GENETIC STUDIES ON THE OXYTETRACYCLINE PRODUCING ORGANISM, STREPTOMYCES ALBOFLAVUS

by

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SUMMARY

The Actinomycete organism, Streptomyces alboflavus, produces the antibiotic oxytetracycline. Treatment with curing agents resulted in S. alboflavus losing the ability to produce oxytetracycline. However, the loss of oxytetracycline production was reversible, and non-producing colonies regained the ability to produce oxytetracycline when the curing agent was removed. Therefore the loss of oxytetracycline production was not due to the irreversible loss of genetic material specifying oxytetracycline production, but was possibly due to genetic instability.

Two extrachromosomal DNA species were isolated from S. alboflavus, (SAP1 and SAP2). SAP1 was approximately 8 - 10 x 10^6 daltons, and appeared to be linear and heterogenous. SAP2 was 20 - 25 x 10^6 daltons and appeared to be covalently closed circular. The functions of SAP1 and SAP2 are unknown, although transformation experiments with SAP1 suggested that it may play a role in the production of an antibiotic-like substance, possibly by acting as a promotor of the genes coding for the antibiotic-like substance.

CHAPTER I

INTRODUCTION

1.1 The genus Streptomyces

The period since World War II has seen the establishment and extremely rapid growth of a major new industry, the use of microorganisms for the synthesis of chemotherapeutic agents, particularly antibiotics. Antibiotics were the first industrially produced microbial metabolites which were not major metabolic end products. Yields, calculated in terms of conversion of the major carbon source into antibiotic, are low and are greatly influenced by the composition of the medium and by other cultural conditions, including the age of the microbe culture. Economical considerations have therefore encouraged research directed towards improving yields. However, knowledge of the genetics of antibiotic production and of the biosynthetic pathways of antibiotics is still disproportionately small when compared with the vast amount of information collected about the Escherichia, Salmonella or Saccharomyces systems with their highly developed experimental genetic systems. Genetic approaches to the problem of increasing yields have been slow, at best, and largely ignored. Mutagenesis has been the main approach, but this is highly ineffecient since it relies on detection of the very low percentage of progeny with beneficial mutations resulting in increased yields. Another reason for this slow development is that much of the research into antibiotic production has been fragmentory, with industrial

laboratories conducting their own research and for obvious reasons not making the advances known, thus undermining other efforts to achieve the same end. Yet another reason is that very few antibiotic-producing microorganisms have well developed genetic systems on which to base genetic approaches to yield improvement. However, it is now possible to look forward to the use of recombinant DNA techniques and the transfer of antibiotic genes to systems whose genetics is better understood.

The order Actinomycetales is unique in the number of antibiotics made from it, and in the diversity of the chemical structures of these antibiotics. Perlman (1977) lists nearly seventy that are produced commercially. The genus Streptomyces contains the majority of the antibiotic producers.

The Streptomyces are gram-positive, mostly soil-living eubacteria, exhibiting the most complex morphology of all the eubacteria: so complex that, in the past, several authors have considered them as intermediates between bacteria and fungi (Waksman, 1950). Streptomyces produces a true mycelium consisting of a continuous system of interconnected hyphae. Within a colony two layers of mycelium can be distinguished - an aerial mycelium and a substrate mycelium (Fig 1.1). The substrate mycelium develops by the branching of one or more germ tubes produced by the germinating spore, and the mycelia grow over the agar surface and penetrate it. At this stage the colony has a smooth, shiny appearance. Aerial hyphae develop from specialized side branches of the substrate mycelium. Spores are produced by a regular fragmentation

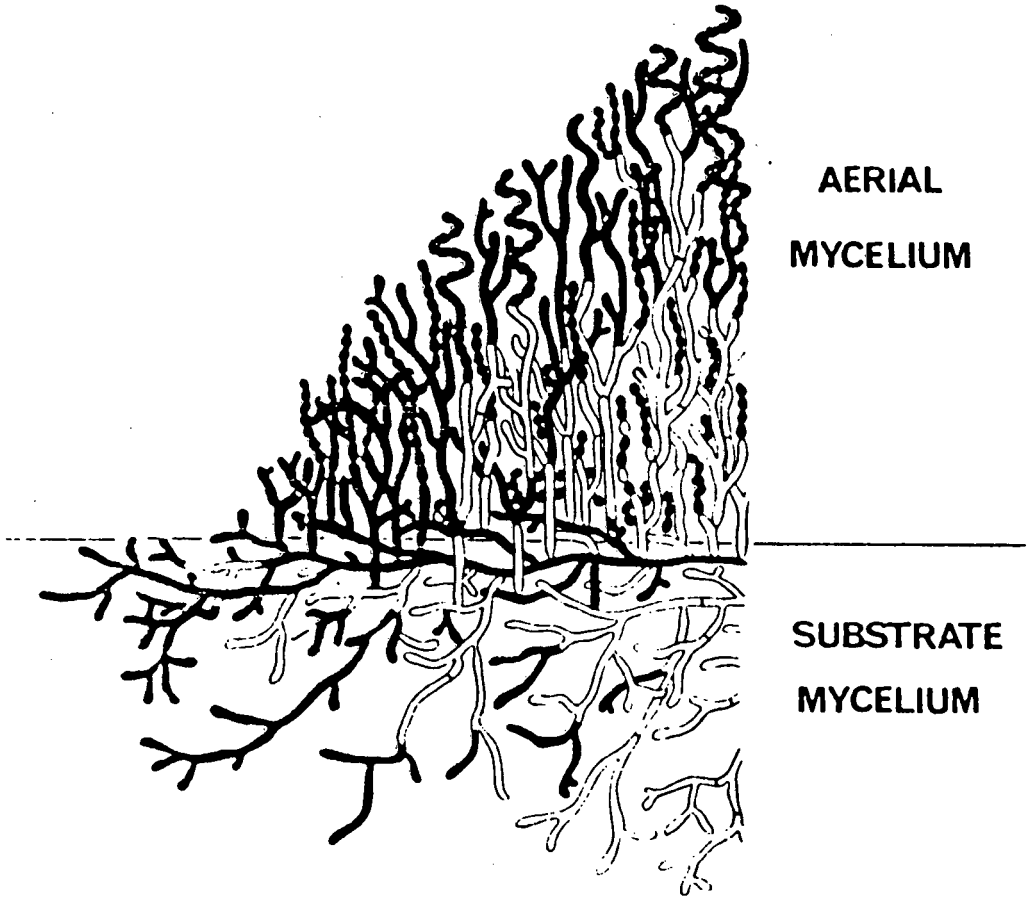

Fig. 1.1: Cross-section of Streptomyces colony.

of the terminal hyphae in the aerial mycelium, giving the colony a fluffy appearance. The spores, which are non-motile and uninucleate, are only slightly more resistant to heat than the mycelium (Stanier, Doudoroff and Adelberg, 1971). In molecular- and ultra-structure, the Streptomyces are typical prokaryotes: they have no nuclear membrane, small hyphal diameters ($< 1,0\ \mu$), are sensitive to lysozyme and antibacterial agents, their flagella, when produced, are of the bacterial type, and their cell wall constituents are diaminopimelic acid and glycine. One factor not common to other eubacteria is the coenocytic nature of their hyphae.

It is interesting that antibiotic-producing Streptomyces are soil organisms, as are all the antibiotic-producing fungi (Hopwood and Merrick, 1977). It is possible therefore, that the soil provides an environment which favours the production of antibiotics. However, it has proved difficult to demonstrate antibiotic production in the soil. Possibly the antibiotics are important at some stage in the colonization process, enabling the organism to establish a niche, and are not, as has been suggested, just waste products produced during metabolism. Their very widespread occurence (amongst various species and families) and activity would suggest that they have some positive effects.

Antibiotic production occurs late in the growth cycle, after most of the cellular growth has already occured. In unicellular bacterial cultures, the growth phase (tropophase) is definable from the production phase (idiophase). The separation between tropophase and idiophase is not so clear

cut in filamentous microorganisms, since dry cell weight continues to increase significantly during the idiophase, although at a lower rate than earlier in the tropophase. However, dry weight is a poor criterion of true growth. Probably the best parameter with which to measure true replicator growth is the increase in DNA. When this is done, cell growth can be clearly dissociated from antibiotic production in filamentous microorganisms (Fig 1.2). The factor that controls the onset of antibiotic biosynthesis is probably the deficiency of one or more nutritional growth limiting components. Depletion of such a component arrests growth and initiates idiolite biosynthesis by mechanisms which are only beginning to be understood (Martin and Demain, 1980).

Several considerations suggest that the ability to synthesise antibiotics may sometimes be plasmid controlled, or might have been transferred on a plasmid sometime in the past. One is the occasional occurence of the same or similar antibiotic in taxonomically diverse bacteria. For example, neomycin is produced by Micromonospora species as well as by Streptomyces fradiae (Hopwood & Merrick, 1977). Conversely, apparently closely related species often produce chemically diverse antibiotics. For example, different strains of Streptomyces griseus produce streptomycin, cycloheximide, candicidus, cephamycins, and several other antibiotics (Umezawa, 1967).

Methods for isolating plasmid DNA have been developed, and most Actinomyces, including Streptomyces, are known to

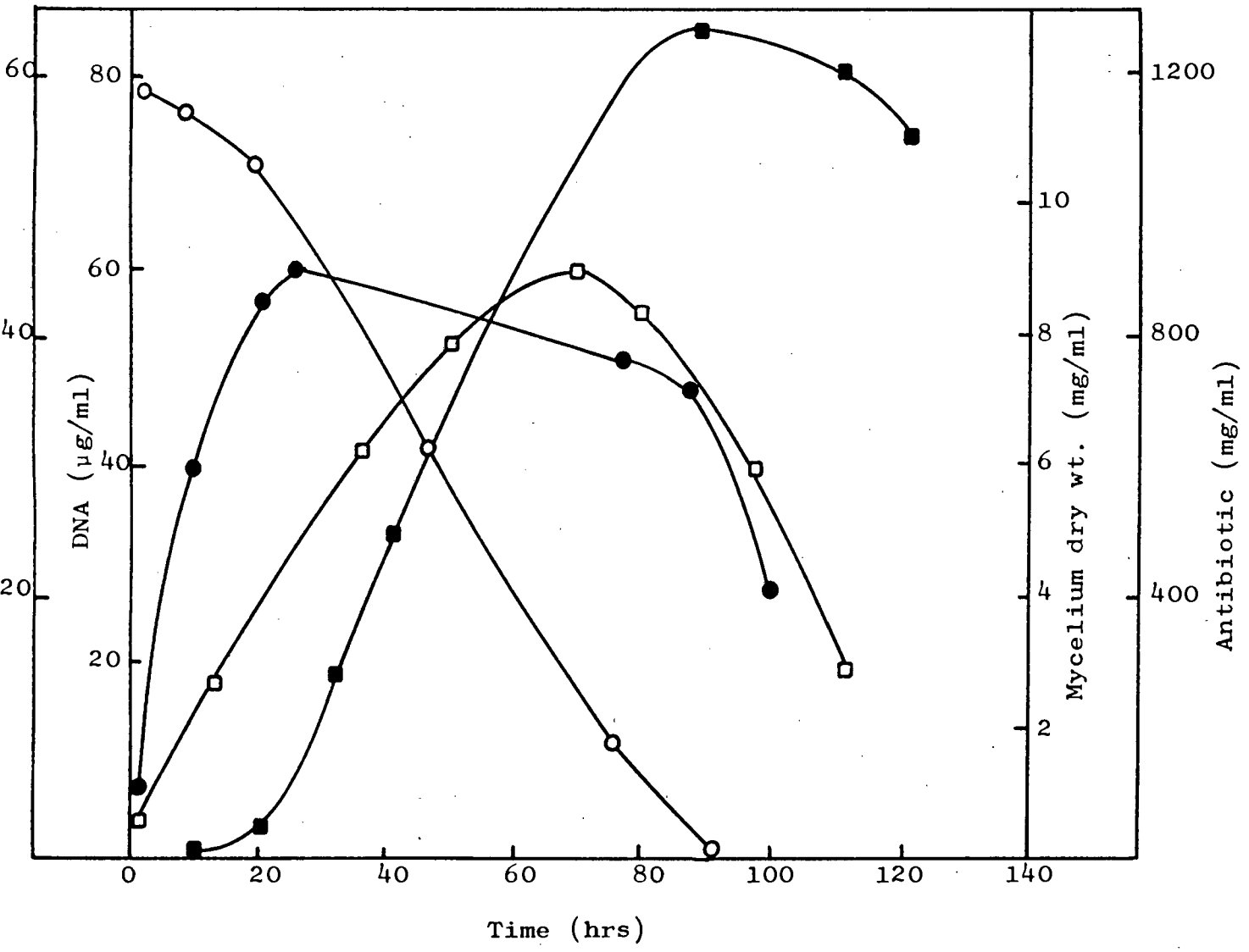

Fig. 1.2: Antibiotic production by a Streptomyces species grown in liquid medium - antibiotic production (■) mycelium dry weight (□), glucose concentration (○), DNA content (●).

have natural systems of genetic recombination. Therefore the potential for the study and exploitation of plasmid-determined antibiotic production in this important antibiotic-producing order is enormous. The isolation of plasmid DNA seems to present problems - there are cases where there is genetic evidence for plasmid involvement, but where it has proved impossible to obtain physical evidence. For example, the SCP1 sex factor of Streptomyces coelicolor A3(2) which codes for methylenomycin synthesis, was not revealed by any of several diverse procedures (Hopwood, 1978). Recently it was isolated and identified as an approximately 100 x 10^6 dalton plasmid (J. Westpheling personal communication). It is also possible that antibiotic production, antibiotic resistance, pigment formation and possibly several other characteristics in some Streptomyces are affected by reversable changes caused by unstable elements similar to transposons. Such a jumping gene has been genetically characterised in S. coelicolor A3(2) (Sermonti, Lanfaloni, and Micheli, 1980).

1.2 Plasmid occurence in Streptomyces

Plasmids have been implicated in many eubacterial characteristics - sex; bacteriocin production; resistance to antibiotics and other agents; pathogenicity. By definition, a plasmid is a circular length of DNA that can exist and replicate in the host cell without relying on chromosomal replication i.e. it is autonomous and extrachromosomal. Therefore it need not necessarily be inherited

by all progeny of the parent, and this characteristic of non-segregation is of primary importance in identifying the presence of plasmid controlled functions. Plasmids can be identified in two ways - genetically or physically, and a correlation between the genetical and physical evidence is necessary before a plasmid can be implicated in any function. Evidence for plasmid presence can be of several kinds (Hopwood, 1978).

a) Segregational evidence. Failure of a character to segregate according to Mendelian ratios is sufficient to implicate plasmid determination. However, further evidence is needed, showing lack of linkage of the character in question to any chromosomally determined characteristics. To employ this approach, a well developed genetic system is necessary, with at least a rudimentary chromosomal linkage map. Such linkage maps have so far been constructed for only a few antibiotic-producing bacteria.

b) Infectious transfer. A plasmid-determined character may be identified by its frequency of transfer to suitably chromosomally marked strains. Transfer is significantly higher than that of chromosomal recombination. Obviously the donor and recipient should have as little chromosomal homology as possible, thereby minimising recombination. However, evidence of infectious transfer is by no means conclusive, since a plasmid may be promoting exchange of chromosomal regions at a frequency higher than that of normal

chromosomal recombination. Similarly, negative evidence could be a result of surface exclusion, incompatability with any plasmids resident in the recipient, or the non-conjugative nature of the plasmid.

c) Correlation with plasmid DNA. Assuming that the plasmid has been isolated, there should be correlation between the presence of the plasmid DNA and the manifestation of the character. All variants lacking the character should also lack the plasmid DNA. Again, negative evidence is inconclusive since well-characterised plasmids may be difficult to detect physically, as was the case with the SCP1 plasmid of S.coelicolor A3(2).

d) Instability and curing. The occurence of variants lacking the character, (e.g. antibiotic production), which arise at frequencies higher than the normal mutation frequency is an indication of plasmid involvement. The suspicion of plasmid involvement is further strengthened when the instability of the character is enhanced by treatment of the bacteria with known curing agents. Curing agents such as intercalating dyes and ultraviolet (UV) light act on the DNA and interfere with its replication. A curing test may fail to reveal a plasmid since some plasmids, especially those with high copy numbers, are lost infrequently, or because the plasmid loss is fatal for the bacteria. The latter explanation raises the difficult question of whether it is in fact a plasmid, or whether it is a second chromosome.

In general, Streptomyces have reduced ability to form aerial mycelium and antibiotics after repeated sequential cultures. This effect is to some extent reversible, since a small percentage of clones will exhibit normal production. This phenomenon is apparent in the case of Streptomyces alboflavus, where one clone will produce an unusually large amount of antibiotic, and another clone from the same parent will produce an average amount. The clone that produced a large amount will, upon subculturing, produce an average amount. This is indicative of a form of genetic instability, since culture and fermentation conditions are relatively constant. A further fact which implicates plasmids in Streptomyces is one mentioned earlier - the occurence of antibiotics having the same or similar chemical structures in totally unrelated species and genera.

Besides the fact that the occurence of plasmids in yet another group of prokaryotes is of enormous academic interest in leading to a better understanding of the organisms' genetics, there is also the impact such plasmids will have in economical terms. They will be an ideal tool for introducing novel genes into the Streptomyces, increasing the gene dosage of the antibiotic biosynthesis genes, and introducing powerful promoters into the organism, thereby providing a positive means of increasing the levels of antibiotic production. In addition, since Streptomyces utilise relatively inexpensive substrates and conditions for their growth are well known, they could be engineered to synthesise products that are economically important but have previously only been produced by organisms with more stringent and

expensive growth requirements.

As a result of these considerations the question of plasmid involvement has been addressed by many people in recent years, and the evidence indicates that plasmids do play a role in Streptomyces. Their role in antibiotic biosynthesis and regulation is of most interest. Although methylenomycin production by S. coelicolor A3(2) was not the first case in which plasmid involvement in antibiotic synthesis was suggested, it is the one in which the strongest evidence exists for plasmid linkage of structural genes encoding antibiotic biosynthesis.

Kirby and Hopwood (1977) reported that the SCP1 plasmid of S. coelicolor A3(2) codes for methylenomycin A (mmy) production and resistance. Using an S. coelicolor strain carrying a SCP1' - cysB$^+$ plasmid, 7 strains carrying SCP1-linked mutations abolishing mmy synthesis were isolated. Plasmid linkage of the mutation was proved by transferring the plasmid from each mutant to a cysB$^-$ SCP1$^-$ recipient, and the recipients all remained mmy negative, while all acquired antibiotic resistance and extrachromosomal cysB$^+$ allele. Transfer of SCP1 to Streptomyces lividans and Streptomyces parvulus, organisms that do not normally produce mmy, resulted in the production of a compound that was biologically and chromatographically identical to mmy. Sixteen mmy mutations were found and all of these were SCP1-linked. In addition, no chromosomal mmy mutations were identified. Recently, this SCP1 plasmid has been isolated and it is approximately 100 x 10^6 daltons (J. Westpheling, personal communication). Interestingly, Okanishi, Manome and Umezawa (1980) recently

screened 32 strains of 30 Actinomycete species for the presence of supercoiled DNA. They reported that Streptomyces violaceus-ruber, a species which produces mmy, is phenotypically identical to S. coelicolor A3(2) and is thought to be of the same species, has a plasmid of 100 x 10^6 daltons (pSV1). This plasmid could not be detected as a satellite band on cesium chloride-ethidium bromide gradients, but was detected by agarose gel electrophoresis of DNA sampled from the base of the chromosomal DNA peak. Similar problems prevented the isolation of SCP1 as supercoiled DNA for many years. Possibly pSV1 and SCP1 are identical plasmids.

There are numerous other examples of plasmid involvement in antibiotic production by Streptomyces, although none of these cases is as well studied as is SCP1 and its role in mmy synthesis. Plasmids have also been implicated in aerial mycelium formation, fertility, and antibiotic resistance.

In 1974 Kähler and Noack reported that treatment of Streptomyces hygroscopicus JA 6599 with acridine orange resulted in 17% of the progeny showing very low antibiotic activity and 14% showing no antibiotic activity at all. The non-producing colonies also showed an altered morphology in that the beginning of aerial mycelium formation was delayed. None of the low or non-producing mutants reverted to wild-type properties. These results seem to implicate a plasmid, if not directly in antibiotic production, then pleiotropically by controlling aerial mycelium formation. However, the antibiotic production character was found to

transfer to non-producing mutants at a much higher frequency than that of chromosomal recombination, suggesting that a plasmid controlled antibiotic production directly (Hopwood and Merrick, 1977). In 1979 Hayakawa et al. isolated a 37,9 x 10^6 dalton plasmid from a different strain of S. hygroscopicus, strain 434, but no function has been reported for this plasmid.

Shaw and Piwowarski (1977) examined the effects of ethidium bromide and acriflavine on Streptomyces bikiniensis. Eleven of the isolates from ethidium bromide treatment and 31 isolates from acriflavin treatment failed to produce detectable streptomycin (str) activity, and isolates were obtained which were inhibited by str at concentrations of 25 and/or 50 µg/ml. The frequency of loss of str production was 2 - 4% with ethidium bromide and 4 - 16% with acriflavin. The loss of str production was stable over 6 generations, but no selective pressure for reversion was applied. All the isolates that had lost the ability to produce str had also become increasingly sensitive to str. The isolates that did produce str, produced it in a range from 13 µg/ml to more than 1000 µg/ml. Str production by the producing isolates was not correlated with the levels of sensitivity of the isolate to str. For example, an isolate sensitive to 9 µg/ml str produced 125 µg/ml of str, and another isolate which produced more than 1000 µg/ml of str was inhibited by 18 µg/ml.

These results, taken together with the known effects of ethidium bromide and acriflavin, were used to suggest that plasmid/s were involved in str production and resistance

in S. bikiniensis. However, Kirby and Lewis (1981) have evidence that suggests that the element involved in str^r is not a plasmid, but something akin to a transposon. The possibility of transposon-like elements occuring in Streptomyces, and their roles, will be discussed in more detail later.

Okanishi, Ohta and Umezawa (1970) observed that the effect of acridine dyes or high temperature on Streptomyces kasugaensis was to increase the incidence of progeny lacking the ability to form aerial mycelium and to produce the antibiotics kasugamycin and aureothricin. Acridine dyes and high temperature have the effect of eliminating plasmids in some bacteria, although many plasmids are not affected by these treatments for reasons unknown at present. S. kasugaensis was reported to harbour 3 different plasmids (Okanishi, 1977); a 1,5 ± 0,5 μ plasmid that is thought to be involved in the biosynthesis of kasugamycin since kasugamycin non-producers lack this plasmid; a 3,35 ± 0,5 μ plasmid associated with biosynthesis of aureothricin; and an uncharacterised 0,59 μ plasmid. Okanishi, Manome and Umezawa (1980) isolated a 6,7 x 10^6 dalton plasmid from S. kasugaensis IMC MB 273 which also produces kasugamycin and aureothricin. At present no function can be assigned to this plasmid.

Yagisawa, Huang and Davies (1978) obtained three antibiotic non-producing variants of Streptomyces fradiae ATCC 10745 from 191 clones tested after treatment with acridine dyes. These non-producers remained stable for 20 generations. Two isolates, AO 80 and AO 83, which were inhibited by

1,56 µg/ml neomycin (neo), did not produce phosphotransferase, but AO 144, an isolate inhibited by 100 µg/ml, and CMP 487, another non-producing mutant obtained from colleagues, showed approximately the same phosphotransferase activity as the parent ATCC 10745. This suggested that resistance to neo requires the phosphotransferase, and that the gene for this enzyme may be carried on a plasmid not present in AO 80 and AO 83. Further, they found that DOS, an intermediate in the neo biosynthetic pathway, when fed to two-day old cultures of the isolates, resulted in AO 144 and CMP 487 producing neo.

The non-producers AO 80 and AO 83 did not produce neo when fed with the same intermediate. These results suggested that there were two plasmids present in the parent strain, one encoding the phosphotransferase which confers resistance to neo, and the other encoding gene(s) for the biosynthesis of DOS. Examination of DNA isolated from the parent, S. fradiae ATCC 10745, revealed two plasmids, pSF 1 and pSF2 with molecular weights of 14,9 and 21,9 x 10^6 daltons respectively. These two plasmids have not been isolated from AO 80 and AO 83. This is consistent with the fact that AO 80 and AO 83 are sensitive to neo and do not produce neo. However, Yagisawa, Huang and Davies (1978), Kirby (personal communication) and Okanishi, Manome and Umezawa (1980) have reported a whole array of plasmids isolated from various strains of S. fradiae. In S. fradiae ATCC 10745 Kirby found plasmids that ranged in size from 1,04 - 53,91 x 10^6 daltons and Davies (1978) found a number of

plasmids from 1,21 - 57 x 10^6 daltons. Kirby (personal communication) also identified a 56 x 10^6 dalton plasmid from S. fradiae NCIB 8235. Okanishi, Manome and Umezawa (1980) identified 2 plasmids of 63 and 42 x 10^6 daltons in S. fradiae ATCC 10745. Many of the smaller plasmids probably represent rearrangement and deletion products of larger parent plasmids to a shorter length. No direct correlation was found between neo resistance and sensitivity and the plasmids pSFI and pSF2. Further study of the plasmids is needed to clarify the situation.

Chloramphenicol (cpp) production by Streptomyces venezuelae was shown to be regulated by a plasmid (Akagawa, Okanishi and Umezawa, 1979). They isolated two classes of mutants: an auxotrophically marked cpp non-producing mutant obtained after acriflavin treatment, was crossed with a cpp producing mutant with different auxotrophic markers. The cpp non-producing mutation did not show linkage with a set of chromosomal markers i.e. the strain had lost cpp production due to losing a plasmid. However, it was shown that this and most other plasmid-less and cpp non-producing mutants still produced a low level of cpp (about 1% of wild type levels). Mutants which had completely lost the ability to produce cpp could be obtained by UV irradiation or NTG treatment at a frequency of 0,2 - 0,5% - and these mutations mapped in at least two genetically separable positions between met and ilv on the chromosome. To detect any plasmids in these chromosomal cpp mutants, they were crossed with suspected plasmid-less cpp non-producing mutants. If a chromosomal cpp mutant carries a

plasmid, strains with a high capacity for cpp production should be obtained amongst the recombinants. High producing recombinants were found in all crosses between chromosomal cpp mutants and plasmid-less mutants. Recombination between the suspected plasmid-less mutants yielded no high cpp producing recombinants. They examined the production of cpp intermediates in the cpp chromosomal and plasmid-less mutants, and found that the increase and decline of intermediates corresponded to cpp levels in the case of the plasmid-less mutants, and no intermediates were present in cpp chromosomal mutants. Therefore, in the case of S. venezuelae, a plasmid appears to play a purely regulatory role with all the structural genes for the cpp biosynthetic pathway residing on the chromosome. Malik and Reusser (1979) have since identified a plasmid, pUC 3, in S. venezuelae, but its role is as yet unknown.

Akagawa, Okanishi and Umezawa (1979) also found that all the mutants, cpp chromosomal and plasmid-less, displayed a lower level of resistance to cpp than the wild type, and that the level of resistance could be temporarily increased to the wild type level by preincubating the mutants in 20 μg/ml cpp. This suggested that a plasmid did not mediate cpp resistance but that the resistance was inducible.

Recently, Nojiri et al., (1980) presented physical evidence for the presence of a plasmid in Streptomyces ribosidificus, which produces ribostamycin. The molecular weight of the plasmid was calculated to be $52 \pm 2 \times 10^6$ daltons, based on a measurement of the contour length.

This was confirmed by Okanishi, Manome and Umezawa (1980), who reported a size of 49 - 56 x 10^6 daltons. Electron micrographs of the plasmid revealed that it was covalently closed circular (ccc). However, the authors found the plasmid to be present in two antibiotic non-producing mutants. It was also shown that the plasmid in the non-producing mutants had an identical restriction pattern to that of the plasmid obtained from the wild type. Consequently, the role of this plasmid is unknown at present, but this is further evidence that plasmids in Streptomyces may play a role in other functions apart from antibiotic production and resistance.

Omura, Ikeda and Kitae (1979) extracted DNA from Streptomyces ambofaciens KA-1028 (spiramycin producing) and spiramycin non-producing mutants of S. ambofaciens obtained after treatment with acriflavin. They could detect a plasmid band in the KA-1028 extract, but none in the spiramycin non-producing mutants AF-30 and S-1. In order to ascertain if a plasmid was involved in the biosynthetic pathway to spiramycin, they examined the plasmid-cured strain for its ability to convert forocidin I, (an important intermediate in the biosynthesis of spiramycin), into spiramycin II. They found that upon addition of forocidin I, AF-30 and S-1 produced spiramycin II, and they concluded that one or more plasmid-encoded functions was required at some stage before the production of forocidin I. In addition, they found that AL-30 and S-1, unlike the parent KA-1028, did not produce aerial mycelium or soluble pigment, and possibly these phenotypic characteristics also require some plasmid-encoded functions.

Treatment of spores of Streptomyces griseus with high temperature resulted in 9% of the progeny lacking aerial mycelium (bald mutants) and the ability to produce str (Yu-gu et al., 1978). It was concluded that a plasmid was controlling both aerial mycelium and str synthesis. However the loss of str synthesis could be a pleiotropic effect of the bald phenotype, as is the case with bald mutants of S. coelicolor (Hopwood and Merrick, 1977). This possibility is borne out by the fact that Streptomyces synthesise antibiotics during aerial mycelium formation. The plasmid indicated in S. griseus could therefore control aerial mycelium formation alone.

Evidence supporting the existance of a plasmid involved with oxytetracycline (otc) synthesis in Streptomyces rimosus was presented by Boronin and Sadovnikova (1972). They identified a class of otc non-producers, which were also sensitive to otc, and which arose at high frequency after acridine orange treatment. The non-producing mutation showed evidence of non-linkage with chromosomal markers. Recently, 7 otc mutations were located on the map of S. rimosus in respect to the already mapped nutritional genes (Pigac and Alačević, 1979). However, the authors pointed out that they did not look for plasmid mutations during this study, so the possibility remains that a plasmid is involved with the regulation of otc production, as in the case in S. venezuelae.

Plasmids have also been implicated in other functions unrelated to antibiotic production. Both S. rimosus and

S. coelicolor A3(2) have plasmids which control fertility. The SRP1 plasmid of S. rimosus determines most of the genetic recombination which occurs between marked derivatives of S. rimosus (Friend, Warren and Hopwood, 1977). High fertility variants of S. rimosus arose at a frequency of 0,3% amongst spores of an $SRP1^+$ strain. This high spontaneous frequency suggested that plasmid loss was responsible for the high fertility observed. Crosses of $SRP1^+$ x $SRP1^-$ were one hundred times more fertile than $SRP1^+$ x $SRP1^+$ crosses, a situation similar to the fertility of $SCP2^+$ strains of S. coelicolor A3(2), where $SCP2^+$ x $SCP2^-$ crosses are at least one order of magnitude more fertile than are $SCP2^+$ x $SCP2^+$ crosses (Bibb, Freeman and Hopwood, 1977). The SCP2 plasmid is capable of mutation to a variant form, SCP2*, with enhanced sex factor activity. From SCP2* strains, $SCP2^-$ cultures were isolated at an average spontaneous frequency of 0,8%. The SCP2 DNA was isolated as ccc DNA of 20×10^6 daltons and was not present in genetically defined $SCP2^-$ strains (Schrempf et al., 1975; Bibb, Freeman and Hopwood, 1977). Both SCP1 and SCP2 share the property of exerting an effect similar to "lethal zygosis" caused by the fertility factor (F) in E. coli: it is shown by $SCP1^+$ strains against $SCP1^-$, and by SCP2* (but not $SCP2^+$) against $SCP2^-$. The lethal zygosis effect is manifested as a zone of growth inhibition around the plasmid-carrying colony.

Crosses between strains of both S. rimosus and S. coelicolor A3(2) lacking the plasmids are not completely sterile. This finding perhaps supports the idea that a

low level of gene exchange in Streptomyces can occur in the absence of a sex factor. Perhaps the main importance of these findings is that sex factors may be responsible for recombination in many Streptomyces species. Although recombination has been reported in many other Streptomyces (Hopwood and Merrick, 1977), sex factors have not yet been implicated in these strains.

Table 1.1 is a summary of all Streptomyces harbouring plasmids, their roles, and the evidence for their presence.

All the evidence to date, though it is largely circumstantial, seems nevertheless to indicate that the absence of a plasmid is an exception rather than the rule. The plasmids seem to play a role mainly in antibiotic production, and to a lesser extent in antibiotic resistance and fertility. Care should be exercised when interpreting results in which both aerial mycelium formation and antibiotic production are lost after curing (e.g. S. griseus) since antibiotic production occurs during aerial mycelium formation. Therefore the loss of antibiotic production may be a pleiotropic effect due to the inability of the organism to form aerial mycelium, and not due to the loss of a plasmid specifying antibiotic production.

The widespread occurence of plasmids amongst the Streptomyces provides a natural vector population for use in cloning. Antibiotic biosynthesis genes carried on plasmids will be more accessible for study and manipulation with the result that plasmid determined antibiotics will probably be the first to be engineered to higher yields.

Table 1.1: Plasmids in Streptomyces.

SPECIES	PHYSICAL PLASMIDS	FUNCTIONS	GENETIC PLASMIDS	REFERENCES
S. ambofaciens	Yes	Spiramycin ? production	-	Omura, Ikeda & Kitao (1979).
S. bikiniensis	No	-	Streptomycin production	Shaw & Piwowarski (1977)
S. coelicolor A3(2)	SCP1 100 md	Methylenomycin production	-	Kirby & Hopwood (1977).
S. fradiae sp	55 md	Unknown		Kirby, personal communication.
S. fradiae sp	pSF1 14,9 md	Unknown		Yagisawa, Huang & Davies (1978).
	pSF2 21,9 md	Unknown		
S. griseus	-	-	Streptomycin production	Yu-gu et al., (1978).
S. hygroscopicus sp	-	-	Tunicamycin production	Kähler & Noack (1974)
S. hygroscopicus sp	37,9 md	-	-	Hayakawa et al (1979)

Table 1.1: Continued.

SPECIES	PHYSICAL PLASMID	FUNCTIONS	GENETIC PLASMID	REFERENCES
S. kasugaensis	6,7 md	Aureothricin production	-	Okanishi, Ohta & Umezawa (1970)
	30 md	Kasugamycin production	-	
	1,2 md	Unknown	-	Okanishi (1977)
S. lividans	SLP1 7,9 md	Lethal zygosis		Bibb
S. ribosidificus	52 md	Unknown	-	Akagawa, Okanishi & Umezawa (1979).
S. rimosus	-	-	Oxytetracycline regulation ?	Boronin & Sadovnikova (1972)
	SRP1	Fertility	-	Friend, Warren & Hopwood (1977).
S. scabies	-	-	Tyrosinase	Gregory & Huang (1964 a & b)
S. venezuelae	pUC3	Unknown	-	Malik & Reusser (1979)
	-	-	Chloramphenicol regulation	Akagawa, Okanishi & Umezawa (1979)
S. violaceus-ruber	pSVI 100 md	-	-	Okanishi, Manome & Umezawa (1980)

1.3 Transposons in Streptomyces.

The first indication that unstable genetic elements were present in Streptomyces was the report by Freeman et al. (1977) that there was an oscillation between chloramphenicol (cml) sensitivity and resistance in S. coelicolor A3(2) and S. lividans 66. Most of the reports involve transposable elements which effect antibiotic resistance.

Cml^s variants arose at a frequency of 0,5 to 2%. Cml^s isolates spontaneously reverted to cml^r at a lower frequency than the $cml^r \rightarrow cml^s$ frequency. The cml^r revertants obtained from cml^s clones again produced cml^s isolates at the normal frequency of several percent.

In crosses between cml^s and cml^r S. coelicolor, transfer of cml^r into the sensitive strain occured independantly of chromosomal recombination. Ccc DNA from cml^s and cml^r strains of S.coelicolor showed no difference in molecular weight and endonuclease cleavage patterns. These findings were considered suggestive of some sort of transposition event - in this case the cml^r genes were carried on a transposon. They suggested this hypothesis since their results were not compatible with the possibility of the cml^r genes on the chromosome having fixed positions and being subject to insertional inactivation, or even of a variable but exclusively chromosomal location, since insertional and transpositional events reversibly inactivating the cml^r gene and occuring at frequencies of a few percent should not have obscured a map location for any particular cml^s variant. Therefore they suggested that some genetic

element capable of continued extrachromosomal existance was involved in cml^r in S. coelicolor.

A different hypothesis was proposed not long afterwards by Sermonti et al. (1977). They observed the spontaneous occurence of cml^s variants from S. coelicolor A3(2) cml^r $SCP1^-$ at a high frequency - too high to be accounted for by gene mutation. However, some evidence suggested that the cml^r factor was located to the right of Arg A on the chromosome. Repeated isolations were required to obtain a stable cml^s clone. This was indicative of a control by multiple elements, which were diluted out during the re-cloning process - a situation not compatible with a chromosomal trait. The recombination rate in cml^r x cml^s crosses was increased up to several thousand-fold more than was usual in $SCP1^-$ x $SCP1^-$ matings. Promotion of recombination by the cml^r factor was thought likely, and they suggested that the cml^r factor was acting like a fertility factor, promoting bidirectional transfer of a chromosome by associating with the chromosome at a site to the right of Arg A.

Sermonti, Lanfaloni and Micheli, (1980) presented evidence for the occurence of a transposon carrying both cml^r and Arg G in S. coelicolor A3(2). Their attempts to map the cml^r locus differed from earlier attempts in that they divided the segregant phenotypes (cml^r and cml^s) into two sub-populations according to some arbitary criterium. (Str A^+ and Str A^- in one case, and Arg A^+ and Arg A^- in another case). This was done because they suspected that previous difficulties in mapping the gene for cml^r were due

to its location at different sites on the chromosome in various mixed sub-populations. The cml^r locus could be mapped within each sub-population, but in distinct sites, indicating that the cml^r parent may bear the cml^r locus in two alternative positions, possible borne in different sub-populations. They mapped very young sub-populations thereby minimising the chance of relocation of the transposon. But the question is whether more alternative positions would be found if the sub-populations were further divided.

Upon analysing revertants, it was found that the transfer ability (tra) connected with cml^r was lost in cml^s variants and not resumed in cml^r revertants. The cml^s variants also frequently lost the Arg G function. They proposed that these losses were a consequence of deletions induced by insertion of the same insertion sequence which switches off the cml^r function. The loci cml^r and Arg G appeared to be closely linked in crosses, suggesting that they both belonged to the same transposon (which they designated SCTn1). The two best documented locations of SCTn1 are between met A and cys A, and right of arg A. In the revertant strain, Arg G^+ and cml^r appear to be independant from each other and mappable in different positions. The two genes occupy the two positions most frequently occupied by SCTn1 in cml^r stock strains. They also reported that a fourth locus, the one for aerial mycelium formation (amy) appeared to be connected or included in SCTn1, because the cml^s variants were isolated as amy^-.

The mapping evidence of Sermonti, Lanfaloni and Micheli (1980) supports a variable location of both the cml^r and Arg G^+ loci, which can be explained by a transposon which inserts at several sites. However, more detailed study is necessary before this can be confirmed.

Similar systems seem to occur in other Streptomyces. In Streptomyces alboniger the amy^- variants were always Arg G^-; in Streptomyces scabies and Streptomyces violaceus-ruber, amy^- variants were frequently Arg G^- (Pogell, 1979). Possibly a transposon regularly or optionally including the Arg G locus was involved in these cases.

Freeman and Hopwood (1978) determined the levels of resistance of about 40 Streptomyces strains to a series of 25 antibiotics and other antimicrobial agents. They were looking for strains which had significantly higher levels of resistance to a certain antibiotic than the majority of the strains. Such behaviour might be expected if some extrachromosomal element, not uniformly present in the majority of strains, conferred some resistance to an antibiotic. Such highly resistant strains were tested for reversion to a less resistant phenotype. They found four cases in which a strain exhibited a higher than average resistance to an antibiotic. S. coelicolor A3(2) has been discussed previously, and is the best understood (Sermonti, Lanfaloni and Micheli, 1980). Streptomyces glaucescens exhibited an oscillation between resistance and sensitivity, with a much higher frequency of $r \rightarrow s$ than of $s \rightarrow r$ events. This is the same as occurs in S. coelicolor A3(2).

S. griseus and Streptomyces acrimycini also exhibited instability of antibiotic resistance, but in these two cases true reversal of the r → s event was not demonstrated. None of these examples can be explained by the simple loss of a plasmid since no irreversable loss of resistance occurs.

Kirby and Lewis (1981) found that str^r of S. griseus was unstable and gave rise to str^s clones at a frequency of 0,5 - 11,7%. The str^s clones were not totally sensitive, but were sensitive to 60 µg/ml of str (the wild type was sensitive to 95 µg/ml). The reversion of str^s to str^r was at a frequency of approximately $1:10^7 - 10^8$, and reversion from reverted str^r back to str^s occured at a frequency of 1-2% spontaneously. No auxotrophic mutations accompanied the str^r - str^s oscillations, and str production remained stable. The S. griseus system resembles the S. coelicolor A3(2) system of cml^r in that no auxotrophs or aerial mycelium variants arose which correllated with loss of str^r. At present there is no chromosomal map for S. griseus so the $str^{r/s}$ phenotype could not be located. However the element possibly carries the str^r genes, or genes controlling str^r. In other strains of S. griseus the oscillation between sensitivity and resistance does not occur, and plasmid-encoded str^r is still a strong possibility (Kirby and Lewis, 1981). Kirby and Lewis (1981) studied a similar situation in S. bikiniensis ISP 5235. Although Shaw and Piwowarski (1977) postulated a plasmid involvement in str^r with plasmid loss resulting in str^s clones, Kirby and Lewis (1981) found that the str^r gave rise to str^s at a frequency of approximately 1%

spontaneously, and 4% after UV treatment. The str^s clones reverted to str^r at a frequency of approximately $1:10^5 - 10^7$. This indicated that there was no loss of the genes. The reverted str^r clones themselves gave rise to str^s at 1 - 4% frequency. Kirby and Lewis (1981) found that some revertants were stable, whereas others were unstable at the above frequencies. The oscillation between str^r and str^s was occasionally accompanied by a change in aerial mycelium production. Some revertants did not produce aerial mycelium (Bld^-), and upon reversion some progeny were Bld^+ whereas some remained Bld^-. There was no correllation between the Bld phenotype and the str^r. They concluded that because there was no loss of resistance, the element responsible was capable of integrating at various sites, one of which could be in the str^r genes. The pleiotropic effects of Bld^- and auxotrophy apparent on reversion to str^r could be due either to imperfect excision of the element, or to the element reintegrating into another site affecting the genes there, in this case the aerial mycelium and auxotrophic genes. The latter explanation was corroborated by the observation that a high proportion of revertant str^r clones were auxotrophic and that the auxotrophs in turn were unstable.

In the case of S. bikiniensis it was suggested that a transposon did not carry the str^r genes, but rather that it had an insertion site which was within the str^r genes. Another possibility was that the proposed transposon could carry controlling genes, and its position and orientation on the chromosome could cause it to switch various genes on or

off (Kirby and Lewis, 1981). Genetic mapping is essential to establish whether this is the case.

It has been suggested that some sort of transposition process occurs in Streptomyces lavendulae. Matsubara-nakano, Kataoka and Ogawara (1980) found that a strain of S. lavendulae gave rise to two distinct variants at a high frequency: one was a β-lactamase non-producing variant and the other was an Arg^- variant. It was previously reported that β-lactamase, one of the antibiotic-inactivating enzymes, was produced by many strains of Streptomyces (Ogawara, 1975). They found that β-$lactamase^-$ clones arose spontaneously at a frequency of 2,7%, and at a frequency of 8,3% after acriflavin treatment. (The β-$lactamase^-$ mutants produced very low levels of β-lactamase - less than 20% of the wild type.) UV irradiation yielded β-lactamase mutants, a number of which were auxotrophic. All of the auxotrophy was Arg^-. Mutagenesis with NTG resulted in 20% of the auxotrophic mutants being Arg^-. The unusually high proportion of β-$lactamase^-$ and Arg^- double mutants suggested a plasmid involvement, and treatment with ethidium bromide corroborated this theory - a high proportion of double mutants resulted.

Upon testing the reversion frequency of Arg^- to Arg^+, they found that reversion occurred, but at the low frequency of 10^{-10}. The 3 Arg^+ revertants had not reverted to β-$lactamase^+$, and in fact the β-lactamase activity was at an even lower level than in the original Arg^- β-lactamase-mutants. All the double mutants (Arg^- β-$lactamase^-$) were

Nutrient broth

Nutrient broth	8 g
distilled H_2O	1000 ml

Restriction endonuclease buffer. (Broach, Strathern & Hicks, 1979).
(for BamHI, BglI and BglII, EcoRI, HindIII, and SalI.)

Tris	6 mM
$MgCl_2$	6 mM
β-mercaptoethanol	6 mM

Adjust to pH 7,8 with HCl.

unable to produce aerial mycelium or spores. The Arg^+ revertants regained the ability to form aerial mycelium and to sporulate. They could not, however, detect any ccc DNA in S. lavendulae wild type, minimizing the possibility of plasmid involvement, but not completely ruling it out since the S. coelicolor A3(2) SCP1 plasmid was not detected by similar methods (Hopwood, 1978). Matsabura-nakano, Kataoka and Osawara (1980) proposed that it was unlikely that the initial β-lactamase mutants were caused by point mutations because of the high frequency at which they arose and because of the concomitant occurence of Arg^- mutants. They also ruled out deletional loss due to the fact that reversion occured. However, this low frequency of reversion to Arg^+ clones could be reversion of point mutations, and all the non-revertant Arg^- clones could be deletion mutants. The frequency of reversion is too low to rule out the possibility that most of the Arg^- mutants are due to deletions. Nevertheless, they suggested that the β-lactamase genes were part of a transposon, similar to the proposed transposon of S. coelicolor A3(2), which possibly carries both the cml^r and the Arg G genes (Sermonti, Lanfaloni and Micheli, 1980).

Nakano and Ogawara (1980) found that all the Arg^- mutants of S. lavendulae showed inhibition of aerial mycelium, repression of β-lactamase production, development of acid pH, low saturation density of growth in liquid culture, a decrease in antibiotic production, an increase in sensitivity to benzylpenicillin and a decrease in pigment production. All these factors are indicative of

a block in secondary metabolism. They suggested that the mutation was not caused by the transposition of β-lactamase structural genes as suggested in the earlier paper, but by the transposition of some common regulatory gene for differentiation.

Puznynina et al. (1980) reported that erythromycin-resistant (ery^r) and erythromycin-sensitive (ery^s) variants of S. coelicolor A3(2) occured. The ery^r strains became ery^s at a frequency of 0,14 - 1,5%. The ery^s variants reverted to resistance at a frequency of 10^{-6} to 10^{-8}. Ethidium bromide treatment failed to increase this frequency significantly, and no correlation was found between ery^r and the plasmids SCP1 and SCP2. In two derivatives of S. coelicolor A3(2), the ery marker was localized in different areas on the chromosome : in the strain A617, between AdeC and ArgA1, and in the strain S18, between pheA1 and NF. They therefore suggested that the genetic instability of ery^r was not associated with loss of a plasmid, but was caused by transposition of genetic material, since ery could be mapped in two positions on the chromosome.

Danilenko et al., (1979) positively identified a transposon at the molecular level. They cloned BamHI fragments of S. rimosus (kanamycin resistant) chromosomal DNA onto BamHI - restricted pBR 322. The hybrid plasmids were transformed into E. coli c600. The DNA of a kanamycin resistant (km^r) transformant of E. coli c600 was isolated and transformed into a recipient E. coli c 600, resistant to rifampicin (rif^r). Upon isolation of plasmid DNA from the km^r transformants, they found 3 plasmids of different

sizes in the transformant. Homoduplex analysis revealed that these plasmids contained transposon-like structures (TPS) consisting of a central single-stranded portion about 1000 nucleotides long, flanked by inverted repeats of about 1000 nucleotides.

The km^r gene was contained in the transposon, and the transposon, TnKm, was capable of transposition to phage λ and the plasmid R6KΔSm. TnKm was characterised by high site-specific integration into λ. The results of Danilenko et al. were the first physical evidence in support of a transposon. However, there have not been any reports of instability of the km^r phenotype in S. rimosus, so possibly this transposon does not exhibit or induce phenotypic instability. This could be possible, since TnKm integrated very site-specifically in λ.

Transposons provide a logical explanation for the many cases of phenotypic instability which have been obserbed in Streptomyces. In addition, the growing body of evidence for transposons in Streptomyces is support for the widespread belief that the natural resevoir for antibiotic resistance may be Streptomyces, which synthesize antibiotics and exhibit resistance to them. Many genes controlling antibiotic resistance in eubacteria are contained on transposons capable of migrating between various replicons. If Streptomyces do act as a resevoir, one would expect the antibiotic resistance genes of Streptomyces to bear some resemblance to eubacterial resistance genes. Eubacteria must also be capable of expressing these genes. The kanamycin transposon, TnKm, of S. rimosus bears some resemblance to the Tn 903 enterobacterial transposon in the

length of the inverted repeat and the central DNA region. As Streptomyces transposons are identified, their genetic sequences will have to be determined before they can be compared with eubacterial transposons carrying antibiotic resistance.

1.4 Genetic manipulation in Streptomyces.

In Streptomyces, genetic exchange can be mediated by natural means e.g. conjugation, and by artificial means e.g. transformation. Gene exchange mediated by conjugation within the Streptomyces appears to be widespread (Hopwood and Merrick, 1977), but the transfer of genetic material by such sexual means is predominantly resitricted to members of the same species. Transfer of genes as free DNA (transformation) has been well documented in some microorganisms (Herdman, 1975) but the efficiency of transformation decreases with increasing divergence of the species involved, as the homology of their DNA sequences decreases. Another restriction on transformation is imposed by the fact that most Streptomyces species do not become competent for DNA uptake in the course of their growth cycle, as opposed to the situation in many other eubacteria where DNA uptake is greatly facilitated by the cells becoming competent for DNA uptake at a characteristic stage in their growth cycle (Cohen, Chang and Hsu, 1972). This situation has hitherto prevented the generation of new combinations of DNA sequences from diverse species of Streptomyces which could have had positive effects on both the range and quantity of antibiotics produced.

In order to overcome the natural barriers preventing conjugation between species, protoplast fusion techniques have been developed (Hopwood et al., 1977). Protoplasts of Streptomyces species were prepared by growing cells in glycine. The protoplasts were fused in the presence of polyethyleneglycol (PEG), regenerated, and the spores of regenerated colonies were assayed for parental and recombinant genotypes. They found that amongst the recombinants, 20% represented multiple crossovers, whereas in a mating multiple crossover classes represented only 2% of the total progeny. This indicates a relaxation of linkage (increased recombination per unit of map length) which would lead to a more random assortment of genotypes in a cross than would normally be the case. This method of recombination also allows the rapid construction of complex genotypes without relying on natural conjugation, whereas previously, in matings, two auxotrophic markers were lost by counter-selection wherever two strains were combined, and at least three markers were needed in each parent before recombinants could be selected carrying more markers than either parent. In 1978, Hopwood and Wright established that up to four strains of S. coelicolor A3(2) could fuse at the same time, and that multiple rounds of recombination occured, involving large fragments of DNA. Interspecific fusion, as opposed to the above mentioned intraspecific fusion, has so far not proved successful. Little genetic exchange seems to occur between species after fusion of the protoplasts, any exchange being limited to small areas of the chromosome. Heterokaryons are very common and unstable, possibly due to a lack of

homology between the two chromosomes.

This lead to the development of a system of transformation whereby the necessity for competence and DNA recombination was overcome. DNA cloning methods potentially permit the insertion of a DNA sequence of any origin into a particular host strain, provided that a suitable vector system is available to propogate the cloned DNA, and the means of introducing the recombinant DNA molecule into the host organism exists. The identification of plasmids in Streptomyces has opened up the possibility of their use as vectors. Ideally, a vector should possess restriction sites in a non-essential region, be selectable, replicate autonomously and have a high copy number.

A restriction endonuclease cleavage map of SCP2* (Bibb, Freeman and Hopwood, 1977; Schrempf and Goebel, 1977) indicates the presence of single, well separated recognition sites for both the enzymes EcoRI and HindIII. One or both of these sites could prove satisfactory for the insertion of DNA sequences from any source, using the recombinant DNA technology effective in other prokaryotes. The presence of multiple sites for the restriction endonucleases BamHI and SalI may facilitate the construction of smaller derivatives of SCP2*. Further, SCP2* is self-transmissable. All these factors qualify SCP2* as a potential vector for introducing foreign DNA into Streptomyces. Unfortunately SCP2* has a low copy number and it can only be selected for indirectly, by its ability to induce "lethal zygosis" in an indicator strain which lacks SCP2*. The presence of

SCP2* in a strain can be detected by growing the strain on a background strain that is $SCP2^-$. The colonies that have SCP2* cause a narrow zone of inhibition on the background strain. This inhibition requires hyphal contact and conjugation between the two strains (hence they should both belong to the same species), resulting in the death of the surrounding $SCP2^-$ cells due to lethal zygosis.

Bibb, Ward and Hopwood (1978) exploited the enhancing effect of PEG on the transformation of E. coli spheroplasts with DNA of bacteriophage ϕX174. They transformed PEG-treated protoplasts of S. coelicolor A3(2) and S. parvulus ATCC 12434 with the SCP2* plasmid, and obtained transformation frequencies of 4 - 20% for S. coelicolor A3(2) and >0,1% for S. parvulus. Bibb, Schottel and Cohen (1980) cloned antibiotic resistance genes onto SCP2*, rendering it directly selectable. They cloned the genes for mmy^r onto SCP2* plasmid from S. coelicolor A3(2), and the SLP1.2 plasmid from S. lividans. (The SLP1.2 plasmid is also indirectly selectable, by its ability to induce lethal zygosis.) Mmy has antibiotic activity against a wide range of Gram-positive eubacteria and many Streptomyces species. S. coelicolor A332 contains the SCP1 plasmid, which codes for mmy^r, integrated into the chromosome. The total DNA of S. coelicolor A332 was digested with PstI endonuclease and ligated to similarly digested SCP2* or SLP1.2 plasmid DNA. The hybrid plasmids were transformed back into their parent strains, and transformants were detected which were mmy^r. The hybrid plasmid (pSCP III) was isolated from one of the S. coelicolor mmy^r

transformants, and tested for interspecific transfer and expression. Both S. lividans and S. parvulus could be transformed with pSCP III, and transformants of both species acquired the mmy^r phenotype. Plasmids isolated from S. lividans and S. parvulus transformants were identical to pSCP III. Further, pSCP III could be transferred from S. coelicolor to S. lividans and S. parvulus by mating.

Another potential Streptomyces cloning system has been studied by Thompson, Ward and Hopwood (1980). The host strain was S. lividans, which lacks a restriction-modification system. A S. lividans plasmid, SLP1.2, was used as a vector : all cleavage sites for the restriction endonucleases BamHI and PstI lie in a dispensable region of SLP1.2. The plasmid also has a copy number of >4. Into these sites they inserted resistance genes from S. fradiae resistant to neo and Streptomyces azureus, resistant to thiostrepton, using the shot-gun technique. The hybrid DNA molecules were transformed into S. lividans protoplasts, successful transformation being indicated by the induction of lethal zygosis in a $SLP1.2^-$ background by regenerated protoplasts. SLP1.2 did act as a vector, and a number of transformants of S. lividans were either thiostrepton resistant or neo^r.

The findings reported here demonstrate the practicality of DNA cloning and heterospecific expression of cloned genes in Streptomyces. Because transformation of Streptomyces protoplasts is so efficient, it is possible to clone genes from the chromosome of donor DNA preparations. The ability to clone genes in Streptomyces should prove valuable

in the analysis and, ultimately, the manipulation of antibiotic biosynthetic genes, differentiation genes and controlling genes, which may now be studied in isolation.

Streptomyces plasmids are not the only potential cloning vectors. Temperate phages of Streptomyces have received attention as vectors. Ideally the phage should have a wide host range and be readily inducible. Such a phage is being studied by Chater and Carter (1979). The phage will eventually be modified by restriction and mutation for use as a cloning vector (Chater, personal communication). Transfection of Streptomyces protoplasts has been achieved (Suarez and Chater, 1980 a and b). They inserted pBR322 into a Streptomyces phage, ϕC31 cts Δ 23, which has about 8% less DNA than the wild-type ϕC31. The pBR322 plasmid was inserted within a non-essential region of the phage. The hybrid phage could be transformed into E. coli and was stably inherited, it could be transfected into S. lividans protoplasts and it was stably inherited, it could be detected by plaque hybridization, and the pBR322 antibiotic resistance genes were expressed in E. coli.

The ability of the hybrid DNA to inhabit either Streptomyces or E. coli will be invaluable in introducing some of the powerful genetic techniques (such as transposon mutagenesis) available in E. coli into the study of Streptomyces genetics.

Attempts have been made to adapt eubacterial plasmids for use as cloning vehicles in Streptomyces. Danilenko et al. (1979) used pBR322 as a vector for cloning Streptomyces genes into E. coli c600. They ligated BamHI DNA

fragments of pBR322 to BamHI chromosomal DNA fragments from S. rimosus km^r. The hybrid plasmids were transformed into E. coli c600 recipient cells. They detected E. coli c600 transformants which were km^r, indicating that the S. rimosus km^r gene was functional in E. coli, and that pBR322 could act as a vector for Streptomyces DNA.

A contradictory report has been published by Horinouchi, Uozumi and Beppu (1980). They constructed hybrid plasmids containing chromosomal DNA fragments from various Streptomyces species ligated to pBR322 (amp^r tet^r), pTA2070 (amp^r) and RSF1010 (Str^r). Attempts to isolate transformants of E. coli c600 in which auxotrophic markers were complemented by Streptomyces DNA were unsuccessful. Nor could they detect any str^r transformants owing their resistance to Streptomyces DNA. They attributed this lack of expression of Streptomyces DNA in E. coli to a number of reasons : the reported occurence of Streptomyces tRNA with no pG or pU structure at the 5' end, and the presence of 4 trace nucleosides not present in E. coli tRNA. The lack of expression could also be due to species barriers preventing heterospecific gene expression. Bacillus subtilis has a barrier against the expression of E. coli genes (Ehrlich et al., 1976), and E. coli has a barrier against the trp genes from Pseudomonas (Hedges, Jacob and Crawford, 1977). But the fact that the km^r genes from S. rimosus are expressed by the same E. coli c600, (Danilenko et al., 1979), suggests that possibly the S. griseus DNA fragments did not contain complete genes. It would be necessary to introduce the

same hybrid plasmids into auxotrophic mutants of Streptomyces and detect complementation in this background to rule out the possibility that all the S. griseus DNA fragments contain incomplete genes.

The development of DNA cloning systems and efficient vectors for Streptomyces will greatly facilitate the detailed genetic analysis of their antibiotic production pathways and of the molecular mechanisms involved in their differentiation. Furthermore, DNA cloning could produce new combinations of DNA sequences from genetically diverse organisms and perhaps result in new types and increased yields of antibiotics.

The general situation in Streptomyces seems to be comparable with the well studied eubacterial systems, in which plasmids and transposons play a large role. The present efforts to increase antibiotic yields will be aided by the development of techniques of genetic manipulation. These techniques rely to a large extent on the techniques already in use for eubacterial genetic studies, and are likely to prove just as successful in Streptomyces. The establishment of cloning systems will result in great advances being made towards the elucidation of economically important antibiotic production, as well as the genetics of differentiation, which is most advanced in Streptomyces.

CHAPTER II

CURING AND PLASMID ISOLATION

2.1 INTRODUCTION

To date, there is a large amount of evidence implicating plasmids in the production and control of antibiotic synthesis in Streptomyces. There is also a growing body of evidence suggesting that in some cases a number of characteristics are controlled by unstable genetic elements (see section 1.2 and 1.3).

The search for extrachromosomal DNA in S. alboflavus was prompted by the erratic behaviour of the organism during the factory fermentation process. A low percentage of the fermentations yielded extremely high amounts of the antibiotic oxytetracycline (otc). However, other subclones obtained from the same parent produced a much lower amount or almost zero levels of otc.

As mentioned in section 1.2, the involvement of a plasmid can be determined either genetically or physically. Genetic evidence can be obtained by treating the cells with curing agents and observing the frequency of loss of the character in question. Covalently closed circular DNA can be isolated and correllated with the presence or absence of the character. In any event, the two approaches are complementary and each on its own is not considered unequivocal evidence for the involvement of a plasmid (Hopwood and Merrick, 1977). Both approaches were utilized to investigate the involvement of a plasmid in otc production in S. alboflavus.

2.2 METHODS

2.2.1 Media, buffers and routine reagents

These are detailed in the appendix.

2.2.2 Spore resuspension from cultures grown on solid medium

Sterile distilled water (10 ml) was pipetted onto the surface of agar plates containing spores which were then scraped off using a sterile loop. The spore/mycelium suspension was whirlymixed, filtered through cotton wool and the spore suspension, free of mycelium, collected. This spore suspension was used for making subcultures, for curing and mutagenesis.

2.2.3 Agar plug test for detecting antibiotic production

Plugs of NFP agar, 10 mm in diameter, were streaked with spores from individual colonies of S. alboflavus. The plugs were incubated for 4 d to allow confluent mycelial growth. Sloppy agar (10 ml) containing 0,1% tetrazolium and 0,2 ml of a log phase culture of the indicator organism, E. coli c600 (RP4) was poured around the plugs, and the plates incubated at 37°C overnight. Zones of inhibition, which were also colourless, around the plugs were noted and measured.

2.2.4 Curing of antibiotic production

Spores of S. alboflavus were inoculated into KL medium containing increasing concentrations of acriflavin (0-10 µg/ml) or ethidium bromide 0-10 µg/ml). After 4-6d incubation the mycelia were harvested from cultures growing in sub-lethal concentrations of the curing agents and transferred to NFP

agar and allowed to sporulate. The spores were scored for ability to produce otc using the agar plug test.

2.2.5 Stability of oxytetracycline negative clones

Oxytetracycline negative (otc^-) clones were passed through further 4 - 6d growth cycles in KL medium containing the minimum inhibitory concentration (m.i.c.) of ethidium bromide or acriflavin, and retested for otc production at the end of each growth cycle as outlined above. Otc^- clones obtained after repeated exposure to curing agents were then grown for a number of generations on NFP agar in the absence of curing agents and retested for otc production using the agar plug test.

2.2.6 Photoreactivation and mutagenesis by UV light

Spores of S. alboflavus were irradiated in the dark at 254nM and $1Jm^2s$ for varying times, using a Piccolo UV lamp (Hanau) Two samples (1 ml) were taken at each time interval; one sample was plated on NFP agar in the dark, and the other sample was exposed to a flourescent light source for 1 h prior to plating on NFP agar, to detect photoreactivation. All the plates were incubated in the dark. The UV dose which resulted in 1-5% survival was used for further mutagenesis studies.

2.2.7 Mutagenesis by N-Methyl-N'-Nitro-N-Nitrosoguanadine (NTG)

Spores of S. alboflavus were added to NTG (1 mg/ml in 0,05 M Tris-maleate buffer, pH 9,0) at a ratio of 1:5 ($^v/_v$). The spore suspension was incubated at 37°C for 50 min and then centrifuged at 4000 rpm for 10 min at 37°C. This gave a total

exposure to NTG of 60 min. The spore pellet was resuspended and washed twice in distilled water before plating the spores on NFP agar.

2,2.8 Growth and lysis of S. alboflavus, and isolation of DNA

The method of Schrempf et al., (1975) was modified and used to isolate the DNA. Sucrose-Casamino acids glycine (SCG) medium (200 ml) was inoculated with spores and shaken on an orbital shaker for 4 - 6d at 28°C. Labelled DNA was prepared by growing the spores in SCG medium containing 10µCi of [Methyl-^{3}H] thymidine per ml (24Ci/mmol). The mycelium was harvested by centrifugation at 15000 rpm for 15 min at 4°C, and resuspended in 25 ml of 25% ($^w/_v$) sucrose in 0,05M Tris-HCl buffer (pH 7,0) and 3,5 ml of 25 mg/ml ($^w/_v$) lysozyme in 0,25M Tris-HCl buffer (pH 7,0). This suspension was incubated at 37°C for 30 min, then placed on ice for 5 min, after which lysis was brought about by the addition of 12,5 ml of 2% ($^w/_v$) SDS in TE buffer, (0,05M Tris, 0,05M EDTA pH 7,5). The suspension was cooled on ice for 20 min, after which the sample became viscous, and then 7,5 ml of 10 M NaCl was added. The lysate was stored at 4°C overnight, centrifuged at 16000 rpm for 30 min at 4°C to pellet a large proportion of the chromosomal DNA while the ccc DNA was recovered in the supernatant. Ground PEG 6000 (8,25g) was added to the supernatant and kept at 4°C overnight. Care was taken to ensure that all the PEG was dissolved. The macromolecules were pelleted by centrifugation at 15000 rpm for 2 min at 4°C, and the pellet was resuspended in 7 ml of TE buffer (0,01M Tris, 0,0001M EDTA, pH 7,0). The PEG 6000 was separated from the DNA by

adding 7ml of chloroform, shaking the mixture gently and centrifuging at 5000 rpm for 5 min to separate the layers. The upper DNA layer was removed and this process was repeated twice.

2.2.9 Density gradient centrifugation

A 7ml sample of the DNA, containing 10g of CsCl, was subjected to a clearing spin of 5 min at 40000 rpm in a Beckman 50Ti rotor, to remove cell membrane precipitated by the CsCl. Ethidium bromide (0,2 ml of 10 mg/ml) was added to the cleared DNA-CsCl mixture, and the DNA was centrifuged in a Beckman 50 Ti rotor at 42000 rpm for 60 h at 4°C (Modification of Schrempf et al., 1975). Unlabelled gradients were visualized under UV light, and DNA bands removed from the top of the gradient with a needle and syringe.

Labelled gradients were sampled by 15-drop fractions collected from the bottom of the gradient in Eppendorf tubes (Schrempf et al., 1975). Samples (0,02 ml) of each 15-drop fraction were spotted on Whatman GF/A filter paper discs.

2.2.10 Sucrose gradient centrifugation

Labelled DNA-containing fractions obtained from CsCl gradients were pooled and dialysed against TE buffer (0,01M Tris 0,0001 M EDTA pH 7,0) for 24 h. Samples (0,15 ml) were layered on either linear neutral 5-25% sucrose gradients in TES buffer, or on linear alkaline 5-25% sucrose gradients which contained 0,2M NaOH and 0,7M NaCl. After centrifugation at 45000 rpm for 50 min at 20°C in a Beckman SW 50.1 rotor, 2-drop fractions were collected on Whatman GF/A filter paper

discs by puncturing a hole in the bottom of the nitrocellulose tube. (Modification of Schrempf et al., 1975).

2.2.11 Radioactive profile of [^{3}H] thymidine labelled gradients

The Whatman GF/A filter paper discs containing samples from the gradients were dried and then washed with cold 10% TCA (10 ml), twice with cold ethanol (10 ml), and finally with cold ether (10 ml). The dried filter paper discs were placed in plastic vials containing 10 ml of Packard Insta-gel scintillation fluid and counted in a Beckman LS-250 scintillation system.

2.2.12 Purification of DNA for electron microscopy and restriction enzyme analysis

Ethidium bromide was removed from the ethidium bromide-CsCl-DNA samples by adding an equal amount of CsCl-saturated isopropanol, shaking the mixture gently, and discarding the top layer. This process was repeated three times. The DNA in CsCl was dialysed against TE buffer (0,01M Tris, 0,0001M EDTA pH 7,0) for 24 h at 4°C.

2.2.13 Restriction analysis of DNA

Under normal conditions 50 µl of DNA, 50 µl of restriction enzyme-specific buffer and 1 µl of restriction enzyme were incubated at 37°C for 2 h. If the DNA concentration was low or the restriction sites numerous, 100µl of DNA, 100 µl of restriction enzyme-specific buffer and 1 µl of restriction enzyme were used. The reaction was terminated by the addition of 50 µl of stop buffer in molten 0,7% (^{w}/v) agarose (40°C).

2.2.14 Vertical agarose gel electrophoresis

Samples of DNA in 0.7% ($^{w}/v$) agarose were layered onto the surface of wells cut into 0,7% ($^{w}/v$) agarose gels containing 1 µg/ml ethidium bromide. The gels were subjected to a constant voltage of 20 mV for 20 h, and the flourescent DNA bands were detected by placing the gel on a horizontal UV light source. Photographs were taken with a Polaroid CU-5 land camera equipped with a 5 inch lens and a red filter.

2.2.15 Electron microscopy

The method of Coetzee and Pretorius (1979) was utilized.

Film preparation

Carbon films were prepared by evaporating carbon onto freshly cleaved mica, in a napthalene atmosphere at $<10^{-3}$ Tort. The carbon films were floated onto triple distilled H_2O and positioned onto 300 -mesh copper grids which had been pretreated with a cellulose adhesive tape - chloroform solution (Bradley, 1965).

DNA preparation

DNA was diluted $^{1}/100$ or $^{1}/1000$ into 100 µl droplet solution. Then 5 µl of benzylalkyldiamineethylammonium chloride (BAC - 0,2% $^{w}/v$ in formamide) was added to the diluted DNA. Droplets of DNA solution (50 µl) were formed on a Parafilm sheet. The droplets were covered to avoid contamination. After 10 min droplets were briefly touched with carbon-coated grids. The specimen grids were floated on triple-distilled H_2O for 5 min. The grids were then immersed in 50 mM uranyl acetate in

90% ethanol for 20 sec, rinsed in 90% ethanol and air-dried. The specimens were rotary shadowed at an angle of 7-10° and 10 cm from the source using gold-paladium wire. The specimens were examined in a Zeiss 109 electron microscope.

2.2.16 Nick translation of DNA

This was done by an adaptation of the method detailed in the Bethesda Research Laboratory catalogue and was based on the method of Rigby et al, (1977). Translation buffer (10 µl of 10 x concn), 2 µl each of dTTP, dCTP, dGTP and dATP, 5 µl of ^{32}P-dATP and 1 µl activated DNase (100 ng/ml) were added to 70 µl of DNA. The mixture was incubated at 15°C for 10 min, 2 µl of DNA polymerase was added and the mixture incubated at 15°C. After 1 h, 10 µl of BRL stop buffer was added to stop the reaction. The ^{32}P-labelled DNA was layered onto a 0,7% (^{w}/v) agarose gel and electrophoresed as described previously. The gel was placed on a Kodac X-omat MA X-ray film and the film was exposed for 2-3 d in a sealed black bag. The film was developed in Kodac liquid X-ray developer for 15 min and fixed in Kodac rapid fixer for 5 min. Adenovirus-2 DNA was labelled in the same way and electrophoresed as a control in parallel with the S. alboflavus DNA.

2.2.17 Determination of DNA base composition.

Labelled total cellular DNA from S. alboflavus was subjected to dye bouyant density centrifugation as described previously (2.2.9). The bouyant density of the DNA peaks was determined. The base composition of the DNA was

calculated as described by Schildkraut, Marmur and Doty (1962), using the equation p = 1,660 + 0,098 (GC), where p = bouyant density and GC represents the guanine + cytosine base content.

2.2.18 Measurement of pigment production

Pigment production was determined visually. The pigment (mel) was orange-brown in colour, and was visible as a zone of colouration in the agar around the colony, and within the colony itself.

2.3. RESULTS

2.3.1 Curing of antibiotic production

The plasmid nature of otc production was investigated by curing with ethidium bromide and acriflavin. The m.i.c. of ethidium bromide and acriflavin was 4 and 5 µg/ml respectively. Loss of otc production was markedly increased by curing and the frequency of otc^- ranged from 12,5 - 21,4% after 1-4 cycles of curing. Control cultures showed no loss of otc production. The results of one curing experiment are shown in Table 2.1.

2.3.2 Stability of otc^- colonies obtained after curing.

The otc^- phenotype was unstable and all otc^- colonies obtained after one cycle of curing reverted to otc^+ after 4 generations on NFP agar. The reversion occured even after repeated growth cycles of otc^- colonies in KL medium containing the curing agent. (Table 2.2).

2.3.3 UV mutagenesis

Photoreactivation was not detected in *S. alboflavus* (Fig. 2.1). Exposure of *S. alboflavus* spores to 60-100 Jm^2 dosage of UV light gave rise to 8,0% otc^- colonies. All the otc^- colonies reverted to otc^+ after 4 generations of growth on NFP agar (Table 2.3).

2.3.4 NTG mutagenesis

Exposure of *S. alboflavus* spores to 1 mg/ml NTG for 60 min. did not give rise to any otc^- colonies. All NTG-induced auxotrophs detected ($\sim$1%) were cys^-met^-.

Table 2.1: Effect of ethidium bromide and acriflavin on otc production by S. alboflavus.

Curing agent	Otc production[a]		% otc^-
	otc^+	otc^-	
Control	150	0	0
Ethidium bromide 4 µg/ml	82	18	18
Acriflavin 5 µg/ml	108	22	16,9

a. Number of colonies after 4 cycles in ethidium bromide or acriflavin.

Table 2.2: Reversion of otc$^-$ to otc$^+$

No. of Growth cycles in ethidium bromide	No. of generations without ethidium bromide			
	1	2	3	4
	% reversion			
1	0	37	87	100
2	0	28	80	100
3	0	30	86	100
4	0	21	77	100

Table 2.3: Effect of UV irradiation on otc production by S. alboflavus, and the reversion of otc$^-$ to otc$^+$.

Viable count after UV irradiation ($60Jm^2$)	% otc$^-$	% otc$^-$ → otc$^+$ after 4 generations on NFP agar.
5×10^5	8,6	100

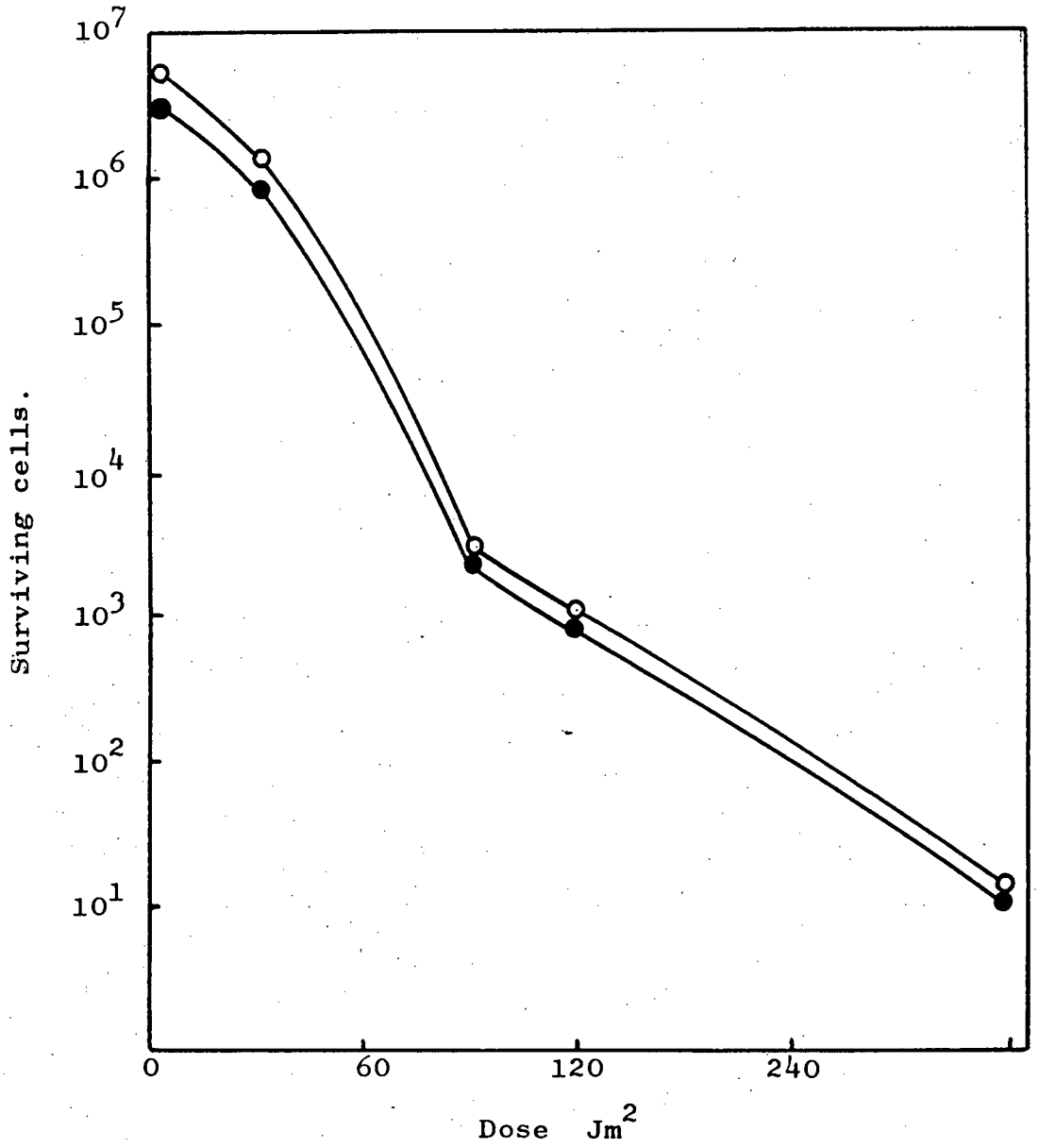

Fig. 2.1: Photoreactivation of S. alboflavus after UV irradiation. Irradiated spores exposed to light (o) before incubation, and spores plated and incubated in the dark (o).

2.3.5 Total DNA profile after density gradient centrifugation

The [^{3}H] thymidine profile of S. alboflavus DNA centrifuged to equilibrium on a density gradient is shown in Fig 2.2. There were 2 species of DNA banding at positions corresponding to higher bouyant densities than most of the extracted cellular DNA. The incorporation of [^{3}H] thymidine into these species of DNA was not very efficient when compared with the high incorporation into the chromosomal DNA. The DNA species were designated SAP1, (S. alboflavus plasmid 1), and SAP2, (S. alboflavus plasmid 2), in order of decreasing density and by analogy with the S. coelicolor A3(2) and S. rimosus plasmids (Friend, Warren and Hopwood, 1978).

2.3.6 Sucrose gradient profiles of [^{3}H] thymidine labelled DNA

Both SAP1 and SAP2 were analysed on linear neutral and alkaline sucrose gradients. However, SAP1 and SAP2 did not incorporate the [^{3}H] thymidine at high enough levels for peaks of radioactivity to be detected on the sucrose gradients.

2.3.7 Characteristics of SAP1

2.3.7.1 DNA base composition

The density to which SAP1 sedimented in a density gradient was 1,84 g/cm^2. From this value the GC content was calculated to be 66,3%.

2.3.7.2 Copy number

The copy number of SAP1 was approximately 4 copies per chromosome, calculated from the ratio of the [^{3}H] thymidine labelled SAP1 and chromosomal peaks obtained after density gradient centrifugation.

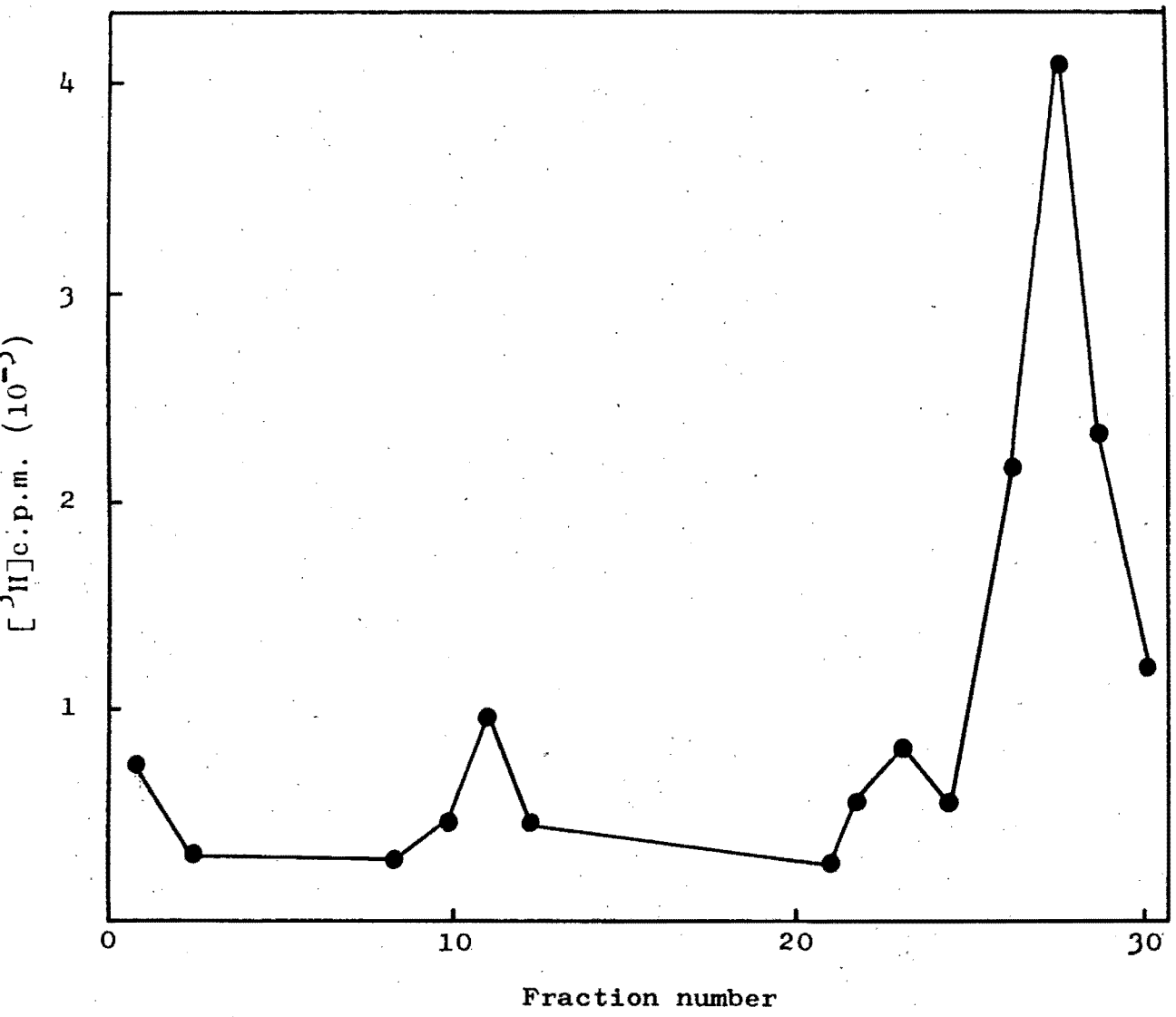

Fig. 2.2: Density gradient of [^{3}H] thymidine labelled total DNA from S. alboflavus.

2.3.7.3 Restriction analysis

SAP1 was sensitive to 4 out of 8 restriction enzymes (Table 2.4). No discrete bands could be identified after agarose gel electrophoresis of restricted SAP1 (Fig. 2.3). SAP1 was approximately 8-10 x 10^6 daltons, measured against BglI restricted λ.

2.3.7.4 Electron micrographs

Electron micrographs revealed linear DNA strands of various lengths (Fig. 2.4).

2.3.7.5 Nick translation

Several attempts to label SAP1 with [^{32}P] -ATP or [^{32}P]-GTP by nick translation, which would have aided in resolving restriction fragments, were unsuccessful. Adenovirus-2 DNA, used as a control, was labelled by nick translation and was visible as a band on X-ray film. Similar bands representing labelled SAP1 were not visible on X-ray film.

2.3.8 Characteristics of SAP2

2.3.8.1 Copy number

There were approximately 3 copies of SAP2 to each chromosome, calculated from the ratio of the [^{3}H] thymidine labelled SAP2 and chromosomal DNA peaks obtained after density gradient centrifugation.

2.3.8.2 Restriction analysis

Only BglII was able to restrict SAP2 and it was restricted into a number of bands. The exact size and position of the bands was impossible to determine since the contaminating chromosomal DNA formed a smear down the gel, obscuring the

Table 2.4: Restriction of SAP1 by restriction endonucleases.

Enzyme	Restriction	Specific bands	Recognition sequence
BamHI	resistant	-	G↓G A T C C
BglI	Yes	none detected yet	G C C N N N↓N G G C
BglII	Yes	none detected yet	A↓G A T C T
EcoRI	resistant	-	G↓A A T T C
HindIII	resistant	-	A↓A G C T T
PstI	Yes	none detected yet	C T G C A↓G
SalI	Yes	none detected yet	G↓T C G A C
XbaI	resistant	-	T↓C T A G A

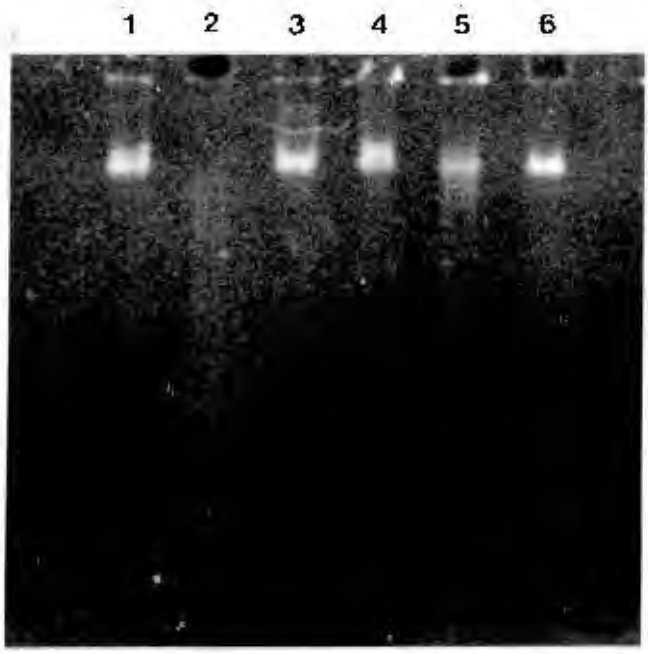

Fig. 2.3: Digestion pattern of SAP1 DNA isolated from S. alboflavus.

1, SAP1 + XbaI; 2, SAP1 + SalI;

3, SAP1; 4, SAP1 + EcoRI;

5, SAP1 + BglII; 6, SAP1 + BglI.

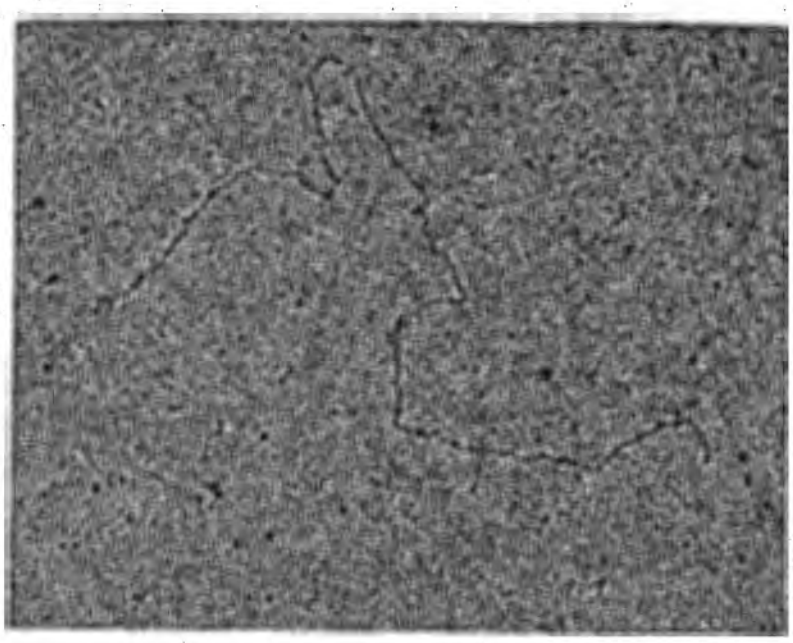

Fig. 2.4: Electron micrograph of SAP1 DNA isolated from S. alboflavus. (x 90 000).

bands (Fig. 2.5). SAP2 was approximately 20-25 x 10^6 daltons, as measured against EcoRI cut λ.

2.3.8.3 Electron micrographs

Electron micrographs of SAP2 revealed at least two small circular DNA structures of approximately the same size (Fig. 2.6). The majority of the DNA was linear. The samples were contaminated with chromosomal DNA (Fig. 2.7).

2.3.9 Pigment production

After UV irradiation resulting in 1-5% survival, approximately 0,1% of the surviving colonies produced no visible pigment, (mel^-), and 1-10,8% of the survivors produced very large amounts of pigment (mel^{++}). NTG- mutagenesis giving 1-5% survival resulted in 0,8% of the survivors being mel^-, and 0,5 - 2,0% being mel^{++}. Mel^- colonies did not occur spontaneously, but mel^{++} colonies arose at a frequency of approximately 1% spontaneously.

Mel^- and mel^{++} colonies were unstable and reverted to mel^+ after subculturing. The mel^{++} colonies produced enhanced levels of otc, and mel^- colonies produced almost zero otc, but otc production returned to a normal level upon reversion of the colonies to mel^+.

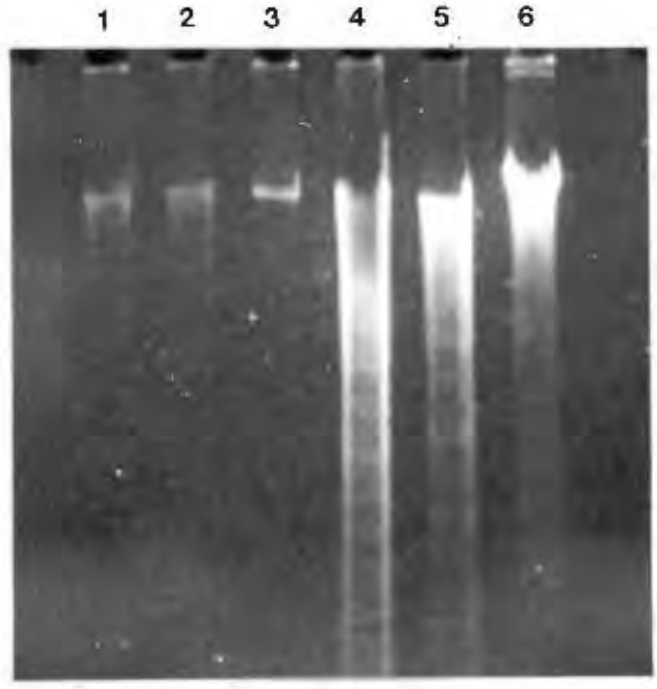

Fig. 2.5: The digestion pattern of SAP1 and SAP2 DNA isolated from S. alboflavus.

1 & 2, SAP1 + BglII; 3, SAP1;

4 & 5, SAP2 + BglII; 6, SAP2.

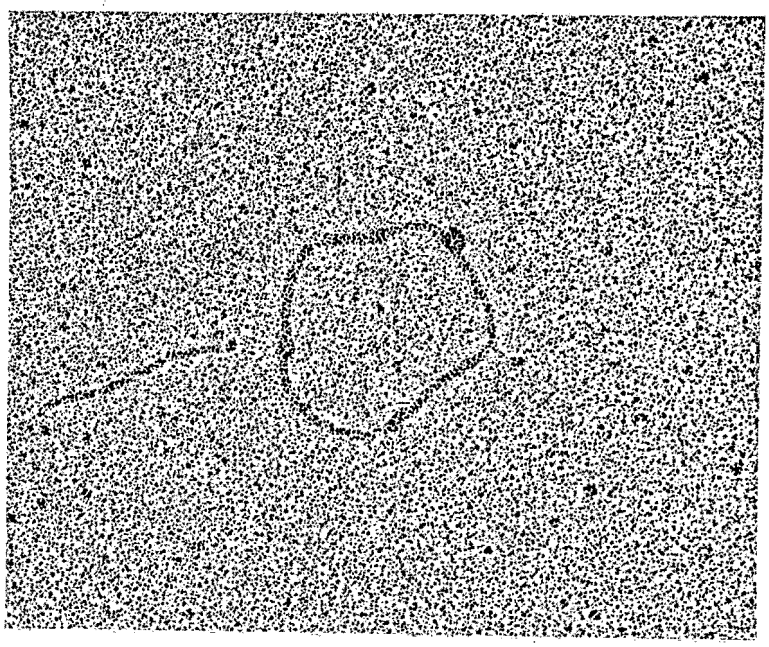

Fig. 2.6: Electron micrograph of SAP2 DNA isolated from S. alboflavus (x 60 000).

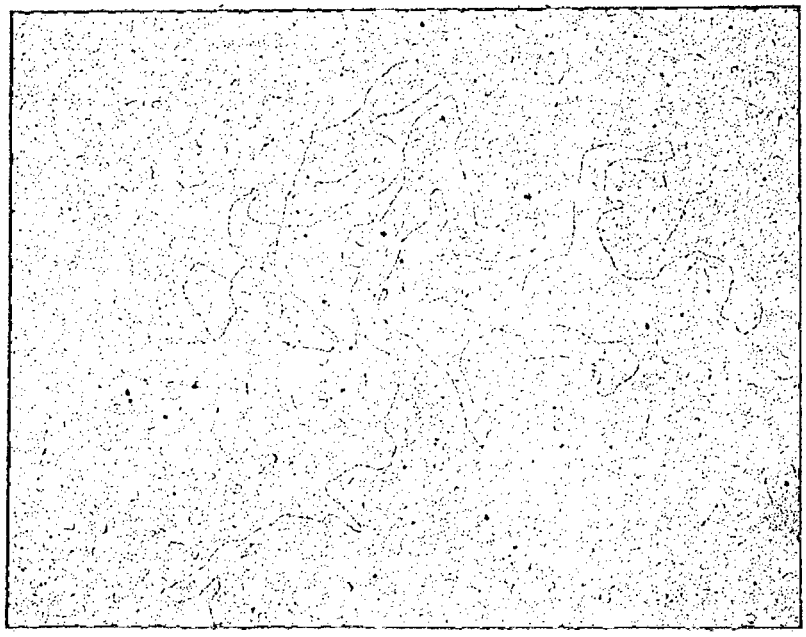

Fig. 2.7: Electron micrograph of SAP2 DNA isolated from S. alboflavus (x 90 000).

2.4 DISCUSSION

Otc production by S. alboflavus was found to be unstable, and treatment with curing agents resulted in 12,5 - 21,4% otc^- colonies. UV irradiation resulted in an 8,0% loss of otc production. However, the loss of the ability to produce otc was totally reversible. This reversion indicated that there was no loss of genetic material, but rather that an unstable element may play a role in otc production.

Mel production by S. alboflavus was unstable. UV-irradiation and NTG mutagenesis of S. alboflavus resulted in colonies which were mel^- and mel^{++}. The mel^- colonies produced almost zero levels of otc, and the mel^{++} colonies produced enhanced levels of otc. However, the mel^- and mel^{++} colonies reverted to mel^+ upon subculturing, with concommittent reversion of otc production to normal levels. This could indicate that both otc production and mel production are affected by the same unstable element, possibly due to the close proximity of the otc and mel genes.

Other cases of unstable mel production have been reported in Streptomyces. When spores of Streptomyces lincolnensis were exposed to UV irradiation, about 1% of the progeny colonies did not produce a pigment (mel^-). Two thirds of the mel^- colonies did not produce the antibiotic normally produced by S. lincolnensis. However, the mel^- colonies were stable and did not revert to mel^+, suggesting that mel^- is due to the loss of genetic material (Freeman, 1978).

Streptomyces scabies has unstable mel production (Gregory and Shyu, 1961; Gregory and Huang, 1964). The

enzyme tyrosinase (tyr) converts tyrosine into a mel precursor. Tyr^- colonies arose spontaneously at a frequency of 0,2%, and after X-ray treatment the frequency of tyr^- increased to 5%. The tyr^- colonies were stable, and no reversion to tyr^+ occured. It appears that tyr production in S. scabies is plasmid controlled.

The SAP1 DNA species was sensitive to a number of restriction enzymes, but was not cut into discrete bands. The GC content in the recognition sites of restriction enzymes which cut SAP1 is $^{16}/_{24}$, and the GC content in the recognition sites of restriction enzymes which do not cut SAP1 is $^{10}/_{24}$ This is in accordance with the calculated GC content of 66,3% in SAP1. SAP1 DNA is linear, and appears to be of varying lengths. The absence of specific bands indicative of specific, unique, restriction enzyme recognition sites could be explained by two possibilities:

a) The sites of recognition are numerous and cannot be resolved on conventional vertical agarose gels.

b) The SAP1 DNA is heterogenous, and is a species of DNA occuring in S. alboflavus which has a very different base composition from the chromosomal DNA. The SAP1 DNA could be similar to the satellite DNA of eukaryotes, which represents multiple copies of a short nucleotide sequence arranged in tandem arrays in large blocks (Bostock, 1980). However, the fact that no discrete restriction bands of SAP1 could be detected argues against the possibility that SAP1 consists of repeated sequences similar to eukaryotic satellite DNA.

It is more possible that SAP1 is a satellite band of DNA comprising the S. alboflavus chromosomal DNA regions with higher GC contents.

The SAP2 DNA species was difficult to isolate, and was always contaminated with chromosomal DNA. The SAP2 DNA could be identified as ccc DNA in some DNA isolates, but restriction bands were difficult to identify because the contaminating chromosomal DNA obscured the bands. The difficulty of isolating plasmid DNA from Streptomyces was demonstrated in S. coelicolor A3(2) (J. Westpheling, personal communication). The SCP1 plasmid of S. coelicolor A3(2) was only isolated recently, using an adaptation of the method used in this study.

CHAPTER III

CLONING & TRANSFORMATION

3.1 INTRODUCTION

Since SAP1 could be isolated attempts were made to determine the phenotype of SAP1 by cloning and transformation studies. The SAP1 DNA was either cloned into E. coli or transformed into Streptomyces albus utilizing the transformation techniques developed for Streptomyces (see Section 1.4).

A strain of E. coli was selected as the recipient of cloned SAP1 since transformation procedures for E. coli are well established (Mandel and Higa, 1970). The strain used was E. coli RRI, which does not have restriction and modification systems, does not produce otc and is otc^s. The vector for carrying SAP1 fragments into E. coli RR1 was the plasmid pACYC184 (Chang and Cohen, 1978), carrying chloramphenicol resistance (cml^r) and tetracycline resistance (tet^r). There is a single SalI restriction site in the tet^r gene of pACYC184. Therefore any DNA inserted in the SalI site of pACYC184 would result in the inactivation of the tet^r gene (insertional inactivation).

Restriction analysis of SAP1 revealed that it was restricted by SalI into many fragments of varying lengths (see section 2.3.7.3). Therefore, SAP1 was partially restricted by SalI to generate fewer, larger fragments for cloning. The SalI DNA fragments of SAP1 were ligated with SalI restricted pACYC184. The ligated SAP1/pACYC184

DNA was transformed into competent E. coli RRI cells, and the transformants were selected for cml^r and tet^s. This phenotype indicated that DNA had been inserted into the SalI site of pACYC184, resulting in the inactivation of the tet^r gene. The E. coli RR1 cml^r tet^s transformants were screened for otc production and mel production. However there are contradictory reports regarding the expression of Streptomyces DNA in E. coli (see section 1.4) and it is possible that the SAP 1 DNA may not be expressed in E. coli RR1.

Interspecies gene expression has been demonstrated in Streptomyces (see section 1.4). Transformation of Streptomyces is well documented and has an efficiency of 4 - 20% (Bibb, Ward and Hopwood, 1978). Attempts were therefore made to transform SAP1 into S. albus CM153766 otc^- otc^s. A restriction and modification system has not been reported in S. albus. The protoplast transformation procedure of Bibb, Ward and Hopwood, (1978) was utilized. The S. albus CM153766 transformants were screened for otc production, otc^r and melanin production.

3.2 METHODS

3.2.1 Isolation of plasmid DNA

E. coli c600 pACYC184 was grown at 37°C overnight in 100 ml nutrient broth containing 300 µg/ml spectinomycin, to amplify the pACYC184 plasmid. The cells were pelleted at 10000 rpm for 15 min, and resuspended in 2,5 ml of 25% sucrose in 0,05M Tris HCl buffer (pH 7,0) and 0,4 ml of 10 mg/ml lysozyme in 0,25 M Tris HCl buffer (pH 7,0). The suspension was incubated at 37°C for 30 min and then cooled rapidly on ice for 5 min, after which 1,25 ml of 0,2 M EDTA was added. After a further 4 min on ice, 2,5 ml of 2% Triton in 0,05 M Tris 0,05 M EDTA and 20 µg/ml RNase was added. This mixture was held on ice for 30 min and then centrifuged at 17000 rpm for 30 min. The supernatant was collected and CsCl was added to give a refractive index of 1,39. A 0,2 ml amount of 10 mg/ml ethidium bromide was added to 7 ml of DNA in CsCl, and the DNA was centrifuged in a 50 Ti rotor at 44000 rpm and 10°C for 36 h. The lower plasmid band was removed and the ethidium bromide and CsCl removed as before (see section 2.2.12). SAP1 was isolated according to the method described in section 2.2.8.

3.2.2 Ligation of pACYC184 and SAP1.

SAP1 DNA (80 µl, approx 15 µg) was added to 20 µl pACYC184 DNA (approx 2 µg), and 2 x concn restriction endonuclease buffer. Restriction enzyme (5 µl SalI) was added and the mixture incubated at 37°C for 2 h. The restriction enzyme was inactivated by placing the mixture at 65°C for 10 min.

Freshly made up ligation buffer (15 µl) was added to 150 µl restricted pACYC184/SAP1 DNA. In addition, 15 µl 10mM ATP and 1 µl ligase was added and the mixture incubated at 12°C overnight. EDTA (16 µl of 0,25M, pH 8,0) was then added and the ligated DNA could then be stored at 4°C for up to one week without loss of transforming ability. Samples of the ligated DNA mixtures were analysed on agarose gel (see section 2.2.14).

3.2.3 Transformation of E. coli RR1

The calcium shock transformation procedure of Mandel and Higa (1970), and Cohen et al., (1972), was adopted. A 50 ml aliquot of Luria broth was inoculated with 5 ml of an overnight culture of E. coli RR1, and incubated with aeration for 2,5 h at 37°C. The culture was cooled and the cells pelleted by centrifugation at 6000 rpm and 4°C for 5 min. The cells were resuspended in 20 ml cold $CaCl_2$ (50mM) and pelleted as before. The cells were finally resuspended in 2 ml of cold 50mM $CaCl_2$, and kept on ice.

A 50 µl aliquot of ligated DNA was added to 100 µl of competent E. coli RR1 cells, and the mixture held on ice for 30 min, followed by a heat pulse of 45°C for 2 min. The cells were then held on ice for a further 30 min, diluted into 3 ml of Luria broth and incubated at 37°C for 3 h. The cells were plated without dilution onto selective Luria agar containing chloramphenicol (25 µg/cml) or cml (25 µg/ml) + tetracycline (25 µg/ml). As a control, competent E. coli RR1 was plated onto the same selective media, and onto Luria agar. In addition, E. coli RR1

was transformed with unrestricted pACYC184 or SAP 1, and with restricted and religated pACYC184 on its own and grown on selective Luria agar.

3.2.4 Transformation of S. albus.

S. albus CM153766 was grown in 25 ml medium S for 5 d. The cells were pelleted at 15000 rpm for 10 min at 15°C, washed twice in 20 ml medium P and resuspended in 1,5 ml medium P containing 1 mg/ml lysozyme. After incubation with agitation at 37°C for 1 h, the cells were pipetted up and down vigorously to release the protoplasts. The protoplasts were filtered through sterile cotton wool, and washed twice in 1,5 ml medium P. Transforming DNA (20 l SAP 1) and 0,5 ml of 20% PEG in medium P was added to the protoplasts. After 1 min, 0,5 ml of 10% PEG in medium P was added, and after a further 3 min, 4 ml of medium P was added. The suspension was mixed gently and then the protoplasts were pelleted and resuspended in 0,2 ml of medium P. The transformed protoplasts were diluted 10^{-1} and 10^{-2} in medium P, and plated on R2 agar. The protoplasts were spread on the agar using a sterile loop, and incubated for 7 d at 30°C. Spores were harvested and plated on NFP agar for isolated colonies. The individual colonies were tested for otc production, using the agar plug test (see section 2.2.3), for resistance to otc, by plating on J1M agar containing 30 g/ml otc, and for pigment production (see section 2.2.18).

3.3 RESULTS

3.3.1 Transformation of E. coli RR1

Transformed cells were selected on Luria agar + cml, and tested for insertional inactivation by replica plating onto Luria agar + cml + tet. E. coli RR1 cells transformed with ligated pACYC184/SAP1 DNA failed to grow on Luria agar + cml. Cells transformed with pACYC184 grew on both Luria agar + cml and Luria agar + cml + tet. Cells transformed with SAP1 or religated pACYC184 failed to grow on Luria agar + cml or Luria agar + cml + tet.

Although SAP1 and pACYC184 DNA were visible as bands on agarose gels, religated SAP1 and religated pACYC184 were not present on agarose gels.

3.3.2 Transformation of S. albus

The spores of transformed S. albus CMI53766 protoplasts were tested for otc production, otc resistance and pigment production.

3.3.2.1 Otc production.

Out of 120 colonies tested, 7 produced an antibiotic-like substance which inhibited the growth of E. coli c600 otc^s and B. subtilis RUB834 otc^s, but did not inhibit the growth of E. coli c600 RP4 otc^r. The ability to produce this antibiotic-like substance was unstable, and subcultures of the 7 transformants failed to inhibit E. coli c600 otc^s and B. subtilis RUB834 otc^s. Control cultures of S. albus CM153766 which had not been transformed did not inhibit the growth of E. coli c600 otc^s or B. subtilis RUB834 otc^s.

Further transformation experiments failed to produce transformants of S. albus CMI53766 capable of inhibiting the growth of either E. coli c600 otc^{s} or B. subtilis RUB834 otc^{s}.

3.3.2.2 Otc resistance

Spores of regenerated S. albus CMI53766 protoplasts which had been transformed with SAP1 were grown on JIM agar + otc. No colonies were detected which were resistant to otc. The 7 transformants which produced an antibiotic-like substance were also sensitive to otc.

3.3.2.3 Pigment production

No mel^{+} colonies were detected amongst the spores of regenerated protoplasts which had been transformed with SAP1.

3.4 DISCUSSION

The *E. coli* RRI cells were competent for DNA uptake since transformation with pACYC184 rendered the cells cml^r and tet^r. The religated pACYC184 could not transform the cells to cml^r and tet^r, and was not present as a band on agarose gel. Transformation with SAP 1 had no detectable effect on the phenotype of *E. coli* RRI. Both SAP1 and pACYC184 were detectable on agarose gel. Therefore it appears that the ligation did not occur. Additional experiments were carried out using different ligase preparations, with similar results.

Studies on transformation of *S. albus* CM153766 with SAP1 resulted in 7 transformants in one experiment which produced an antibiotic-like substance which was inhibitory to otc^s indicator organisms, *E. coli* c600 and *B. subtilis* RUB834. It is possible that this antibiotic-like substance was otc, since *E. coli* c600 RP4, which is otc^r, was not inhibited by the same 7 transformants. However, the 7 transformants were sensitive to 30 μg/ml otc, suggesting that the antibiotic-like substance was not otc. It is also possible that the transforming SAP1 DNA was acting as a promotor to switch on silent genes which are present in *S. albus* CMI53766 and which specify the production of the antibiotic-like substance. However it was not possible to analyse the nature of the antibiotic-like substance since its production by *S. albus* CMI53766 transformants was unstable, and was lost after subculturing. The antibiotic-like substance was not produced spontaneously by *S. albus*

CMI53766. Attempts to repeat the transformation using different SAPI preparations failed to produce transformants which could inhibit E. coli c600 otc^{s} or B. subtilis RUB834 otc^{s}.

The transformation frequency of 5,8% observed compares favourably with the transformation frequencies reported for other Streptomyces species (Bibb, Ward and Hopwood, 1978).

CHAPTER IV

4.1 CONCLUSION

The aim of this study was an attempt to locate the genes of S. alboflavus, with the long term goal of increasing and stabilizing the levels of production of otc. Since a number of antibiotic producing Streptomyces have plasmid controlled antibiotic production (see section 1.2), the S. alboflavus strain was exposed to curing agents to determine whether the otc genes were located on a plasmid. At the same time, S. alboflavus was investigated for the physical presence of extrachromosomal DNA. The function of the extrachromosomal DNA was investigated by cloning and transformation.

Otc production by S. alboflavus was lost at a significant frequency when single colonies were examined after curing and UV irradiation. Upon subculture, all the otc^- isolates regained their ability to produce otc. Therefore this instability of otc production was not due to the loss of a genetic element, since loss of otc production was totally reversible.

A similar result was reported concerning aureothricin production by S. kasugaensis (Freeman, 1978). Colonies cured of aureothricin production reverted upon subculture.

The occurence of mel^{++} colonies which had enhanced levels of otc production, and mel^- colonies which had almost zero levels of otc production, and the linkage of mel and otc during reversion suggested that both mel and otc genes were under the same control, possibly due to their being

adjacent genes. However, there is no chromosomal map for S. alboflavus, therefore the linkage of the genes is unknown.

A theory can be advanced to account for the reversible loss of otc production by S. alboflavus. This model is based on existing knowledge from eubacteria and Streptomyces. The simplest model would propose that there is an insertion element present in S. alboflavus, and that the S. alboflavus chromosome has hot spots for integration of this element. The element could be similar to IS2 (Nevers and Saedler, 1977), which is present in E. coli. The E. coli chromosome has hot spots for integration of the insertion elements (Chadwell and Starlinger, 1978). When IS2 is integrated in one orientation, it acts as a promotor and genes located downstream are expressed constitutively at a level three times higher than normal. When IS2 is integrated in the opposite orientation, it reduces operon expression, or abolishes it if integrated within a structural gene.

A similar insertion element could have a relatively stable site of integration on the S. alboflavus chromosome, where it has no detectable effect on the phenotype (Fig 4.1.1). Transposition of the insertion element to the area upstream of the otc and mel genes could have the effect of increasing levels of otc and mel production, if the insertion element is in the promotor orientation (Fig. 4.1.2), and abolishing otc and mel production if the insertion element is in the opposite orientation (Fig. 4.1.3). Transposition of the insertion element to an area within the otc genes could interrupt the continuity of the gene and abolish otc production, irrespective of the orientation of the insertion

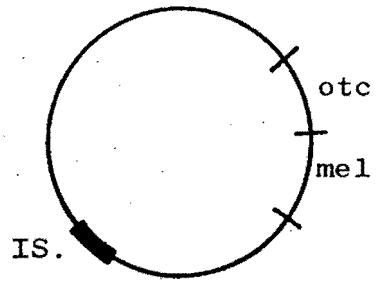

Fig. 4.1.1: $otc^{+}mel^{+}$

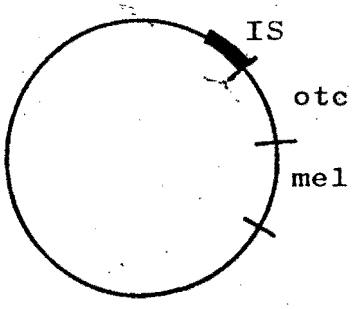

Fig. 4.1.2: $otc^{++}mel^{++}$

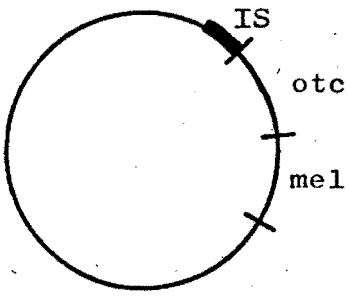

Fig. 4.1.3: $otc^{-}mel^{-}$

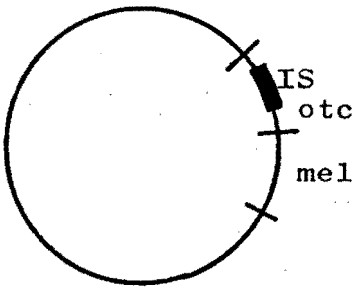

Fig. 4.1.4: $otc^{-}mel^{+}$

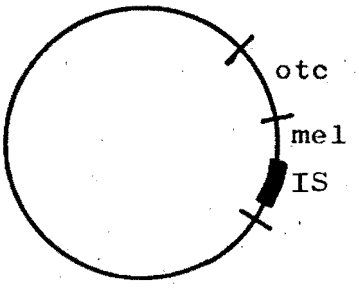

Fig. 4.1.5: $otc^{+}mel^{-}$

Fig. 4.1: Model for instability of otc production in S. alboflavus.

element (Fig. 4.1.4), and transposition to an area within the mel genes could abolish mel production, irrespective of the orientation of the insertion element (Fig. 4.1.5). Exposure of S. alboflavus to curing agents could promote the insertion of the element in a particular site or orientation resulting in the prevention of otc production (Fig. 4.1.3 and 4.1.4). After removal of the curing agent the insertion element could revert to its normal site of insertion (Fig. 4.1.1).

Failure of the curing agents to successfully cure S. alboflavus of otc production does not necessarily imply that a plasmid is not involved in otc production. It may indicate that the plasmid is extremely resistant to curing, and can reinfect the hyphae once the curing agent is removed, resulting in a reversion to otc^+. But this is unlikely, since otc^- colonies were repeatedly grown in the presence of curing agents, in an attempt to cure all the cells of the plasmid, and 100% reversion to otc^+ occured, even after 6 purification cycles in the presence of the curing agent.

The two extrachromosomal DNA species SAP1 and SAP2, isolated from S. alboflavus, have unknown functions. The SAP1 DNA appeared to be linear and heterogeneous, and could possibly represent a fraction of chromosomal DNA with a higher GC content than the majority of the chromosome. This could be verified by reanealing SAP1 with the chromosome. The isolation of 7 S. albus transformants suggested that SAP1 could be involved in the production of an antibiotic-like substance, possibly by acting as a promotor of the S. albus chromosomal genes, or by carrying the genes

specifying the production of the antibiotic-like substance. Further transformation experiments should be carried out to verify this possibility.

Agarose gel electrophoresis of SAP2 showed it to be ccc DNA. However, no ccc DNA was detected under the electron microscope. This may indicate that SAP2 is sensitive to the methods of isolation utilized. Coencentric electrophoresis of larger samples of SAP2 DNA should enable the ccc DNA bands to be isolated for further study, and this method of electrophoresis will enable the restriction fragments to be isolated free of chromosomal DNA. Coencentric electrophoresis enables fragments of DNA to be isolated which vary in position on the gel by about 5 mm. (Hoefer 1980). Once purified SAP2 has been obtained its function could be elucidated by transformation experiments.

MEDIA, BUFFERS AND ROUTINE REAGENTS

Enzymes

Restriction enzymes and ligase enzymes were obtained from Bethesda Research Laboratories.

Agarose gel

Agarose	0,7 g
Tris-borate buffer	100 ml

CsCl-saturated isopropanol

CsCl	10 g
distilled water	5 ml
isopropanol	150 ml

Add additional CsCl until no more can dissolve.

Droplet solution

NaAc	100 mM
Triethanolamine (pH 8,0)	50 mM
Formamide	30% ($^{v}/_{v}$)

J.I.M. Agar

Asparagine	0,5 g
K_2HPO_4	0,5 g
KOH	0,5 g
$MgSO_4.7H_2O$	0,2 g
$FeSO_4.7H_2O$	0,01 g
Agar	15 g
distilled H_2O	1000 ml

Autoclave. Add 20 ml of sterile 20% glucose.

After autoclaving, the following sterile solutions were added to each 80 ml aliquot:

KH_2PO_4 (0,5%)	1 ml
$CaCl_2.2H_2O$ (3,68%)	10 ml
TES (N-Tris-(hydroxymethyl)-methyl-2 aminoethanesulphonic acid) buffer (0,25M pH 7,2, filter sterilize)	10 ml

Medium R2

Sucrose	103 g
K_2SO_4	0,25 g
$MgCl_2.6H_2O$	10,12 g
Glucose	10 g
Casamino acid	0,1 g
Agar	22 g
distilled H_2O	800 ml

After autoclaving, the following sterile solutions were added to 80 ml aliquots:

Trace element solution	0,2 ml	
KH_2PO_4 (0,5%)	1,0 ml	
$CaCl_2.2H_2O$ (3,68%)	8,02 ml	
L-proline (20%)	1,5 ml	
TES buffer (0,25M pH 7,2)	as above	10 ml
NaOH (1N)	0,5 ml	

Medium S

Glucose	10 g
Peptone	4 g
Yeast extract	4 g
$MgSO_4.7H_2O$	0,5 g
KH_2PO_4	2 g
K_2HPO_4	4 g
distilled H_2O	800 ml

After autoclaving, 5 ml of sterile 20% glycine was added to 20 ml aliquots in 250 ml flasks.

Ligation buffer

Tris	0,66 M
$MgCl_2$	0,066 M
1,4-Dithiothreotol	0,1 M
distilled H_2O	10 ml

Luria agar

Tryptone	10 g
Yeast extract	5 g
NaCl	0,5 g
NaOH (1M)	2 ml
distilled H_2O	1000 ml
Agar	15 g

Adjust to pH 7,0 with 1M NaOH.

Medium P

Sucrose	103 g
K_2SO_4	0,25 g
Trace element solution (see below)	2 ml
$MgCl_2.6H_2O$	2,03 g
distilled H_2O	800 ml

Restriction endonuclease buffer (for XbaI) (Bethesda Research Laboratory catalogue).

Tris	6 mM
$MgCl_2$	6 mM
NaCl	100 mM

Adjust to pH 7,4 with HCl.

Restriction endonuclease buffer (for PstI) (Bethesda Research Laboratory catalogue).

Tris	20 mM
$MgCl_2$	10 mM
$(NH_4)_4SO_4$	50 mM
BSA	100 µg/ml

Adjust to pH 7,5 with HCl.

Stop buffer (Bethesda Research Laboratory catalogue)

Na_2EDTA	0,3 M

Adjust to pH 8,0 with HCl.

Stop buffer

Sucrose	40 g
Bromophenol blue	
EDTA (0,1M)	

Sucrose-Casamino acid-glycine medium

Sucrose	340 g
Casamino acid	24 g
$MgCl_2$	10 g
Glycine	5 g
Glucose	5 g
distilled H_2O	1000 ml

TES buffer

Tris	0,03 M
EDTA	0,005 M
NaCl	0,05 M

Adjust to pH 8,0 with HCl.

Trace element solution

$ZnCl_2$	0,04 g
$FeCl_3.6H_2O$	0,2 g
$CuCl_2.2H_2O$	0,01 g
$MnCl_2.4H_2O$	0,01 g
$Na_2B_4O_7.10H_2O$	0,01 g
distilled H_2O	1000 ml

Translation buffer (10 x conc)

Tris	0,5 M
$MgCl_2$	0,05 M
2-mercaptoethanol	0,1 M
BSA (nuclease free)	0,5 mg/ml

Adjust to pH 7,8 with HCl.

Tris-borate buffer

Tris	10,8 g
EDTA	0,93 g
Boric acid	5,5 g
distilled H_2O	1000 ml

Tris-maleate buffer

Tris	0,05 M
Maleic acid	0,05 M

Adjust to pH 9,0 with NaOH.

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