

## Plasmid Curing and Generation of Mutations Induced with Ethidium Bromide in *Streptomyces*

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The DNA-intercalating agent ethidium bromide was used to eliminate plasmid DNA from streptomycetes. Other mutational events associated with chromosome changes occurred at high frequency; they resulted in phenotypic changes such as loss of enzyme activity, antibiotic resistance or ability to sporulate.

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### INTRODUCTION

Following reports of plasmid 'curing agents' which implied the possible involvement of extrachromosomal elements in the biosynthesis of secondary metabolites (Gregory & Shyu, 1961), many studies of unstable genetic elements have been published. Plasmid involvement in the genus *Streptomyces* has been postulated for numerous characteristics such as fertility (Vivian, 1971; Friend *et al.*, 1978), melanin formation (Gregory & Huang, 1964*a, b*; Shaw & Piwowarski, 1977), differentiation (Okanishi *et al.*, 1970; Pogell, 1979), antibiotic sensitivity (Shaw & Piwowarski, 1977) and antibiotic production (Hopwood, 1978; Hopwood & Merrick, 1977; Schrempf, 1981). It has been argued that a plasmid has been involved in the expression of a given phenotype when that phenotype has shown spontaneous instability and/or curing, infectious transfer during crosses, non-linkage to chromosomal markers, and correlation with presence or loss of plasmid DNA. The pros and cons of these arguments have been discussed extensively (Hopwood, 1978), and with a few notable exceptions it has been difficult to provide a conclusive demonstration of the role of plasmids in the expression of a given phenotype.

One of the best illustrations is the case of chloramphenicol production in *Streptomyces venezuelae*. The ability to produce chloramphenicol in this strain is sensitive to curing agents (Okanishi & Umezawa, 1978; Michelson & Vining, 1978) and the first genetic analysis of crosses showed no linkage of production genes with chromosomal markers (Akagawa *et al.*, 1975). *S. venezuelae* contains plasmid DNA (Malik, 1977), and a correlation between plasmid presence and production of chloramphenicol has been found (Okanishi & Umezawa, 1978). A more accurate analysis of crosses involving chloramphenicol-producing and non-producing strains showed that the genes responsible for chloramphenicol production were, in fact, localized chromosomally (Akagawa *et al.*, 1979), a result that was confirmed by DNA analysis of producing and non-producing strains (Ahmed & Vining, 1983). Moreover, Malik (1980) demonstrated that plasmid-less, chloramphenicol non-producing *S. venezuelae* strains acquired the ability to produce chloramphenicol after mutagenic treatment. This excluded the possibility that the biosynthetic genes for chloramphenicol were plasmid coded.

A contrary example is the case of *S. glaucescens*. This strain shows a high spontaneous instability, especially of the genes required for streptomycin resistance and melanin formation (Freeman & Hopwood, 1978; Hütter *et al.*, 1981). It was demonstrated that the genes controlling

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both of these functions were linked to chromosomal genes (Cramer *et al.*, 1983, 1984) and that the instability was due to independent, large deletions of chromosomal DNA (Hintermann *et al.*, 1984; Hintermann & Hütter, 1984).

These studies left open the question of whether ethidium bromide was a useful agent for plasmid-curing of streptomycetes and in this paper we have examined this point.

#### METHODS

**Bacterial strains and plasmids.** The *Streptomyces* strains used were *S. lividans* 1326 (Lomovskaya *et al.*, 1972) and *S. glaucescens* GLAO (Hütter *et al.*, 1981). The multicopy plasmids pIJ303 (thiostrepton resistance), pIJ211 (neomycin resistance) (Kieser *et al.*, 1982), pIJ702 (Katz *et al.*, 1983) and the low copy number plasmid pIJ61 (Thompson *et al.*, 1982*b*) were used. pIJ702 contains the thiostrepton-resistance gene and a tyrosinase gene from *S. antibioticus* which confers the ability to form black melanin haloes on indicator plates (Katz *et al.*, 1983). pIJ61 confers resistance towards thiostrepton and neomycin (Thompson *et al.*, 1982*b*).

**Media and culture conditions.** Strains were grown on yeast extract/malt extract agar (Pridham *et al.*, 1956/7) at 30 °C until sporulation. Spore suspensions were prepared either from agar slants or plates using 50% (v/v) glycerol and stored at -20 °C. Yeast extract/malt extract medium was used as complete medium for all experiments described. Minimal medium was the standard medium used for segregational analysis in *S. glaucescens* (Cramer *et al.*, 1983). Liquid cultures for plasmid isolations were in tryptic soya broth CM-129 (Oxoid) according to Kieser (1984).

**Transformation procedure and selection of transformants.** Plasmid DNA was introduced into *S. lividans* or *S. glaucescens* protoplasts (Thompson *et al.*, 1982*a*) and transformants detected by overlaying plates with 2.5 ml soft agar (Kieser *et al.*, 1982) containing thiostrepton (200 µg ml<sup>-1</sup>) or neomycin (100 µg ml<sup>-1</sup>).

**Plasmid stability.** This was determined by measuring the stable inheritance of antibiotic resistance. The percentage of thiostrepton- or neomycin-resistant colonies per sporulation round on antibiotic-free complete medium plates was determined (Kieser *et al.*, 1982).

**Curing experiments.** These were done using ethidium bromide (Sigma) at various concentrations in complete medium plates inoculated with serial dilutions of filtered stock spore suspensions.

**Detection of plasmid loss.** This was done by replica-plating sporulated colonies on to complete medium agar containing thiostrepton (20 µg ml<sup>-1</sup>) or neomycin (10 µg ml<sup>-1</sup>). Physical absence of plasmid DNA was demonstrated by agarose gel electrophoresis after alkaline lysis and phenol/chloroform extraction (Kieser, 1984).

**Detection of other mutational events.** Streptomycin sensitivity in *S. glaucescens* was determined according to Cramer *et al.* (1983); the inability to produce melanin was detected as described by Cramer *et al.* (1984). Chloramphenicol sensitivity in *S. lividans* was detected after replica-plating on media containing chloramphenicol (10 µg ml<sup>-1</sup>), and arginine auxotrophy after replica-plating on minimal medium containing arginine (1 mM) (Altenbuchner & Cullum, 1984).

#### RESULTS

##### Plasmid stability

In the absence of ethidium bromide, the test plasmids pIJ61 and pIJ702 in *S. lividans* as well as pIJ303 in *S. glaucescens* were completely stable after several rounds of growth and sporulation (Table 1). Plasmid stability was determined by comparing the number of c.f.u. on complete media with and without antibiotic. Between 2500 and 5000 colonies per sporulation cycle on antibiotic-free solid medium were tested. Since pIJ211 is highly unstable in *S. glaucescens* (Table 1), curing experiments with this strain were done using the stably inherited plasmid pIJ303.

Table 1. *Plasmid stability*

Strain	Transformation by plasmid*	Percentage antibiotic-resistant colonies after round(s) of growth without antibiotic						
		0	1	2	3	4	5	6
<i>S. glaucescens</i>	pIJ211 (Neo <sup>R</sup> )	100	67	44	33	22	15	12
<i>S. glaucescens</i>	pIJ303 (Thio <sup>R</sup> )							
<i>S. lividans</i>	pIJ702 (Thio <sup>R</sup> )	100	100	100	100	100	100	100
<i>S. lividans</i>	pIJ61 (Neo <sup>R</sup> Thio <sup>R</sup> )							

\* Neo, neomycin; Thio, thiostrepton.

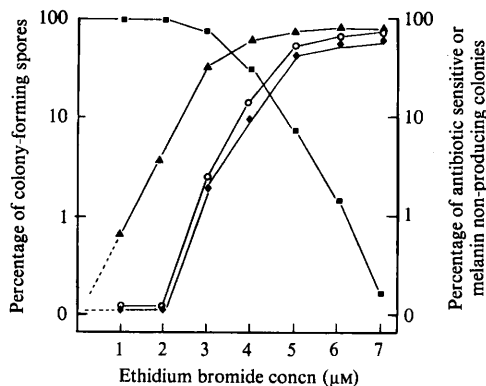


Fig. 1. Curing of pIJ303 from *S. glaucescens* and generation of melanin-negative and streptomycin-sensitive colonies. The surviving colonies (■) are given as a percentage of the original number of colonies; thioestrepton-sensitive (▲), melanin-negative (○) and streptomycin-sensitive (◆) colonies are given as a percentage of the surviving colonies.

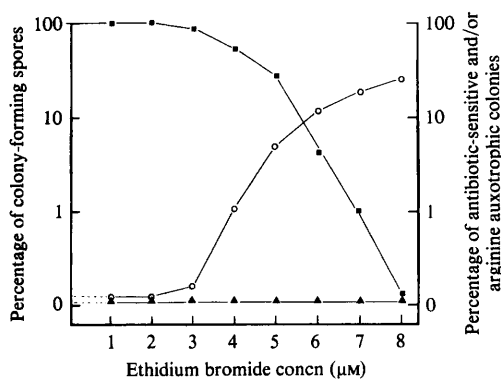


Fig. 2

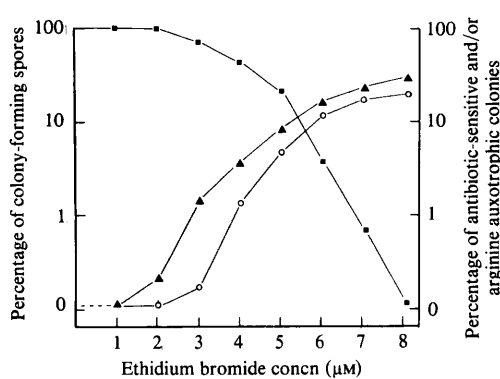


Fig. 3

Fig. 2. Effects of ethidium bromide in *S. lividans* carrying pIJ702. The surviving colonies (■) are given as a percentage of the original number of colonies; thioestrepton-sensitive (▲) and chloramphenicol-sensitive arginine-negative (○) colonies are given as a percentage of the surviving colonies.

Fig. 3. Effects of ethidium bromide in *S. lividans* carrying pIJ61. For explanation of the symbols see Fig. 2.

#### *Killing effect of ethidium bromide, plasmid-curing and generation of other mutations*

*S. glaucescens*. Fig. 1 summarizes the results of a typical curing experiment using pIJ303-containing *S. glaucescens* spores. This strain can be almost 100% cured of the plasmid by ethidium bromide concentrations that result in more than 90% of either killed spores or colony inhibition. Under these conditions ethidium bromide generated melanin-negative and streptomycin-sensitive colonies at high frequencies. Twenty thioestrepton-resistant and twenty thioestrepton-sensitive colonies from plates containing 4  $\mu\text{M}$ -ethidium bromide were analysed for the presence of plasmid DNA on agarose gels after mini preparations by alkaline lysis. A strict correlation between plasmid presence and thioestrepton resistance was found (data not shown).

*S. lividans*. The results obtained routinely with this strain are summarized in Figs 2 and 3. Plasmid pIJ702 is completely refractory to curing. In addition, among 85 637 surviving colonies grown in the presence of 7  $\mu\text{M}$ -ethidium bromide, no thioestrepton-sensitive or melanin-negative colonies could be detected. On the other hand, plasmid pIJ61, a low copy number plasmid, could be cured efficiently at high ethidium bromide concentrations (Fig. 3), but less efficiently than

pIJ303 in *S. glaucescens*. When plasmid mini preparations of thiostrepton- and neomycin-resistant colonies were analysed by agarose gel electrophoresis, plasmids of different sizes were observed, suggesting that plasmid deletions (between 1–10%) occurred in the presence of ethidium bromide (see Discussion). A restriction nuclease digestion analysis of purified plasmids confirmed this hypothesis (data not shown).

During this experiment it was noticed that a substantial number of the survivors were unable to sporulate (Figs 2 and 3); 25 such sporulation-negative colonies were studied. All were highly sensitive to chloramphenicol ( $8 \mu\text{g ml}^{-1}$ ) and were able to grow on minimal medium supplemented with arginine (1 mM) or argininosuccinate (2 mM) but not on media supplemented with ornithine (2 mM) or citrulline (2 mM). This suggests the loss of argininosuccinate synthetase, an event that occurs spontaneously in this strain at low frequencies (Altenbuchner & Cullum, 1984).

#### DISCUSSION

We have demonstrated that the DNA-intercalating agent ethidium bromide can be used to eliminate certain plasmids at high efficiency from *Streptomyces* strains. The availability of selectable plasmid vectors (Kieser *et al.*, 1982; Thompson *et al.*, 1982*b*) has facilitated this work substantially, because putative 'cured' colonies can be screened as antibiotic-sensitive clones. However, ethidium bromide does not only eliminate plasmids and *S. glaucescens* shows multiple effects of treatment. This strain has a high genetic instability with respect to several phenotypes (Hütter *et al.*, 1981), including streptomycin resistance. This resistance marker is lost spontaneously (Freeman & Hopwood, 1978; Cramer *et al.*, 1983); biochemically, streptomycin-sensitive mutants lack a streptomycin-6-phosphotransferase (Ono *et al.*, 1983; Hintermann *et al.*, 1984). Treatment of spores with ethidium bromide can increase the number of sensitive mutants to a level of 90% of surviving colonies (Suter, 1978; this work). The mutant strains are genetically homogeneous, and the *strS* mutation maps chromosomally (Cramer *et al.*, 1983). Moreover, *strS* mutants show large deletion(s), including deletion of the structural gene coding for a streptomycin-6-phosphotransferase as demonstrated by DNA hybridization experiments using the cloned gene (Hintermann *et al.*, 1984). Analogous behaviour is seen for the ability to produce melanin, a black pigment produced by tyrosinase-catalysed oxidation of tyrosine. In this case, also, the mutants appear to be genetically homogeneous and can be mapped chromosomally (Cramer *et al.*, 1984). Phenotypic instability of this trait occurs spontaneously (Hütter *et al.*, 1981) and can be increased significantly by ethidium bromide treatment (Suter, 1978; this work). In the case of tyrosinase-negative variants, analysis of DNA using a cloned gene as probe showed that ethidium bromide generated mutants which lacked the structural gene coding for tyrosinase and its flanking regions (Hintermann & Hütter, 1984). Similar mutations in another melanin-producing streptomycete, *S. reticuli*, are also due to deletion of the structural gene coding for tyrosinase (Schrempf, 1983). No physical evidence exists for a plasmid-like molecule present in the wild-type strain of *S. glaucescens* (Hintermann *et al.*, 1984).

The best-investigated strain for *in vitro* genetic manipulations of streptomycetes, *S. lividans* (Hopwood *et al.*, 1983), reacts to ethidium bromide treatment essentially in the same way as *S. glaucescens*. The fact that pIJ702 was not eliminated with ethidium bromide could be due to its extremely high copy number in this strain (Kieser *et al.*, 1982). The low copy number plasmid pIJ61 derived from the SLP1.2 replicon (Thompson *et al.*, 1982*a*) was efficiently cured by ethidium bromide. In these cases, the intercalating agent did not only cure plasmids, but in some cases generated plasmid deletions, a phenomenon known for *S. fradiae* (Komatsu *et al.*, 1981) and for *S. reticuli* plasmids (Schrempf & Goebel, 1979). In addition to these two 'mutational' events, other phenotypic changes occurred, or were increased above the spontaneous rate: loss of sporulation ability, increased chloramphenicol sensitivity and arginine auxotrophy. In spite of the fact that *S. lividans* contains several plasmids (Hopwood *et al.*, 1983) it seems improbable that genes responsible for primary metabolites such as amino acids are plasmid borne. The type of mutation increased by ethidium bromide treatment in *S. glaucescens* and in *S. lividans* also occurs spontaneously at high frequencies when compared with 'classical' chromosomal markers (Hütter *et al.*, 1981; Altenbuchner & Cullum, 1984).

We should point out that these 'mutations' do not occur randomly, but appear to be limited to plasmids and to other regions of DNA showing an increased spontaneous genetic instability. All examples of ethidium bromide-generated 'mutations' so far investigated have proved to be deletion events, as demonstrated by DNA hybridization experiments using the cloned structural genes (Schrempf, 1983; Hintermann *et al.*, 1984; Hintermann & Hütter, 1984). This could suggest an interaction between ethidium bromide and specific DNA regions. Sequence determination of such regions of DNA could be useful in elucidating the type of molecular interaction between 'curing agents' and DNA, and may lead to an understanding of the reasons for the high natural genetic instability in this genus. We conclude that in streptomycetes, ethidium bromide, and probably other DNA-intercalating agents, are potent and probably region-specific mutagens that are also capable of inducing high rates of plasmid loss (curing).

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