Unstable Naturally Occurring Resistance to Antibiotics in *Streptomyces*

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INTRODUCTION

Two sets of observations suggested that extrachromosomal elements conferring antibiotic resistance might be widespread in streptomycetes. One was the evidence for plasmids affecting various other phenotypic properties in these organisms. *Streptomyces coelicolor* A3(2) contains a plasmid, SCP1, which acts as a conjugal sex factor and also codes for methyl-enomycin production and resistance (Kirby, Wright & Hopwood, 1975; Wright & Hopwood, 1976); less direct evidence implicates plasmids in the control of antibiotic production in *Streptomyces kasugaensis* (Okanishi, Ohta & Umezawa, 1970), *Streptomyces hygroscopicus* (Kähler & Noack, 1974) and *Streptomyces venezuelae* (Akagawa, Okanishi & Umezawa, 1975), melanin production in *Streptomyces scabies* (Gregory & Huang, 1964), *S. venezuelae* (Okanishi *et al.*, 1970) and *Streptomyces glaucescens* (Baumann & Kocher, 1976), and aerial mycelium production in *S. venezuelae*, *S. kasugaensis*, *S. scabies* (Okanishi *et al.*, 1970) and *Streptomyces alboniger* (Redshaw *et al.*, 1976); and a plasmid is apparently also involved in resistance to oxytetracycline in a strain of *Streptomyces rimosus* which produces it (Boronin & Sadochnikova, 1972). Recently a second sex factor, SCP2, has been found in *S. coelicolor* A3(2) (Bibb, Freeman, & Hopwood 1977) and a sex factor, SRP1, has been revealed in a strain of *S. rimosus* (Friend, Warren & Hopwood, 1978).

A second line of evidence was the consideration that antibiotic resistance in clinical isolates of eubacteria is usually due to plasmid-borne genes and it has been suggested (Walker & Walker, 1970; Benveniste & Davies, 1973) that such genes may have originated in the bacteria that produce the antibiotics, notably members of the genus *Streptomyces*, which synthesize the majority of all known antibiotics (Bérdy, 1974), and may have been transferred to unrelated bacteria. The main argument for this theory is the presence of similar antibiotic-inactivating enzymes in antibiotic-producing streptomycetes and in resistant eubacteria (Benveniste & Davies, 1973; El-Kersch & Plourde, 1976; Shaw & Hopwood, 1976; Nakano *et al.*, 1977). Although this theory does not require the genes to be permanently plasmid-linked in their original streptomycete hosts, their dissemination to other bacteria might have been facilitated if this had been the case.

As a preliminary approach to the problem (Freeman, 1976) we determined the level of resistance of about 40 streptomycete strains, chosen essentially at random from various culture collections, to a series of 25 antibiotics and other antimicrobial agents, giving some 1000 tests. Some strains were relatively resistant to the majority of the growth inhibitors while some inhibitors were inactive against all, or nearly all, of the strains. We selected for study four cases in which a particular strain was significantly more resistant to a certain antibiotic than were most of the strains, since such behaviour might be expected if an extrachromosomal element, not uniformly present in the set of cultures, conferred some resistance to an antibiotic.

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Suggestive preliminary evidence for extrachromosomal determination of resistance was then sought by testing each of the four strains for the frequent occurrence of colonies which had become more sensitive to the antibiotic in question. One of the four examples, in the genetically well-known *S. coelicolor* A3(2), was subjected to detailed genetic analysis which suggested that some transposable, possibly extrachromosomal, factor affected chloramphenicol resistance (Freeman, Bibb & Hopwood, 1977); data on the other three examples – streptomycin resistance in *Streptomyces griseus* and *S. glaucescens* and erythromycin/chloramphenicol resistance in *Streptomyces acrimycini* – are described here.

METHODS

Strains. Of the 40 strains in the preliminary survey (Freeman, 1976), four were studied in detail: *S. coelicolor* A3(2); *S. griseus* (Collection of the University of Bradford no. 609, also known as Waksman 3475); *S. acrimycini* (Istituto Patologia Vegetale, Milan, no. 1610); and *S. glaucescens* (E.T.H., Zürich, no. 22794).

Cultural methods. Complete (CM) and minimal medium (MM) and general methods were those described by Hopwood (1967). In some experiments, glycerol (1%, v/v) was substituted for glucose as carbon source in case some form of catabolite repression affected antibiotic resistance, but no obvious evidence for this was found. Stock cultures were grown on CM at 30 °C and stored at 4 °C, or suspensions of mycelial fragments and spores were stored in 20% (v/v) glycerol at -17 °C. Ultraviolet (u.v.) irradiation was to a survival of about 1%.

Preliminary tests for antibiotic resistance. These were made by measuring zones of growth inhibition (on lawns of each strain on MM containing glycerol) produced by antibiotic test discs (Mast Laboratories, Liverpool) containing known amounts of each of 25 antibiotics or antimicrobial agents. In some cases, when growth of the lawn was slow, the plates were pre-incubated for 24 h before receiving the test discs.

Isolation of antibiotic-sensitive variants. The three strains grew almost without inhibition on MM containing the following levels of antibiotic: *S. griseus*, 10 µg streptomycin sulphate ml⁻¹ (Glaxo Laboratories); *S. acrimycini*, 2 µg erythromycin ml⁻¹ (Sigma); *S. glaucescens*, 1 µg streptomycin sulphate ml⁻¹. Sensitive isolates, which arose spontaneously and after u.v. irradiation, were obtained by plating spores on CM at dilutions that gave well separated colonies and then replicating these colonies, when they were well sporulated, to MM containing the appropriate concentration of antibiotic. Colonies that failed to grow on replication were streaked on MM and re-tested. Most were confirmed as being antibiotic-sensitive.

RESULTS

Antibiotic-sensitive variants occurred spontaneously in all three strains at frequencies between about 0.1 and 1%; after u.v. irradiation the frequency range was increased by a factor of 10 (Table 1). The frequencies of origin of streptomycin-sensitive (Sm^s) variants of *S. griseus* and *S. glaucescens* and of erythromycin-sensitive (Ery^s) variants of *S. acrimycini* were remarkably similar and also resembled those of chloramphenicol-sensitive (Cml^s) variants in the two closely related strains *S. coelicolor* A3(2) and *S. lividans* 66 (Freeman *et al.*, 1977).

In a study initiated with a different objective, Wright & Hopwood (1977) found that the same strain of *S. acrimycini* gave rise to Cml^s variants at a similar frequency (e.g. 1% spontaneously and 4% after u.v. irradiation) to that of Ery^s variants in the present work. In a further experiment, we therefore sought variants that were sensitive to either of these antibiotics, or both. Out of 18 such variants, 16 were Ery^s Cml^s, two were Ery^s Cml^r and none was Ery^s Cml^r.

The antibiotic-sensitive strains proved difficult to preserve. Of 17 Sm^s derivatives of *S. glaucescens*, eight were found on subculture to have produced Sm^r colonies. Four of the subcultures consisted almost entirely of Sm^r colonies; the remainder gave mixtures of Sm^s and Sm^r colonies. Re-streaking an Sm^s colony from such a mixture gave rise to a pure Sm^s subculture. Instability was also observed with the Sm^s *S. griseus* and Ery^s *S. acrimycini* cultures; both required continued re-purification.

In *S. coelicolor* A3(2) and *S. lividans* 66, revertibility of the Cml^s to the Cml^r phenotype showed that permanent loss of an extrachromosomal element could not explain the

Table 1. *Origin of antibiotic-sensitive variants in three streptomycetes*

Treatment	Number of colonies		Antibiotic-sensitive (%)
	Total	Antibiotic-sensitive	
(a) <i>Streptomycin sensitivity in S. griseus</i> CUB609			
Control	1143	2	0.2
u.v.	2500	44	1.8
u.v.	95	10	10.5
(b) <i>Erythromycin sensitivity in S. acrimycini</i> IPV1610			
Control	1943	12	0.6
u.v.	1250	65	5.2
Control	921	0	< 0.1
u.v.	995	7	0.7
(c) <i>Streptomycin sensitivity in S. glaucescens</i> ETH22794			
Control	2889	8	0.3
u.v.	1211	35	2.9
Control	550	2	0.4
u.v.	1130	30	2.7
u.v.	350	39	11.1
(d) <i>Streptomycin sensitivity in Sm^R revertants from an Sm^S variant of S. glaucescens</i> ETH22794			
u.v.	213	10	4.7
u.v.	269	14	5.2

Cml^R → Cml^S transition, even though segregation of Cml^S versus Cml^R in crosses was incompatible with simple chromosomal linkage. We therefore attempted to characterize the reversion or otherwise of the Sm^S and Ery^S phenotypes. Two sensitive variants of each of the three species were studied by plating spores on antibiotic-containing plates. In *S. glaucescens*, one of the Sm^S variants yielded Sm^R colonies at reproducible frequencies of about 10⁻⁵ spontaneously and 10⁻⁴ after u.v. irradiation, and two of the Sm^R revertants yielded Sm^S colonies again at frequencies of 4 to 5% after u.v. irradiation (Table 1); thus the Sm^S 'mutation' in this strain appeared to be truly reversible. The second Sm^S variant failed to revert (detection level, 10⁻⁶ spontaneously and 10⁻⁵ after u.v.).

In *S. griseus*, the two Sm^S variants gave Sm^R colonies at frequencies of about 10⁻⁶, both spontaneously and after u.v. irradiation. However, two Sm^R 'revertants' failed to yield Sm^S variants (< 0.3%) after u.v. irradiation. Thus true reversal of the original Sm^S 'mutation' was not involved. In the case of Ery^S (Cml^S) variants of *S. acrimycini*, background growth on plates containing erythromycin or chloramphenicol prevented the selection of possible Ery^R (or Cml^R) colonies.

DISCUSSION

The most striking aspect of the results of this survey is the finding that, in all four of the chosen cases in which a strain exhibited a higher level of resistance to an antibiotic than the majority of strains, this resistance was found to be unstable. Since selection of the four cases was, to a considerable extent, arbitrary, it is very likely that many more examples were present in the original matrix of tests. In two of the chosen examples – *S. coelicolor* (and *S. lividans*) and *S. glaucescens* – there was an oscillation between resistance and sensitivity, at least in a proportion of occurrences, with a much higher frequency of R → S than of S → R events. In the other two examples, true reversal of the R → S event was not demonstrated, although it was not excluded.

Another example of unstable antibiotic resistance has recently been published (Shaw &

Piowowski, 1977); Sm^s variants of *S. bikiniensis* arose with high frequencies after acriflavine or ethidium bromide treatment (many of them also failed to synthesize streptomycin). The Sm^s variants were not highly unstable – they remained sensitive after six transfers – but no attempt to select rare Sm^r ‘revertants’ was described. In another report, an unnamed *Streptomyces* sp. was found to give rise to frequent β -lactamase non-producing variants after acriflavine treatment, although they were apparently not more sensitive than the original strain to β -lactam antibiotics (Ogawara & Nozaki, 1977).

All these results point to the presence in many streptomycetes of determinants of antibiotic resistance which can be inactivated or lost with high frequency. Much more detailed studies will be required in order to understand the genetic mechanisms involved. Even in *S. coelicolor* A3(2), with a comparatively well-developed experimental genetic system, it has not yet been possible to provide a detailed model of the Cml^r $\rightarrow$ Cml^s $\rightarrow$ Cml^r oscillation, although it seems probable that some kind of transposition of genetic material is involved, rather than its permanent loss (Freeman *et al.*, 1977). The Sm^r $\rightarrow$ Sm^s $\rightarrow$ Sm^r oscillation in *S. glaucescens* seems to be similar, although the genetic system in this strain (Baumann & Kocher, 1976) has not yet been used to analyse it.

In summary, none of the examples of unstable antibiotic resistance described in this paper can be completely explained by simple loss of a plasmid, since return to resistance could always take place (albeit in certain cases not by a true reversion of the change to sensitivity). It is, of course, possible that more than one mechanism is at work in different strains. Certain other cases of frequent origin of sensitive strains may well involve genuine plasmid loss: A. M. Boronin has recently found (personal communication) extrachromosomal DNA in his *S. rimosus* strain which is absent from the oxytetracycline-sensitive variants. Moreover, in *S. coelicolor* A3(2), simple loss of the SCP1 plasmid certainly gives rise to methylenomycin sensitivity. It may be significant that in these two examples, resistance is in respect of the organism's own antibiotic.

Detailed analysis of several of these systems involving antibiotic resistance may very well illuminate the many poorly documented examples of ‘instability’ of desirable characteristics which plague strain improvement programmes in industrial fermentations.

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