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DNA Amplification and Genetic Instability in *Streptomyces*

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Introduction

Streptomyces species are of considerable industrial importance because they produce about 60% of known antibiotics (Bérdy, 1974). They are prokaryotes which grow in a filamentous form and produce spores. They have a genome size of about 10 000 kb, i.e. about three times as large as *E. coli* (Benigni, Antonov and Carere, 1975) and a G + C content of 70–75% in their DNA (Gladek and Zakrzewska, 1984; Usdin, Gertsch and Kirby, 1984).

Genetic instability is very common in *Streptomyces* species and can affect a variety of genes. However, only specific genes are affected in any one strain. The sort of genetic instability discussed in this review is probably responsible for the 'degeneration' of commercial strains, a process in which strains irretrievably lose useful properties (Miyoshi *et al.*, 1980).

Table 1 contains a list of some of the unstable genetic systems that have been studied in *Streptomyces* and which initially, were often attributed to plasmid loss. However, in many species, evidence has now accumulated of chromosomal DNA changes including deletions and DNA amplifications. The characters that are subject to instability mainly affect secondary metabolic functions such as pigment production or antibiotic production. Frequently, pleiotropic mutants arise that are deficient in many aspects of secondary metabolism including aerial mycelium formation and sporulation. Sometimes, however, primary metabolic processes such as arginine biosynthesis may be unstable. In this review we discuss some selected systems and point out their similarities and differences. A unique feature of instability in *Streptomyces* is the frequent amplification of chromosomal DNA sequences

Abbreviations: EtBr, ethidium bromide; IS, insertion sequence.

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Table 1. Examples of genetic instability in *Streptomyces*

Species	Unstable properties	Reference
S. alboniger	Arginine biosynthesis	Redshaw *et al.* (1979)
S. aureofaciens	Chlortetracycline production	Okanishi (1980)
S. bikiniensis	A-factor synthesis	Hara and Beppu (1982)
S. cattleya	Arginine biosynthesis	Usdin *et al.* (1985)
S. coelicolor A3(2)	Chloramphenicol resistance	Freeman, Bibb and Hopwood (1977)
	Arginine biosynthesis	Sermonti *et al.* (1978)
S. fradiae	Tylosin production	Baltz and Stonesifer (1985)
S. glaucescens	Streptomycin resistance	Freeman and Hopwood (1978)
	Melanin production	Hütter *et al.* (1981)
S. griseus	A-factor synthesis	Hara and Beppu (1982)
S. hygroscopicus	Turimycin production	Roth and Noack (1982)
S. kasugaensis	Arginine biosynthesis	Nakano, Ozawa and Ogawara (1980)
61	Kasugamycin production	Okanishi (1980)
	Aureothricin production	Okanishi (1980)
S. lavendulae	Arginine biosynthesis, β-lactamase	Nakano and Ogawara (1980)
S. lividans 66	Chloramphenicol resistance	Freeman, Bibb and Hopwood (1977)
	Arginine biosynthesis	Altenbuchner and Cullum (1984)
S. reticuli	Melanin production	Schrempf (1982)
S. supporonensis	Bicyclomycin production	Miyoshi *et al.* (1980)
S. scabies	Melanin production	Gregory and Huang (1964b)
S. venezuelae	Chloramphenicol production	Okanishi (1980)
S. violaceus-ruber	Arginine biosynthesis	Redshaw *et al.* (1979)

to a high copy number (often over 100) in the absence of any obvious selective pressure for amplification.

Unstable genes in *Streptomyces*

MELANIN PRODUCTION IN *S. SCABIES*

Melanin production in many *S. scabies* strains and in some other strains was unstable, giving rise to spontaneous Mel$^-$ mutants at a frequency of about 1% of spores (Gregory and Huang, 1964b). Treatment of mycelial fragments with acriflavine increased the frequency of Mel$^-$ mutants, sometimes to more than 25% of surviving spores. The Mel$^-$ phenotype is caused by loss of the enzyme tyrosinase (monophenol monooxygenase, EC 1.14.18.1).

As intercalating agents, such as acriflavine, were known to 'cure' (i.e. lead to loss of) some plasmids, it was suggested that the high frequency of Mel$^-$ mutants was due to loss of a plasmid carrying the tyrosinase gene. This interpretation was supported by conjugation experiments which showed that, in matings of Mel$^+$ × Mel$^-$ strains, the tyrosinase gene was transferred independently of chromosomal markers (Gregory and Huang, 1964a). Moreover, nearly all recombinants for chromosomal markers (94–100%) produced melanin, suggesting high-frequency transfer of the melanin gene independently of chromosomal markers.

This work, the first detailed examination of the phenomenon of instability in *Streptomyces* revealed properties typical of many instances of instability later studied. It has been found that, as a general rule:

1. Only specific genes are affected;
2. Instability is increased by treatments that 'cure' (i.e. eliminate) plasmids:
3. The genes affected cannot be mapped to the chromosome;
4. The wild-type allele is over-represented in transconjugants.

These characteristics were, however, also found in studies of the tyrosinase gene of *S. glaucescens* (*see below*) where there is convincing evidence that the gene is chromosomally encoded.

MELANIN PRODUCTION IN *S. RETICULI*

A 70 kb plasmid was found in a *S. reticuli* strain that was unstable, giving rise to mutants unable to produce melanin and also defective in aerial mycelium formation and the production of secondary metabolites (Schrempf, 1982). The Mel^- mutants had either lost the plasmid or showed gross changes in the plasmid DNA. However, hybridization experiments, using a cloned tyrosinase gene, showed that, although the tyrosinase gene had been lost from the Mel^- mutants, it was *not* encoded by the plasmid (Schrempf, 1983).

Closer analysis of the DNA of Mel^- mutants demonstrated that some chromosomal DNA sequences had become amplified. The amplifications were of high copy number (> 100) and varied from isolate to isolate, although there seemed to be some common subsequences. It was also found that some plasmid DNA sequences had integrated into the chromosome in Mel^- strains, but it is not clear whether the plasmid is intimately involved in the instability events or is only another target for DNA rearrangements.

MELANIN PRODUCTION AND STREPTOMYCIN RESISTANCE IN *S. GLAUCESCENS*

S. glaucescens is unstable for melanin production and for streptomycin resistance. Both genes have been cloned: the *mel* C gene (codes for tyrosinase, the melanin-producing enzyme; Hintermann, Zatchej and Hütter, 1985) and the *str* S gene (codes for streptomycin 6-kinase (EC 2.7.1.72) that inactivates streptomycin; Hintermann *et al.*, 1984). The Mel^- strains have deleted the *mel* C gene and the Str^S strains have deleted the *str* S gene; in both cases large deletions have occurred. The frequency of Mel^- and Str^S mutants is increased by treatment with ethidium bromide (EtBr); after treatment with 8 μg/ml EtBR nearly 100% of survivors are Mel^-Str^S (Hütter *et al.*, 1981). At lower doses, the proportion of Mel^- is higher than that of Str^S and the response to EtBr treatment suggests that the two mutation events are independent. Storage of spores in glycerol at −20°C also stimulates instability, but UV irradiation seems to have no effect.

The *str S* gene can be mapped to a chromosomal location in conjugational crosses between Str^S mutants and Str^R strains (Crameri *et al.*, 1983). Some *mel* C mutants can also be mapped in $Mel^+ \times Mel^-$ crosses; they map, however, to a location distinct from the *str* S gene and separated from it by several amino-acid biosynthesis genes (Crameri *et al.*, 1984). Some *mel* C

mutants can not be mapped to a definite chromosomal location in Mel$^+$ × Mel$^-$ crosses. In such cases the crosses show abnormally high apparent fertility for transfer of the *mel* C$^+$ marker and consequently give unbalanced crosses that cannot be properly analysed.

The promotion of instability by treatment with plasmid 'curing' agents such as EtBr has been interpreted in other cases (e.g. in *S. scabies*, *see above*) as evidence that unstable genes are plasmid-borne and that instability involves plasmid loss. The example of *S. glaucescens* where *str* S and *mel* C are plainly chromosomal genes yet their loss (by deletion) is promoted by EtBr treatment shows that 'curing' data must be interpreted very cautiously in the absence of physical evidence of plasmid involvement. It should be noted that the anomalous conjugation behaviour of some *mel* C mutants described above could also have been interpreted as evidence that *mel* C was plasmid-borne and transmitted at a much higher frequency than normal chromosomal genes during conjugation.

Some, but not all, Mel$^-$ and Str^S mutants of *S. glaucescens* carry DNA amplifications (Ono *et al.*, 1982; Hasegawa *et al.*, 1985). The DNA amplifications consist of tandem repeats (often with a copy number of over 100) of chromosomal sequences. Different isolates had different amplifications that varied in length from 2·9 kb to 35 kb. Most of the amplifications were not very stable, being lost on subculturing, but one was stable enough to be used as a marker in conjugation experiments; this showed that the amplification mapped near the *str* S gene (Hasegawa *et al.*, 1985). The different amplifications proved to involve sequences in one of two chromosomal regions of approximate lengths 12 kb and 30 kb. However, the different amplifications involved different sequences that sometimes did not overlap, i.e. there was no common sequence to the different amplifications. If specific sequences exist within the amplifications that are necessary for amplification there must be at least five different ones. In most cases there appear to be deletions to one or both sides of the amplified sequences after amplification.

TYLOSIN PRODUCTION IN *S. FRADIAE*

Two strains of *S. fradiae* which no longer produced the antibiotic tylosin were found after a protoplast-regeneration cycle (Baltz and Stonesifer, 1985): this procedure consists of forming protoplasts from a strain and allowing them to regenerate; it seems to eliminate plasmids and induce instabilities, but the mechanisms are not known. Both mutants were also sensitive to tylosin and chloramphenicol. One strain (JS82) had also become sensitive to mitomycin C and hygromycin B, but was unstable, giving rise to hygromycin B-resistant derivatives at high frequency. The other mutant (JS87) was also unstable giving methionine–histidine double auxotrophs.

Fishman and Hershberger (1983) found that JS82 carried an amplification of a 10·5 kb sequence and they used quantitative hybridization to estimate its copy number as 500 ± 150. It was assumed that this sequence was carried chromosomally because there is no evidence of plasmid involvement. The amplifiable sequence was examined in the wild-type parent and proved to

consist of an inner sequence of 8·3 kb flanked by two direct repeats of a 2·2 kb sequence (Fishman, Rosteck and Hershberger, 1985). In the amplified strain there were tandem repeats with the repeat unit consisting of one copy of the flanking sequence and one copy of the inner sequence. This is reminiscent of the structures in *E. coli* that give rise to amplification by unequal crossing over between the flanking sequences (Schmitt *et al.*, 1981). It is not known if the sequences adjacent to the amplifiable region in the wild-type strain have undergone DNA rearrangements in the amplified strain (cf. *S. glaucescens* and *S. lividans* where deletions occur adjacent to amplifications).

The flanking sequence was also present at two other sites in the wild type and thus seemed to be similar in properties to insertion sequences in *E. coli* (*see* Cullum, 1985) although in this case transposition has not been proven. In the other tylosin non-producing mutant, JS87, there is no amplification and all four copies of the flanking sequence have been deleted (Hershberger and Fishman, 1985). This suggests that all copies may be in the same region of the chromosome.

A-FACTOR GENES IN *S. GRISEUS* AND *S. BIKINIENSIS*

S. griseus and the related species *S. bikiniensis* give rise at high frequency to non-sporulating derivatives. Sporulation is restored by a diffusible substance, the A-factor, produced by sporulating colonies (Khokhlov *et al.*, 1973). The A-factor is 2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone (Mori, 1983) and is necessary for many aspects of secondary metabolism including streptomycin production and resistance.

A-factor non-producing mutants arise at high frequency if strains are grown at a high temperature (37°C) or treated with intercalating agents (Hara and Beppu, 1982). In protoplast fusion experiments using A-factor-producing strains and non-producing mutants of *S. griseus*, the A-factor genes could not be mapped to a chromosomal location and they were transferred independently of chromosomal genes, suggesting that it was a plasmid-borne character (Hara *et al.*, 1983). However, there is as yet no physical evidence for this and, in *S. coelicolor* A3(2), two A-factor genes (*afs* A and *afs* B) can be mapped to the chromosome. Horinouchi, Kumada and Beppu (1984) cloned genes from *S. bikiniensis* that complemented A-factor non-producing mutants of *S. bikiniensis* and *S. griseus*. In hybridization experiments they showed that the mutants had lost sequences homologous to the cloned genes which would be consistent with either plasmid loss or deletion of chromosomal genes.

It is possible that another mechanism of instability might occur in these species as Kirby and Lewis (1981) observed cyclical behaviour of Str^S mutants, with reversion of Str^R which, in its turn, gave rise to Str^S mutants. It is not clear if these mutants affect A-factor synthesis, but some are pleiotropic, affecting sporulation and streptomycin production as well as resistance; they are thus similar in phenotype to A-factor mutants.

ARGININE INSTABILITIES

Many species from a range of taxonomic groups give rise to arginine auxotrophs at high frequency, either spontaneously or after various treatments (*Table 2*). These mutants usually lack the enzyme argininosuccinate synthase (EC 6.3.4.5) and thus can grow on argininosuccinate but not on citrulline (Sermonti *et al.*, 1978; Redshaw *et al.*, 1979; Matsubara-Nakano, Kataoka and Ogawara, 1980). Recently it has been shown that the Arg$^-$ mutations in several species arise from deletion of the gene for argininosuccinate synthase, *arg* G (Ishihara, Nakano and Ogawara, 1985; Usdin *et al.*, 1985).

In at least two cases, arginine instabilities have been correlated with the presence or absence of plasmids. In *S. kasugaenisis*, Arg$^-$ mutants had lost a plasmid that reappeared in Arg$^+$ revertants (Nakano, Ozawa and Ogawara, 1980). In *S. cattleya*, one Arg$^-$ mutant had transferred part of the *arg* G gene to a plasmid (Usdin *et al.*, 1985).

Table 2. Arginine instabilities in *Streptomyces*

Species	Conditions*	Deletion of *arg* G
S. alboniger	Spontaneous, EB, AF (1)	Yes (6)
S. cattleya	Spontaneous, EB (7)	Yes (7)
S. coelicolor A3(2)	Cml^S (3)	Yes (6)
S. lavendulae	Spontaneous, EB, RF, UV (2)	Yes (6)
S. kasugaensis	AF (4)	?
S. lividans 66	Cml^S (5)	Yes (6)
S. scabies	AF (1)	?
S. violaceus-ruber	AF (1)	?

* EB: ethidium bromide; AF: acriflavine; RF: rifampicin; UV: ultraviolet radiation; Cml^S: chloramphenicol-sensitive mutants.
References: (1) Redshaw *et al.* (1979); (2) Matsubara-Nakano, Kataoka and Ogawara (1980); (3) Sermonti *et al.* (1978); (4) Nakano, Ozawa and Ogawara (1980); (5) Altenbuchner and Cullum (1984); (6) Ishihara, Nakano and Ogawara (1985); (7) Usdin *et al.* (1985).

CHLORAMPHENICOL RESISTANCE AND ARGININE INSTABILITIES IN *S. COELICOLOR* A3(2) AND *S. LIVIDANS* 66

S. coelicolor A3(2) and the closely related species *S. lividans* 66 have unstable chloramphenicol resistance (Cml^R) genes and produce spontaneous Cml^S (chloramphenicol-sensitive) mutants at a frequency of up to 1% of spores (Freeman, Bibb and Hopwood, 1977). This instability was stimulated by ultraviolet irradiation and ethidium bromide treatment. In both species reversion to Cml^R was seen and for *S. coelicolor* it was shown that the Cml^R revertant gave rise again to Cml^S derivatives at a similar frequency to the Cml^R parent strain: thus, the revertants seemed to be true revertants rather than caused by suppression by a second site mutation. *S. lividans* 66 has a repetitive DNA element RES1 which undergoes deamplification in most spontaneous Cml^S mutants (Danilenko and Starodubtseva, 1986). Cml^R revertants had reamplified RES1. RES1 seemed to encode some sort of

control gene as its copy number also modulated expression of a cloned kanamycin resistance gene from *S. rimosus* (Potekhin and Danilenko, 1985).

In contrast to these observations, Altenbuchner and Cullum (1984) were unable to obtain full revertants of their Cml^S mutants of *S. lividans* 66 and attributed the partial revertants found to a second site mutation. F. Flett and J. Cullum (unpublished work) investigated the molecular basis for the Cml^S instability in *S. coelicolor* and *S. lividans*. It was not possible to clone the Cml^R gene directly because the Cml^S mutants could not be transformed at high efficiency. Therefore, an alternative approach was used of selecting for higher chloramphenicol resistance in the expectation that the resistance gene would have become amplified; such an approach had proved successful for cloning a kanamycin resistance gene from *S. rimosus* (Potekhin and Danilenko, 1985). After serial subculture in increasing concentrations of chloramphenicol, a mutant of *S. coelicolor* was found with a minimal inhibitory concentration for chloramphenicol of over 200 μg/ml (the wild type has an MIC of 20 μg/ml). This mutant had amplified a long DNA sequence (larger than 50 kb) to a low copy number (about 20). Parts of the amplified sequence were cloned and used as hybridization probes to investigate spontaneous chloramphenicol-sensitive mutants of *S. coelicolor* and *S. lividans*. This showed that in both species the Cml^S mutants had large deletions (these deletions covered the whole length of the probes used and were, therefore, at least 30 kb in length. As the probes covered only a small part of the amplified sequence in the highly resistant mutant, it is likely that the deletions are much larger than 30 kb). Cml^R 'revertants' were selected from the Cml^S mutants of *S. coelicolor*, but, unlike the revertants described by Freeman, Bibb and Hopwood (1977), they did not give rise to Cml^S mutants at high frequency. The reasons for these discrepancies are unknown: perhaps there are two mechanisms for the production of Cml^S mutants, one involving deletion of the Cml^R gene and the other, deamplification of element RES1. The differences in protocols used in the different laboratories might lead to preferential selection of one of these classes of mutants. This will be resolved only by comparing the Cml^S mutants isolated in the different laboratories.

Freeman, Bibb and Hopwood (1977) were unable to map the Cml^R gene to a chromosomal location in $Cml^R \times Cml^S$ crosses and found very unbalanced crosses with high apparent fertility for transfer of Cml^R. This is very similar to the situation found with some of the *mel* C mutants of *S. glaucescens* (*see above*). Sermonti *et al.* (1977) interpreted $Cml^R \times Cml^S$ matings as showing evidence of high-frequency transfer of the Cml^R gene independently of chromosomal gene transfer and concluded that the Cml^R gene was carried on an extrachromosomal element; however, Freeman, Bibb and Hopwood (1977) showed that the Cml^R gene was not carried on any known extrachromosomal element in *S. coelicolor*. It was subsequently suggested that the Cml^R gene was on a transposable element that transposed between different chromosomal locations at such a high frequency that any population of cells contained individuals with the Cml^R gene at different locations, thus confounding normal mapping procedures (Sermonti *et al.*, 1978; Sermonti, Lanfaloni and Micheli, 1980). These authors tried to overcome this problem

by splitting transconjugants into different classes where each class should be derived from parents with the Cml^R in a different particular location. However, Chater and Hopwood (1984) pointed out that this analysis did not unambiguously prove that the Cml^R gene had different chromosomal locations in different sub-populations. Sermonti, Lanfaloni and Micheli (1983) described experiments suggesting that the Cml^R gene can transpose to the plasmid SCP1 to give very unstable insertions. Unfortunately, SCP1 cannot be isolated as extrachromosomal DNA so it has not been possible to check whether there are DNA insertions into SCP1 consistent with the genetic evidence. It therefore remains to be proved that the Cml^R gene is anything other than a normal chromosomal gene in a region prone to frequent deletion and that the strange results obtained in conjugation experiments are not artefacts due to the presence of large deletions.

Sermonti *et al.* (1978) showed that some Cml^S *S. coelicolor* mutants gave rise to arginine auxotrophs at high frequency (about 5% of spores). Arg^- mutations are due to deletion of the *arg* G gene (Ishihara, Nakano and Ogawara, 1985; F. Flett, J. Platt and J. Cullum, unpublished work) and result in loss of the penultimate enzyme of the arginine biosynthesis pathway (argininosuccinate synthase) but the previous enzyme (ornithine carbamoyl transferase, EC 2.1.3.3) and the subsequent one (argininosuccinate lyase, EC 4.3.2.1) are both retained. In *S. lividans*, Cml^S mutants also give rise to arginine auxotrophs, but at the extremely high frequency of 25% of spores (Altenbuchner and Cullum, 1984). The *arg* G gene is also deleted in this case (Ishihara, Nakano and Ogawara, 1985; J. Altenbuchner, unpublished work).

Altenbuchner and Cullum (1984) showed that the *S. lividans* Δ(*arg* G) mutants had all amplified the same 5·7 kb DNA sequence to over 100 tandem copies. In the Arg^+ parent, the amplifiable region consists of three copies of a 1 kb sequence flanking two copies (directly repeated) of a 4·7 kb sequence (*Figure 1*; Altenbuchner and Cullum, 1985). The repeat unit of the 5·7 kb amplified sequence consists of one copy of the 1 kb flanking sequence and one copy of the 4·7 kb internal sequence (*Figure 1*). This structure is similar to that found in *S. fradiae* (*see above*) except that there is an extra copy of the repeat in the wild type. Altenbuchner and Cullum (1985) observed that, in DNA prepared from Cml^S strains, there was often an extra band in Southern hybridizations which showed that some members of the population carried only two copies of the flanking sequence and one of the internal sequence giving a structure like *S. fradiae*. It is not known whether this variant form is an intermediate in amplification or just the product of a side pathway.

In *S. lividans* Δ(*arg* G) strains there is a deletion of sequences to one side of the amplification, but sequences to the other side remain intact (*Figure 1*; Altenbuchner and Cullum, 1985). It is tempting to postulate that the *arg* G gene lies within these deletions, but the occurrence of at least two deletions in *S. glaucescens* Mel^- Str^S mutants discussed above shows that care must be taken in interpreting such data. The occurrence of the 1 kb

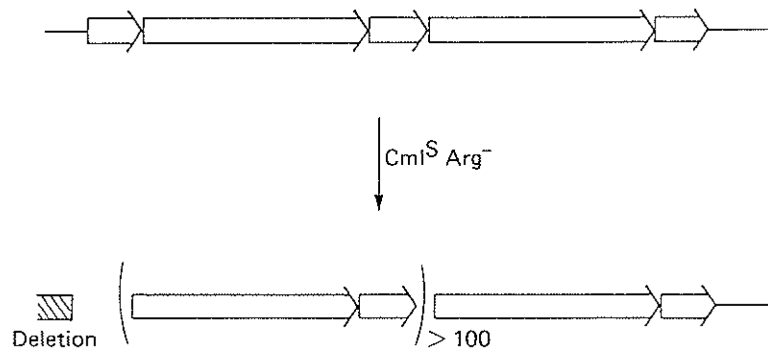

Figure 1. DNA amplification and deletions in spontaneous Arg$^-$ mutants of *S. lividans* 66 produced at high frequency from spontaneous Cml^S mutants.

element in three copies and the formation of deletions adjacent to the element on amplification (*Figure 1*) suggest that it might resemble an *E. coli* insertion sequence (*see* Cullum, 1985). However, restriction analysis (Altenbuchner and Cullum, 1985) and DNA sequence analysis (W. Piendl, unpublished work) show that the three copies of the element differ in sequence. This suggests that the structure in the amplifiable region has existed long enough to allow sequence divergence and argues against frequent transposition (no other copies exist in the *S. lividans* chromosome).

Cml^{S}Arg$^-$ mutants of *S. coelicolor* A3(2) do not show extensive DNA amplification. However, hybridization experiments using the *S. lividans* 5·7 kb amplified sequence as a probe show that this sequence also occurs in *S. coelicolor* and that DNA rearrangements in this region have occurred in all Arg$^-$ mutants examined (F. Flett, J. Platt and J. Cullum, unpublished work). The events observed are more varied than in *S. lividans* and fall into three classes which occur with approximately equal frequency:

1. Deletion of all sequences hybridizing to the probe;
2. Amplification (to 5–10 copies) of a region of at least 20 kb in length flanking the probe on both sides;
3. Deletion of sequences to one side of the probe.

The experiments described above for *S. lividans* used strain TK64 that has been 'cured' for its normal plasmids SLP2 and SLP3. When an SLP2$^+$ SLP3$^+$ strain is used, as well as the normal amplification of the 5·7 kb band there is amplification of another sequence which is much longer (>20 kb) and amplified to a lower copy number (about 20). There is some circumstantial evidence linking this second amplification to the presence of plasmid SLP3. However, the only unambiguous property identified is that the second amplification includes a region coding for mercury resistance (J. Altenbuchner, unpublished work).

TURIMYCIN PRODUCTION IN *S. HYGROSCOPICUS*

S. hygroscopicus produces the antibiotic turimycin. During long continuous culture experiments, the population was sometimes taken over by non-turimycin-producing strains (Roth and Noack, 1982). The mutants did not revert, suggesting that they had arisen by loss of genetic material. It was found that manipulation of fermenter conditions such as choice of limiting substrate or dilution rate could prevent appearance of non-producing strains. It was thought that these effects were on the frequency of production of non-producing mutants because competition experiments showed that non-producers outgrew producing strains even when fermenter parameters corresponded to those for stable cultures. It was interesting that raising the temperature from 30°C to 37°C led to stabilization. In *E. coli*, the insertion sequence (IS) IS1 is deletogenic only at lower temperatures (*see* Cullum, 1985) and other transposable elements also show temperature sensitivity. In fact, an IS-like element of 1·14 kb with 100 bp inverted repeats at the ends has been observed in phage SH3 of *S. hygroscopicus* (Klaus *et al.*, 1982). The observation of this element at different sites is consistent with a high frequency of transposition and the authors suggest that it might be involved in the turimycin instability.

The *S. hygroscopicus* strain used has a low copy number 9 kb CCC–DNA plasmid species. There do not seem to be any gross changes in this plasmid between turimycin-producing and non-producing strains (Zippel, Neigenfind and Noack, 1983). However, these authors showed that in crosses between producing and non-producing strains, it was not possible to map the production genes to a chromosomal map position; the crosses employed both conjugation and protoplast fusion. The crosses showed transfer of the turimycin production allele at frequencies characteristic of chromosomal markers rather than the high frequencies seen in some *S. glaucescens* Mel crosses and *S. coelicolor* Cml crosses (*see earlier*). However, in view of the known problems in mapping some *Streptomyces* genes that are chromosomal, it would be dangerous to conclude that any of the genes needed for turimycin production are extrachromosomal in the absence of molecular evidence.

Genetic instability and DNA amplification in applications

THE PROBLEM OF GENETIC INSTABILITY AND STRAIN DEGENERATION IN ANTIBIOTIC FERMENTATIONS

Genetic instability can be a serious problem when producing antibiotics. For example, *S. aureofaciens* (producing chlortetracycline) and *S. rimosus* (producing oxytetracycline) are commercially important species that are subject to genetic instability (Okanishi, 1980; *see* discussion of Hopwood, 1983). Frequently, strains developed to achieve high antibiotic production grow slowly, so that mutants of low productivity often outgrow the production strain.

When a bicyclomycin-producing strain of *S. sapporonensis* was grown in pilot plant fermenters of 4000 litres, it gave a lower yield of antibiotic than expected from the shake-flask data (Miyoshi *et al.*, 1980). This proved to be attributable to the accumulation of aerial-mycelium-negative (Amy$^-$) low-producing mutants. The production of Amy$^-$ mutants was stimulated by treatment with acriflavine, but was suppressed by growth of cultures at 40°C (cf. *S. hygroscopicus*, above). Thus, this strain 'degeneration' resembles the genetic instabilities described earlier.

Roth and Noack (1982) showed that, in *S. hygroscopicus*, careful choice of growth media and conditions may eliminate the problem of instability (*see above*). However, in many fermentations, such an approach may not be feasible or desirable. In the case of the batch cultures used for commercial antibiotic production, the problem of genetic instability may be overcome by careful testing of the inocula. If a 100 000 litre fermenter is inoculated using a litre starter culture, then the fermenter volume is about 2^{17} times as large as that of the starter culture. Thus, there will only be about 20 generations of growth in the large fermenter, so, if the number of mutants in the starter culture is low enough, genetic instability may not pose a problem.

DNA AMPLIFICATIONS ARISING IN STRAIN IMPROVEMENT PROGRAMMES

Koller, Engels and Uhlmann (1984) reported work on increasing the expression of the α-amylase inhibitor 'Tendamistat' (Hoe467, Hoechst) in *S. tendae*. This is a polypeptide produced by a single gene. One overproducing strain (about 20-fold overproduction) was selected after mutagenesis with acriflavine and proved to have amplified a region of 37 kb including the Tendamistat gene. The overproduction presumably arose as a direct consequence of increased gene dosage and can also be achieved by cloning the gene in a high-copy-number plasmid. The amplified overproducing strain was stable, making this amplification a useful step in strain development.

In the case of antibiotics and other secondary metabolites produced by multigene pathways, it is far from obvious whether amplifying particular biosynthetic genes will result in increased production. However, it is likely that in some cases improved production in commercial strains has arisen from fortuitous amplification of genes during strain development. Orlova and Danilenko (1983) reported that a strain of *S. antibioticus* that had been developed to produce high levels of oleandomycin had undergone amplification of a 32·3 kb chromosomal sequence they named eSA1. eSA1 was present as a single chromosomal copy in the parent strain before the strain development programme and had undergone tandem amplification in the production strain. Some copies of eSA1 (about one per chromosome) were present as extrachromosomal circular DNA, but they may have been derived from the amplification by recombination giving 'loop out'. The role of the amplification in overproduction is not yet clear. One possibility is that amplification of some sort of control gene has occurred, similar to that of

RES1 in *S. lividans* (Starodubtseva, Taisova and Danilenko, 1985), the amplification of which is needed for efficient expression of Kan^R and Cml^R (*see earlier*).

Robinson, Lewis and Napier (1981) observed DNA amplifications in strains that were thought to derive from interspecific protoplast fusion. Closer analysis suggested that the amplified strains were not related to those used for protoplast fusion but were contaminants (Evelyn Lewis, personal communication). The presence of amplifications could be correlated with the property of resistance to tunicamycin, suggesting that the amplifications had arisen as a result of selection for tunicamycin resistance in the supposed recombinants.

DIRECTED AMPLIFICATION OF CLONED GENES

The ability to amplify cloned genes may prove useful in constructing stable highly expressing clones. The major problem in harnessing the ability of *Streptomyces* species to amplify chromosomal DNA fragments is to find systems that give reproducible amplification. One approach to this problem has been to find suitable marker genes which can be selected for the higher expression that would come from an increased copy number. Potekhin and Danilenko (1985) showed that selection for higher kanamycin resistance (Kan^R) in a strain of *S. rimosus* led to a 300-fold amplification of a 15·6 kb chromosomal fragment carrying the Kan^R gene. The molecular basis for kanamycin resistance in this species is not clear because, although the amplified fragment codes for kanamycin kinase (EC 2.7.1.95) that confers resistance to neomycin and paromomycin, there is some evidence that this enzyme is not responsible for the observed kanamycin resistance (Starodubtseva, Taisova and Danilenko, 1985). The Kan^R gene could be cloned into *S. lividans* using the plasmid SLP1.2 as a vector. Selection of higher levels of Kan^R in *S. lividans* carrying such plasmids led to tandem amplification of the plasmid DNA (Danilenko *et al.*, 1984; Potekhin and Danilenko, 1985) and this could be used to coamplify other foreign DNA fragments cloned into the plasmids (Starodubtseva, Taisova and Danilenko, 1985; Danilenko, Starodubtseva and Navashin, 1985). Amplification seemed to result in greater plasmid stability. In the amplified strains the plasmids could no longer be recovered as CCC–DNA species and it was assumed that they had integrated into the chromosome. However, SLP1.2 lacks the normal SLP1 attachment site that allows it to integrate into the chromosome of *S. lividans* (Omer and Cohen, 1984) and it can not be ruled out on the published data that the DNA exists as large multimers of the plasmids that would not be recovered intact as CCC–DNA. It is also possible that the Kan^R fragment carries sequences capable of promoting chromosomal integration.

We have used a different approach, based on the observation (Altenbuchner and Cullum, 1984—*see earlier*) that spontaneous Cml^S strains of *S. lividans* (that arise at a frequency of about 1% of spores) are extremely unstable, giving rise to amplification of a particular 5·7 kb chromosomal fragment at a frequency of 25% of spores; the amplified strains are easily recognized because they are deficient in sporulation due to deletion of the

arg G gene. We cloned the 5·7 kb amplified fragment into plasmid pIJ702 and showed that when such plasmids were introduced into *S. lividans* it was possible to obtain spontaneous Cml^S Arg^- derivatives (Cullum and Altenbuchner, 1985). These derivatives were extremely stable for the plasmid $Thio^R$ marker and contained only small amounts of CCC–DNA, although restriction analysis of total DNA showed that there was a high concentration of plasmid sequences. It was concluded that the plasmid had integrated and amplified in the chromosome. The agarase (EC 3.2.1.81) gene of *S. coelicolor* (Kendall and Cullum, 1984) was cloned into such an integrating plasmid and this allowed coamplification of the agarase to give relatively stable $Agarase^+$ clones.

In some cases it might not be desirable to have a high-copy-number plasmid replication origin like pIJ702 within the amplification. If a cloned gene is ligated to the 5·7 kb amplified sequence and transformed into *S. lividans*, it can integrate into the chromosome by recombination between the 5·7 kb sequence and the chromosomal amplifiable region (*Figure 2*; J. Altenbuchner and J. Cullum, unpublished work). The cloned genes are then amplified in Cml^S Arg^- derivatives of the transformants (*Figure 2*).

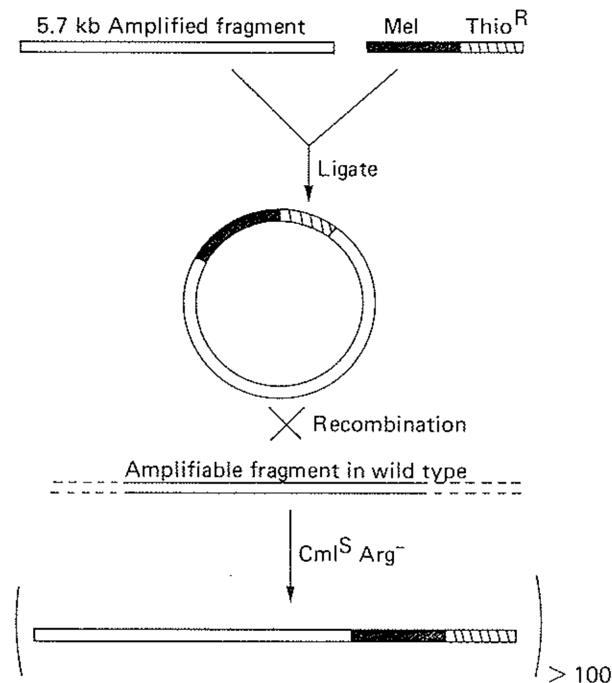

Figure 2. Amplification of a cloned tyrosinase gene in *S. lividans* 66. The tyrosinase gene (Mel) is linked to a thiostrepton-resistance gene (Thio). DNA from a fragment carrying Mel and Thio is purified from an *E. coli* plasmid and ligated together with the 5·7 kb amplified fragment also propagated in an *E. coli* plasmid. The transformants that are rescued by recombination into the chromosome using the homology of the amplified fragment are selected as thiostrepton resistant. Many of the spontaneous Cml^SArg^- derivatives of such a strain have also coamplified the tyrosinase gene to give stable high production of melanin.

Mechanisms of instability and DNA amplification

PLASMID LOSS OR CHROMOSOMAL DELETIONS?

In nearly every well-characterized case of genetic instability in *Streptomyces*, the loss of gene function is due to deletion of the gene. In the case of *S. glaucescens* it is clear that the genes involved are chromosomally encoded. In the other cases, the location of the unstable genes is usually not clear from mapping experiments. However, there is no physical evidence that the genes are plasmid encoded despite experiments to try to detect plasmid involvement. The genetical data that have been taken as evidence of plasmid involvement ('curing' experiments, mapping attempts) also occur in *S. glaucescens* for genes that are definitely chromosomally encoded. We therefore believe that, in the majority of cases, unstable genes will prove to be chromosomally encoded and subject to high frequencies of deletion. Normally we would expect the presence of large deletions to be associated with lowered frequencies of recombination. However, it is possible that the high frequency of deletions results from recombinogenic sequences and that these are also very active in recombination after conjugation. It is also possible that the deletion strains are less viable, leading to a higher recovery of wild-type strains and thus giving unbalanced crosses and a high apparent frequency of transfer of the wild-type allele. The mutants are often defective in aerial mycelium formation which would favour recovery of wild-type recombinants.

Little can be said about the mechanisms of deletion because the endpoints have not yet been characterized. Many *Streptomyces* species have a significant amount (5–10%) of repetitive DNA (Antonov, Ivanov and Markov, 1977; Usdin, Gertsch and Kirby, 1984). If these repetitive sequences are dispersed, they might provide homology for recombination systems to create deletions. Recombination would probably be stimulated by treatment with UV radiation or intercalating agents which increase the frequency of mutants in many unstable systems. Another possibility is that the deletions are caused by transposable elements. In the case of *S. lividans* 66 there are deletions adjacent to the amplifiable region which contains IS-like elements (*see above*; Altenbuchner and Cullum, 1985). In the cases of *S. hygroscopicus* (Roth and Noack, 1982; *see above*10) and *S. sapporonensis* (Miyoshi *et al.*, 1980), instability is reduced by growth at higher temperature; this is reminiscent of the effect of temperature on transposable-element function in *E. coli* (*see* Cullum, 1985). However, in other systems, growth at high temperature may promote instability (Hara and Beppu, 1982).

The sizes of the deletions are unknown, but evidence from *S. glaucescens*, *S. coelicolor* and *S. lividans* suggests that they are large (i.e. much larger than 10 kb and possibly even several hundred kilobases).

CORRELATION OF DELETION WITH AMPLIFICATION

In *S. glaucescens* (Hasegawa *et al.*, 1985) and *S. lividans* (Cullum and Altenbuchner, 1985) there are deletions adjacent to amplified sequences. In

other systems with DNA amplification it is not known whether deletion of adjacent sequences occurs. It seems likely that amplification must involve overreplication of DNA sequences (*see later*) and it is possible that deletion may occur before, after or during amplification:

1. *Deletion occurs before amplification*: the deletion may activate a replication origin, e.g. by fusing a promoter to a potential origin sequence. The overreplication from the newly activated origin would then cause DNA amplification.
2. *Deletion occurs during amplification*: if amplification involves a combination of replication and recombination, then recombinogenic intermediates might interact with distant sequences, resulting in deletions. In this case deletion would be a non-essential accidental side-effect of amplification.
3. *Deletion occurs after replication*: if amplification occurred without deletion, it would make the genome significantly larger (10–50% larger in many cases). This might be very deleterious (e.g. by increasing the time needed for a round of DNA replication). There would be strong selection pressure for a reduction in genome size and this could be achieved by deletion. The deletions could, presumably, be either adjacent to the amplification or elsewhere in the genome.

One of the most striking properties of genetic instability in *Streptomyces* is the variety of events observed in any one strain. An apparent exception is the reproducibility of the DNA amplification in *S. lividans* 66 (Altenbuchner and Cullum, 1984). However, it is possible to find other events in this strain: if spores of the Cml^S mutants are stored in glycerol at −20°C for several months, they still produce Arg^- mutants at high frequency, but these mutants often lack highly amplified DNA (J. Altenbuchner, unpublished work). Other examples of instability and DNA rearrangements have also been observed (Paul Dyson and Hildgund Schrempf, referred to in Schrempf, 1985).

In *S. lividans*, instability seems to be a two-step process: in the first step, Cml^S mutants are formed and these then give rise to Arg^- mutants in a second step (Altenbuchner and Cullum, 1984). The Cml^S mutants have large deletions (>30 kb). If these deletions are responsible for the extreme arginine instability, this could arise in one of two ways:

1. There is a specific gene in the deleted region that represses the arginine instability;
2. The large deletion affects the structure of the chromosome and leads to the switching-on of functions (e.g. replication origins or insertion sequences) that are normally dormant.

It appears that there is some mechanism that normally switches off replication of integrated plasmids. Omer and Cohen (1984) showed that plasmid SLP1 can exist stably as a single copy integrated into the chromosome in

contrast to autonomous derivatives which have a copy number of 3–5. In this case, the differences could also be explained by the fact that the autonomous plasmids lack some sequences that are present on the integrated plasmid. This would not explain a similar observation using a derivative of the multicopy plasmid pMT660 (Birch and Cullum, 1985). This plasmid can exist autonomously at a copy number of over 100, but can also integrate stably into the chromosome and be maintained at a low copy number (Birch, 1985). Integrated plasmids seem to be common in *Streptomyces* species (Hopwood *et al.*, 1984; Cohen *et al.*, 1985). If a disturbance of chromosome structure by large deletions activated various elements, it would explain the large variety of events seen in many strains.

It is not known if the two-step process in *S. lividans* is typical of genetic instability in *Streptomyces*. It may well be that intermediates corresponding to Cml^S mutants exist in other systems, but do not have a convenient phenotypic property to identify them.

MECHANISMS OF DNA AMPLIFICATION

DNA amplification often occurs in mammalian cells after selection for over-expression of genes (*see* Schimke, 1984). However, the structure of the amplified DNA is much more complicated and irregular than the simple tandem repeats seen in *Streptomyces*. In the cases of *S. lividans* and *S. fradiae* the repeat unit for amplification is already present as a duplication in the wild-type strains; this is not true of *S. glaucescens*. However, in this case, the initial duplication might be formed by a mechanism different from that amplifying the DNA once a duplication is present. Such a mechanism might be recombination at short direct repeats like that responsible for a duplication observed in a plasmid in *S. lavendulae* (Nakano, Ogawara and Sekiya, 1984). The variation in the sequences amplified in different mutants of *S. glaucescens* would then be explained by their having different initial duplications; a similar mechanism explains the differing duplication end-points during amplification of the *amp* C gene in *E. coli* K12 (Edlund and Normark, 1981). The validity of these ideas will become clearer when sequence data are available for the end-points of the amplifications.

Once a duplication is established, amplification formally resembles the amplification due to unequal crossing-over observed in some *E. coli* systems (Schmitt *et al.*, 1981; *see* Cullum, 1985). However, the high frequency of amplification in some *Streptomyces* strains and the high copy number argue for some sort of overreplication of the amplified sequences and not just unequal crossing-over. The simplest explanation would be if the duplication 'looped out' a circular extrachromosomal copy by recombination and replicated copies reintegrated by recombination. There is no evidence for such extrachromosomal intermediates and it is possible that overreplication occurs *in situ* in the chromosome and some sort of recombination process resolves the DNA copies generated by multiple replication forks into linear tandem repeats. It is not useful to put forward a detailed model for such processes in our present state of knowledge.

Summary

Genetic instability is very common in *Streptomyces* species, but only affects specific genes in any one strain. It sometimes occurs at high frequency spontaneously, but may be stimulated by treatments such as UV irradiation or intercalating agents. Deletion of genes occurs and may be accompanied by DNA amplifications. It is unlikely that there is plasmid involvement in most cases. Little is yet known about the molecular mechanisms of deletion and DNA amplification.

Genetic instability can be a problem during commercial antibiotic production. DNA amplification of cloned genes is potentially useful for achieving both stability and high gene dosage.

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