performed in a different way from that used traditionally in *Streptomycetes*, because the plating of filtered spore suspensions on MM agar failed to produce phenotypically prototrophic colonies. The primary colonies grown from unfiltered mixed cultures have to be heteroclones or heterokaryons, since their progenies were heterogeneous in respect of productivity, and parental segregants could be isolated from the first shake generation at a frequency of 0,1–1 %. The prototroph cultures isolated from the first shake generation were considered to be haploid recombinants, because further segregation of parental phenotypes and prototrophs with opposite *sis* allele was not observed.

Table 1. *Distribution of* sis *alleles among prototrophic recombinants*

	Recombinants checked			Parents in mixed culture	
Cross	Total	*sis*$^-$	%	Initial	Final
(1) Leu$^-$*sis*$^-$xAde$^-$*sis*$^+$	600	596	99	1:1	8:1
(2) Leu$^-$*sis*$^+$xAde$^-$*sis*$^-$	540	142	26	1:1	1:2·5

Frequency of primary colonies: 10^{-4}.
The recombinants originated from 27, and 30, resp. primary colonies (see Methods).

It might be supposed that competition between producer and non-producer prototrophic recombinants during growth on solid and in liquid media could cause a change in their ratio. This assumption was, however, ruled out by a control experiment: using a mixed culture of producer and non-producer prototrophic recombinant strains inoculated in equal amounts either on a solid (CM or MM) or into liquid (production) medium, their ratio exhibited no significant change during subsequent growth.

On the basis of the starting premises, our results mean that the *sis* gene in question has chromosomal localization.

It is surprising, however, that the *sis*$^-$/*sis*$^+$ ratio in these reciprocal crosses was not reciprocal as it would be expectable, if it depends only on the linkage relations.

Some other crosses were made with *sis*$^-$ and *sis*$^+$ mutants having different auxotrophic markers, and various *sis*$^-$/*sis*$^+$ ratios among the prototrophic recombinants was observed (data not shown). Trying to clear up the linkage between *sis* and the various auxotrophic markers some controversies were experienced.

A possible explanation for these discrepancies may be provided by a rather unexpected finding: the *sis* allele of that parent strain was always predominant which had an over-growing ability in the mixed culture. In cross 1 of the experiment presented the Leu$^-$*sis*$^-$ parent strongly overgrew its partner, while in cross 2 the Ade$^-$*sis*$^-$ parent did so to a rather moderate degree. The actual proportion of the parents, varied by means of different inoculum concentrations, was irrelevant. We suppose primary colonies to be hetero-karyons in which multiple crossing over may occur as in the products of protoplast fusion (Hopwood & Wright, 1978). The chromosome of the parent with overgrowing capacity participates more actively in multiple crossovers with primary recombinants so that the *sis* allele of this chromosome comes to predominate in the final recombinant population.

The authors are indebted to professor D. A. Hopwood for the criticism on the manuscript and his encouragement. We wish to thank Mrs Ilona Garai, Vera Pallos and Ilona B. Varga for the excellent technical work.

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