The Cloning and Analysis of the Genes Specifying Geldanamycin Biosynthesis from *Streptomyces hygroscopicus*

Thesis submitted in accordance with the requirements of The University of Liverpool for the degree of Doctor in Philosophy by

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This thesis is dedicated to my family,

Mum, Dad and Karen, without whom.....

Abstract

The ansamycin antibiotic geldanamycin is produced by the soil actinomycete *Streptomyces hygroscopicus* N.R.R.L. 3602. Geldanamycin is synthesised by the successive condensation of carboxyllic acid "building blocks" (principally propionate and acetate units) to form a polyketide chain bridging non-adjacent carbon atoms on an aromatic amino acid starter unit (3,5-aminohydroxybenzoic acid). The method of this synthesis is analogous to that for fatty acids and is mediated by a polyketide synthase (PKS).

An initial attempt was made to isolate the genes specifying geldanamycin biosynthesis by probing "total" DNA preparations from *S. hygroscopicus* 3602 with DNA sequences known to be involved in polyketide synthesis, namely the *act*I and *act*III sequences cloned from the actinorhodin biosynthetic cluster of *S. coelicolor* A3(2). However, very little homology was detected between these probes and the *S. hygroscopicus* 3602 target DNA.

As an alternative cloning strategy a gene library of large (>15 kb) fragments of *S. hygroscopicus* 3602 "total" DNA was constructed in a low copy number plasmid vector, pIJ61, and individual *S. lividans* TK24 transformants selected for (plasmid determined) thiostrepton resistance then screened for the acquired ability to exhibit antibiosis against *Bacillus subtilis.*

Three antibiotic producing colonies were isolated (pIA101, pIA102 and pIA103). Plasmid DNA was isolated from each of these transformants but proved refractory to

genetic mapping due to their structural instability. Investigation of the antibiotic compounds produced by these transformants by thin-layer chromatography, bioautography, high performance liquid chromatography and mass spectrometry led us to the conclusion that they were synthesising geldanamycin (and probably some analogues of this compound).

Confirmation that part of the geldanamycin biosynthetic cluster had been cloned was obtained by insertion mutagenesis utilising segments of the cloned DNA to make geldanamycin non-producing mutants from "wild-type" *S. hygroscopicus* 3602 using a øC31 based phage vector (øC31 KC515, which is *att*-site deleted).

The DNA inserts in the clones exhibited homology with polyketide synthase genes isolated from the erythromycin A biosynthetic cluster (*ery* ORF A) of *Saccharopolyspora erythraea* perhaps indicating that the PKS specifying geldanamycin is rather more like this generalised type of system (Type I PKS analogous to the vertebrate fatty acid synthase) than that for the previously mentioned actinorhodin (Type II PKS analogous to the FASs found in bacteria and higher plant plastids).

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Abbreviations

ABA	-	3,5-aminohydroxybenzoic acid.
ATP	-	adenosine triphosphate.
DNA	-	deoxyribonucleic acid.
DNase	-	deoxyribonuclease.
dNTP		deoxyribonucleotide triphosphate.
EDTA	-	ethylenediaminetetraacetic acid.
HPLC	-	high pressure liquid chromatography.
O.D.	-	optical density.
RNA	-	ribonucleic acid.
RNase	-	ribonuclease.
SDS	-	sodium dodecyl sulphate.
SSC	-	salt (NaCl)/sodium citrate.
TLC	-	thin-layer chromatography.
Tris	-	2-amino-2-hydroxymethylpropane-1,3-diol.
u.v.	-	ultraviolet.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Actinomycetes

Several microorganisms which we now ascribe to the actinomycetes were described by early microbiologists as fungi because of their mycelial colony organisation and the capacity for sporulation. Subsequent genetic studies indicated that the actinomycetes were not fungi but many workers placed them taxonomically between the bacteria and the fungi. Further studies revealed, however, that the actinomycetes closely resembled the bacteria in a number of important ways - they had a Gram-positive cell wall, were sensitive to specific antibacterial agents, lacked a nuclear membrane and possessed a single, circular chromosome. Williams *et al.* (1982) defined the actinomycetes as Gram-positive bacteria with the ability to form branching hyphae that may develop into a mycelium. This mycelial habit presumably, therefore, evolved independently in the actinomycetes and in the fungi, but possibly in response to similar selective pressures, and though it is ideally suited to growth on insoluble organic matter or within tissues of other organisms it requires the development of specialised dispersal systems away from the mycelial colonisation. In actinomycetes this is brought about by one of two means:

i) by hyphal disintegration into fragments, or ii) by the production of spores in specialised mycelial areas.

Actinomycetes occur in many and diverse habitats including fresh- and salt-water, within animals and composts but particularly in the soil where viable counts of 10^6 per gram are not uncommon and at least 25 different genera have been isolated (Lechavalier and Lechavalier, 1967). Of these, the genus *Streptomyces* is the most widespread and important in the soil (Williams, 1978).

1.2 Streptomycetes

The streptomycetes are amongst the most complex of the actinomycetes and their life cycle has been studied in great depth. The mycelium exhibits two very distinct forms. A substrate or vegetative mycelium which develops initially from a spore giving rise to aerial mycelia, parts of which subsequently differentiate into spores. The period of vegetative development is an active growth phase whereas the change to the production of aerial mycelium is a non-growth phase of the life-cycle usually seen in response to a decline in the levels of available nutrients from the environment.

During this "transition phase" of the life cycle, the hyphae of the substrate mycelium lyse and so release nutrients that can be utilised for the synthesis of the aerial mycelium and spores. It is at this step of differentiation that substances which are not apparently required for vegetative growth of the mycelium are produced (Drew and Demain, 1977). This has led to the concept of primary and secondary metabolism where primary

metabolism describes the vegetative growth of the mycelium and primary metabolites are defined as catabolic substances essential to this growth, e.g. DNA, RNA, amino acids, polysaccharides, etc. Secondary metabolism is typified by the production of substances that are not essential for growth and their detection is often concommitant with the change from the growth of substrate mycelium to the production of aerial hyphae. Bu'Lock (1975) proposed a nomenclature to distinguish these metabolic stages - the tropophase, describing the span of vegetative or trophic growth and the idiophase, typified by the production of idosyncratic, secondary metabolic products.

Chater (1984) hypothesised that whilst the hyphae of the substrate mycelium are lysing to release nutrients to synthesise the aerial hyphae and spores the streptomycetes would be at a competitive disadvantage with regard to other soil microbes. If this was a selective pressure it may lead to evolutionary changes to allow the streptomycetes to compete. Thus streptomycetes may have evolved the ability to produce antibiotics to inhibit competing microorganisms. This could explain why they typically appear at this transitional period in the life cycle and not earlier, where they would compete with primary nutrition. This argument, however, is open to question in view of the finding that a large number of secondary metabolites are in fact without antibiotic activity. The interest in antibiotics and other secondary metabolites arises therefore not only from

their known pharmacological and antibiotic activities but also to understand the reasons for their production *per se*.

1.3 Streptomycete Differentiation and Secondary Metabolism

Streptomycete biology has been an area of intense active interest in the past half-century or so, principally because of the evidence that the secondary metabolites produced by these organisms often exhibited interesting pharmacological activities. These compounds were principally screened for antibacterial and antifungal properties but many have since been found to have other useful properties, e.g. anti-parasitic, insecticidal, herbicidal and as antitumour chemotherapeutics (Demain, 1983).

The temporal synchrony between the two unique biological properties of the streptomycetes, secondary metabolite production and morphological differentiation has long been established and recent evidence suggests a genetic basis for this synchrony. Work on mutants of *S. coelicolor* A3(2) that were phenotypically bald (Bld), that is they fail to develop aerial mycelia. Chater and Merrick (1979) showed that a number of these mutants (*bld* A, D and G) were pleiotropic, also abolishing the production of most, or all, of the five known antibiotics produced by wild-type *S. coelicolor* A3(2). Chater (1984) therefore considers that "... it is logical to think of 'physiological' and morphological differentiation as two sides of the same coin...", when attempting to under-

stand the control mechanisms which temporally and spatially separate the growth phases of streptomycetes and their respective metabolic priorities.

Thus, the factors which affect and effect the differential switch in the streptomycete life cycle have become an active area of interest.

1.3.1 The Control of Streptomycete Differentiation

A number of "environmental" factors have been demonstrated to affect the balance between primary and secondary metabolism, and therefore differentiation, including catabolite repression and inhibition. Carbon source and availability have been shown to regulate the production of a number of antibiotics (Review: Demain *et al.*, 1983) and nutrient limitation affects antibiotic yields particularly with respect to phosphate and nitrate levels (Martin, 1983). Indeed actinorhodin production by *S. coelicolor* A3(2), the best understood example of antibiotic biosynthesis, was found to be relatively insensitive to carbon source concentration but was elicited by both nitrogen and phosphate depletion (Doull and Vining, 1990).

1.3.2 Genetic Control of Streptomycete Differentiation

A great deal of knowledge has been amassed about the *in vivo* genetics of streptomycetes from the identification of recombinational events in the 1950's (Sermonti and Spada-Sermonti, 1955) to the work on sexual biology and extrachromosomal genetics in the 1970's (e.g. Hopwood

et al., 1973) and the analysis of primary metabolism (e.g. Hodgson, 1982) in the 1980's (Review: Chater and Hopwood, 1983). 1980 also saw the first reports of successful gene cloning experiments in streptomycetes (Bibb *et al.*, and Thompson *et al.*) and in 1983 Gil and Hopwood cloned the first gene from an antibiotic biosynthetic pathway. Since that time many more genes have been identified and cloned that are known to play a role in secondary metabolic processes and some of these have been examined, even down to the sequence level, to try to determine what controls the shift from primary to secondary metabolism.

Westpheling *et al.* (1985), using *B. subtilis* promoters of two types, *veg* and *ctc*, as templates for *in vitro* transcription deduced the existence of two types of RNA polymerase in preparations from extracts of *S. coelicolor*. The promoter specificities of the two species of RNA polymerase holoenzyme seemed to be determined by the presence of differing σ factors. The *S. coelicolor* polymerase incorporating σ^{35} (Mr $\approx$ 35,000) had a similar specifity to the typical eubacterial RNA holoenzyme, that is it efficiently utilised a promoter of the eubacterial canonical form (with -10 and -35 sequences). The other holoenzyme described contained σ^{49} and appeared to utilise promoters specific for the unique $E\sigma^{37}$ holoenzyme previously only found in *B. subtilis*, where it is implicated in the transcription of developmentally regulated genes.

Buttner *et al.* (1988) elicited the existence of a further RNA polymerase holoenzyme, containing σ^{28}, as well as confirming the existence of enzymes featuring σ^{35} and σ^{49} and they also proposed the existence of a fourth holoenzyme because their studies on the gene encoding agarase (*dag*A) from *S. coelicolor* contained four promoters operating in tandem each of which probably specifying one of the alternative available RNA polymerase holoenzymes.

This is taken as evidence to support the view of Westpheling *et al.* (1985) that the differing types of promoter found in streptomycetes might be recognised by alternative RNA polymerase holoenzymes as part of a strategy of temporal regulation of gene expression somewhat analogous to the ordered cascade of gene expression known to occur in *B. subtilis*.

The work of Errington and Mandelstam (1986), Turner *et al.* (1986), Carter and Moran (1986), Clarke *et al.* (1986), Zuber and Losick (1987), Labell *et al.* (1987), Kennedy and Moran (1987) and Dubnau *et al.* (1988) on genes involved in the sporulation of *B. subtilis* (*spo*) led Losick *et al.* (1989) to postulate a sequence of genetic events that result in the synthesis of the sporulation septum proteins. Much of the work is based on promoter analysis derived from the results of portable gene fusions to the *lacZ* reporter gene to detect transcription from these constructs.

Some of the genes involved in *B. subtilis* sporulation were found to operate in the following temporal array:

*spo*0H $\cdots\rightarrow$ *spo*IIG $\cdots\rightarrow$ *spo*IIE $\cdots\rightarrow$ *spo*IID.

The *spo*0H gene product was found to be a sigma factor, σ^H (σ^{30}), different from the sigma factor of the RNA polymerase holoenzyme responsible for the transcription of *B. subtilis* "housekeeping" genes, σ^A (σ^{43}), which acts on only one, P_1 of the two promoters, P_1 and P_2, of the *cis*-acting regulatory region of *spo*IIG to allow transcription of this gene.

In turn, the promoter of *spo*IIE would appear to be transcribed by a RNA polymerase holoenzyme containing a minor variant of σ^A, the principal form in the vegetative state of *B. subtilis*, because the "-10" and "-35" consensus sequences of the *spo*IIE promoter have a significantly larger spacing than those of typical vegetative genes. The gene products of *spo*IIE are required to allow the proteolytic processing of σ^E (σ^{29}), an important event later in sporulation.

*spo*IID is transcribed later in sporulation and seems to require an RNA polymerase with an associated σ^E (σ^{29}) which in turn is a gene product of *spo*IIG, pro-σ^E, which must be processed by the gene products of *spo*IIE to the active σ^E. Processing of pro-σ^E is thought to be performed by a membrane protein within the properly assembled sporulation septum, the morphogenesis of which would depend on the products of *spo*IIE (as discussed

above) and *spo*IIA, which is thought to encode an RNA polymerase sigma factor which directs the synthesis during sporulation of proteins also involved in the formation of the sporulation septum.

This model describes an orderly cascade of gene transcription events whereby subsequent genes are transcribed by RNA polymerase holoenzymes whose respective sigma factor sub-units are determined by genes that are expressed earlier in the cycle of differentiation and which are altogether different from the sigma factor of the RNA polymerase holoenzyme responsible for the transcription of vegetative or "housekeeping" genes.

That a similar model might control the activation of genes with functions associated with streptomycete differentiation is supported by the fact that the gene product of *whi*G, a gene that acts in the earliest stages of spore formation in *S. coelicolor* (Chater and Merrick, 1979), is an RNA polymerase sigma factor (Chater *et al.*, 1988) as deduced from its presumed amino acid sequence evinced from DNA sequencing data. Furthermore, Hopwood (1989) reports the work of Takahashi who has found four separate genes in *S. coelicolor* with sequences homologous to the *rpo*D gene from *Escherichia coli* which encodes the major form of RNA polymerase holoenzyme sigma factor, σ^{70}, which recognises the transcription promoters of *E. coli* "housekeeping" genes.

A surprisingly large number of promoters have been found associated with the antibiotic resistance determining genes in streptomycetes. These resistance genes

are often contained within the cluster of biosynthetic genes which specify antibiotic production, a genetic organisation to which there is currently no proven exception (Hopwood, 1988: Table 1.1).

Antibiotic resistance genes within biosynthetic clusters are typically found to contain two, or more promoters, some of which show consensus with the conventional bacterial "-10" and "-35" regions, but others have proved to differ (Bibb *et al.*, 1985; Bibb and Janssen, 1986; Neil, 1987 and Vögtli and Hütter, 1987). Of these promoters one, or more operated on the resistance gene itself with the remaining promoters, still being upstream of the resistance genes, yet controlling transcription in the opposite direction. These divergent transcripts have either been proved, or are guessed, to be directly involved in the synthesis of antibiotic biosynthetic enzymes (Figure 1.1). Of the four clusters intensively studied, those for methylenomycin, erythromycin, neomycin and streptomycin, the divergent transcripts overlap in two cases and in the other two there is only a short untranscribed region between them. This would make unidirectional transcription of the genes extremely unlikely.

Chater and Hopwood (1988) proposed that, in the case of methylenomycin resistance (*mmr*), a regulatory cascade may be in operation whereby the promoter for the resistance determinant itself would be initially transcribed and translated until its gene product exceeded a threshold level, whereupon transcription could take place, in

Table 1.1

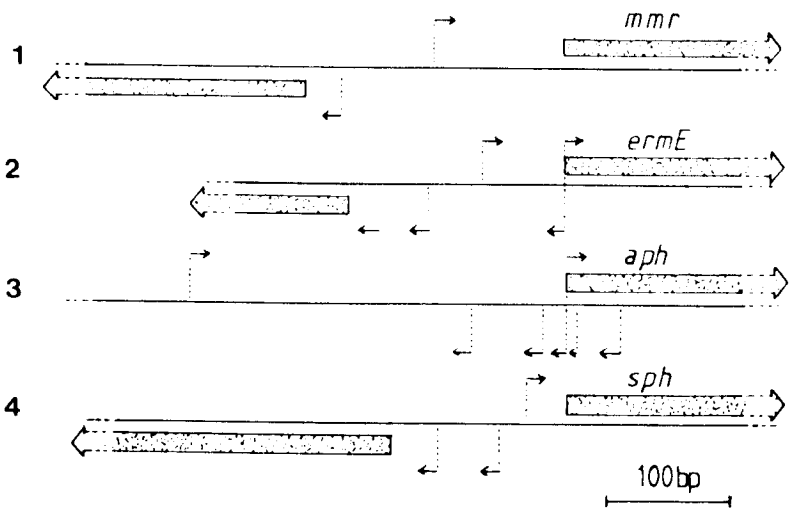
GENE CLUSTERS FOR ANTIBIOTIC BIOSYNTHESIS IN *STREPTOMYCES*

actinorhodin†, ‡, §	milbemycin
bialaphos‡, §	oxytetracycline†, ‡
cephamycin†	streptomycin‡, §
erythromycin†, ‡	tetracenomycin†, ‡
granaticin/dihydrogranaticin	tylosin‡
methylenomycin†, ‡, §	undecylprodigiosin†, §

† Clusters known to contain complete sets of biosynthetic genes.
‡ Clusters known to contain resistance genes.
§ Clusters known to contain regulatory genes.

Figure 1.1

The promoter organisation from various antibiotic biosynthetic clusters.
(from Chater & Hopwood, 1988)



1 = methylenomycin

2 = erythromycin

3 = neomycin

4 = streptomycin

the opposite direction, of the presumed biosynthetic genes. The cascade may involve a number of steps or the *mmr* gene product itself might activate divergent transcription.

Certainly the number and differing type of promoters associated with such antibiotic resistance genes must play a role in the temporal control of antibiotic (secondary metabolite) production and it does not seem unreasonable to assume that at least one level of this control is via RNA polymerase holoenzymes with differing specifities for the various promoters.

In addition to this Neil (1987) drew parallels between the arrangement of the transcription pattern around the *mmr* gene and that of bacteriophage λ between the divergent transcripts for *c*I (repressor) and *cro*. The promoters for these two regions are separated by a short, untranscribed region (82 bp, or 81 bp for *mmr*) which contains three operator genes for the products of *c*I and *cro*. The relative concentrations of the *c*I (repressor) and *cro* gene control transcription from only one, or the other, of these promoters (Gussin *et al.*, 1983; Ptashne, 1986).

This direct linkage between the resistance and structural determinants for an antibiotic may have come about for one or more possible reasons:

i) Hopwood and Wright (1973) showed that such an arrangement would facilitate the interspecific lateral spread of the ability to produce the antibiotic methylenomycin, which is plasmid determined on the conjugative

plasmid SCP1, because resistance must be transferred along with the ability to produce an antibiotic to maintain cellular viability.

There is no direct evidence, however, that lateral transfer would be facilitated when the pathway genes were chromosomally located, which, despite the inherent instability of antibiotic production - usually a sign of a plasmid location - is generally the case (Chater and Hopwood, 1988).

Interestingly, certain workers (Hopwood *et al.*, 1986 and Distler *et al.*, 1987) have postulated a further reason for the inclusion of the resistance determinant(s) within the biosynthetic cluster with such a complex array of divergent promoters. In addition to conferring resistance to an organism to a potentially lethal gene product the resistance determinants may play a role in controlling the activation of the biosynthetic genes. It is speculated that the promoter for the resistance determinant initiates transcription of this gene and the product of this gene somehow interacts, either directly or indirectly, with the divergent promoter(s) for the biosynthetic pathway. Thus resistance to an antibiotic would always be established before its production.

Yet another level of gene control of known significance in the differential expression of streptomycete genes is the phenomenon of selective codon usage. A typical genome size for the *Streptomyces* might be of the order of 10^4 kb, though there is a considerable specific variation (Hütter and Eckhardt, 1988) with the genome

size of the archetypal *S. coelicolor* A3(2) being estimated at about 6 kb from pulsed-field electrophoresis experiments (Hopwood, 1988). (Of this DNA, greater than 1% may be dedicated to antibiotic production in some species (Hopwood *et al.*, 1983).) This DNA shows an unusually high guanine plus cytosine (G+C) content, 70-76% (Enquist and Bradley, 1971 and Gladek and Zahrzewska, 1984) approaching the maximum theoretical limit of Woese and Bleyman (1972), but there are adenine- (A) and thymine- (T) rich repeated sequences widely spread in the genome (representing up to 12% of total DNA) (Chater, 1982).

Work on the Bld mutants of *S. coelicolor* A3(2) led to the sequencing and analysis of *bldA* (Lawlor *et al.*, 1987). The gene product of *bldA* was deduced to be a tRNA-like molecule that, if involved in protein synthesis, would translate the TTA codon for leucine (represented as $tRNA^{Leu}_{UUA}$). This codon, TTA, is not frequently represented in the G/C-rich DNA of the *Streptomyces* genome (occurring only once in the first 5195 codons to be sequenced (Hopwood, 1988)). Chater *et al.* (1988) deduced that the TTA codon does not occur in *Streptomyces* vegetative genes because:

(i) *S. coelicolor bldA* mutants are phenotypically "wild-type" during this phase of growth.

(ii) Cloned genes known to contain TTA codons are not expressed well, if at all, when transformed into *bldA* mutants but do so normally when transformed into an *S. coelicolor* strain known to be "wild-type" at this locus.

This supports the hypothesis of Lawlor *et al.* (1987) that TTA occurs in genes involved in the switch from primary to secondary metabolism, that is differentiation, and these genes can only be expressed when $tRNA^{Leu}_{UUA}$ is available, that is following the expression of *bldA*, which is known to occur relatively late in the cycle (see Chater and Merrick, 1979).

An interesting codicil to this hypothesis is that in addition to this pleiotropic *bldA*-dependent regulatory system there appears to be a second route to gene activation, which is inactivated by inorganic phosphate (Guthrie and Chater, 1990) for genes in the undecylprodigiosin pathway (*red*). This phosphate repression is not seen in $bldA^{+}$ strains, so the *bldA*-dependent regulatory system overrides the phosphate effect on the *red* genes and does not act upon genes in the actinorhodin pathway.

Therefore we have come full circle and are beginning to see how environmental factors such as inorganic ion levels play a role in determining the growth phase of a *Streptomyces* colony. The picture we have of the genetic control systems of differentiation is still rather incomplete but that there are a number of levels to this control has become evident, and indeed, was not unexpected.

Much more work will have to be done before we understand fully how even one of these rather complex control systems operates and only then may we deduce how the intricate interplay between these systems governs the morphological and "physiological" state of any given

streptomycete colony under any given set of conditions. However, with the coming of this understanding will also come a quantum leap forward in our ability to manipulate these organisms and so their importance to man may yet again increase.

1.4 Secondary Metabolites as Antibiotics

That a large number of microbial secondary metabolites are useful to man, not in the least as pharmacologically active antibiotics is undeniable, but their role in the host organism, and so their reason for being produced is not so clear.

We have already discussed the idea that they are of adaptive value by competing against other soil microbes at a crucial point in the life cycle (section 1.2) and found this argument a little wanting, but the fact that the vast majority of naturally occurring antibiotics are associated with spore-forming organisms and are produced at the "transitional" stage in the life-cycle between vegetative growth and sporulation is probably important, possibly indicating a role in differentiation.

It seems logical to assume some adaptive value of secondary metabolites in view of the investment of energy in their production. Woodruff (1966) suggested that they may be shunt products, possibly to allow the detoxification of primary metabolic pathways. Because the secondary metabolite is generally exported from the cell, they cannot usually be re-utilised for growth precluding the idea that secondary metabolites act as food stores.

There would seem, however, to be easier ways of exporting toxic compounds than by using entire secondary metabolic pathways and one might expect less diversity amongst the types of secondary metabolite produced.

It seems likely that microbial secondary metabolites do not all perform one function, but as they tend to be very bioactive molecules they may have come to fill a variety of roles which, though not essential to the growth of the organism, are beneficial under certain circumstances.

1.4.1 Secondary Metabolites and Man

Since the 1940's, and Fleming's discovery of penicillin, man has been using microbial secondary metabolites as antibacterial and anti-fungal agents in ever increasing numbers and against an ever widening scope of infections. However, streptomycete secondary metabolites have since been found to be useful in other pharmacological applications.

As early as 1968, monensin, a polyether product of *Streptomyces cinnamonensis* was found to be active against the poultry disease coccidiosis, whose causative organism is the parasitic protozoan *Eimeria* (Shumard and Callender, 1967). In addition, the streptomycetes also provide a group of compounds with the generic name the "avermectins", which are macrolides with bioactivity against many intertebrates. These have found use in veterinary practice as antihelminthic agents.

Agricultural uses of microbial secondary metabolites also extend to insecticides and herbicides of which the ansamycin compounds milbemycin and herbimycin are respective examples. Interestingly these compounds are structurally closely related and are both produced by *S. hygroscopicus* strains despite their quite different biological specificities (Omura *et al.*, 1979; Iwai *et al.*, 1980).

Secondary metabolites are also used as antitumour agents and as immunosuppresants in modern medicine and Demain (1983) describes no less than 38 different and diverse pharmacological activities of microbial secondary metabolites ranging from antidepressant to spasmoloytic agents.

The streptomycetes, as producers of secondary metabolites are already very important to us. They are responsible for the production of about two-thirds of currently known antibiotics (Hütter and Eckhardt, 1988) (an additional seventh are made by members of the related genera *Nocardia* and *Micromonospora*) and at least seventy of the approximately 100 marketed antibiotics for human usage are, or are derived from, streptomycete secondary metabolites (Lago and Streicher, 1982). Despite the forty years of intensive screening for useful secondary metabolites the range of new and interesting compounds is still growing. Novel compounds are usually produced by new organisms recently isolated from natural sources and placed into one or more of the vigorous screening programmes now in use, typically in an industrial micro-

biology laboratory. If a useful and, most importantly, novel compound is isolated the rules for optimising its production are largely empirical, being based on the production data for other compounds. This process can take a long time, involving the manipulation of both the growth conditions and the strain, the latter being chiefly based on the "hit and miss" approach of mutagenesis followed by screening for enhanced production.

The application of recombinant DNA technology, or genetic engineering, to streptomycetes may greatly facilitate the discovery and production of potentially useful secondary metabolites in many ways, some of which will be discussed later.

1.5 Polyketides

Amongst the diverse range of secondary metabolites produced by *Streptomyces* species are a group of compounds called the polyketides. Polyketide production is not, however, limited to the streptomycetes, but is widespread in both prokaryotes and eukaryotes (fungi and plants) where their occurrence is almost as variegated as their many functions. Polyketide structures are also amazingly diverse but they are grouped together because of a commonality of their syntheses, that is the successive addition of carboxylic acid building blocks into a polyketide chain. These building blocks are commonly residues of acetate, propionate or butyrate but other, more complex possibilities are known. Each building block, or extender unit, adds two carbon atoms to the

chain, the ß-carbon of which always carries a keto-group, which may subsequently undergo processing, but the common occurrence of unaltered keto-residues on the chain has lead to this class of compounds being named the polyketides.

In addition to the ß-keto-group processing that occurs the other carbon atom of each of the building blocks can also carry different substituents, for example hydrogen alone if acetate is the extender or a methyl or even an unsaturated ethyl group for propionate. Other groups are also possibilities for the more esoteric building blocks. Add to this the fact that chirality may occur at some of the chain carbon atoms and that the chains themselves may be of differing lengths then one can see how one relatively simple method of molecular construction can account for the vast array of polyketide compounds that are known to occur.

A number of reviewers have since extended the hypothesis of Birch (1967) that the synthesis of polyketide compounds has analogies in the biosynthesis of saturated fatty acids (Sherman *et al.*, 1988 and Hopwood and Sherman, 1990).

1.5.1 The Biosynthesis of Saturated Fatty Acids (FAs)

The biologically most abundant class of fatty acids are not only fully saturated along their carbon chains but these chains are also unbranched, making them also the simplest class of these compounds. The fatty acid chains are synthesised by the consecutive addition of

two-carbon units, devised from malonyl Co-A, by condensation initially to an acetyl "starter" unit. The condensation reactions involve the loss of one carbon atom as CO_2, forming an even-numbered chain. The ß-carbon of each extender carries an oxygen atom, which is removed subsequent to the condensation in three stages:-

i) Reduction to an hydroxyl (OH).

ii) Dehydration to remove the hydroxyl, resulting in the formation of a double bond in the carbon chain.

iii) Reduction of the carbon-carbon double bond to restore the enoyl functionality.

The three reactions listed above are catalysed by the fatty acid synthase (FAS). In addition to these reactions the FAS is also responsible for four other functions; the attachment of the first malonate unit to the acetate "starter" unit, the attachment of subsequent malonate units to the growing chain, the detachment of the complete chain from the FAS and since the "acyl" building units are attached not directly to the FAS but to a phosphopantetheine "arm" linked to a serine residue within the active site of an acyl carrier protein (ACP) which is part of the FAS. This "arm" passes the extending malonate units to a cysteinyl residue within the active site of the condensing functionality of the FAS for incorporation into the fatty acid chain.

The FAS therefore encompasses eight catalytic functions:-

i)	Ketoacyl synthase	-	(condensing enzyme)
ii)	Ketoacyl reductase	-	(= O ▶- OH)
iii)	Dehydratase	-	(- c - $\overset{OH}{\underset{\vert}{c}}$ -▶- c = c -)
iv)	Enoyl reductase	-	(- c = c -▶- c - c -)
v)	Acetyl transferase	-	(attaches first malonate to acetyl "starter" unit)
vi)	Malonyl transferase	-	(attaches subsequent malonyl units to the chain)
vii)	ACP	-	(mediates in extender unit handling).
viii)	Palmityl transferase	-	(detaches complete fatty acid chain)

In this, the simplest of schemes the FAS is required to make only one "choice", that is the absolute length of the carbon chain built. Hopwood and Sherman (1990) refer to the ability of a FAS to make such choices as the programming of that particular FAS. In nature fatty acid chains are more complex than the above case, for example branched fatty acids exist resulting from the selection by the FAS of branched starter or extender units. In addition to this unsaturated fatty acids are commonplace in nature. In mammalian systems these double bonds are not part of the FAS's scope of activity but occur because of the action of specific desaturases after chain elongation is complete but in bacteria the occurrence of such bonds relies on a lack of enoyl reductase activity

elongation is occuring and so this function must be programmed into the FAS, especially as it occurs only at specific places in the carbon chain.

FASs are organised in one of three ways:-

i) Type I, found in vertebrates where fatty acid biosynthesis occurs free in the cytsol and all of the functions are carried out by separate domains on one large multifunctional polypeptide.

ii) Type II, found in most bacteria and plant plastids (which are derived from ancestral prokaryotes), where fatty acid synthesis may be membrane associated and the functions are defined by separate polypeptides forming a multi-enzyme complex.

iii) The fungal system, representing an intermediate between Type I and II. The FAS consists of two large polypeptides specifying three and five of the required enzymic functions. The two separate sub-units aggregate to form a functional FAS. (For a review of the above see: McCarthy and Hardie, 1984.)

1.5.2 The Biosynthesis of Polyketides

From the foregoing discussion we can see an interesting parallel between the construction of long carbon chain fatty acids and the primary carbon chain found in polyketide structures (Figure 1.2). (We shall see later that this similarity extends beyond the theoretical to the genetical level.) One important factor that must be borne in mind is that the number of diversity of natural polyketide structures would logically

Figure 1.2

Schematic representation of the similarities between fatty acid and polyketide synthesis. (from Hopwood & Sherman, 1990)

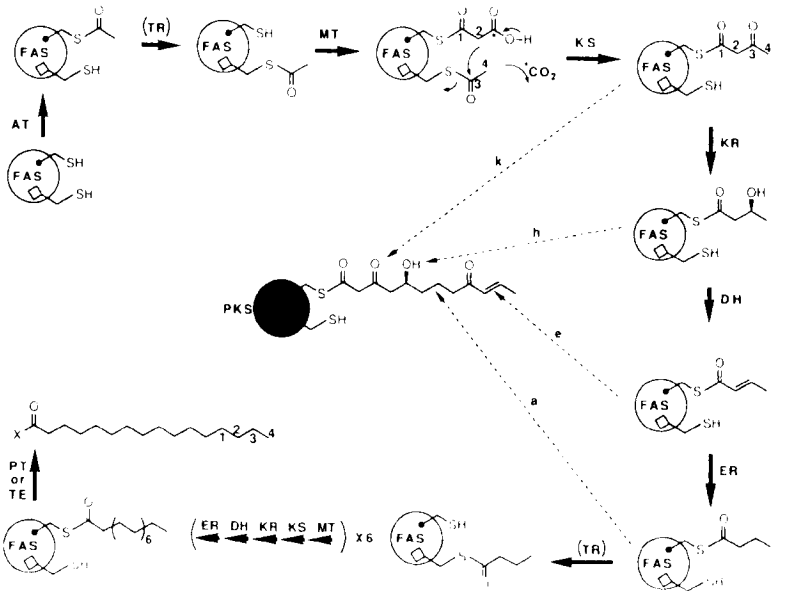


Figure 1.2 Schematic representation of fatty acid and polyketide biosynthesis. The circle labelled FAS or PKS represents the fatty acid or polyketide synthase, carrying two thiol groups, one on the β-ketoacyl synthase (condensing enzyme) (○) and the other on the acyl carrier protein (●). The reaction steps are labelled: AT, acetyl transferase; (TR), acyl transfer reaction, not unambiguously assigned to a specific enzyme component; MT, malonyl transferase; KS, β-ketoacyl synthase; KR, ketoreductase; DH, dehydrase; ER, enoyl reductase; PT, or TE, palmityl transferase or thioesterase, involved in chain termination to produce palmityl CoA (X = CoA) or free palmitic acid (X = OH) respectively. 1,2,3,4 designate carbon atoms of malonate and acetate that contribute to chain building, while the asterisk labels the carbon of malonate that is eliminated as CO_2. k,h,e,a, represent the various possibilities that follow each condensation step to give keto, hydroxyl, enoyl or alkyl functionality at specific points in the product of a polyketide synthase.

indicate an extension of the programming of the system of polyketide synthesis over the comparatively small number of choices made by FASs.

1.5.3 The Genetics of Actinorhodin Biosynthesis

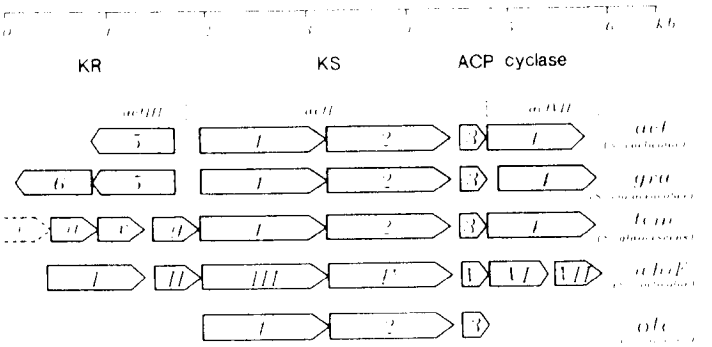
The work of Rudd and Hopwood (1979) and Malpartida and Hopwood (1984 and 1986) led to the cloning of the entire biosynthetic pathway for the polyketide antibiotic actinorhodin, and the identification of seven functional groups of actinorhodin non-producing (blocked) mutants. Two of these genes, *act*I and *act*III, were deduced from complementation analysis to define steps early in the pathway. *Act*I mutants are deficient in certain condensation functions (and possibly transferase and ACP functionalities) and *act*III mutants are unable to perform ketoreduction reactions. These condensing enzyme and reductase (*act*III) gene sequences were used as hybridisation probes (Malpartida *et al.*, 1987) to isolate segments of total DNA from other polyketide producing streptomycetes. This led to the identification of polyketide synthase (PKS) genes for tetracenomycin (*tcm*) from *S. glaucescens*, oxytetracycline (*otc*) from *S. rimosus* and granaticin (*gra*) from *S. violaceoruber*. The majority of these genes have subsequently been sequenced (Sherman *et al.*, 1989, Bibb *et al.*, 1989, and Sherman, in press). The genes for the production of a spore coat pigment of *S. coelicolor* A3(2) (*whi*E) were also sequenced and found to be similar to those of the already known PKS genes (Davis and Chater, 1992).

In their review, Hopwood and Sherman, (1990) indicate that all of these PKS gene clusters, *act*, *gra*, *tcm*, *otc*. and *whiE* contain a group of three characteristic open-reading frames (ORFs) (Figure 1.3). The primary amino acid sequences deduced from the DNA sequence data of the first two ORFs exhibited a high degree of similarity to that of the condensing enzyme of the *E. coli* FAS. The gene products of these two ORFs, 45 and 42 kDa respectively, probably represent a heteromultimer, i.e. one single condensing enzyme because only one potential active site is indicated and the DNA sequences overlap suggesting translational coupling. The third ORF in each case showed similarities with ACP amino acid sequences from bacteria and plant FAS (Type II) and the equivalent domains from vertebrate FASs (Type I), particularly with the required serine residue for phosphopantetheine binding. This, Hopwood and Sherman argue, indicates that polyketide synthesis occurs along a pathway requiring an ACP.

Downstream and adjoining the above three ORFs in all of the clusters described is a fourth ORF with rather more limited similarities. These similarities tend to be restricted to specific regions of the deduced protein sequences. The *act* and *gra* ORF4s are both deduced to encode a cyclase/dehydrase enzyme because they are largely similar throughout and they both complement mutants blocked in actinorhodin cyclisation and subsequent dehydration. The other ORF4s studied are rather more divergent and fulfil slightly different roles along

Figure 1.3

Open reading frame organisation of various PKS gene clusters. (from Hopwood & Sherman, 1990)



with cyclase activity, as required in the synthesis of the polyketide.

It has already been mentioned that *act* ORF5 (*act*III) encodes a putative ketoreductase and the deduced protein sequences of the corresponding granaticin genes are very similar. The *tan* sequences, however, exhibit no such similarities in keeping with an absence of requirement of ketoreductase activity in tetracenomycin biosynthesis.

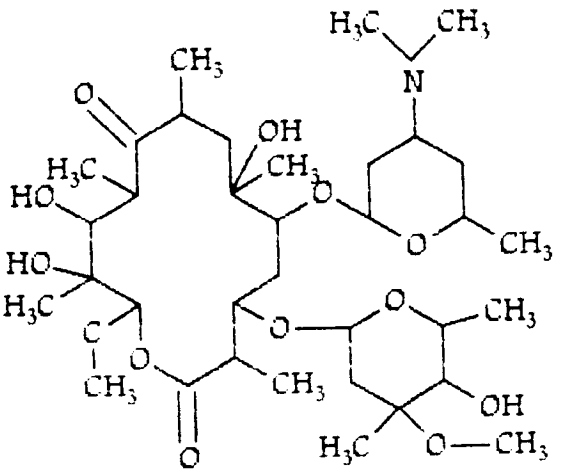
Gramajo *et al.* (1991) indicate that a further understanding of the enzymology and mechanism of polyketide biosynthesis requires the production of sufficiently large amounts of the various proteins involved in the processes. To this end they have performed experiments involving the over-expression of PKS genes in *E. coli* using the pT7 system of Tabor and Richardson (1985). These studies showed that the gene products of the *tcm* ORF1 and ORF2 had a membrane location and they suggested that a membrane association for the PKS may indicate an efflux resistance mechanism for tetracenomycin. Post-translational processing of the ORF2 gene product was also indicated prior to membrane association.

1.5.4 The Genetics of Erythromycin Biosynthesis

Erythromycin A (Figure 1.4) is a complex, macrolide antibiotic produced by *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus* NRRL 2338) which is the drug of choice for infected patients who are allergic to ß-lactam antibiotics. Genetic analysis of the genes involved in the biosynthesis of erythromycin began with

Figure 1.4

The structure of erythromycin A.



the cloning of the resistance determinant, *ermE* (Thompson *et al.*, 1982). The remainder of the biosynthetic gene cluster has been the subject of intensive investigation (Dhillon *et al.*, 1989; Vara *et al.*, 1989; Weber *et al.*, 1989 and Paulus *et al.*, 1990).

The group of Weber *et al.* at Abott Laboratories published a review of the known genetics of erythromycin biosynthesis (1990) including the results of their own detailed studies. They used mutagenesis studies to define a 30 kb segment of *S. erythraea* chromosomal DNA around the *ermE* gene as being responsible for erythromycin biosynthesis. The largest transcription unit, *eryA1*, (9 kb) was transcribed convergently with *ermE* beginning 21 kb downstream of it.

Sequencing of the *eryA1* region had indicated that it encoded a synthase for the polyketide aglycone core of erythromycin 6-deoxyerythronolide B (Cortes *et al.*, 1990). The predicted protein sequence showed similarities to active site sequences from known fatty acid and polyketide synthases, including those mentioned above for tetracenomycin and granaticin. The observed order of the active sites in *ery* ORFA1 was identical to those in vertebrate fatty acid synthases. The production of 6-deoxyerythromolide B requires six cycles of polyketide chain extension and that the polypeptide encoded by *ery* ORFA1 is capable of catalysing two of these cycles, probably numbers 5 and 6, in the chain (Cortes *et al.*, 1990). These factors indicate that the organisation of the erythromycin PKS is somewhat different to the Type II

organisation of PKSs proposed for actinorhodin, granaticin, tetracenomycin and oxytetracycline (see section 1.5.3 above).

Weber *et al.* (1991) extended their mutagenesis techniques, using integrative plasmids for the targeted gene disruption of erythromycin biosynthetic genes, and thin-layer chromatography to identify resulting mutations. One such mutant, *eryF*, was found to produce 6-deoxyerythromycin A as a "shunt" by-product due to the inactivation of the C-6 hydroxylase, indicating that the enzymes required for the later biosynthetic steps are unable to discriminate between substrates with variations at C-6 (the practical implications of this will be discussed in the final chapter of this thesis).

Further to this work Donadio *et al.* (1991) showed that *eryA* extends for 32 to 35 kb and is likely to consist of one, or possibly two transcripts. Sequencing this 35 kb of DNA established that *eryA* consisted of a total of six repeated units each of which was 4.3 to 6.5 kb and had a nucleotide similarity of at least 64%.

EryA consists of three large ORFs (of which ORFA1 is one), each representing two of the six previously mentioned repeated units. The deduced protein sequences of these ORFs indicate the following FAS-like domains : seven acyl carrier proteins (ACP), six keto-acyl-ACP synthases (KS), eight acyl transferases (AT), six keto-reductases (KR), one dehydratase (DH) and one enoyl reductase (ER). The information for these functionalities is laid out on the genetic map in precisely the

correct order that their corresponding synthase activities are required for the six polyketide chain elongation steps required for the synthesis of 6-deoxy-erythronolide B.

Analysis by the same workers also indicates that the predicted polypeptide from one of these ORFs, ORF3, resembles a Type I FAS system of eukaryotic origins, that is, the functions required for synthesis reside within a single polypeptide, in contrast to the systems (Type II) previously discussed.

This information has led to the "module hypothesis" of polyketide biosynthesis where each segment of DNA encoding a repeated unit, for example, *eryA*, is designated a "module". Each module, therefore, is specific for one step in polyketide chain elongation, that is they each comprise one functional synthase unit (SU). Evidence for this hypothesis is provided by the fact that selective mutations in given segments of *eryA* should effect a single biochemical event in a predicted synthase function. This, as we have already seen, is the case with the prodcution of the "shunt" metabolite 6-deoxy-erythromycin. Similarly, a selective mutation within the ketoreductase encoding domain of module 5, which is normally responsible for the introduction of the C-5 hydroxyl group in the completed polyketide, led to the production of 5,6-dideoxy-5-oxoerythronalide B as predicted, but a more complete erythromycin analogue could not be constructed because the hydroxyl group at C-5 is required for attachment of sugar groups in the

complete compound.

The "programming" of such a Type I or modular system would seem much more straightforward than that of a Type II PKS, because the order in which the reactions are performed is the same as the given order of genes on the chromosome. That each module is trans-acylated in the correct order is probably also a function of steric considerations. Selection of the correct starter and extender units for the chain may be a function of the acyltransferase domains because mutations in AT domains affect the fidelity of the PKS and the production of 6-deoxyerythromycin indicates a lack of specificity of the enzymes following the C-6 hydroxylase in the *eryF* mutant.

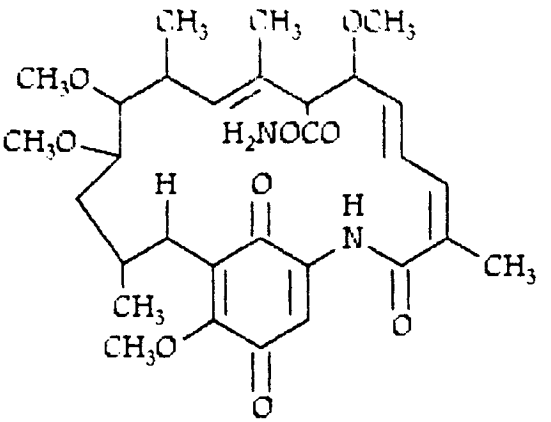
Leadley (1991) has managed to express some of the erythromycin PKS functions in *E. coli*. The polypeptide produced was used to prove the actual existence of the large proteins required by the "module hypothesis" by immunological means and Western blotting.

1.6 The Ansamycins and Geldanamycin

Geldanamycin (Figure 1.5) is an ansamycin antibiotic containing the characteristic aliphatic bridge connecting two non-adjacent carbon atoms of an aromatic chromaphore of this group (named from the Latin *ansa* = handle) which is built as a polyketide chain. The ansamycins are structurally complex with an interesting range of bioactivities, typically binding to and inactivating DNA-

Figure 1.5

The structure of geldanamycin.

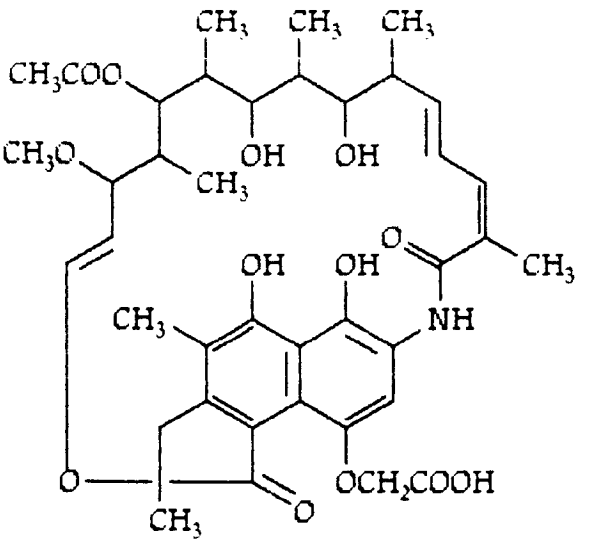


dependent RNA polymerases, inhibition of reverse transcriptase and powerful antitumour activities (giving antibacterial, anti-viral and anti-cancer properties respectively to the compounds) (Rinehart and Shield, 1976). Lancini (1983) distinguishes between ansamycins with a benzenic chromophore (e.g. geldanamycin) and those with a naphthalenic chromophore (e.g. rifamycin, Figure 1.6) and states that these structural differences are mirrored by differences in their biological activities, the former being typified by actions interfering with eukaryotic cell replication and the latter by inhibiting bacterial RNA polymerases.

Geldanamycin was first described by De Boer *et al.* (1970) as a substance produced in submerged culture by an actinomycete identified as *S. hygroscopicus* var. *geldanus* var. *nova* (UC-5208) (later categorised as NRRL.3602) by A. Dietz in the laboratories of The Upjohn Company, Kalamazoo, USA. Geldanamycin was isolated during a screening process for substances active against protozoans and was patented in the USA by the same group in 1971 because of its activity against the nematode, *Syphacia obvelata*. A range of other biological activities was also noted - activity against several genera of fungal plant pathogens (e.g. *Alternaria*, *Pythium*, *Botrytis* and *Penicillium*; a low order of activity against Gram-positive bacteria and an extreme activity against KB cells (human epidermoid carcinoma cells) but no activity against the Coxsackie, Parainfluenza and Herpes simplex virus tested.

Figure 1.6

The structure of rifamycin.



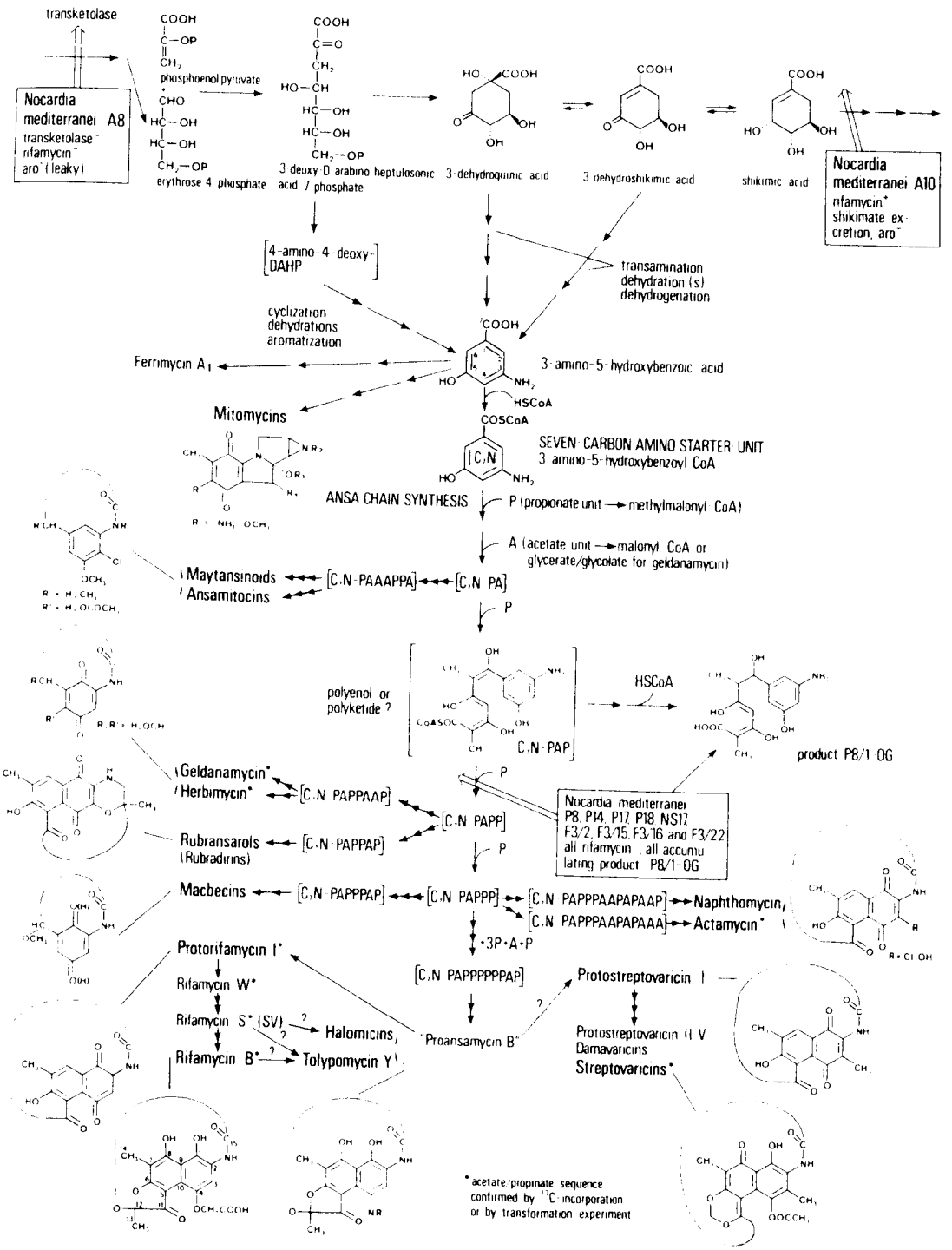
Geldanamycin was described by De Boer *et al.* as being a yellow, crystalline solid with a melting point of 253 - 257^{o}C. The molecular mass was considered to be 560 and the pure solid relatively soluble in organic solvents. (Various other physical and chemical characteristics are described in the literature.) The structure of geldanamycin was assigned by X-ray crystallography (Rinehart and Shield, 1976).

The ansa chain of geldanamycin was shown to be derived from acetate and propionate units by feeding experiments with radio-labelled precursors (Ghisalba *et al.*, 1981) except that two of the seven C_2-units were found to derive from incorporation of glycolate in the ansa chain.

The remainder of the molecule is a seven-chain amino unit (C_7N) which is also found in a large number of other ansamycins (see Figure 1.7). The C_7N unit is the putative starter unit for a diverse group of ansamycins with different ansa/polyketide chains and radio-labelling experiments with precursors plus genetic studies using mutants blocked in certain biochemical pathways has led to the hypothesis that this starter unit is derived from the shikimic acid pathway.

Figure 1.7

A chematic overview of ansamycin biosynthesis.



CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains

The bacterial strains used in this work are listed in Table 2.1.

2.2 Plasmids and Phages

The plasmids and bacteriophages used in this work are listed in Table 2.2.

2.3 Reagents

Chemicals were purchased from several sources, including the Sigma Chemical Company, and Merck PLC, Poole. Restriction endonucleases were obtained from Boehringer Mannheim (UK), and radioactively labelled compounds from Amersham International PLC. Thiostrepton was a gift from E. R. Squibb & Sons, and geldanamycin was a gift from Dr. E. M. Wellington, University of Warwick.

2.4 Media

2.4.1 R2YE Medium

Sucrose, 103 g; K_2SO_4, 0.25 g; $MgCl_2.6H_2O$, 10.2 g; glucose, 10 g; Difco casamino acids, 0.1 g; distilled H_2O to 800 ml. The solution was aliquoted into 400 ml volumes to which 11 g of Difco Bacto agar were added and autoclaved. When required the media was re-melted and supplemented with KH_2PO_4(0.5%), 5 ml; $CaCl_2.2H_2O$ (3.68%), 40 ml; L-proline (20%), 7.5 ml; TES buffer (5.73%) pH 7.2, 50 ml; trace element solution (see below* - p.43) 1 ml; NaOH (1 N), 2.5 ml; Difco yeast extract (10%), 25 ml.

Table 2.1: Bacterial Strains

Strain	Source
Streptomyces lividans TK24 (used as a cloning host in this work).	John Innes Institute Culture Collection
S. lividans 1326 (used as a host for øC31 derivatives in this work).	John Innes Institute Culture Collection
S. lividans JT46 (used as a cloning host in this work).	John Innes Institute Culture Collection
S. coelicolor M145 (DNA from this strain used in hybridisation experiments - Chapter 3).	John Innes Institute Culture Collection
S. hygroscopicus 3602 (the *Streptomyces* strain under study in this work).	Dr. P. G. Logan.
S. glaucescens 5155 (DNA from this strain used in hybridisation experiments - Chapter 3).	Professor S. T. Williams.
S. rimosus (DNA from this strain used in hybridisation experiments - Chapter 3).	Professor S. T. Williams.
S. antibioticus 524 (DNA from this strain used in hybridisation experiments - Chapter 3).	Professor S. T. Williams.
S. parvulus 524 (used as a cloning host in this work).	Professor S. T. Williams.

Table 2.1: Bacterial strains (continued).

Strain	Source
S. hygroscopicus 3672 (DNA from this strain used in hybridisation experiments - Chapter 3).	Dr. P. G. Logan.
S. aureofaciens (used as an indicator strain in this work).	Dr. J. Muchovà.
Bacillus subtilis (used as an indicator strain in this work).	Liverpool Culture Collection
Amycalatopsis mediterranei (DNA from this strain used in hybridisation experiments - Chapter 3).	Professor S. T. Williams.

Table 2.2: Plasmids and phages

Plasmid	Source
pIJ61	John Innes Institute.
pIJ709	"
pIJ699	"
pIJ2345	"
pIJ2346	"
pBROC397	SmithKline Beecham PLC.
pIA101	This work.
pIA102	"
pIA103	"
Phage	
øC31 KC515	John Innes Institute.

* Trace Element Solution

$ZnCl_2$, 40 mg; $FeCl_3.6H_2O$, 200 mg; $CuCl_2.2H_2O$, 10 mg; $MnCl_2.4H_2O$, 10 mg; $Na_2B_4O_7.10H_2O$; $(NH_4)_6\ Mo_7O_{24}.4H_2O$, 10 g; distilled H_2O to 1 l.

2.4.2 Difco Nutrient Agar (DNA)

Difco Nutrient Broth Powder, 8g; agar, 12 g; distilled H_2O to 1 l. Mix and heat until completely dissolved, dispense into suitable aliquots (400 l).

2.4.3 Soft Nutrient Agar (SNA)

Difco Nutrient Broth Powder, 8 g; agar, 6 g; distilled H_2O to 1 l. Mixed and heated until completely dissolved, dispensed into suitable aliquots (50 ml).

2.4.4 L Agar

Difco Bacto tryptone, 5 g; Difco yeast extract, 5 g; NaCl, 5 g; glucose, 1 g; distilled H_2O to 1 l. The solution was aliquoted into 400 ml volumes to which 6 g of agar was added prior to autoclaving.

2.4.5 Yeast and Malt Extract Agar (YEM)

Difco yeast extract, 3 g; Oxoid malt extract, 3 g; Difco Bacto peptone, 5 g; glucose, 10 g; distilled H_2O to 1 l. The solution was aliquoted into 400 ml volumes, the pH adjusted to 7.1 - 7.2 with 10 N NaOH and 6 g of agar added to each aliquot prior to autoclaving.

2.4.6 Yeast Extract-Malt Extract Medium (YEME)

Difco yeast extract, 3 g; Difco Bacto peptone, 5 g; Oxoid malt extract, 3 g; glucose, 10 g; sucrose, 350 g; distilled H_2O to 1 l. The solution was aliquoted into 400 ml volumes prior to autoclaving. 800 μl $MgCl_2.6H_2O$ (2.5 M) and 10 ml glycine (10%) (only added if protoplasts were required) were added to each aliquot prior to use.

2.4.7 Difco Nutrient Broth (DNB)

Difco nutrient broth powder, 8 g; distilled H_2O to 1 l.

2.4.8 L Broth

Difco Bacto tryptone, 10 g; Difco yeast extract, 5 g; NaCl, 5 g; glucose, 1 g; distilled H_2O to 1 l.

2.4.9 Supplements

For work with øC31 the media and buffers were supplemented with 10 mM $MgSO_4$ and 8mM $Ca(NO_3)_2$ (4 mls of a 1 M and a 0.8 M stock respectively per 400 ml medium). When required thiostrepton (50 mg/ml stock solution in DMSO) was added to liquid media at a final concentration of 5 μg/ml and to solid media at 50 μg/ml.

2.5 Buffer

2.5.1 Protoplast (P) Buffer

Sucrose, 103 g; K_2SO_4, 0.25 g; $MgCl_2.6H_2O$, 2.02 g; trace element solution, 2 ml; distilled H_2O to 800 ml. This solution was dispensed into 80 ml aliquots and autoclaved. Prior to use the following sterile solutions were added, in order - KH_2PO_4 (0.5 %), 1 ml; $CaCl_2.2H_2O$ (3.68%), 10 ml; TES buffer (5.73%; pH 7.2), 10 ml.

2.5.2 TNE Buffer

Tris-HCl (2 M; pH 8), 5 ml; NaCl (5 M), 20 ml; sodium EDTA (0.25 M; pH 8), 4 ml; distilled H_2O to 1 l.

2.5.3 TE Buffer

Tris-HCl (2 M; pH 8), 5 ml; sodium EDTA (0.25 M; pH 8), 4 ml; distilled H_2O to volume. Often made up as a 10 x TE stock and diluted when required.

2.5.4 SM Buffer

Tris-HCl (1 M; pH 7.5), 20 ml; Mg_2SO_4 (1 M), 1 ml; NaCl (5 M), 20 ml; gelatine, 1 g (dissolved in 10 ml distilled H_2O by autoclaving); distilled H_2O to 1 l. Dispensed into suitable aliquots (200 ml) prior to autoclaving.

2.5.5 20 x SSC

NaCl, 175.32 g; trisodium citrate, 88.23 g; distilled H_2O to 1 l. (Often diluted before use, e.g. 2 x SSC).

2.5.6 Tris-Borate Buffer: (TBE) x 10.

Tris-HCl, 108 g; boric acid, 55 g; sodium EDTA (0.5 M; pH 8), 40 ml; distilled H_2O to 1 l. Diluted to 1 x strength before use.

2.5.7 Stop Buffer : (Gel-loading Buffer) x 6.

60% (w/v) sucrose containing 100 mM EDTA (pH 8); 0.25% bromophenol blue. Added to samples at a 1 in 6 dilution.

2.5.8 Ligation Buffer x 10.

Tris-HCl (1 M; pH 7.5), 0.3 ml; $MgCl_2$ (2.5 M), 25 μl; distilled H_2O to 1 ml. The solution was filter sterilised and stored as 100 μl aliquots.

2.5.9 Hybridisation Buffers

(a) NaCl (1 M), 6.75 ml; sodium phosphate buffer (0.2 M; pH 7.7), 1.875 ml; sodium EDTA (0.5 M), 0.3 ml; SDS (10%), 2.5 ml; PEG 6000, 1.5 g; distilled H_2O to

25 ml. The solution must be warmed and mixed prior to use when 0.125 g of "Marvel" skimmed milk was added.

(b) Sodium phosphate buffer (0.5 M; pH 7.7), 2.8 ml; sodium EDTA (0.5 M), 200 µl; SDS (10%), 7 ml; distilled H_2O to 10 ml.

(c) "Rapid Hybridisation Buffer" (Amersham UK) 10 ml aliquots a) was used when hybridisations were performed in heat-sealable plastic bags, b) and c) were used in conjunction with hybridisation ovens.

2.5.10 TE Sucrose

34% sucrose was added to TE buffer, i.e. 34 g sucrose per 100 ml TE.

2.5.11 M9

NH_4Cl, 2 g; $Na_2HPO_4.7H_2O$, 11.66 g; KH_2PO_4, 6 g; NaCl, 10 g; distilled H_2O, 1900 ml. Dispensed into 190 ml aliquots prior to autoclaving.

2.6 General Methods for Streptomycetes

(All after Hopwood *et al.* [1986])

2.6.1 Preparation of Spore Suspensions

A petri dish containing approximately 25 ml of agar medium was spread with a large number of spores from either a frozen spore sample or from a plate spore stock. The plate was incubated at 30°C until sporulation occurred (usually 4 - 7 days). 10 ml of sterile distilled H_2O was poured onto the surface of the plate. A thick loop was used to separate the spores from the substrate mycelium, and this crude spore suspension poured into a plastic universal container (30 ml),

vortexed thoroughly to separate spores from debris and then filtered through non-absorbent cotton wool in a pre-prepared filter unit. The filtered suspension was then centrifuged at 3,000 rpm for 10 mins, the supernatant discarded and finally the spore pellet resuspended in 20% (w/v) glycerol. Spores prepared by this method are suitable for immediate use or for use after storage at -20°C or -70°C.

2.6.2 Preparation of Protoplasts

Streptomyces protoplasts were used for protoplast fusion, plasmid transformation and phage transfection.

Mycelia from approximately 25 ml of liquid culture was centrifuged at 3,000 rpm for 10 mins, the resulting pellet washed in sterile 10.3% (w/v) sucrose, again pelleted at 3,000 rpm, for 10 mins and resuspended in a total of 5 ml lysozyme solution (2 mg/ml in P buffer). The suspension was incubated at 30°C until turbid (15-60 mins) to allow protoplast formation. The process was accelerated by triturating with a 10 ml disposable pipette at 15 min intervals to separate aggregated mycelia. The proportion of protoplasted units was estimated by light microscopy using a haemocytometer. The protoplasted mycelia were diluted with 5 ml P buffer, and filtered through non-absorbent cotton wool. After transfer to a 30 ml plastic universal container the protoplasts were sedimented at 3,000 rpm for 7 min, the supernatant discarded and the pellet gently resuspended in an appropriate volume of P buffer to give about

4×10^5 protoplasts per ml as determined by direct counting using an haemocytometer. Aliquots of this suspension were either used immediately or placed on ice before being stored at -20°C. (Protoplasts should be frozen slowly but thawed quickly to ensure maximum viability.)

2.6.3 Preparation of Bacteriophage Stocks

A plate containing fresh plaques was used to prepare a phage stock by using a sterile pasteur pipette to suck up a single plaque. This plaque was placed in 5 ml of DNB supplemented with divalent cations and the phages allowed to soak out for 2-6 hrs at room temperature. This stock was filtered through a 0.45 μm nitrocellulose filter and then titred against a suitable indicator strain using serial dilutions of the phage stock. This method produced phage suspensions with titres of between 10^7 - 10^9 pfu/ml which were stored at 4°C or used in phage infection experiments.

2.6.4 Screening *Streptomyces* Colonies for Antibiotic Production

Recombinant, transformed *Streptomyces* colonies were replicated to YEM (without selection), allowed to grow at 30°C for 7 days and the plates overlayed with SNA cooled to 45°C and seeded with cells of *Bacillus subtilis*, the indicator strain. The plates were incubated at 37°C overnight and the colonies scored for presence/absence of a surrounding zone of growth inhibition. Two refinements of this technique were also used - the use of a

thiostrepton-resistant *B. subtilis* indicator strain, to allow the maintenance of thiostrepton-selection on the recombinant *Streptomyces* colonies, and the killing of the *Streptomyces* colonies by exposure to chloroform prior to overlaying to rule out nutrient depletion effects, etc.

An agar layer seeded with *B. subtilis* indicator cells was also used to assay antibiotics produced by colonies transferred to this layer on agar plugs from plates on which they were previously grown, and antibiotic solutions applied to the seeded layer on sterile filter paper discs. The colonies/discs were again scored for antibiosis by the presence/absence of a zone of inhibition of growth of the indicator strain around the colonies/discs.

2.7 Isolation of Chromosomal, Plasmid and Phage DNA

2.7.1 Isolation of *Streptomyces* "Total" DNA (modified from Hopwood *et al.* [1985])

This method is applicable without significant modification, for the isolation of chromosomal DNA, from most *Streptomyces* species.

Mycelia from about 25 ml liquid culture was centrifuged at 3,000 rpm for 10 mins and the supernatant discarded. The resulting pellet was washed in 10.3% sucrose, again pelleted at 3,000 rpm for 10 mins and the supernatant discarded. The mycelial mass (about 1 g) was resuspended in TESucrose to a total volume of 5 ml. 10 mg of lysozyme was added (i.e. to 2 mg/ml) as was RNase to a final concentration of 40 μg/ml and the mixture incubated at 30°C with trituration every 15 mins until a

drop of the suspension placed on a microscope slide showed complete clearing following the addition of a drop of 10% SDS. At this point 1.2 ml of 0.5 M EDTA was added and gently mixed into the suspension followed by 0.2 mg/ml of pronase solution, whereupon the mixture was incubated at 30°C for 5 mins. SDS was added to a final concentration of 1% (i.e. 0.7 ml), the suspension immediately mixed by inversion, then incubated at 37°C until complete lysis of the mycelia was seen (up to 2 hrs). 6 ml of phenol solution was added to the suspension and mixed thoroughly by inversion for 10 mins at room temperature. 6 ml of chloroform was then added and the tube contents mixed continuously for a further 5 mins. The phases were separated at 3,000 - 4,000 rpm for 10 mins, the upper aqueous phase was carefully removed using a wide-bore pasteur pipette into a clean glass universal. This phenol/chloroform extraction was repeated until the interphase was reduced and a final chloroform extraction was used to remove any residual phenol. The aqueous supernatant was treated with 0.1 volume of 3 M Na acetate and 1 volume of isopropyl alcohol to precipitate the DNA. The DNA was spooled on a sealed pasteur pipette, dried and transferred to a fresh bottle where it was washed, first in 70% ethanol, then absolute ethanol followed by drying. The DNA was dissolved in a suitable volume of sterile TE buffer (1-5 ml) and chloroform added to 1% to maintain sterility.

2.7.2 Isolation of *Streptomyces* plasmid DNA (after Kieser [1984])

Plasmid DNA was isolated by alkaline lysis, a procedure which can be scaled up or down as required.

Fresh or frozen mycelia, harvested from liquid culture by centrifugation and washed in 10.3% sucrose, were used. Lysozyme solution (2 mg/ml) in TESucrose) was added to a final volume of about 10 ml, to approximately 2 g mycelial pellet and RNase was added to a final concentration of 50 μg/ml. The mixture was incubated at 37°C for 30 mins. 5 ml of SDS-NaOH (2% and 0.3 M respectively) was added and mixed thoroughly by sucking up and down with a 25 ml pipette or syringe, and the sample incubated at either 55°C for 35 mins, or 70°C for 15 mins for smaller plasmids. The sample was allowed to cool to room temperature and then mixed with 1.6 ml acid phenol/chloroform for about 1 min. The phases were separated by centrifugation at 3,000 rpm for 10 mins. 0.1 volume of 3 M Na acetate was added to the supernatant in a clean glass universal followed by 1 volume of isopropyl alcohol. The contents of the tube were mixed carefully and left at room temperature for 5 mins. The precipitated DNA was collected by centrifugation (3,000 rpm; 10 mins) after the supernatant was discarded. The DNA pellet was redissolved in the smallest volume possible of TE buffer. This solution was treated with a phenol/chloroform solution equilibrated to approximately pH 7.5, the phases separated by centrifugation (3,000 rpm; 10 mins) and DNA precipated from this supernatant

using Na acetate and isopropyl alcohol as described above. The resulting pellet was further purified using ethanol and spermine hydrochloride precipitations if there was relatively little DNA present, i.e. smaller preparations, or purified using a caesium chloride gradient for the larger preparations.

DNA was purified on caesium chloride gradients by re-suspending the DNA pellet in 6 mls TE buffer to which 6.3 g of caesium chloride was added and ethidium bromide to 200 μg/ml. The gradients were then ultra-centrifuged at 50,000 rpm for 20 hrs at 17°C.

The discrete band of plasmid DNA was visualised on a U.V. transilluminator and removed using a syringe fitted with a wide-bore needle. The ethidium bromide was removed from the DNA solution by the addition of butanol, which is immiscible with aqueous solutions but has a higher partition coefficient for ethidium bromide. The plasmid DNA was precipitated with absolute ethanol (-20°C; 2 hrs) and finally re-suspended in a suitable volume of TE buffer.

2.7.3 Isolation of *Escherichia coli* plasmid DNA (from Maniatis *et al.* [1982])

A 10 ml overnight culture of *E. coli* cells was grown in Luria broth supplemented with relevant selecting antibiotics, and this was used to seed a 100/200 ml culture which was grown at 37°C on an orbital incubator until the $O.D._{600nm}$ was approximately 0.8. The bacterial cells were harvested by centrifugation at 10,000 rpm for 5 min and the resulting pellet washed in 15 ml M9 buffer. The

cells were pelleted as described above and the pellet suspended in 4 ml 25% sucrose containing 25 mM Tris buffer and transferred to ice, 1 ml lysozyme solution (5 mg/ml in TE buffer) was added and the cells incubated for 30 min followed by the addition of freshly made SDS/ NaOH solution (1% and 0.2 M respectively) and the sample mixed thoroughly by inversion. 7.5 ml of 3 M potassium acetate (pH 4.8, adjusted using glacial acetic acid) was added with constant agitation and the mixture incubated on ice for 30-60 min. The resulting precipitate was pelleted at 10,000 rpm for 20 min and the supernatant carefully decanted. The DNA was precipitated from this solution using 5 M NaCl (0.1 volume) and isopropanol (1 volume) and centrifuged at 5,000 rpm for 5 min. The resulting pellet was purified on a caesium chloride gradient as described in section 2.7.2.

2.7.4 Isolation of *Streptomyces* phage DNA (after Hopwood *et al.* [1985])

Phage was grown overnight in a total of 120 ml of SNA as a top layer of total area 0.6 m^2, inoculated with spores of *S. lividans* 1326 on a basal layer of DNA spread with 1.5 ml DNB containing 1.5 x 10^5 p.f.u. at 28^oC. A glass slide was used to scrape off the top layers into 2 x 70 ml DNB in 250 ml centrifuge bottles, these were mixed by shaking and allowed to stand at room temperature for 2 hr. The bottles were centrifuged at 10,000 rpm for 20 min to remove the agar. The supernatants were pooled and stored at 4^oC whilst the agar was further extracted with 30 ml DNB. The agar was spun out and the super-

natants all pooled and then dispensed into ultracentrifuge tubes and centrifuged at 25,000 rpm for 75 min at 4°C using a swing-out rotor to sediment the phage particles. The supernatant was discarded and each of the pellets suspended in 0.5 ml of SM buffer in an orbital incubator at 37°C for 15-30 min. These suspensions were pooled, the volume made up to 10 ml total, 8.5 g of caesium chloride added and these gradients centrifuged in disposable ultracentrifuge tubes at 36,000 rpm for 18 hr at 20°C. The phage was seen as a discrete blue band under natural light and was removed from the gradient through a wide-bore syringe needle. The solution was dialysed for 4 hr at room temperature against 2 x 200 ml SM buffer. The resulting solution was heated to 60°C for 10 min following the addition of 1/20th volume of 20 x SSC, and allowed to cool to room temperature. 5 M NaCl was added to a final concentration of 0.25 M and an equal volume of phenol/chloroform solution was added and the whole inverted 50 times to mix. The phases were separated by centrifugation at 4,000 rpm for 10 min and the aqueous phase carefully removed using a wide-bore pipette. This phenol extraction was repeated two further times, and each of the phenol/chloroform phases re-extracted with an equal volume of TE buffer containing 0.25 M NaCl as above. All of the aqueous phases were pooled and the phenol extracted by gentle mixing with an equal volume of ether. The phases were separated by centrifugation at 3,000 rpm for 5 min and the ether phase discarded. This ether extraction was repeated twice more

and 0.1 volume 3 M sodium acetate added to the aqueous phase. This was mixed with 2.5 volumes of absolute ethanol until the DNA precipitated as a fibrous clot. This clot was removed, washed with 70% ethanol, dried and finally dissolved in a suitable volume of TE buffer (chloroform must not be added to purified actinophage DNA).

2.8 *In vitro* Methods for DNA

2.8.1 Quantitation

DNA concentrations were estimated by relative fluorescence of ethidium bromide stained DNA samples viewed under U.V. on an agarose gel alongside markers of known DNA concentrations.

Accurate quantitations and estimations of purity were performed on purified samples by measuring the relative optical density of the DNA solution at $^{260nm}/_{280nm}$. Pure DNA has an $^{O.D.260}/_{O.D.280}$ of 1.8 and an $O.D._{260}$ of 1 is equivalent to 50 μg/ml for single-stranded DNA.

2.8.2 Endonuclease restriction

All restriction digests were done using the enzyme manufacturers recommended buffers and conditions.

2.8.3 One-step restriction enzyme - alkaline phosphatase treatment of DNA

The DNA was restricted as above, to completion. Twenty minutes before the theoretically predicted end of the digestion, 6-8 units of calf intestinal alkaline phosphatase were added and the incubation completed at

20°C. The reaction was stopped by heating the sample at 65°C for 10 min in the presence of 20 mmol EGTA. The DNA was extracted with phenol/chloroform and the phases separated in a microfuge at 14,000 rpm for 3 min. The aqueous phase was removed and the DNA precipitated using 0.1 volume 3 M sodium acetate and 2.5 volumes absolute ethanol at -20°C for 2 hr. The redissolved DNA was then ready for use.

2.8.4 Agarose Gel Electrophoresis (after Maniatis *et al.* [1982])

Agarose gel electrophoresis was performed using commercially available electrophoresis kits (Bio-Rad, Pharmacia) using agarose gels of 0.5 - 0.7% concentration, TBE buffer to volume and electrophoresed under TBE buffer. Gels were typically run at 40 V for 2-4 hr for mini-gels and 25-40 V for 12-20 hr for full-size gels, and were stained using ethidium bromide either added to the molten agarose prior to casting (0.5 μg/ml final concentration) or by soaking the gel in dilute ethidium bromide solution for about 20 min followed by de-staining under running water for apparoximately 20 min. Completed gels were viewed under U.V. to visualise the DNA which could be photographed using a Polaroid hand camera or a video camera and printer unit.

2.8.5 Isolation of DNA Fragments from Agarose Gels

Fragments of restriction enzyme cleaved DNA were separated on agarose gels and the bands visualised using ethidium bromide under U.V. light. The chosen band was

excised from the gel with a scalpel and the DNA extracted from the agarose using the protocols of "Geneclean" (NEN DuPont).

2.8.6 Establishing Conditions for the Partial Digestion of High Molecular Weight DNA (after Maniatis et al. [1982])

In order to create representative "gene libraries" high molecular weight (chromosomal) DNA must be cleaved using a restriction enzyme into a set of overlapping fragments of approximately uniform size.

A reaction mixture was prepared containing 10 μg chromosomal DNA and restriction enzyme buffer in a final volume of 150 μl. 30 μl of this mixture was dispensed into Tube 1 and a further 15 μl into each of Tubes 2-8, and the remainder into Tube 9, all on ice. 4 units of restriction enzyme were added to Tube 1, 15 μl of the reaction mixture was transferred to Tube 2 and mixed, thus diluting the enzyme concentration. This two-fold serial dilution was continued to Tube 8, and Tubes 1-8 incubated at 37^{o}C for 1 hr. The reactions were stopped by the addition of gel-loading buffer and the samples analysed by gel electrophoresis using accurate size markers in the relevant size range. The amount of enzyme required to produce maximum fluorescence in the desired size range was ascertained. (Intensity of fluorescence is directly proportional to the mass distribution of DNA, therefore to obtain the maximum number of molecules in this size range half the amount of enzyme required to produce maximum fluorescence was used.)

2.8.7 Size Fractionation of DNA by Sucrose Centrifugation

Having produced a representative population of restricted DNA fragments (Section 2.8.6) these were size-selected by sucrose gradient centrifugation.

Sucrose gradients were formed by carefully layering 5 ml of 10% (w/v) sucrose TEN on top of 5 ml 40% (w/v) sucrose TEN in a swing-out ultracentrifuge tube. The tube was frozen at -20°C overnight and allowed to thaw thoroughly without perturbation for at least one hour before use. The restricted DNA sample, in a maximum volume of 500 µl, was applied to the top of the gradient and centrifuged at 35,000 rpm for 16 hr at 17°C in a swing-out rotor. The gradient was removed from the bottom of the tube, using a peristaltic pump, in 500 µl aliquots. A sample from every second of these aliquots was assayed by gel electrophoresis and the aliquots containing the required fragments pooled where necessary. The DNA was removed from the sucrose solution by ethanol precipitation.

2.8.8 Ligation of DNA Molecules

In this work only DNA molecules with complementary staggered ends were ligated together following their cleavage with suitable restriction enzymes. This is more efficient than ligating blunt-ended DNA fragments. Also, only bi-molecular ligations were performed where possible. Plasmid vectors, being the smaller of the DNA species involved in most of the ligations performed, were pretreated with calf intestinal alkaline phosphatase to

remove 5′ terminal phosphate groups and thus prevent vector recircularisation during the ligation. Ligation conditions were based upon the optimal DNA concentrations described in the J.I.I. Handbook (Table 2.3), but a series of reactions were always performed with DNA ratios surrounding the optimum described for any given experiment. Ligations were performed using T4 polynucleotide kinase (Boehringer Mannheim) in the presence of 1 x ligation buffer containing 0.5 mM ATP to activate the reaction. Where possible the success of the ligation was assayed by gel electrophoresis prior to transformation/ transfection.

2.8.9 Radioactive Labelling of DNA Probes

DNA probes for DNA-DNA hybridisations were radioactively labelled with ^{32}P-labelled dCTP using the method of random-primed labelling using the "Random Primed DNA Labelling Kit" (Boehringer Mannheim). Unincorporated dNTPs were separated from labelled probes using a Sephadex G50 column with a tracking dye (1% orange G in concentrated blue dextran; 50 mM NaCl) to follow the elution of the fractions.

2.8.10 "Dot Blot" Transfer of DNA (modification of Hopwood *et al.* [1985])

The "Dot Blot" is a quick, simple method of immobilising DNA target sequences for DNA-DNA hybridisation studies. 5 μg of the target DNA was dissolved in 1 M NaCl; 0.1 M NaOH; 10 mM EDTA and denatured for 5 min at 95^{o}C. This DNA was applied in varying amounts (normally

Table 2.3 Conditions for the ligation of DNA

	Vector DNA		Insert DNA		Final Total DNA Concentration (μg/ml)
	Size (kb)	Quantity (μg)	Size (kb)	Quantity (μg)	
Plasmid	ca. 5 (eg. pIJ702)	1	c.2-30	5	c.15
	ca.15 (eg. pIJ61)	1	c.2-30	5	c.18
	ca.25 (eg. pIJ922)	1	c.2-30	5	c. 6
Phage	ca.35 (eg. KC404) a) *cos*-site not co-valently joined.	1	1-10	0.2	ca.100-200
	ca.35 (eg. KC404) a) *cos*-site co-valently joined.	1	1-10	1	ca. 5

(From Hopwood *et al.*, 1985)

3 µl, 2 µl and 1 µl) to separate spots on a suitable piece of hybridisation membrane ("GeneScreen" [NEN DuPont] or "Hybond N" [Amersham International plc, UK]) and dried between two sheets of "Whatman 3MM" paper for 30 min. The membrane was rinsed in 3 x SSC for 2 min, blotted dry and the DNA cross-linked to the membrane with U.V. from a transilluminator for 2 min. The membrane was then used in hybridisation experiments.

2.8.11 "Southern" Transfer of DNA (Southern [1975])

The DNA target molecules were separated by agarose gel electrophoresis and stained and photographed as described above. The gel was trimmed to the minimum size required and a small piece removed from the bottom right-hand corner to assist orientation. Large DNA targets (> 10 kb) were treated by soaking the gel in 0.25 M HCl (2 x 10 min) to partially depurinate the fragments. The DNA was then denatured using alkali (2 x 15 min, 0.5 M NaOH; 1 M NaCl), rinsed 3 times in distilled H_2O and the gel neutralised in 0.5 M Tris-HCl; 3 M NaCl at pH 7.5 for 20 min. Hybridisation membrane was cut to the size of the gel as were two pieces of "Whatman 3MM" paper, and the membrane pretreated according to the manufacturers instructions. 20 x SSC was poured into a tray containing a suitable, raised support for the gel and wicks of Whatman 3MM paper placed on this support so that they dipped into the 20 x SSC. A final piece of 3MM paper was placed on the support over the wicks, soaked in 20 x SSC and all air bubbles were removed using a glass pipette.

The gel was placed on top of the support and excess moisture removed with a gloved hand. The hybridisation membrane was carefully placed directly on to the gel and the bottom corner cut away, corresponding to that of the gel. Cling film was placed over the tray right up to the sides of the gel and then 3MM paper placed on top of the membrane followed by a stack of paper towels. An 800 g weight was applied to the top of the stack and DNA transfer allowed to proceed for 4-16 hr. The apparatus was dismantled and the DNA cross-linked to the membrane by exposure to U.V. for 2 min on a transilluminator.

In the latter stages of the project alkali transfer of DNA was used with "Hybond N" (Amersham International plc) membranes as described by the manufacturer. The process is the same as described above, except that the transfer buffer is alkaline.

2.8.12 DNA-DNA Hybridisations of Hybridisation Membranes

The earlier work in this thesis used the protocols for DNA-DNA hybridisations described in the J.I.I. Handbook, i.e. hybridisation was performed in heat-sealable plastic bags at 70^{o}C, as were high stringency washes using 0.2 x SSC; 0.1% SDS. Later work used the more convenient hybridisation ovens (Hybaid; Techne) according to the instructions supplied by the manufacturer's of both the ovens and the membranes used. The later work also used different hybridisation buffers, as described in the "Hybond N" protocols or as supplied by Amersham International plc (Rapid-Hybridisation Buffer),

which were used as described by the manufacturer's instructions.

2.8.13 Autoradiography of Hybridisation Membranes

Membranes holding target DNA hybridised against specific radioactive probes were analysed by autoradiography using X-ray film (Kodak "X-OMAT"; Amersham International "Hyperfilm") exposed at -70°C for 1-10 days in light-proof cassettes containing intensifying screens where necessary. Exposure times were estimated from the activity of the membranes as detected by a Geiger-Müller tube connected to a counter. The autoradiographs were developed as described in the manufacturer's instructions.

2.9 *In vivo* Methods for DNA

2.9.1 Plasmid Transformation (Rapid) (modification of Hopwood *et al.* [1985])

Fresh or frozen *Streptomyces* protoplasts were used, pre-washed in P buffer and pelleted at 6,500 rpm in an Eppendorf tube. The pellet was resuspended in the drop of liquid left following removal of the supernatant by pouring. 5 µl of DNA solution (containing a maximum of 1 µg DNA) was added to the protoplasts and mixed by tapping. 250 µl of 25% PEG in P buffer was added and mixed by pipetting up and down three times. The PEG was diluted by mixing the protoplast suspension with 200 µl P buffer, and 100 µl aliquots of this mixture spread on each of five R2YE plates. The plates were incubated at 30°C for 4-7 days to form colonies and where necessary

overlaid with selecting antibiotic after 16 hr.

2.9.2 Bacteriophage Transfection (after Rodicio and Chater [1982])

Freshly prepared *Streptomyces* protoplasts were washed in P buffer, pelleted at 6,500 rpm for 1 min in an Eppendorf tube after which the supernatant was poured off and the protoplasts re-suspended in the drop of remaining liquid. The DNA solution (up to 50 μl of TE buffer containing a maximum of 0.5 μg DNA) was mixed with previously prepared DNA-free liposomes (100 μl) in a separate Eppendorf tube and this mixture added to the protoplasts. 0.5 ml of 60% PEG in P buffer was immediately added and pipetted up and down three times to mix. The mixture was left at room temperature for 1 min. 0.1 ml of undiluted and of ten-fold serial dilutions of the transfection mixture in P buffer were plated on R2YE plates. The plates were overlayed with 5 ml of molten "soft R2" agar (R2 mixed with an equal volume of P buffer) at 45^{o}C and seeded with *S. lividans* 1326 spores (approximately 10^{7} ml^{-1}). The plates were incubated at 30^{o}C for 18 hr.

2.9.3 Preparation of DNA-Free Liposomes (Rodicio and Chater [1982])

2.5 mg of L-phosphatidyl choline and 1.2 mg stearylamine were separately weighed into two 5 ml bijoux bottles. Each was dissolved in 5 ml chloroform and 0.1 ml of the stearylamine solution was added to the phosphatidyl choline solution. The mixture was transferred to a round-bottom flask, diluted with 8 ml chloroform and

reduced to a thin film on a rotary evaporator *in vacuo* at 55°C. The film was resuspended in 0.5 ml G buffer and the liposomes separated in a plastic tube by the addition of 2 ml 5.2% KCl (dissolved in 10% ethanol) and centrifugation at 6,000 rpm for 15 min at room temperature. The supernatant was decanted to a fresh plastic tube in which it could be stored at 4°C for up to one month prior to use.

Note - all glassware must be washed in ethanol and then with chloroform before use.

2.10 Physical Methods

2.10.1 Thin-Layer Chromatography (TLC) - for antibiotic assays

Solutions of purified geldanamycin were prepared in either ethyl acetate or methanol for use as a standard. Colonies to be assayed were grown in YEME at 30°C for 4-7 days, harvested and the mycelium removed by centrifugation. Spent broth from liquid cultures was extracted with ethyl acetate or chloroform and the phases separated by centrifugation at 3,000 rpm for 5 min. The solvent extract was evaporated to dryness *in vacuo* and the residuum redissolved in a small volume of ethyl acetate or methanol. The samples were applied to the base-line (1-2 cm from the bottom of the chromatoplate) of a pre-coated and activated TLC plate (silica-gel containing a 254 nm fluorescent marker, Sigma Chemical Co. Ltd.). The plates were developed in a glass chromatography tank in one of the following mixtures:-

ethyl acetate:n-hexane:dichloromethane:methanol; 9:6:6:1, or chloroform: methanol; 9:1.

The solvent front was marked, the plate removed from the tank and the residual solvent allowed to evaporate from the plate. Substances on the plate were visualised either: under U.V.; by spraying with acid-vanillin (5% vanillin in ethanol including 5 ml cH_2SO_4 per 100 ml) and heating *in vacuo*; or by overlaying the plates with SNA at 45°C containing an indicator strain and incubating overnight at 37°C to score for zones of growth inhibition.

Broth extracts were compared with purified antibiotics by co-chromatography on the same plate.

2.10.2 High-Performance Liquid Chromatography (HPLC) Screening

All HPLC analyses were carried out on an LKB single pump system, using an LKB programmable gradient maker, an LKB uvicord fixed wavelength spectrophotometer, an LKB two channel chart recorder and an LKB super rack fraction collector, where necessary. Reverse-phase chromatography was the chosen method using an Apex Octadecyl 5 μ (C18) column supplied by Jones Chromatography. A variety of buffering systems were used, including isocratic and gradient systems of acetonitrile/water or methanol/water as appropriate. Purified geldanamycin and spent broth extracts were prepared as described (Section 2.10.1) and eluted from the above system in an attempt to characterise the materials present.

CHAPTER 3

CLONING GENOMIC DNA FROM *Streptomyces hygroscopicus* 3602

A variety of cloning strategies are available for *Streptomyces* and have been successfully used to clone genes involved in the biosynthesis of antibiotics. These are discussed below together with their applicability to the cloning of the geldanamycin biosynthetic genes.

3.1 Cloning of Antibiotic Resistance Determinants

It is now generally accepted that the genes involved in antibiotic biosynthesis in *Streptomyces* are organised as closely linked clusters (Sherman *et al.*, 1988) and that these clusters typically contain the corresponding genes which determine resistance to that antibiotic. In some cases the resistance genes play a role in controlling the expression of the structural genes (Hopwood and Chater, 1989).

This gene clustering enormously facilitates the cloning of whole sets of antibiotic biosynthetic genes via the initial cloning of the corresponding resistance genes where phenotypic identification is theoretically simpler, because of this the cloning of resistance genes is probably the most often used approach.

A simple strategy might involve the shotgun cloning of genomic DNA from the antibiotic-producing strain into a suitable plasmid vector. The resulting gene library is used to transform a host strain known to be sensitive to the antibiotic. Transformants are selected for the

acquisition of an antibiotic-resistant phenotype. Any segments of DNA seen to confer resistance can then be used as probes in "chromosome-walking" experiments to identify adjacent regions in the cluster. This procedure has been used to successfully clone over 60 kb of DNA which encode the resistance to and biosynthesis of erythromycin A from *Saccharopolyspora erythraea*. The *ermE* gene which specifies erythromycin resistance was originally cloned by Thompson *et al.* (1982), principally for use in vector construction and the "chromosome-walking" was subsequently performed by Weber *et al.* (1990).

This approach was not suitable for cloning the geldanamycin biosynthetic genes as, at the start of this work no suitable geldanamycin-sensitive host strains were known. *Escherichia coli* is resistant to geldanamycin and while *Bacillus subtilis* was known to be geldanamycin-sensitive no expertise in the genetic manipulation of that organism existed in this laboratory. At that time we believed that none of the *Streptomyces* species suitable as cloning hosts were sensitive, however we subsequently found a *S. aureofaciens* strain that is geldanamycin-sensitive.

3.2 Complementation of Antibiotic Non-producing Mutants

This procedure again relies upon the fact that antibiotic biosynthetic genes lie on the chromosome in clusters, so that when part of this cluster is identified the remainder of the pathway can be isolated by

"chromosome-walking". A pre-requisite for this cloning procedure is the existence of a set of mutants of the antibiotic-producing strain that are phenotypically identifiable as being antibiotic non-producers and are preferably well characterised.

The cloning strategy involves the transformation of these mutants with a library of shotgun cloned DNA sequences followed by examination of the resultant transformants for restoration of the antibiotic-producing phenotype. This approach was used successfully to isolate the complete set of biosynthetic genes for the production of the polyketide antibiotic actinorhodin from *S. coelicolor* (Malpartida and Hopwood, 1984), using actinorhodin non-producing mutants previously isolated and characterised by Rudd and Hopwood (1979). Although a set of geldanamycin non-producing mutants were available, having been made in this laboratory by Yu Bai Song (unpublished data), this strategy was not pursued because these mutants were not well-characterised and those that were tested were noticeably refractory to protoplasting techniques, thus rendering them unsuitable, as yet, as cloning hosts.

3.3 Mutational Cloning

Chater and Bruton (1983) reported the use of the actinophage øC3 KC400, an attachment-site deleted derivative of the *Streptomyces* temperate phage øC31, in a procedure called "mutational cloning", to clone DNA fragments involved in the biosynthesis of methylenomycin

A from *S. coelicolor* A3(2). The principle of mutational cloning depends on the use of a phage vector which is *att*-site deleted and therefore incapable of initiating lysogeny of a host through the normal process of site-specific recombination. The phage can, however, integrate into the host chromosome when the phage has cloned into it a fragment of host DNA by recombination between homologous DNA sequences. øC31 KC400 libraries containing shotgun clones of DNA from an antibiotic-producing strain are therefore used to transfect the wild-type, antibiotic-producing strain from which the clones were isolated. Lysogens are selected and screened for mutants which have lost the capacity for antibiotic production. This should occur where the cloned DNA mediating the recombinational event represents part of the antibiotic biosynthetic pathway and where that fragment of DNA contains neither a transcription initiation or termination sequence (a more complete account of the rationale of mutational cloning and its uses will be given in a later Chapter).

The appropriate recombinant phage DNA can be recovered by replication of the lysogens to a lawn of indicator strain on which the phage can form plaques. This DNA can then be used as a probe to isolate adjacent sequences of the remainder of the pathway.

An attempt was made to identify DNA sequences involved in geldanamycin biosynthesis by mutational cloning with phage øC31 KC515, however no lysogens were obtained carrying the antibiotic resistance genes (*vph*

and *tsr*) specified by the phage. This was probably due to poor phage yields and a low regeneration frequency of *S. hygroscopicus* 3602 protoplasts, despite a published report to the contrary (Hooley and Wellington, 1985). Also, there were problems assaying the success of the shotgun ligations between a large vector (ca. 35 kb) and small inserts (<4 kb) on an agarose gel, i.e. the yield of recombinant phage DNA molecules may have been so low as to render the whole protocol inefficient.

3.4 DNA Probing for Homologous Sequences

Malpartida and Hopwood (1984) hypothesised that since actinomycetes carry genes encoding polyketide synthase (PKS) complexes which synthesise a number and variety of different polyketides they could not reasonably have evolved independently. Consequently genes specifying the production of one polyketide antibiotic might be used as DNA probes to detect *pks* genes for other antibiotics. Malpartida *et al.* (1987) proved this to be the case. Using DNA probes of the *act*I and *act*III genes from the actinorhodin biosynthetic pathway previously deduced to be part of the actinorhodin *pks*, they showed that the *act*I probe hybridised with 14 of the 17 known polyketide producers tested, but with only 2 of the 8 polyketide non-producers (both subsequently found to be cryptic polyketide-producers). The *act*III probe hybridised with bands in most of the strains to which *act*I hybridised. In some of the cases examined Malpartida *et al.* confirmed that the hybridising bands represented

parts of polyketide biosynthetic cluster. For example, cloned homologous DNA from the dihydrogranaticin producer, *S. violaceoruber* Tu22.

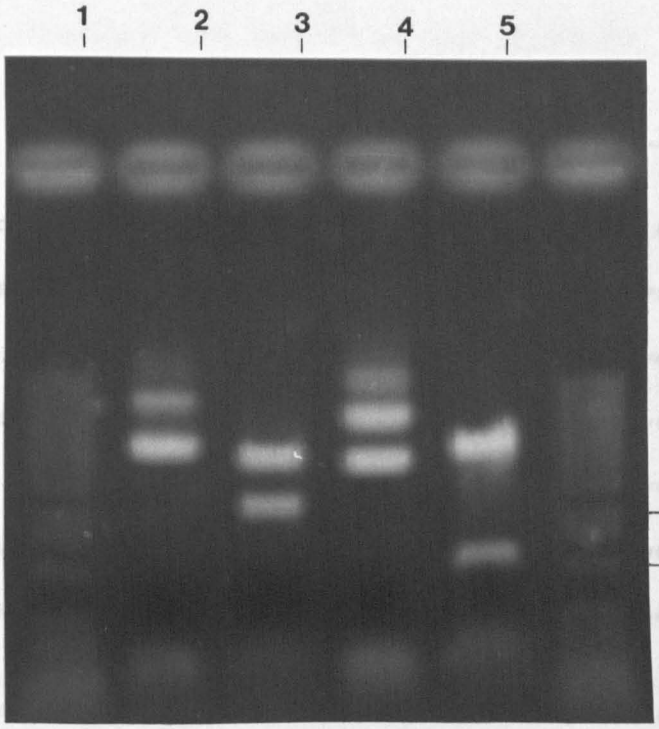
3.4.1 Results

This last approach was used as our primary attempt to clone DNA involved in geldanamycin biosynthesis. The *act*I and *act*III probes used in the above work (as *Bam*HI restriction fragments of 2.2 kb and 1.1 kb respectively) in the recombinant plasmids pIJ2345 and pIJ2346 respectively were used. Plasmid DNA was prepared from *E. coli* by alkaline lysis. This plasmid DNA was cut with *Bam*HI to separate the *act* sequences and the resulting DNA fragments separated by agarose gel electrophoresis (Figure 3.1). The *act*I and *act*III fragments were isolated from the gel using the "Geneclean" procedure and labelled with ^{32}P for use as probes in Southern hybridisations with "total" DNA extracted from *S. hygroscopicus* 3602. A variety of other target DNA species were also included in this study, as shown in Table 3.1.

Total cellular DNA from the various streptomycete strains was prepared from 7d - 10d cultures grown in YEME at 28^{o}C, cut with BamHI under conditions of complete digestion and the resulting fragments were separated on an agarose gel and then immobilised on a hybridisation membrane by Southern transfer. The actI and actIII probes, labelled with ^{32}P, were hybridised individually against these membranes, after which the membranes were

Figure 3.1

Isolation of the *act* Probes



Lane 1 = 1 kb ladder

2 = pBR2345 (undigested)

3 = " (*Bam*HI digest) - lower band = *act*I

4 = pBR2346 (undigested)

5 = " (*Bam*HI digest) - lower band - *act*III

Table 3.1

Target DNA species hybridised against *act* probes

DNA Species

"Total DNA"	Antibiotic Produced	Reason for Inclusion
S. hygroscopicus 3602	Geldanamycin	Strain under study.
S. glaucescens 5155	Tetracenomycin	Positive control *
S. coelicolor M145	Actinorhodin	Positive control *
S. hygroscopicus 3672	Herbimycin	Positive control *
S. griseus	Streptomycin	Negative control *
S. antibioticus 524	Actinomycin	Negative control *
S. lividans TK24	Undecylprodigiosin	Negative control **
Amycolatopsis mediterraneae	Rifamycin	Unknown, but a polyketide ansamycin producer.

Probe DNA, i.e. *act*I and *act*III were also included as positive controls.

* As indicated in the study by Malpartida *et al.* (1987).

** Since found to cryptically encode actinorhodin (E. M. Wellington, University of Warwick, personal communication).

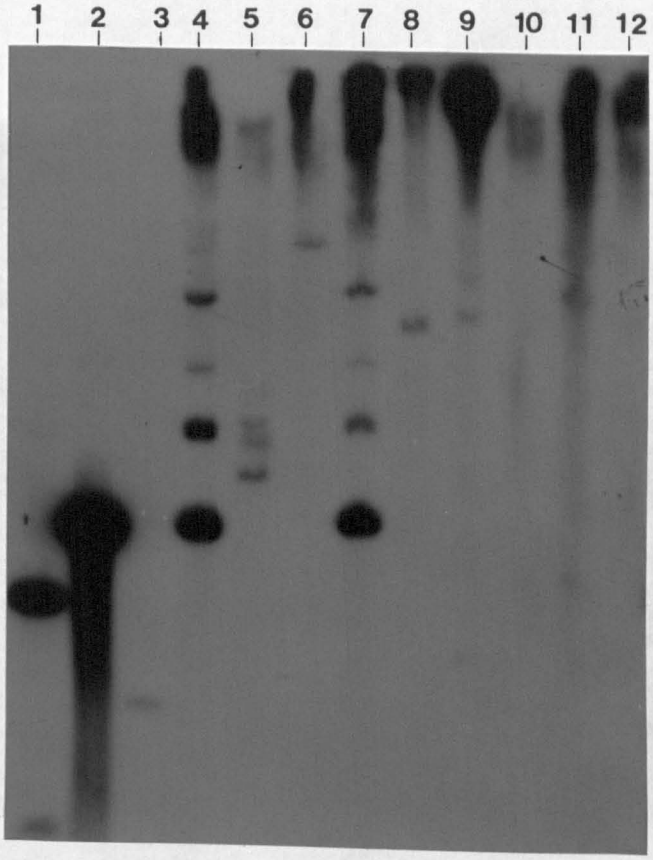
washed at low and high stringencies, i.e. under conditions that require about 65% and 85% DNA-DNA homologies, respectively between target and probe to maintain duplexing. After washing the membrane was exposed to X-ray film for autoradiography. The results are presented in Figures 3.2 - 3.5 and are summarised in Table 3.2.

The data indicate an exact correlation between our work and the results published by Malpartida *et al.* (1987), where the same strains were used in both studies, if one examines our results at high stringency. Certain strains used in our study were not incorporated in the Malpartida *et al.* study including *Amycolatopsis mediterranaea* (the rifampicin producer), which indicates some hybridisation with the *act* probes at low stringency only and *S. hygroscopicus* 3602 (the geldanamycin producer) where we see only very weak hybridisation with the *act*I probe under stringent conditions.

The weakness of the signals obtained with *S. hygroscopicus* 3602 DNA at high stringency led us to conclude that little homology existed and we were not convinced the weak homology detected at low stringency was evidence that these bands represent part of the geldanamycin pathway. Quite possibly they identified other DNA sequences with some homology to the actinorhodin *pks* genes, e.g. fatty-acid synthase sequences. In retrospect this was a sound decision as other groups have encountered problems attempting to isolate genes from antibiotic biosynthetic pathways in this way, e.g. by isolating homologous DNA sequences that were involved

Figure 3.2

Autoradiograph of Chromosomal DNA Probed with *actI* (low stringency).

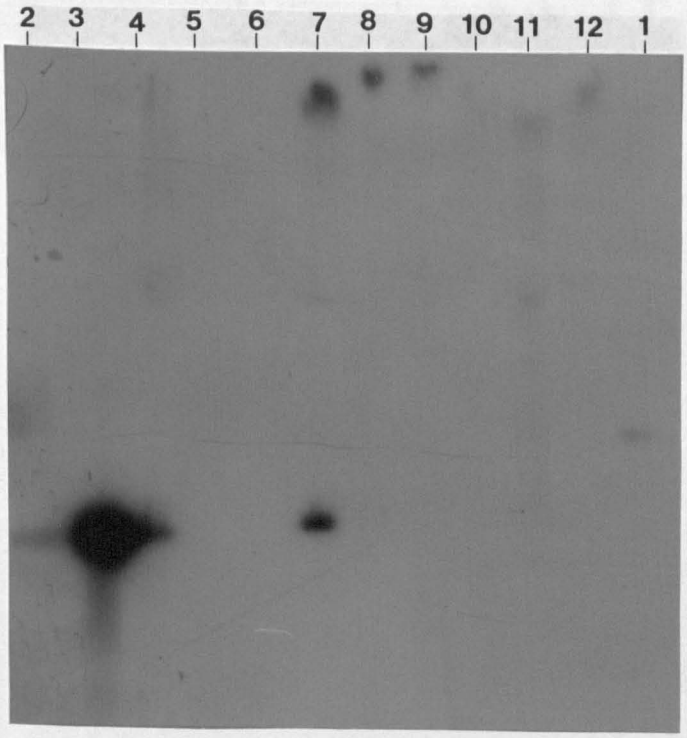


Target DNA species:

Lane 1 = 1 kb ladder
2 = *actI*
3 = *actIII*
4 = *S. lividans* TK24
5 = *S. hygroscopicus* 3602
6 = *S. hygroscopicus* 3672
7 = *S. coelicolor* M145
8 = *A. mediterraneae*
9 = *S. antibioticus*
10 = *S. griseus*
11 = *S. glaucescens*
12 = *S. rimosus*

Figure 3.3

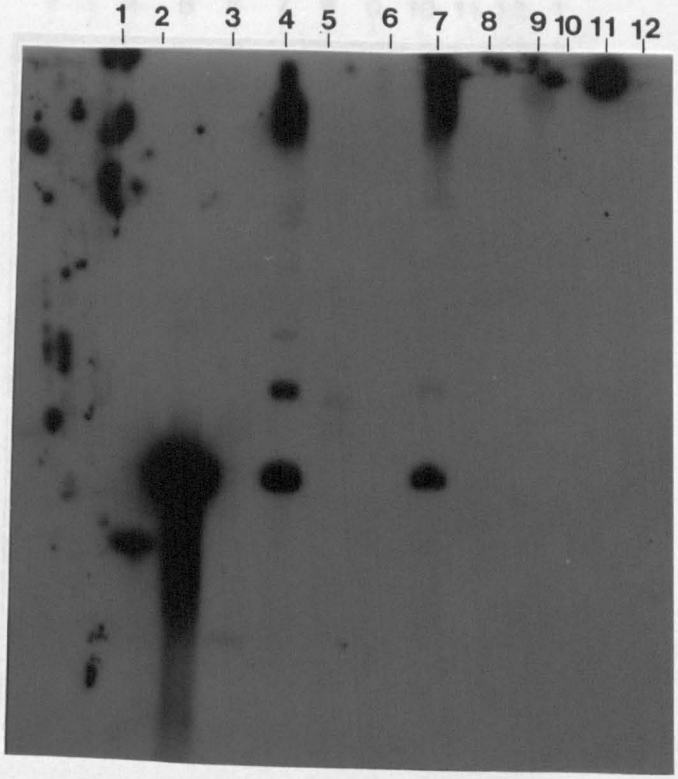
Autoradiograph of Chromosomal DNA Probed with *act*III (low stringency).



Legend as for Figure 3.2

Figure 3.4

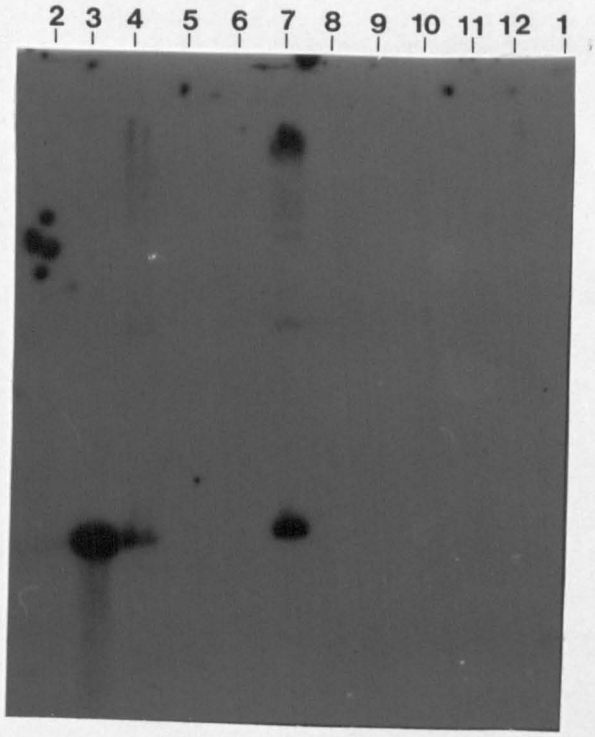
Autoradiograph of Chromosomal DNA Probed with *actI* (high stringency).



Legend as for Figure 3.2

Figure 3.5

Autoradiograph of Chromosomal DNA Probed with *actIII* (high stringency).



Legend as for Figure 3.2

Table 3.2

DNA-DNA Hybridisations with "*act*" probes.

Strain	Probe				
	actI		*actIII*		
	low	high	low	high	: stringency
S. hygroscopicus 3602	++	+	-	-	
S. glaucescens 5155	+++	++	+	-	
S. coelicolor M145	+++	+++	++	++	
S. hygroscopicus 3672	++	-	-	-	
S. griseus	+	-	+	-	
S. antibioticus 5524	+	-	++	-	
S. lividans TK24	+++	+++	++	++	
Amycolatopsis mediterraneae	++	-	+	-	
Controls					
actI	+++	+++	-	-	
actIII	+	+	+++	+++	

Footnote to Table 3.2 -

(as determined by the degree of blackening of the autoradiographs, where target DNA concentrations were approximately equal.

- = no hybridisation
+ = weak hybridisation
++ = moderate hybridisation
+++ = strong hybridisation

in a polyketide pathway not concerned with the antibiotic whose pathway they hoped to clone (B. Rudd; personal communication).

Further information relating to the apparent lack of homology between the *act* probes and "total" DNA isolated from *S. hygroscopicus* 3602 and its possible significance in the biosynthesis of geldanamycin will be discussed in a later Chapter. For the present these results suggested that this route was not appropriate for isolating the *geld* pathway genes.

3.5 Screening Gene Banks for Phenotypic Markers, etc.

It is possible to isolate antibiotic biosynthetic genes from a gene bank shotgun-cloned into a plasmid vector and transformed into a host which does not display the phenotype under study, e.g. if the antibiotic biosynthetic pathway of interest is known to encode an enzymatic step for which there is an assay the gene bank in a heterologous host can be screened for enzymic activity. Where the enzyme is present the recombinant plasmid must contain the gene(s) responsible and these can then be used to find the remainder of the pathway. This, in essence, is the procedure eventually adopted to isolate geldanamycin biosynthetic genes, however it was the acquisition of the ability to produce an antibiotic that was assayed. The remainder of this chapter describes this work in detail.

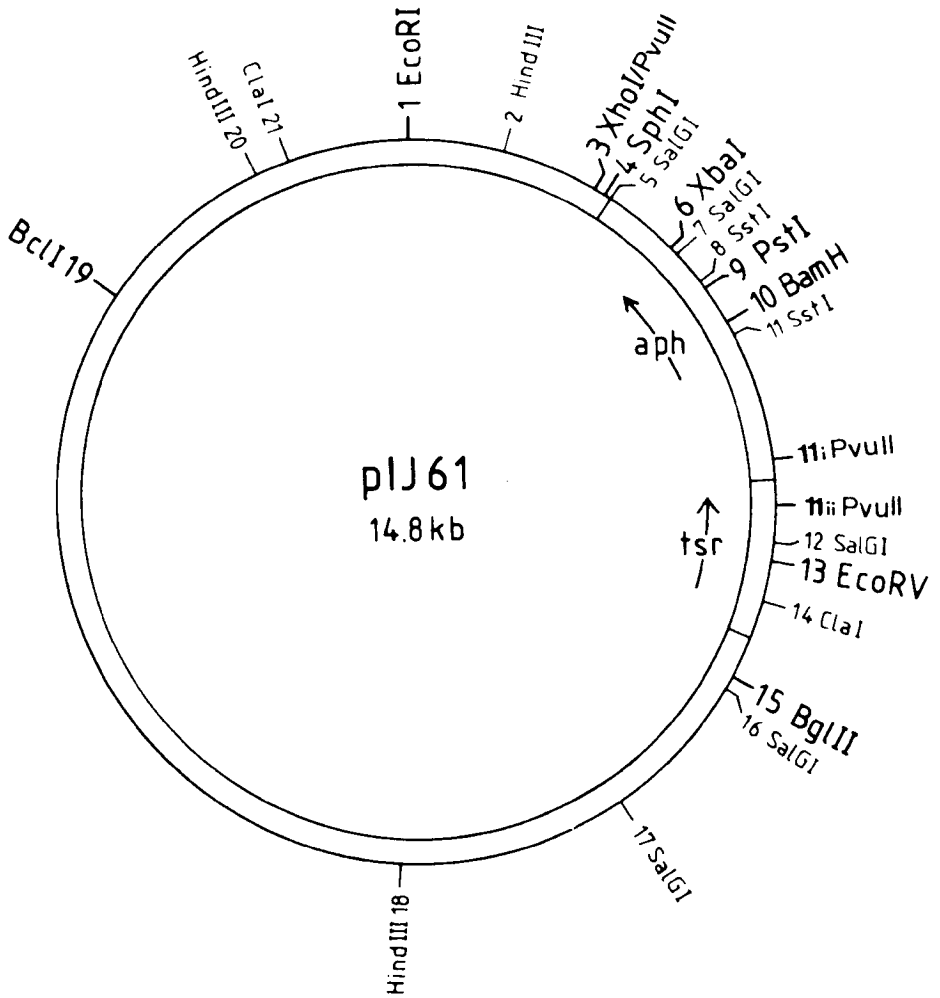
3.6 Shotgun Cloning of Genomic DNA from *S. hygroscopicus* 3602.

The results described in sections 3.1 - 3.5 showed that the more direct routes to isolating geldanamycin biosynthetic genes by cloning sequences homologous to the *S. coelicolor* polyketide synthase probes and by cloning geldanamycin sequences were not suitable. As a next approach the isolation of geldanamycin encoding sequences from a shotgun cloned DNA library was attempted. In the absence, at this stage of the work, of means to identify pathway genes other than by their ability to produce geldanamycin a protocol was chosen to optimise the chances of cloning the entire functional biosynthetic pathway. This required the use of a low copy number vector, to avoid any possible deleterious effects on the host strain brought about by over-expression of genes cloned into a high copy number vector (Malpartida and Hopwood, 1984) and *S. hygroscopicus* 3602 genomic DNA size-fractionated to give DNA fragments of about 20 kb average size. Plasmid pIJ61 (Figure 3.6: Thompson *et al.*, 1982) which is about 15 kb in size, with a copy number of 4-5 as an autonomous plasmid, carries resistance to viomycin (*vph*) and thiostrepton (*tsr*) was selected as the vector. The single *Bam*HI cloning site was suitable for the insertion of DNA fragments derived from *Sau*3a digests.

250 μg total genomic DNA isolated from *S. hygroscopicus* 3602 was digested with 0.025 units of *Sau*3a per μg DNA to produce a population of partially digested molecules, the majority of which were in excess of 15 kb

Figure 3.6

Restriction Map of pIJ61
(after Hopwood *et al.*, 1985)



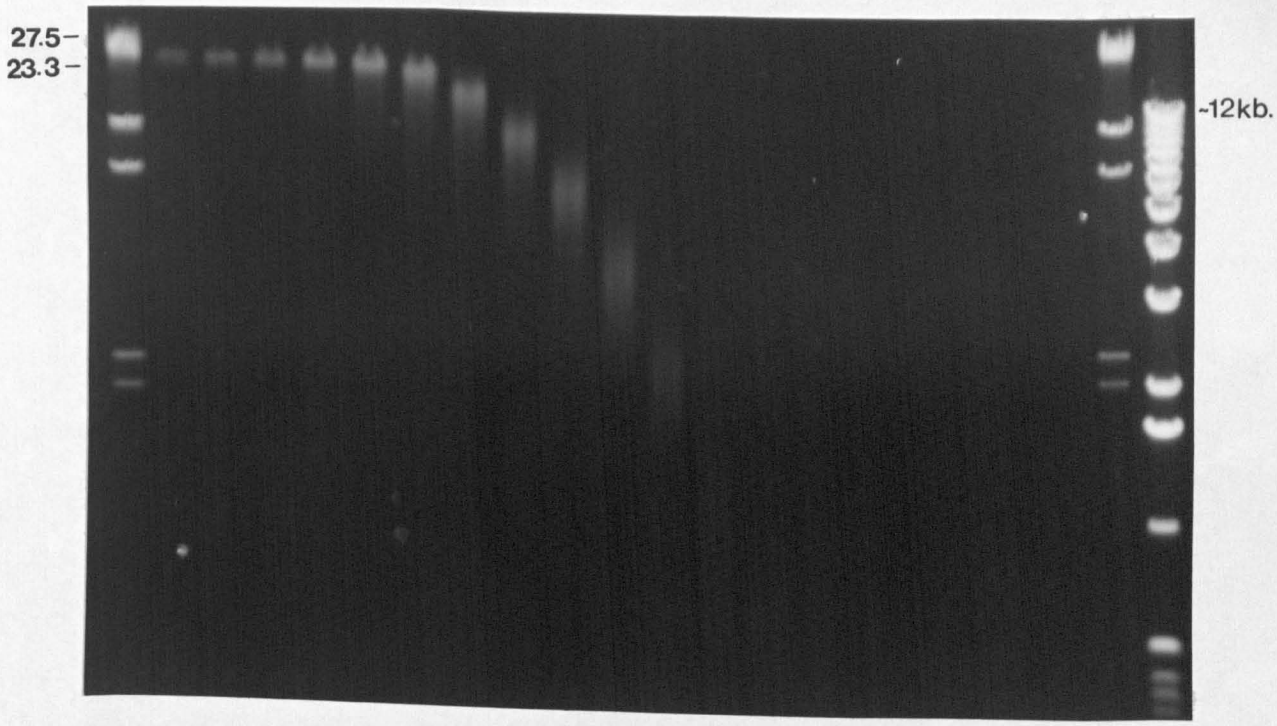
because previously workers (Malpartida and Hopwood, 1984; Chater and Bruton, 1985 and Murakami *et al.*, 1986) had isolated complete sets of antibiotic biosynthetic and resistance genes (for actinorhodin, methylenomycin and bialaphos respectively) on DNA segments of 22 kb, "at least" 17 kb and 16 kb (respectively). The restriction fragments were size-fractionated by sucrose density gradient centrifugation. The collected fractions were analysed by gel electrophoresis (Figure 3.7). Aliquots containing fragments of DNA in the size range 23 kb - 15 kb were pooled together and the DNA recovered from them.

The size-fractionated *Sau*3a genomic DNA fragments were ligated with *Bam*HI-cleaved, alkaline phosphatased pIJ61 vector DNA at vector DNA:chromosomal DNA ratios of 1:5, 1:3 and 1:2 in a total DNA concentration of 18 μg/ml. The effectiveness of these reactions was assayed by subjecting an aliquot from each reaction mixture to agarose gel electrophoresis alongside cleaved, unligated pIJ61 and insert fragments. In each case the ligation reaction showed an upwards shift in the size of the DNA fragments from that of the vector alone and a diminution in intensity of the vector band as compared to the unligated control, indicating successful ligation.

S. lividans TK24 was chosen as the cloning host because it is a relatively widely studied strain which is simple to protoplast, transform and regenerate. It is not, strictly, a heterologous host as it contains actinorhodin (polyketide) genes, though these are not normally expressed. This means that there is a possibility of

Figure 3.7

Aliquots of Size-Fractionated (Sau3a)
S. hygroscopicus 3602 DNA



(Outer lanes represent DNA size standards.)

some functional interaction between the host involving the actinorhodin genes and any geldanamycin biosynthesis genes from the donor DNA.

Protoplasts of *S. lividans* TK24 were transformed with recombinant pIJ61 containing a putative gene library of DNA from 3602. Approximately 24 h after the transformation, transformant colonies were selected by overlaying the plates with 500 μg/ml thiostrepton and allowed to grow for a further 5-7 days at 30°C. A transformation frequency of ca. 1.5×10^4 transformants/μg DNA was thus obtained.

3.7 Screening the Gene Library

Transformants were either replicated or picked off to YEM plates containing thiostrepton to maintain selection and 50 μg/ml of the aromatic amino acid 3,5-aminohydroxybenzoic acid (AHB), a known precursor for geldanamycin biosynthesis, so that we maximised our chances of finding geldanamycin producing clones. These plates were overlayed with SNA containing the indicator strain, *B. subtilis* (*tsr*^{r}; *geld*^{s}) and, following incubation, examined for *Streptomyces* colonies showing antibiosis. 3 of 800 screened were found to produce a zone of inhibition of growth of the indicator strain. These colonies X, Y and Z were isolated, from the master plates, for further examination.

3.8 Preliminary Investigation of X, Y and Z

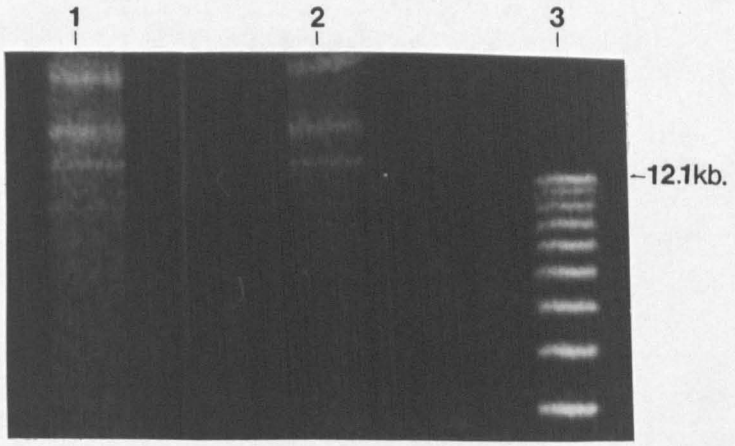
The colonies were individually cultivated in liquid culture with thiostrepton to maintain selection for the plasmid. After 7 days the mycelium was harvested and plasmid DNA isolated by alkaline lysis. This DNA was characterised physically and also used to retransform protoplasts of *S. lividans* TK24 to assess the stability of the plasmid. Plasmid DNA was also isolated from the *S. lividans* re-transformants.

3.9 Physical Characterisation of pIA101, pIA102 and pIA103 DNA

Difficulty was encountered in analysing the physical characteristics of the plasmid DNA isolated from the colonies X, Y and Z. Variability was seen in restriction digest patterns, particularly when comparing one plasmid preparation to another. From restriction patterns it is clear that plasmids co-existed with deletion products. However we have been able to obtain a limited amount of information about the clones. Figures 3.8 - 3.9 show restriction digest patterns for the clones each digested with either *Sst*I alone or *Sst*I/ *Bcl*I together.

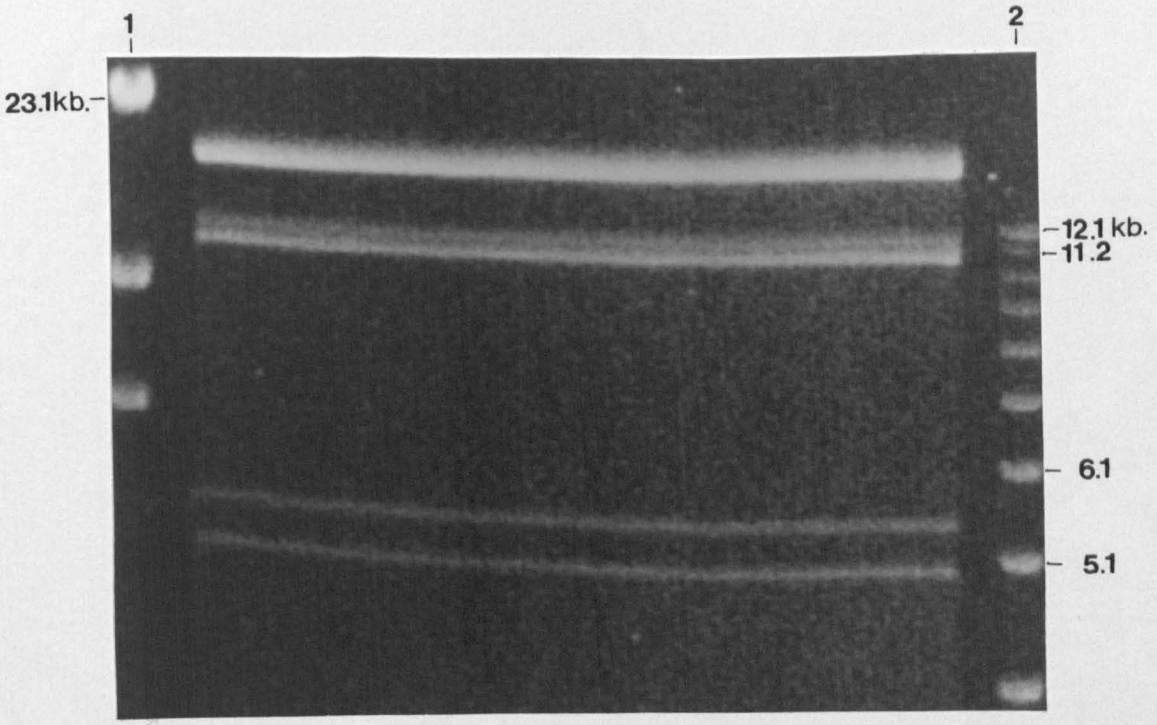
These restriction patterns suggest a clone size of ca. 15 kb (as estimated from alongside size markers). The fact that the clones all seem to restrict similarily in this respect may indicate that they represent the same segment of S. *hygroscopicus* 3602 DNA, though this awaits confirmation. The fragment pattern shown in Figure 3.8b was investigated further - the upper fragment was isolated from this gel (this fragment presumably represents

Figure 3.8



(a) pIA101 and pIA103 digested with *Sst*I

Lane 1 = pIA101 (*Sst*I digest)
Lane 2 = pIA103 (*Sst*I digest)
Lane 3 - 1 kb ladder

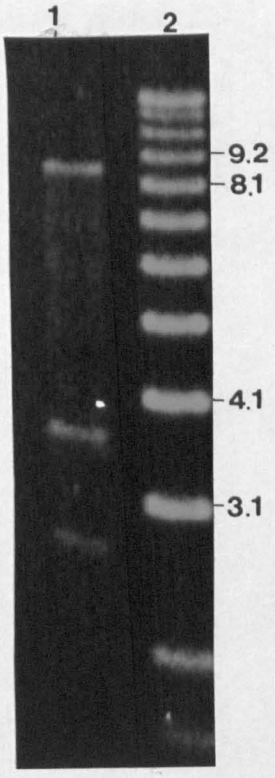


(b) Preparative gel of pIA102 digested with *Sst*I/*Bcl*I.

Lane 1 = \ *Hind*III digest
Lane 2 = 1 kb ladder

Figure 3.9

*Pvu*II digest of the upper band (presumptive *S. hygroscopicus* cloned DNA) from Figure 3.8(b).



Lane 1 = *Pvu*II digest of cloned DNA.
Lane 2 = 1 kb ladder.

cloned *S. hygroscopicus* DNA) and restricted with *Pvu*II. The results of this reaction are shown in Figure 3.9 (lane 1) and show a unique restriction pattern different ot the pattern which would represent fragments of the vector and which also total a size of ca. 15 kb of DNA.

The problems encountered in characterising the cloned DNA may have several sources:

i) Degradation of the plasmid DNA on electrophoresis. Zhou *et al. (1988) reported that S. lividans* DNA was degraded site-specifically on electrophoresis in buffers contaminated with ferrous (Fe^{2+}) iron. It may have been the case that traces of ferrous iron were somehow contaminating either our plasmid samples or, more likely, our electrophoresis kits, buffers or even the agarose used in constructing the gel (trace amounts of Fe^{2+} are known to occur in some batches of EDTA).

ii) Intraplasmid recombination. Both homologous and illegitimate intraplasmid recombination are known to take place in *Streptomyces* (Tsai and Chen, 1987). pIJ61 is a low copy number vector carrying transfer (tra^{+}) functions, it may be that intraplasmid recombinational events were occuring in our clones and thus affecting their stability. It should be noted that a number of restriction experiments were conducted that showed DNA fragments in sub-molar concentrations on electrophoresis (data not presented here), this may be indicative of a mixture of plasmid populations produced by plasmid recombination and for instability. To attempt to overcome this problem the clones have now been transformed into

S. lividans JT46 (kindly supplied by T. Kieser) which is recombination-deficient.

iii) Recombination between the plasmid and the host chromosome. As previously stated *S. lividans* TK24 contains DNA sequences involved in polyketide biosynthesis, the lack of homology between *act*I/*act*III and the "total" DNA from *S. hygroscopicus* 3602 notwithstanding, which may be partly homologous to the DNA cloned into pIJ61. This may allow a Campbell-type recombination to occur and thus any plasmid DNA isolated from the clones may have undergone rearrangments, deletions, etc. Interestingly *S. lividans* JT46 does not prevent chromosomal-plasmid recombination (Kieser *et al.*, 1989) so the use of this strain as a host may tell us more about the actual causes of the perceived plasmid instability we encountered.

3.10 The Bioactivity of pIA101, pIA102 and pIA103

The plasmid clones, both the original transformants and the retransformed strains, were grown on agar in the presence and absence of thiostrepton selection, and in the presence and absence of AHB. Agar plugs bearing colonies from each of these plates were transferred to a pre-seeded lawn of *B. subtilis* and the plugs were scored for the presence or absence of zones of antibiosis following incubation (Figure 3.10). The results of this study are presented in Table 3.3.

The table shows that all of the transformed and retransformed strains exhibit antibiosis against *B. subtilis*. This occurs regardless of whether or not

Legend to Figure 3.10: Antibiosis testing of transformants of *S. lividans vs. B. subtilis*.

			Media		Antibiosis
1.	*S. lividans* TK24		$^{+}$ABA	$^{-}$thio	no
2.	Trans^{d}.	pIA102	"	"	"
3.	Retrans^{d}.	pIA101	"	"	"
4.	"	pIA102	"	"	"
5.	"	pIA103	"	"	"
6.	Trans^{d}.	pIA101	"	"	"
7.	"	pIA102	"	"	"
8.	"	pIA103	"	"	"
9.	"	pIA102	$^{-}$ABA	$^{-}$thio	"
10.	"	pIA101	"	"	"
11.	Retrans^{d}.	pIA101	"	"	"
12.	"	pIA102	"	"	"
13.	"	pIA101	"	"	yes
14.	"	pIA102	"	"	"
15.	"	pIA103	"	"	"
16.	Trans^{d}.	pIA101	"	"	"
17.	"	pIA102	"	"	"
18.	Trans^{d}.	pIA101	$^{+}$ABA	$^{+}$thio	no
19.	"	pIA101	$^{-}$ABA	$^{+}$thio	yes
20.	"	pIA102	"	"	no
21.	"	pIA103	"	"	yes
22.	Retrans^{d}.	pIA102	"	"	no
23.	"	pIA103	"	"	yes
24.	Trans^{d}.	pIA102	$^{+}$ABA	$^{+}$thio	no
25.	"	pIA103	"	"	"
26.	Retrans^{d}.	pIA103	$^{-}$ABA	$^{+}$thio	yes
27.	*S. lividans* TK24		$^{-}$ABA	$^{-}$thio	no

Trans^{d}. = *S. lividans* TK24 transformed with the named plasmid.

Retrans^{d}. = indicates where plasmid has been isolated from *S. lividans* TK24 and reintroduced to determine stability of the acquired phenotype.

Figure 3.10

Antibiosis testing of transformants of *S. lividans vs. B. subtilis*.

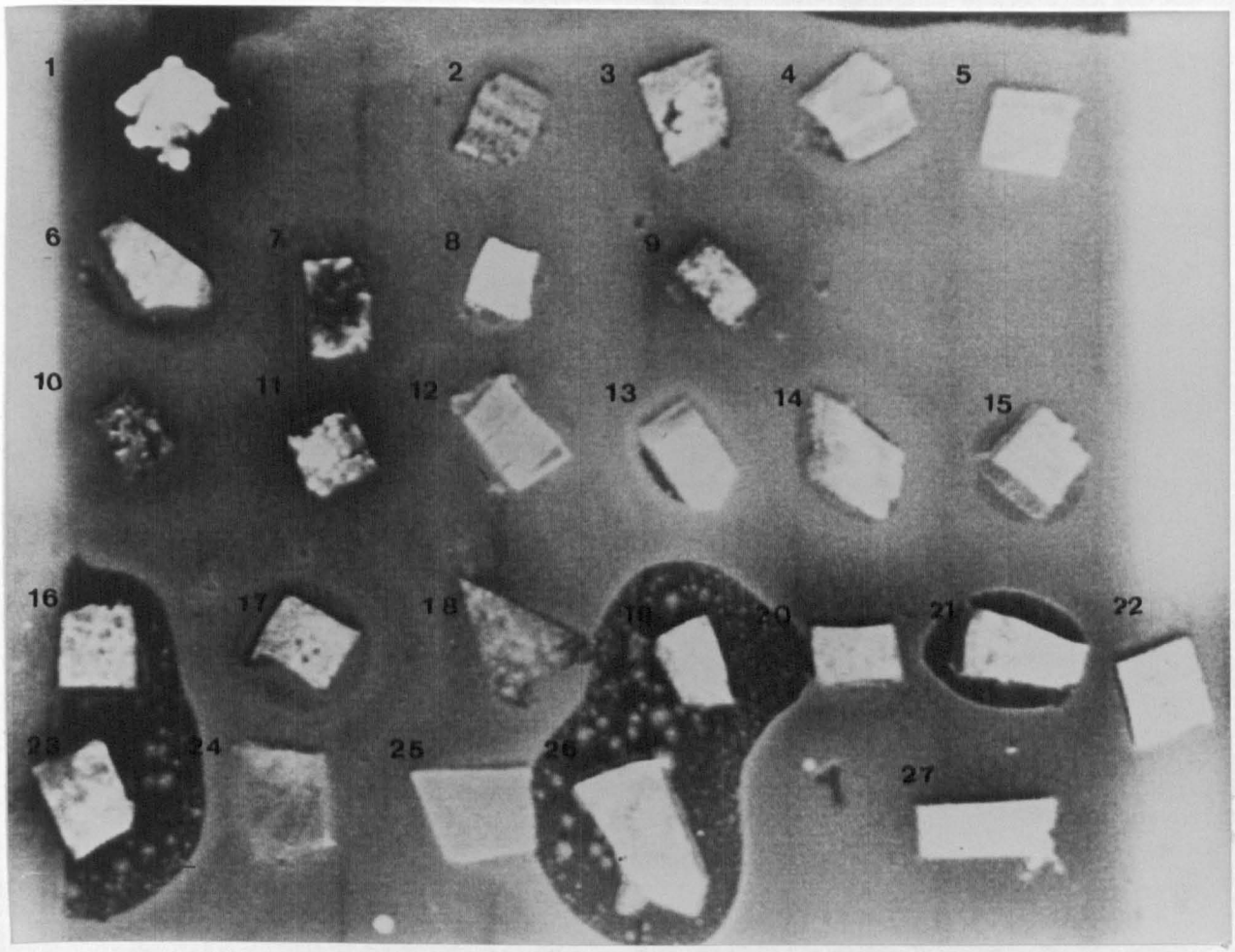


Table 3.3

Antibiosis test for clones pIA101, pIA102 and pIA103

Strains	Antibiosis on agar plugs vs. *B. subtilis*			
	$^{-}$AHB/$^{-}$thio	$^{+}$AHB/$^{-}$thio	$^{-}$AHB/$^{+}$thio	$^{+}$AHB/$^{+}$thio
S. lividans TK24	-	-	NT	NT
Trans^{d}. with pIA101	+	-	+	-
Trans^{d}. with pIA102	+	-	+	-
Trans^{d}. with pIA103	+	-	+	-
Retrans^{d}. with pIA101	+	-	NT	NT
Retrans^{d}. with pIA102	+	-	+	NT
Retrans^{d}. with pIA103	+	-	+	NT

\+ = presence of zones of antibiosis.

\- = absence of zones of antibiosis.

NT = not tested.

thiostrepton is present in the growth media thus ruling out the possibility of toxicity effects caused by this drug. Interestingly, antibiosis only occurs where AHB is absent from the growth media. It is possible that AHB induces resistance to the antibiotic produced by the strains or, more likely, it is interfering with the production of the antibiotic, e.g. by acting as a negatively regulatory factor. The *S. lividans* TK24 control does not exhibit this antibiosis effect either in the presence or absence of AHB, but as it is thiostrepton-sensitive could not be tested in the presence of thiostrepton.

3.11 Discussion

The data presented in this chapter suggest that a fragment or fragments of *S. hygroscopicus* 3602 have been cloned via the vector, pIJ61, into the host *S. lividans* TK24. This fragment(s) has proved difficult to physically characterise but would appear to be approximately 15 kb in size. For reasons explained above the *S. hygroscopicus* DNA is not stably maintained in *S. lividans* TK24, but we hope it will be in the recombination deficient host *S. lividans* JT46. Although not physically stable the transformants and retransformants of pIA101, pIA102 and pIA103 all appear phenotypically stable in that they all exhibit antibiosis on the indicator, *B. subtilis*, a phenomenon not normally attributed to the host strain. This antibiosis is seen to be independent of the addition of the geldanamycin precursor AHB. We therefore conclude that we have successfully cloned

fragments of *S. hygroscopicus* 3602 DNA into a host and that these fragments are directing the synthesis of an antibiotic not normally produced by the host.

Further studies of these clones were performed in an attempt to characterise the antibiotic more fully and the results of these studies will be presented in the next chapter. We obviously hoped that we had cloned the genes for geldanamycin biosynthesis but we were aware of several other possibilities, which will also be discussed in the next chapter.

CHAPTER 4

FUNCTIONAL CHARACTERISATION OF CLONES pIA101, pIA102 and pIA103

4.1 Introduction

Chapter 3 described the isolation of clones pIA101, pIA102 and pIA103 which contain inserts of *S. hygroscopicus* 3602 DNA and exhibit antibiosis against *B. subtilis*. This chapter reports the results of various tests designed to characterise the substance or substances that determine this antibiotic effect. It was hoped that the clones were producing geldanamycin however, there are several other possibilities.

(i) The antibiotic expressed by the clones was unrelated to geldanamycin, e.g. the genes were for another unrelated antibiotic, e.g. nigericin, which is also produced by *S. hygroscopicus* 3602.

(ii) The introduction of "foreign" *S. hygroscopicus* DNA into *S. lividans* could have switched on a cryptic antibiotic biosynthetic pathway encoded on the host chromosome.

(iii) The antibiotic expressed was not geldanamycin but was either an intermediate(s) in the geldanamycin biosynthetic pathway or a shunt-product(s) produced because only a partial set of geldanamycin genes were represented by the clones. This was considered a likely possibility in view of the small size of the cloned DNA inserts.

(iv) There was functional interaction between gene products encoded on the cloned DNA and by the host chromosome.

These resulting substances might be hybrid structures comprising part of the geldanamycin molecule and part of a host encoded antibiotic, e.g. actinorhodin. This is not impossible given that actinorhodin and geldanamycin are both polyketide antibiotics and that Hopwood *et al.* (1990) had shown that "hybrid" antibiotics could be manufactured using recombinant DNA techniques.

Assays were therefore sought that would identify the antibiotic produced by these clones and distinguish between these possibilities. The problem of identification was compounded because Streptomycetes are known to produce a variety of extracellular compounds from which the antibiotics expressed by the clones must be separated. The primary method chosen for separation and analysis was thin-layer chromatography.

4.2 Thin-Layer Chromatography (TLC)

TLC is a commonly used separation procedure, being both relatively rapid and simple, and has been successfully employed to separate compounds from diverse chemical groups, e.g. lipids and nucleic acids; it was quickly adapted for the separation of a great variety of compounds, particularly pharmaceuticals. TLC can be used in antibiotic research in a variety of ways: 1) to follow antibiotic production; ii) to monitor antibiotic purification; iii) to characterise and classify both pure

antibiotics and impure antibiotic preparations; and iv) to assay antibiotics quantitatively.

The TLC method used in this study was that of adsorption chromatography. This physiochemical process allows the separation of mixtures of compounds according to the numbers and types of their functional groups. Separation occurs because of differences in the reversible physical forces exerted by the stationary phase on the functional groups of each of the components of the mixture. Functional groups that influence adsorption include polar groups such as: alcohol, aldehyde, carboxyl, and amine. The formation of transient hydrogen bonds and the occurrence of stoichiometric effects markedly influence adsorption, e.g. *trans* isomers are more strongly adsorbed than their respective *cis* counterparts. Many adsorbent materials are available and from these silica gel was chosen. For elution, apolar solvents or mixtures of polar and apolar solvents may be used, and the use of several solvent systems for the elution of any single mixture of compounds is advisable. The two solvent systems utilised in this study were both mixtures of polar and apolar solvents: i) ethyl acetate: dichloromethane: hexane:methanol (9:6:6:1) [the TLC eluent used for the purification of herbimycins - a benzoquinoid ansamycin closely related to geldanamycin, Omura *et al.* (1979), Iwai *et al.* (1980)], and ii) chloroform:methanol (9:1) (DeBoer *et al.*, 1970).

An ascending technique of chromatography was employed, i.e. the eluent mixture was placed in the

bottom of a lined glass tank into which the chromatoplate, loaded with sample, is placed with the sample about 0.5 cm above the level of the developing mixture. The developer therefore travels up the plate separating the sample in the process.

When the chromatogram has been developed the mobile phase must be allowed to evaporate from the chromatoplate before the spots on the plate can be visualised. A variety of means were used to visualise the chromatograms and these will be discussed in detail later in this chapter.

4.2.1 A Note on Rf Values and Reproducibility

Compounds separated by TLC migrate to specific spots on the chromatoplate and the distance from the point of sample application to the site of migration is a characteristic of the particular compound when developed by any given system. The standard method of presenting this data is by giving the Rf value of the compound. The Rf is defined as the distance a given spot migrates relative to the distance that the solvent front migrates.

There are several factors that affect the reproducibility of Rf values of which solvent equilibrium in the absorbent and in the vapour phase is perhaps the most important. This can be attributed to several physiochemical events depending on factors such as vapour pressure, relative affinities of the absorbent material for the components of the solvent system, conditions and geometry of the TLC tank and solvent saturation

parameters. These factors affect one another in a complicated way especially at the solvent front where the separation of components occurs. At this point the solvent preferentially adsorbed from the vapour phase, which, being mixed, may be different from the solvent which, as a liquid, is the fastest moving constituent of the developing mixture and which constitutes therefore the solvent at the boundary. The qualities of the absorbent also influence the reproducibility of Rf values. These qualities include, particle size, pore volume and diameter and number of active sites at the adsorbent surface. These qualities should be reasonably standardised for pre-coated chromatography plates manufactured to specific tolerances, particularly from plates in the same batch. However, one quality which is not so easy to standardise is the adsorption capacity of the adsorbent particularly with respect to its moisture content. This physisorbed water prevents the adsorption of other less-polar solvents and therefore makes the adsorbent effectively less active. The amount of physisorbed water in silica gel depends on the number of surface hydroxyl groups and the extent of exposure of the plate to water vapour (moisture) before and during chromatography. Other factors known to affect Rf reproducibility are temperature, running distance, sample load, application technique and the thickness of the adsorbent layer.

Taking all of these factors into account it becomes clear that Rf values quoted in the literature for a given

compound should not be regarded as the definitive proof of identify for that compound. Moreover, whereas it should be possible to obtain reproducible Rf values in one laboratory it may be difficult to reproduce these values exactly in another laboratory, particularly as the exact experimental conditions are not always reported. For these reasons samples separated by TLC are best examined alongside purified standards which in this study was co-chromatography with purified geldanamycin. For a full account of the theoretical considerations of TLC see reviews by Aszalos and Frost (1975) and Niederwieser and Pataki (eds.) (1970).

4.2.2 TLC of Clone Broth Extracts

Cultures of *S. lividans* containing the clones pIA101, pIA102 and pIA103 were grown in YEME, the mycelia removed by centrifuge and the spent broth extracted with ethyl acetate, in which geldanamycin is readily soluble. The spent broth extracts were separated by TLC as described in Chapter 2.

Geldanamycin and other compounds can be visualised on these TLC plates under U.V. light, due to the presence of a U.V. fluorescent marker included in the adsorbent layer of the chromatoplate. However, analysis of the clone broth extracts by this method proved to be difficult. This was principally due to the complexity of the TLC profiles of the clone broth extracts and to differences in loading factors. It is impossible to know the concentration of constituents of the spent broth extracts

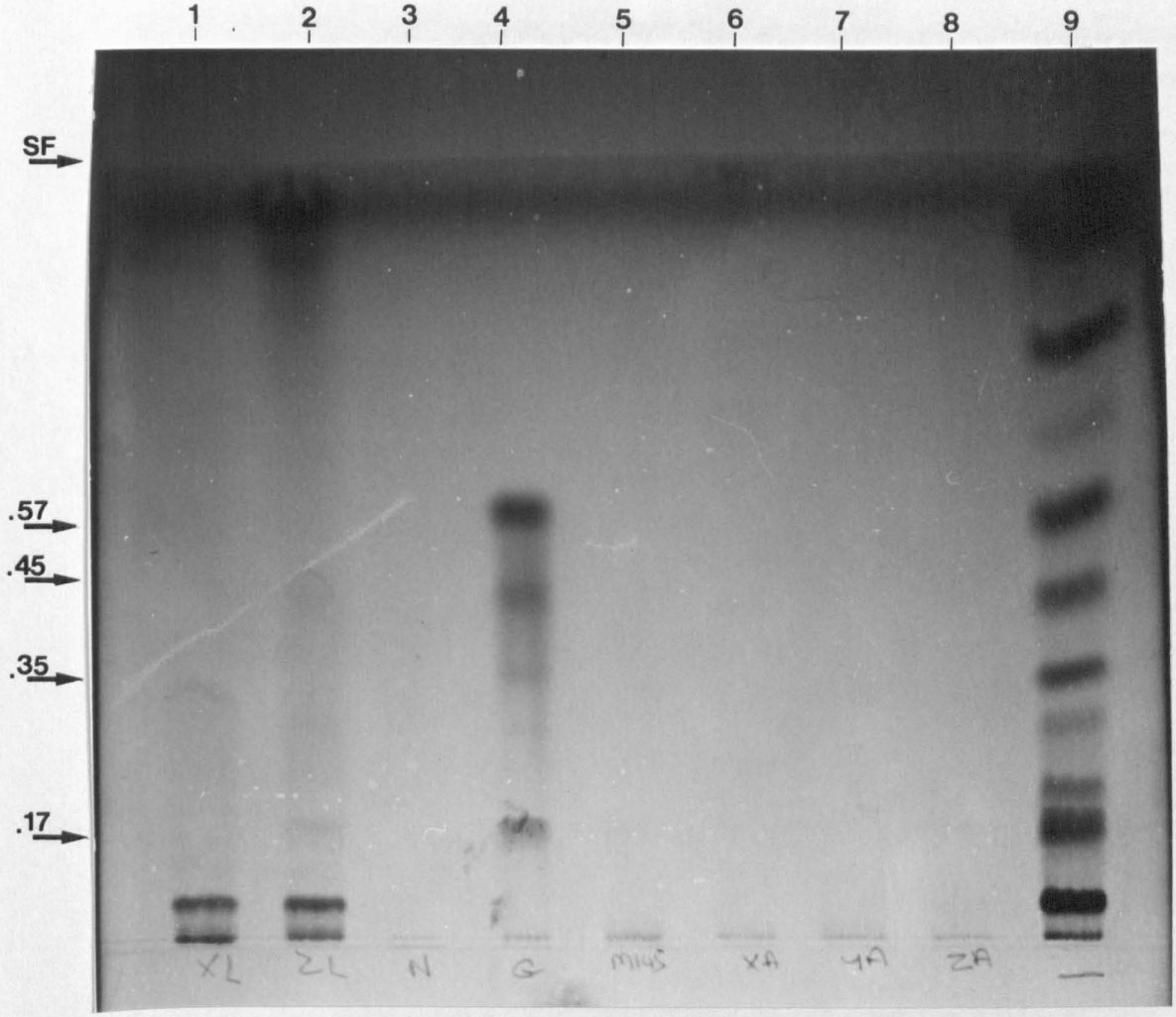
or their relative concentations prior to TLC. Some examples of these separations can be seen in Figures 4.1 to 4.4. In general, some similarities can be seen between the separations of the clone broth extracts and those of purified geldanamycin however, the data is not totally convincing.

An alternative method to identify the compounds on the chromatoplates was therefore sought. To this end plates loaded and developed as described above were treated with acid-vanillin and heated *in vacuo*. Under these conditions acid-vanillin reacts with geldanamycin to produce a blue-green colour. Therefore this reaction is potentially suitable to the detection of the presence of geldanamycin on TLC plates. However, the acid-vanillin is not specific for geldanamycin and will react with many compounds to give a coloured product. This fact confirmed the analysis of the TLC separations of the clone broth extracts, since these comparatively crude extracts contained many compounds that reacted with acid-vanillin to produce spots of various colours throughout the length of the separation. Again the broth extracts showed spots that might have indicated the presence of geldanamycin, but the "background" level of colour from the other spots on the chromatoplate reduced the confidence of these data.

In an attempt to clarify the situation a further method which combined the physiochemical technique of TLC and the biological technique of a bioassay, a process called bioautography, was used.

Figure 4.1

TLC chromatography of broth extracts of *S. lividans* containing pIA101, pIA102 and pIA103.



TLC chromatoplate developed in -

ethyl acetate:dichloromethane:hexane:methanol
9 : 6 : 6 : 1

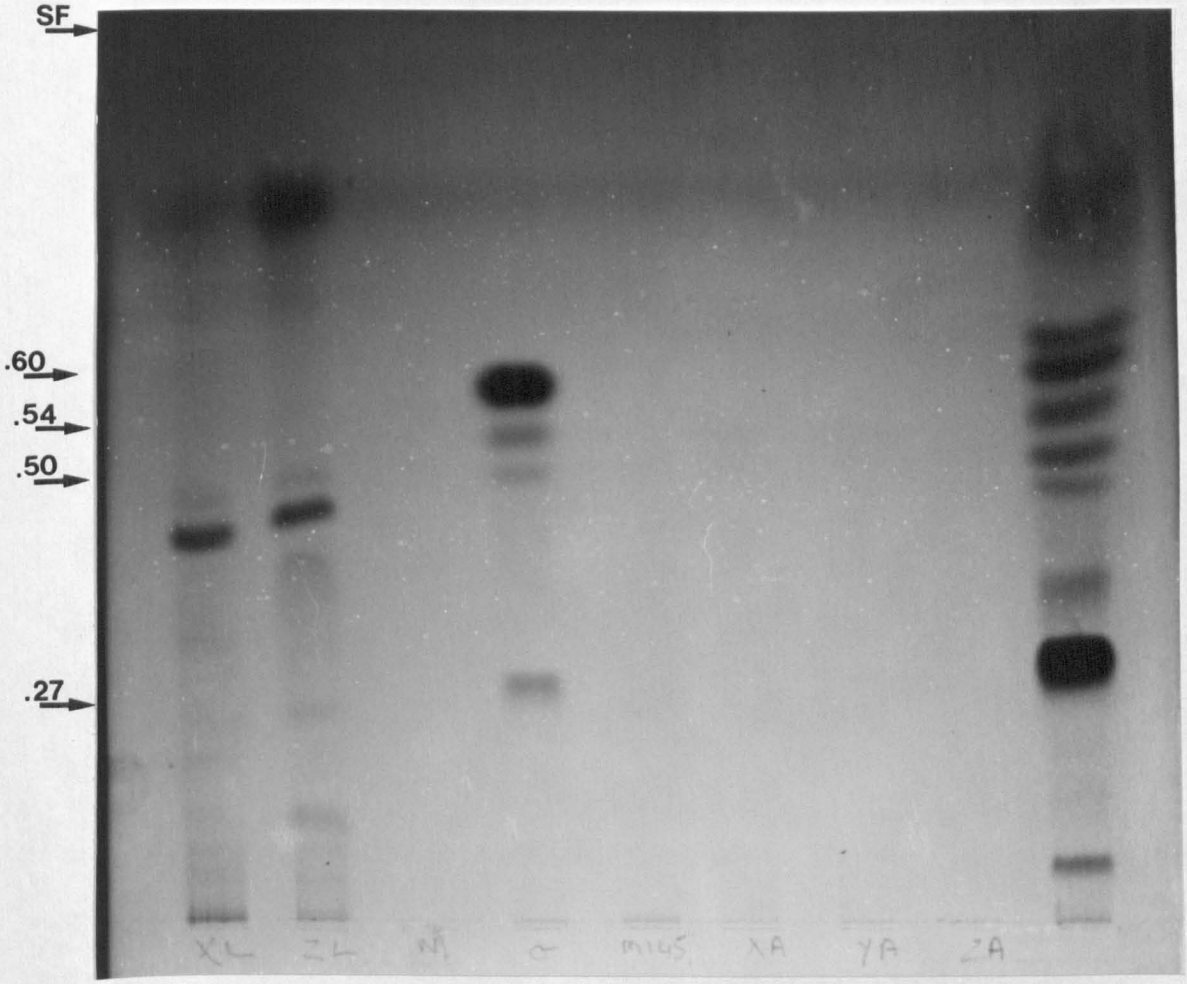
1	Clone broth extract of X
2	" " " " Y (pIA103)
3	Nigericin
4	Geldanamycin
5-8	Next 4 lanes underloaded
9	Geldanamycin producer

SF = solvent front.

Note the similarities between pIA103 and the purified geldanamycin standard at Rfs. 0.45 and 0.17, however, other bands present in the purified geldanamycin are not present in the clone broth extracts.

Figure 4.2

TLC chromatography of broth extracts of *S. lividans* containing pIA101, pIA102 and pIA103.



T.L.C. chromatoplate developed in chloroform:methanol.
9 : 1

Lanes as for Figure 4.1

SF = solvent front.

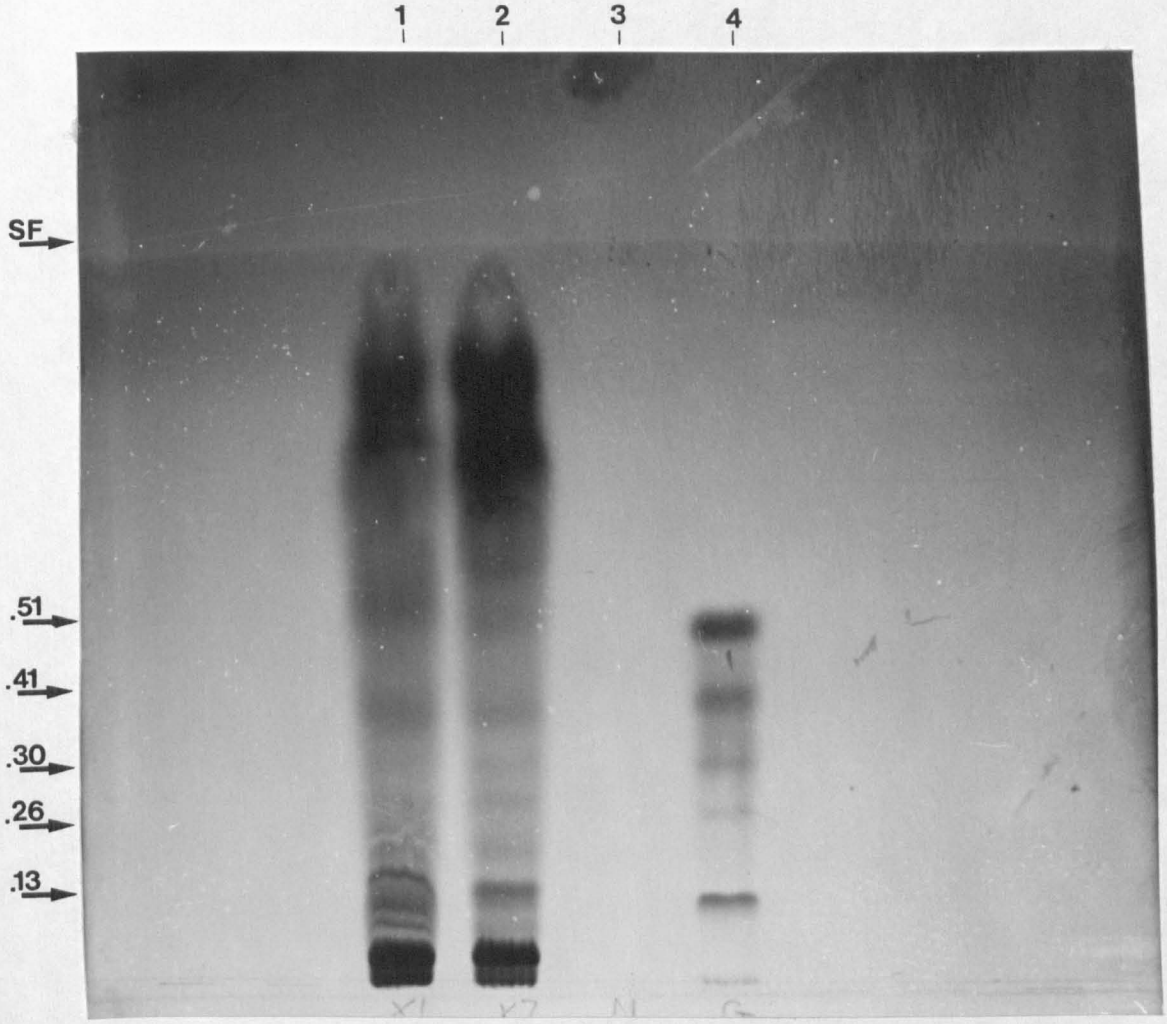
Note the similarities between pIA103 and purified geldanamycin at Rfs 0.61 and 0.34, however apart from these the lanes look dissimilar.

(It is probable that the chromatograms represented by Figures 4.1 and 4.2 are underloaded and that a greater concentration of broth extract applied to the plate may be more illuminating.)

DeBoer *et al.* (1970) give the Rf value of geldanamycin as 0.56 when chromatographed under similar conditions (but for the reasons given in Section 4.2.1 not identical conditions).

Figure 4.3

TLC chromatography of broth extracts of *S. lividans* containing pIA101 and pIA103.



TLC chromatoplate developed in -

ethyl acetate:dichloromethane:hexane:methanol
9 : 6 : 6 : 1

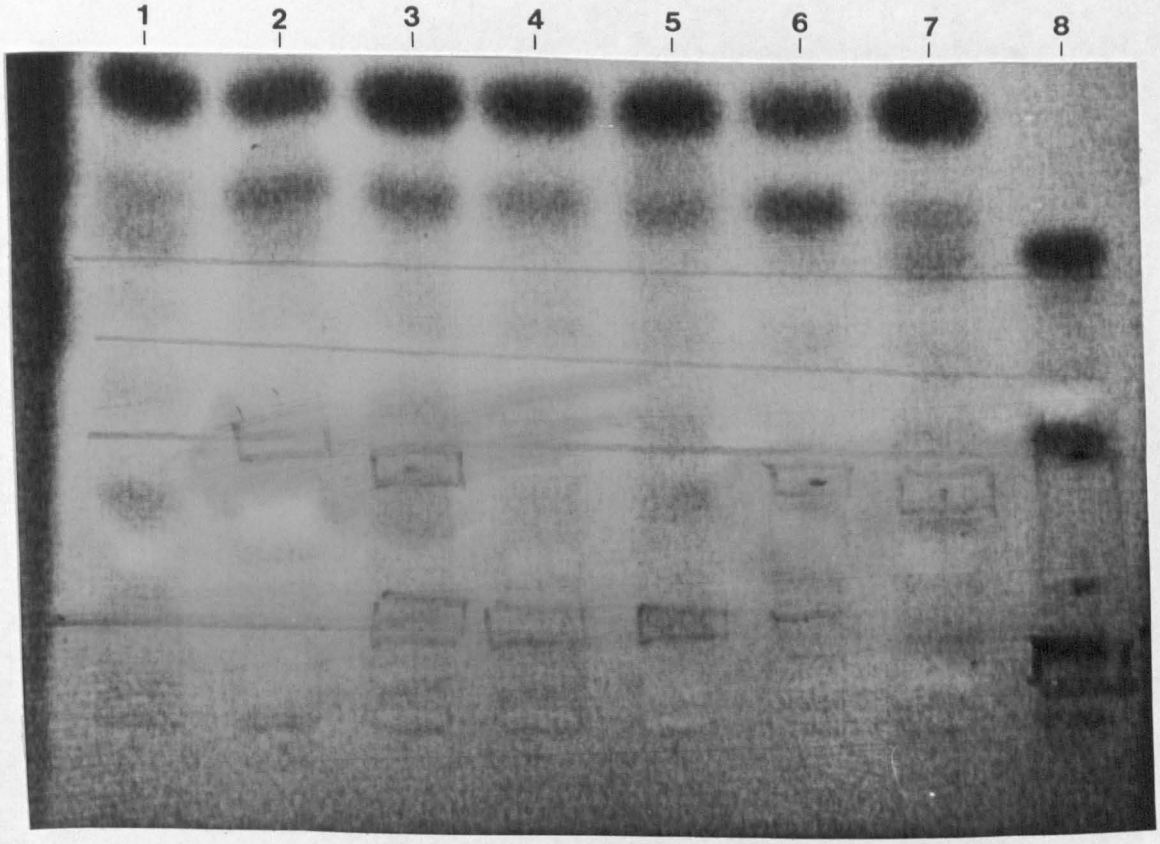
1 Clone broth extract of pIA101.
2 " " " " pIA103.
3 Nigericin
4 Geldanamycin
5-8 Next 4 lanes underloaded.
9 Geldanamycin producer.
SF = solvent front.

This experiment is a re-run of the experiment presented in Figure 4.1 but with an increased sample load. Bands can be seen in the clone broth extracts of pIA101 and pIA103 at Rfs corresponding to those of all of the bands seen in the purified geldanamycin separation (namely Rfs. 0.51, 0.41, 0.30, 0.26 and 0.13).

It may also be noted that the Rf values from geldanamycin on this chromatoplate are different to those in Figure 4.1 despite the samples being identical and the plates having been developed in the same tank on the same day. The loading factor is probably affecting reproducibility.

Figure 4.4

TLC of various clone broth extracts *vs.* geldanamycin.



TLC chromatoplate developed in -

ethyl acetate:dichloromethane:hexane:methanol
9 : 6 : 6 : 1

1. pIA101
2. pIA102
3. pIA103
4. pIA101
5. pIA102
6. pIA103
7. Geldanamycin producer
8. Geldanamycin

The solvent front is not visible on this plate as it has been over-developed, i.e. the solvent was allowed to run right to the top of the plate to increase separation.

Again similarities can be seen between all of the clone broth extracts and the separated components of the purified geldanamycin but the results could not be seen as absolute proof of the presence of geldanamycin in the clone broth extracts and so further evidence was sought.

4.2.3 Bioautography of Clone Broth Extracts

TLC plates were loaded and developed in ethyl acetate:dichloromethane:hexane:methanol (9:6:6:1) as described in section 4.2.2, but following evaporation of the developing mixture the plates were overlaid with soft agar seeded with geldanamycin-sensitive *B. subtilis* indicator cells. Following incubation these plates were scored for zones of inhibition of the *B. subtilis* indicating the presence of an antibiotic at that position on the chromatoplate. The combined results for a series of such experiments (Table 4.1) indicate a considerable similarity between the patterns of all three clone broth bioautographs and that for purified geldanamycin. In particular these samples all exhibited antibiosis against *B. subtilis* at positions with Rf values of 0.15 (± 0.02), 0.33 (± 0.02) and 0.68 (± 0.02).

A variation of ± 0.02 calculated from 5-7 individual Rf values is sufficiently low to be accounted for by the inherent variability of Rf values [Aszalos and Frost (1975)], especially as this variation falls within the standard errors calculated for each of the individual Rf values presented (Table 4.1). These results are a good indication that the clones derived from *S. hygroscopicus* were expressing biosynthetic genes involved in geldanamycin production in *S. lividans* TK24. As a further test of the identity of the antibiotic produced by the cloned inserts we analysed the clone broth extracts by high-performance liquid chromatography.

Table 4.1

Results of TLC Bioautography for Clone Broth Extracts

Rf values for TLC bioautographs - geldanamycin and clone broth extracts. (Rfs given are means of 5-7 repeats.)

Geldanamycin		pIA101		pIA102		pIA103	
Rf.	se.	Rf.	se.	Rf.	se.	Rf.	se.
0.15	0.01	0.15	0.01	0.13	0.00	0.15	0.02
0.33	0.02	0.31	0.01	0.35	0.02	0.31	0.02
0.68	0.01	0.69	0.01	0.70	0.02	0.69	0.02

The host strain *S. lividans* TK24 was seen to produce zones of inhibition of growth of the *B. subtilis* at positions corresponding to Rfs of 0.08 and 0.12. The former of these spots was also present in all of the clone samples but the presence or absence of the latter could not be determined with any surety because geldanamycin displays such a "killing" spot at Rf 0.15 and so it was impossible to ascribe the reason for the zones of growth inhibition in samples pIA101, pIA102 or pIA103 at approximately Rf = 0.15 to either a host encoded factor or a plasmid encoded factor.

4.3 High Performance Liquid Chromatography (HPLC)

HPLC is now considered to be the best of the available methods for the analysis of natural products present in biological matrices such as fermentation broths. This method is applicable to a diverse variety of compounds and in separating these compounds from complex mixtures the protocols used can be optimised to be selective, sensitive and quantitative.

HPLC equipment consists of a constant volume pump or pumps which pressurise a liquid (either a mixture of con-

stant proportions - isocratic, or a mixture of varying proportions - a gradient) at a flow rate of 1-2 ml/min^{-1} through a separating column at a working pressure of 2-300 bar. The separating column is typically a stainless-steel tube containing silica particles 3 to 10 μm in diameter. Reversed-phase columns, as used in this work, are packed with silica bonded to hydrocarbon chains, most commonly C_{18}-octadecylsilane, these chains in turn being bonded to highly polar groups, e.g. phenyl, cyclo-hexyl and fluorocarbon groups.

Many types of detector can be used to analyse the eluant from the column, but the most common is U.V. light, because a large number of analytes contain one or more groups (chromophores) that absorb U.V. light. The benzene moiety of the geldanamycin molecule is such a U.V. absorbing chromophore. The U.V. absorbance of the analyte, as measured by the detector, can be displayed on a strip type chart recorder as a function of time, and so the retention times of compounds on a particular column eluted with a particular mobile phase can be found. Retention times are therefore a convenient method of expressing the results of a HPLC separation.

All of the sample applied to a HPLC column should be recoverable so a mechanised fraction collector can be used to collect the eluant from the column. The chart recording can be used to calculate which fractions contain the compounds of interest and these can be recovered from the solvent phase and used for further analysis, e.g. by biological methods. Alternatively, this type of

system could be used to purify single compounds from mixtures, as in preparative HPLC.

4.3.1 HPLC of Clone Broth Extracts

Broth extracts of clones pIA101, pIA102 and pIA103 in *S. lividans* were prepared as previously described and separations of these mixtures were attempted by HPLC. The retention times of these broth extract separations were compared with purified geldanamycin using an identical system. Several sets of HPLC conditions were used. Initially a mobile phase consisting of acetonitrile/water was used as such a system had been used by Gidoh *et al.* (1981) to separate rifampicin from its main metabolites.

i) Mobile phase (degassed). 20% acetonitrile (w/v)

Flow rate 1 $ml.min^{-1}$.

Pressure 71 bar.

Detector U.V. at 254 nm.

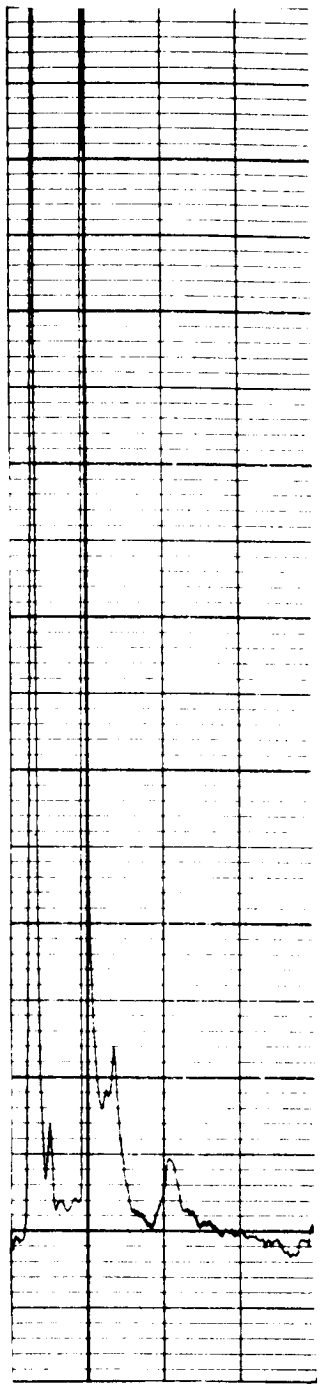
Peaks were seen on the chart at the following retention times -

Purified geldanamycin	30s ; 180s ; 300s (with an additional minor peak at 120s)
Clone pIA102 extract	30s ; 170s ; 300s (with an additional minor peak at 120s)

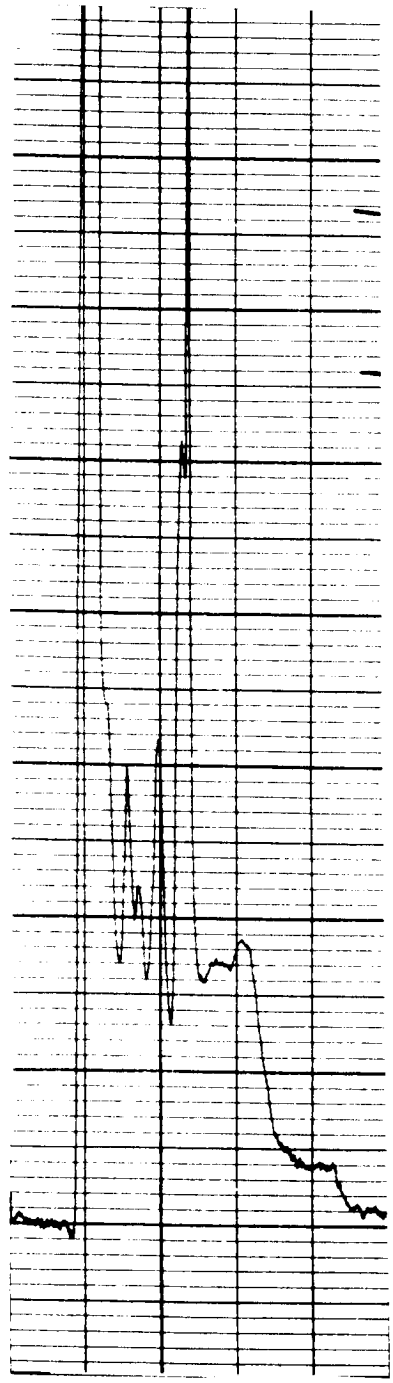
These results are shown in the chart recordings, Figures 4.5a and 4.5b.

Figures 4.5a and 4.5b

HPLC of geldanamycin (Figure 4.5a) and clone broth extract pIA102 (Figure 4.5b).



a



b

The fractions eluted from the column were collected and those corresponding to the peaks on the chart recording were evaporated to dryness *in vacuo*, the solids resuspended in ethyl acetate and analysed by TLC bioautography as described in section 4.2.3. The bioautography results (Table 4.2) show that one of the compounds separated by HPLC (Rt = 180s) gives a zone of growth inhibition of *B. subtilis* at Rf 0.70 which is a similar Rf value to one of the growth inhibition zones found for purified geldanamycin on TLC bioautographs (Table 4.1). The absence of any other zones of growth inhibition may be due to insufficient antibiotic loaded to the chromatoplate to be detected by this assay.

While the chart recordings (Figures 4.5a and 4.5b) do show a separation of the components of geldanamycin and a similar separation for the clone broth extract, the possibility of increasing the resolution was tested by altering the mobile phase of the system.

ii)	Mobile phase	10% to 40% acetonitrile (w/v) gradient in 20 mins.
	Flow rate	1 $ml.min^{-1}$.
	Pressure	70 bar.
	Detector	U.V. at 254 nm.

The use of a gradient instead of an isocratic system should increase the resolution of the separations. The results from a system run with a gradient of mobile phase can be represented by expressing the percentage of the gradient at the point at which a compound elutes from the

Table 4.2

Rf values for TLC bioautographs - geldanamycin and pIA102 broth extract as separated by HPLC.

Retention time on column(s)	Sample Rf	
	Geldanamycin	pIA102 extract
30	-	-
170/180	0.70	0.70
300	-	-

An Rf of 0.70 for a zone of growth inhibition of *B. subtilis* is the same as one of the growth inhibition Rf values previously determined for geldanamycin (Table 4.1). Previous bioautographs of geldanamycin indicated that three zones of growth inhibition should have been present - it is possible that we did not elute all three of these compounds from the column, or, more likely that not high enough concentrations of the other two compounds represented by peaks with retention times of 30s and 300s respectively were applied to the chromatoplate to exhibit antibiosis.

column, i.e. at which a peak occurs on the chart recording. The results of this analysis are shown in Table 4.3 and Figures 4.6 to 4.10.

These results, at first glance, seem to be contradictory for three reasons.

(i) The *S. lividans* TK24 control, gave three "peaks" with this HPLC system, two of which (14.8% and 32.2% acetonitrile) coincided approximately with two of the "peaks" in the geldanamycin trace.

(ii) The values of *S. lividans* TK24 and for *S. lividans* containing clone pIA101 appeared to be almost

Table 4.3

HPLC separations of geldanamycin and clone broth extracts.

Geldanamycin	S. lividans TK24 (cloning host)	Clone	
		pIA101	pIA103
15.4	14.8	15.1	15.1
-	-	-	16.9
-	28.6	28.9	28.3
31.3	33.2	33.1	-
35.5	-	-	35.2

(Results expressed as the percentage acetonitrile content of the mobile phase at the point of elution of the peak.)

identical, suggesting that pIA101 is not producing any substances additional to those encoded and produced by the host.

(iii) Whereas pIA103 has two "peaks" in common with geldanamycin, at 15.1% and 35.5% acetonitrile, there was no peak present at 31.3% acetonitrile for pIA103, though this was present for geldanamycin, and pIA103 exhibits a peak at 16.9% acetonitrile which was not present in any of the other analates.

Figure 4.6

HPLC of purified geldanamycin.

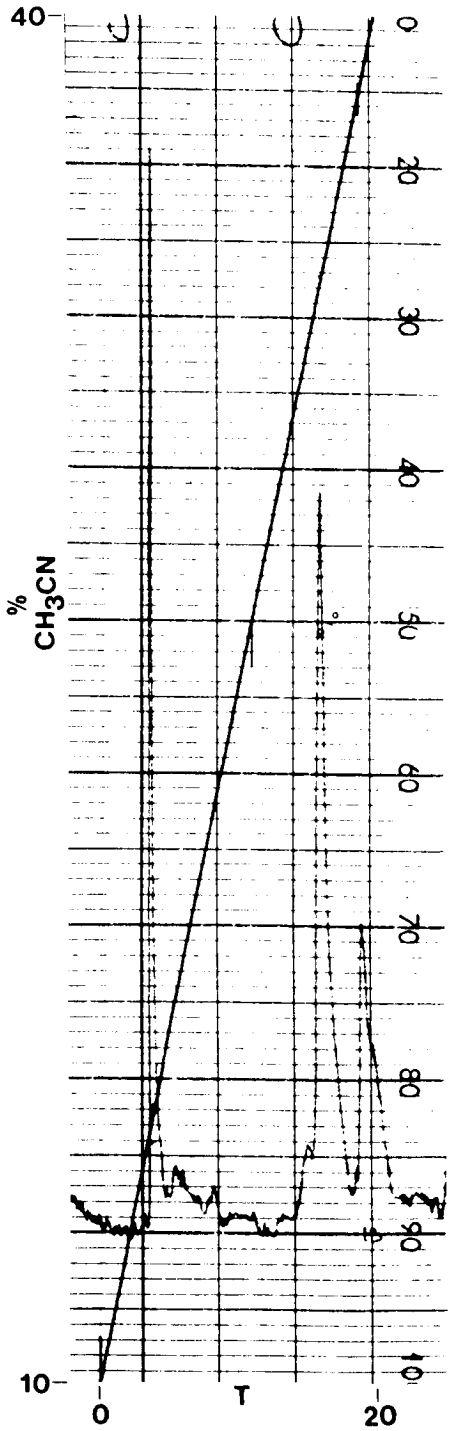


Figure 4.7

HPLC of a *S. lividans* T24 broth extract.

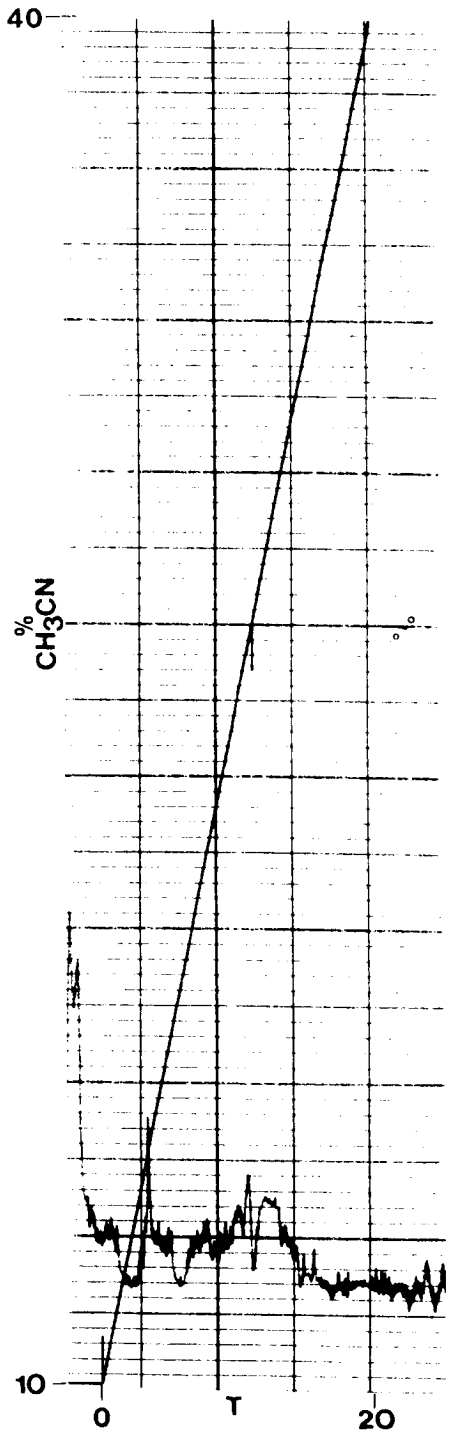


Figure 4.8

HPLC of a clone broth extract (*S. lividans* + pIA101).

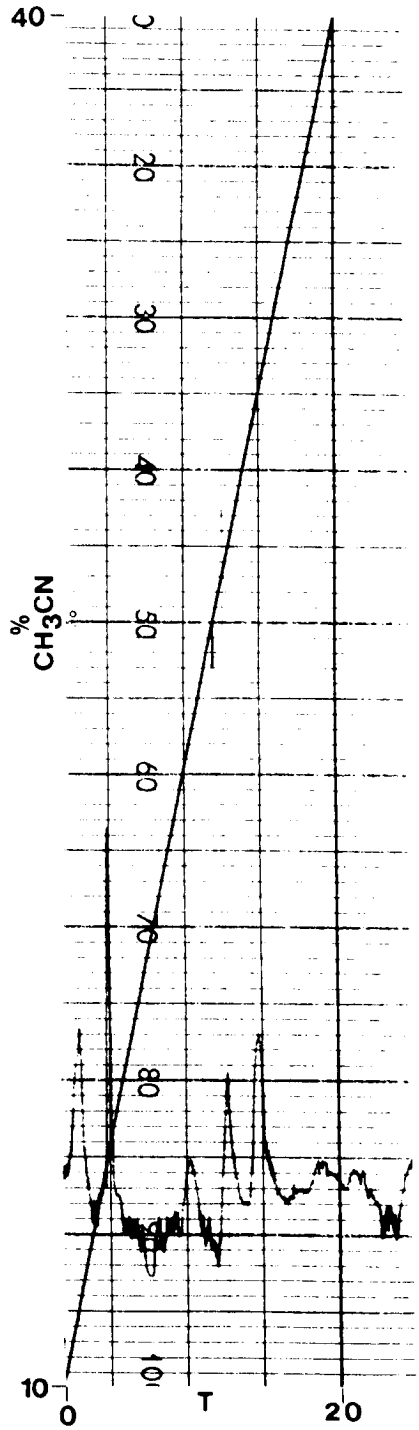


Figure 4.9

HPLC of a clone broth extract (*S. lividans* + pIA103).

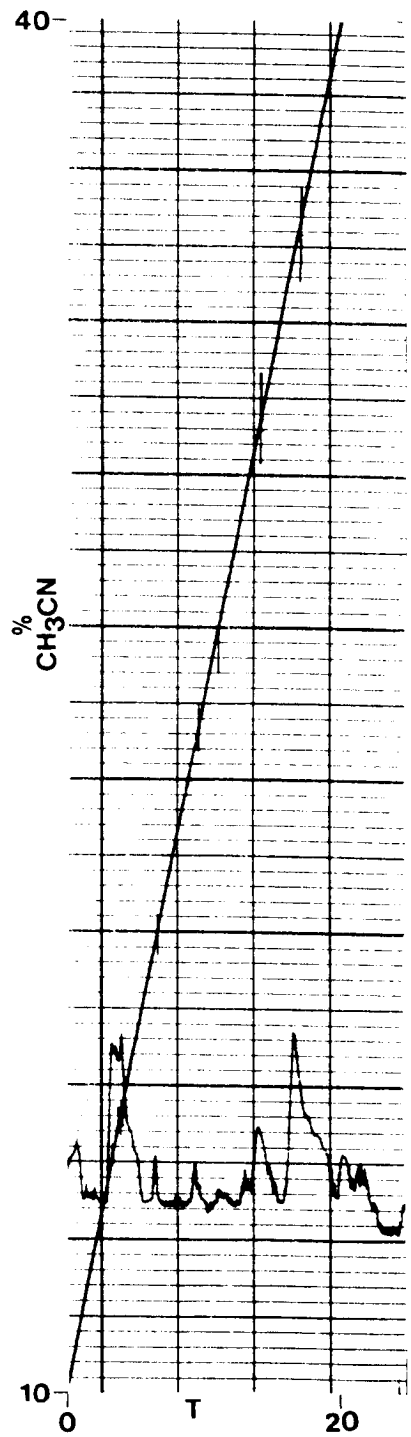
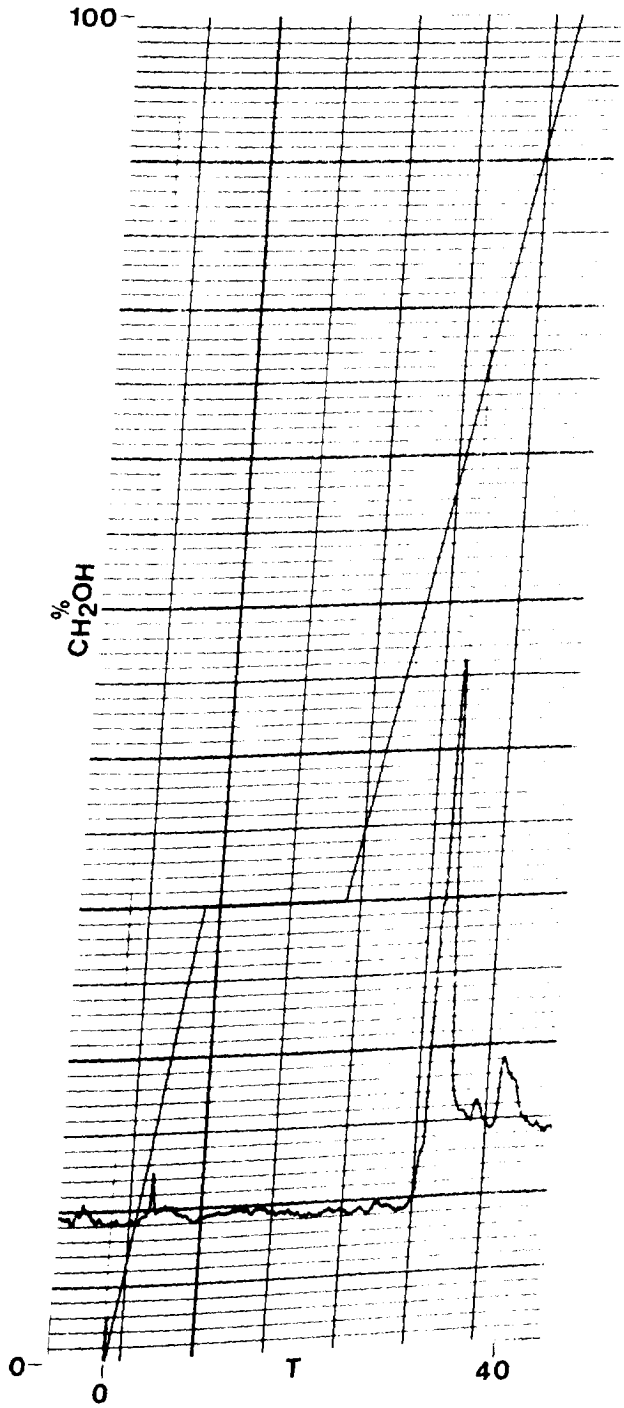


Figure 4.10

HPLC of purified geldanamycin.



There are several possible explanations for these results.

(i) *S. lividans* TK24 is not a producer of the compounds that appear in the purified geldanamycin separation and the separation provided by this particular HPLC system does not sufficiently resolve the difference between the compounds in the different preparations. This is supported by the fact that a number of peaks shown in he chart recordings (Figures 4.6 to 4.9) are not discrete peaks but display "shoulders" which probably indicate the existence of further peaks. In fact the "15.1% acetonitrile peak" (Figure 4.9) provides a good example of a "shoulder" and may therefore represent two peaks (one possibly representing a geldanamycin factor, the other a factor provided from the host). It is interesting to note that the corresponding peaks for purified geldanamycin (Figure 4.6), *S. lividans* TK24 (Figure 4.7) and pIA101 (Figure 4.8) appear not to have this shoulder, perhaps indicating that they represent a purified, single compound. This HPLC system may therefore require modification to increase the resolution.

(ii) The apparent similarities between the broth extracts for *S. lividans* TK24 and clone pIA101 might be explained by the fact that clone pIA101 in this particular preparation did not express the genes for geldanamycin production. Antibiotic biosynthesis is inherently unstable and we know from work on plasmid pIA101 that it is probably physically unstable and so it is not too surprising to find an absence of geldanamycin in some of

the fermentations.

(iii) Little is known about the constituents of the sample of purified geldanamycin supplied to us. Rothrock and Gottlieb (1984) state that geldanamycin (supplied by the Upjohn Company) consisted of three compounds, as separated by HPLC, a finding generally supported by our TLC and HPLC results but what the relationships of these compounds to one another are is not known and so perhaps, the absence of one or more of these compounds from our clone broth extracts should not be viewed as unusual, i.e. some of the "peaks" may represent breakdown products which have not had time to accumulate in the clone broth extracts or, alternatively, they could represent intermediate components which for one reason or another are not accumulating.

In response to these results the mobile phase of our HPLC system was changed in an attempt to improve resolution. A comprehensive search of the available literature did not provide us with a published HPLC protocol for the separation of geldanamycin. However, Deboer *et al.* (1970) reported that geldanamycin is soluble in alcohols but only very poorly soluble in water. Thus a mixture of methanol/water should therefore serve as an ideal eluent for the HPLC of geldanamycin. In addition a gradient system was chosen to give the maximum possible resolution from this mobile phase. The system was optimised experimentally to run as a gradient from 0% to 100% methanol in 40 mins. with a 10 min. isocratic "step" in the gradient at 30% methanol to increase the separation

of the peaks on the chart recordings. The parameters utilised were therefore:

iii)	Mobile phase	0% to 100% methanol (w/v) gradient in 40 mins., with a 10 min. isocratic "step" at 30% methanol.
	Flow rate	1 $ml.min^{-1}$.
	Pressure	72 bar.
	Detector	U.V. at 254 nm.

The results of these analyses can be seen in Table 4.4 and Figures 4.10 - 4.13. The results of our HPLC experiments, i.e. the similarities in profiles between geldanamycin and clone broth extracts, lead us to conclude that the clone broth extracts contained some components in common with the components of the purified geldanamycin that was supplied to us. On consideration of the HPLC data in conjunction with the data from TLC (especially the bioautography) we conclude that it is likely that our clones were producing, in liquid culture, compound(s) that were at least very closely related to geldanamycin, if not in fact geldanamycin itself. The fact that the host organism *S. lividans* TK24 did not show such extensive similarities with geldanamycin suggests that we have successfully cloned at least part of the geldanamycin pathway into this host and that this pathway is expressing genes for the manufacture of geldanamycin.

Table 4.4

HPLC separations of gedanamycin and clone broth extracts.

(Results given as retention times (R_t) for the compounds on the column.)

Geldanamycin	pIA101	pIA101	pIA103
2.92	2.00	2.25	2.50
	4.25	21.00	19.50
	7.00		
22.58	23.875	22.80	22.50
			23.75
25.15	24.87	25.00	24.75
27.07			25.75
29.36	28.25	28.25	28.00

Geldanamycin results represent the means of four runs.

Clone broth extracts represent the means of two runs.

Quite marked similarities can be seen between the profile for purified geldanamycin and those for the clone broth extracts.

It is not possible at this stage to conclude that we have cloned the whole of the geldanamycin biosynthetic pathway. We cannot rule out the possibility of some biochemical input from the host organism in the early stages of geldanamycin production, especially when one considers that *S. lividans* TK24 is known to possess polyketide synthesis genes.

Figure 4.11

HPLC of a clone broth extract (*S. lividans* + pIA101).

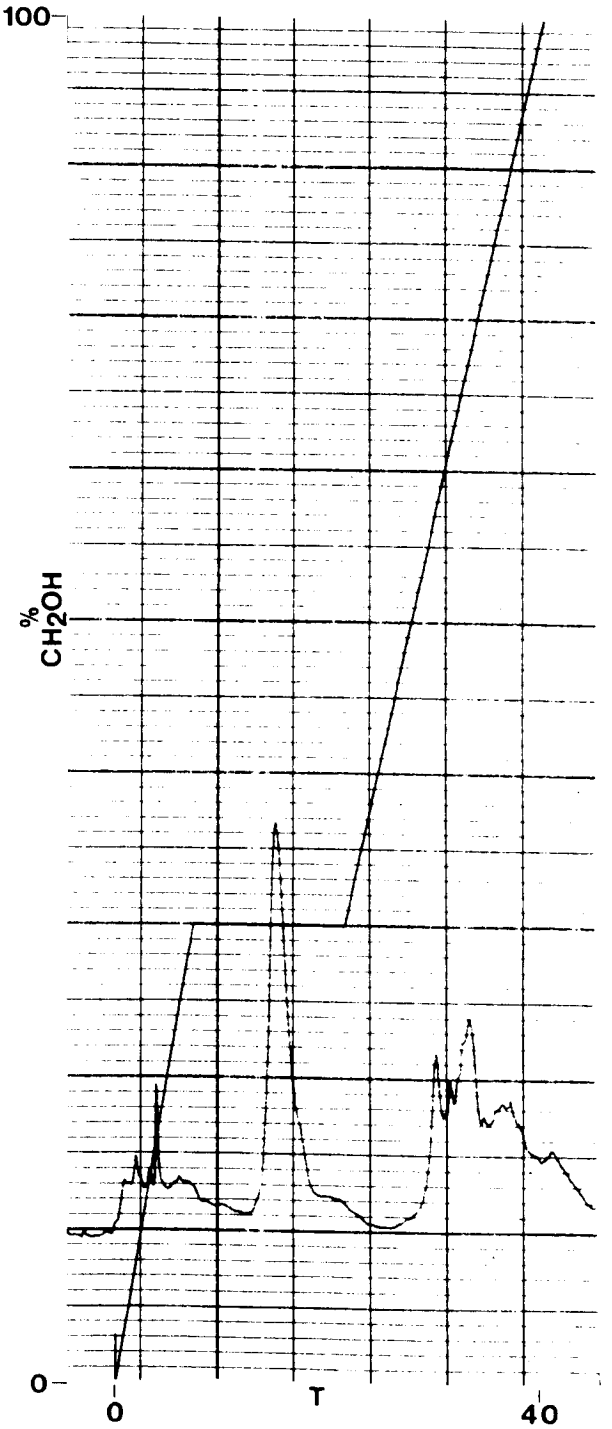


Figure 4.12

HPLC of a clone broth extract (*S. lividans* + pIA102).

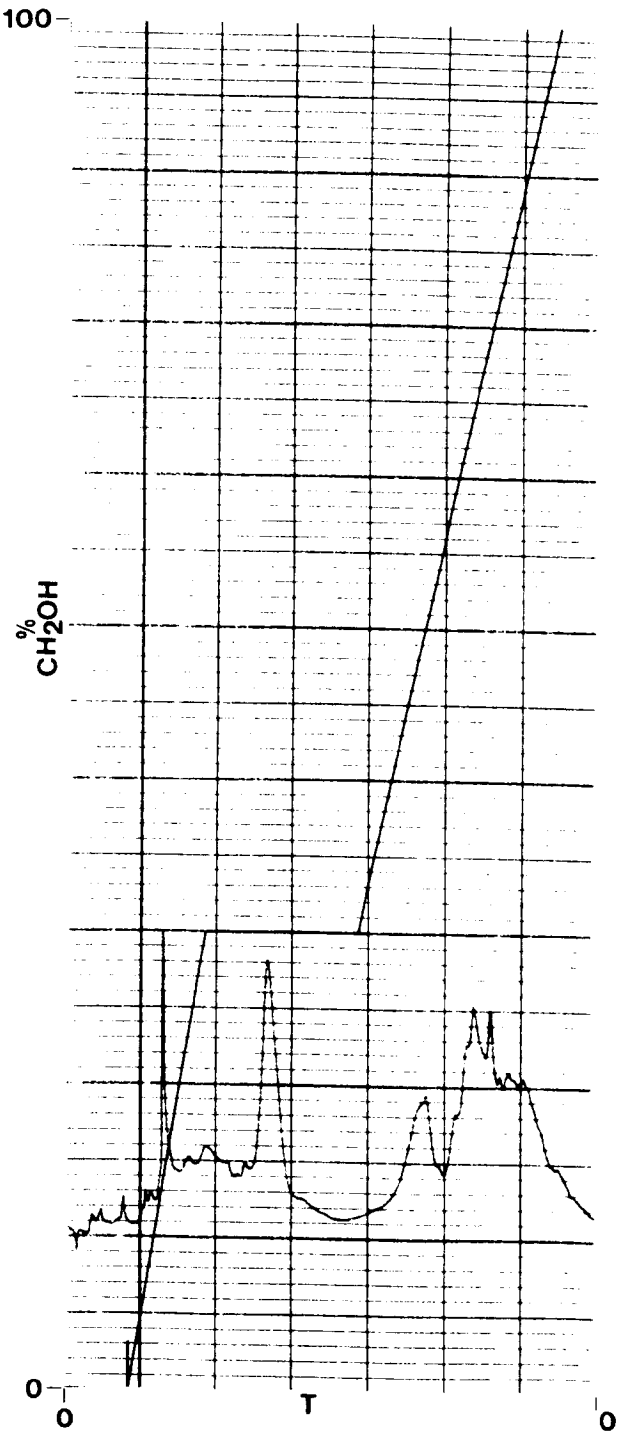
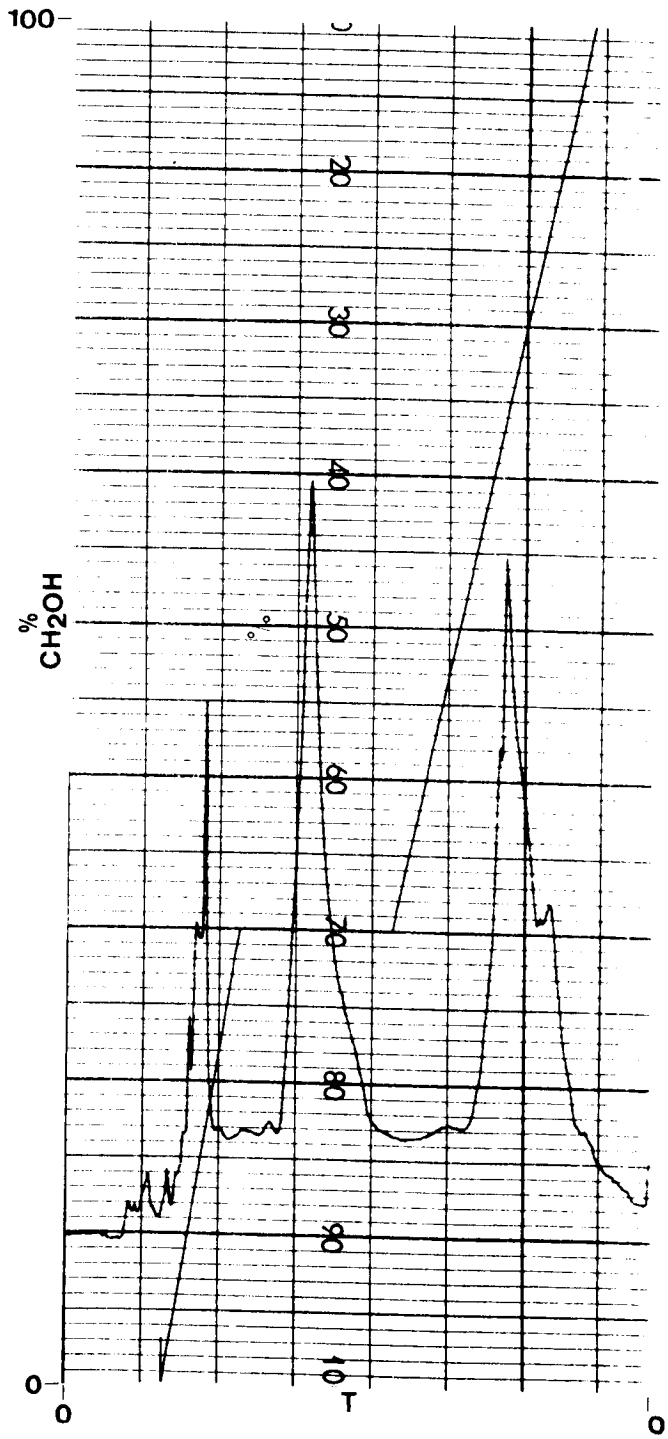


Figure 4.13

HPLC of a clone broth extract (*S. lividans* + pIA103).



To confirm whether the antibiotic in question was actually geldanamycin or, for instance, a closely related analogue with similar characteristics, the clone broth extracts were investigated by mass spectroscopy. This procedure is considered to be sensitive enough to distinguish geldanamycin from other closely related compounds and so to confirm its present or absence in the clone broth extracts.

4.4 Mass Spectrometry

Mass spectrometry is a method for the analysis of organic molecules which can reveal structural information about a molecule by effectively presenting the mass of the individual fragments into which that molecule can be easily split by ionisation. It will also reveal the molecular mass of the total molecule.

A mass spectrometer consists of an ion source which converts molecules into ions, usually positive ions, a mass analyser (strictly speaking a "mass to charge ratio", m/z, analyser but as charge is usually +1, m/z is equivalent to m) which distinguishes between ions with regard to their mass and these separated ions are detected typically using an electron multiplier to produce a small, but measurable current.

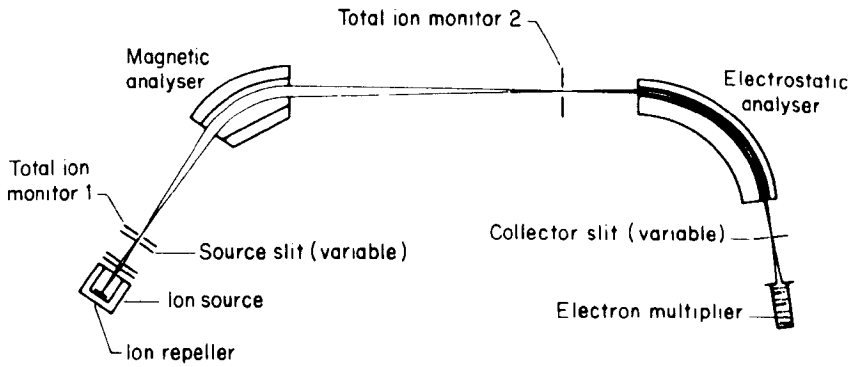
The instrument used in our work was a single focusing magnetic sector mass spectrometer with an electron impact (EI) ion source. The solid samples were individually admitted to the ion source using a direct inlet probe via a vacuum rod where they were volatilised by

heating to a temperature of about 150°C. Ionisation was brought about by bombarding the sample molecules with high energy electrons emitted from a heated metal filament. The electrons are drawn into a well-defined beam using a potential gradient and a series of collimating magnets and this beam of electrons is brought into close proximity with the sample molecules where the field the electrons generate interacts with electrons of the molecule leading to ionisation by removal of an electron. The potential across the ion source was held at 70 volts producing 70 eV electrons. The positive ions are removed from the ion source using a negative potential and accelerated by a potential difference of about 6,000 v to increase their kinetic energy. The ions then enter the analyser which is a magnetic field which causes the ions to describe a circular path, the radius of which is proportional to the m/z of that ion. The analyser can thus distinguish between ions of differing m/z by varying the strength of the magnetic field applied to the ion stream and only allowing ions of a certain m/z to reach the detector (see Figure 4.14). The detector employed was an electron multiplier connected to a computer which is also used to present the data.

If one simply bombarded the sample molecules with electrons with just enough energy to cause one ionisation event, i.e. the first ionisation potential of the molecule (usually in the range 8-15 eV for organic molecules) one would simply detect the molecular ion, M^+ of the molecule. However, an ionisation energy of 70 eV was

Figure 4.14

Schematic representation of a mass spectral analyser.



used in our experiments (as it typically is) because this allows the spectrophotometer to be used at maximum sensitivity, and this is in excess of the energy required simply to ionise the molecule. Some of this surplus energy is transferred to the ion as it is formed and this can lead to the ions existing in an electronically excited state and/or having excess vibrational energy. The former are often unstable and prone to dissociation and, if the vibrational energy is large enough, it will also lead to dissociation by causing vibrations greater than the elastic limits of some of the bonds in the molecule. This dissociation is commonly called fragmentation and invariably leads to the formation of new, smaller ions. Thus mass spectra do not typically display the mass of the molecular ion and the intact molecule, but also show the masses and relative abundance of any fragments of the molecule arising from the above processes which provides information about the sample's molecular structure.

4.5 Mass Spectrometry of Geldanamycin and Clone Broth Extracts

DeBoer *et al.* (1970) performed mass spectrometric analyses on geldanamycin. The spectra were obtained using a direct inlet probe to insert the sample into the ion source at about 100^{o}C and the temperature was slowly raised until ions were produced.

The resulting mass spectrum (Figure 4.15) shows a large peak at 517 and weak peaks of about equal intensity at 560 and 562. The peaks at 560 and 562 were initially

thought to be two real separate entities differing by two hydrogen atoms but they argued that other compounds containing a quinone moiety, as geldanamycin does, were known to yield apparent M^+ +2 peaks in the ion source and so the actual molecular weight of geldanamycin was 560 and they confirmed this by elemental analysis. The peak at 517 corresponds to loss of HOCN from the $C_{29}H_{40}N_2O_9$ at mass 560.

Figures 4.16 - 4.18 show our spectral analyses of purified geldanamycin. A certain number of differences can be seen between the DeBoer *et al.* spectrum and ours but, by examining the enlargements of our spectrum we can see that all of the relatively intense peaks identified by DeBoer *et al.*, namely 138, 358, 469, 501 and 517 (and 519) peaks are present in our geldanamycin sample. The difference between the spectra is in the relative intensities of the peaks seen and this is probably the effect of differences in the way the spectra were produced, e.g. the ionisation energies used may have been different as may have been the temperatures applied to the sample as DeBoer *et al.* did not state the former and we used a constant temperature applied to the inlet probe.

A clone broth extract of pIA102 was prepared and subjected to TLC and developed in the 9:6:6:1 solvent system. Regions of the plate corresponding to Rfs 0.13 and 0.33 (previously determined to contain bioactive components of both the clone broth extract and galdanamycin [see section 4.2.3]) were scraped off and the compound extracted with solvent from the silica gel. The

Figure 4.15

Mass spectrum of geldanamycin.
(DeBoer *et al.*, 1970)

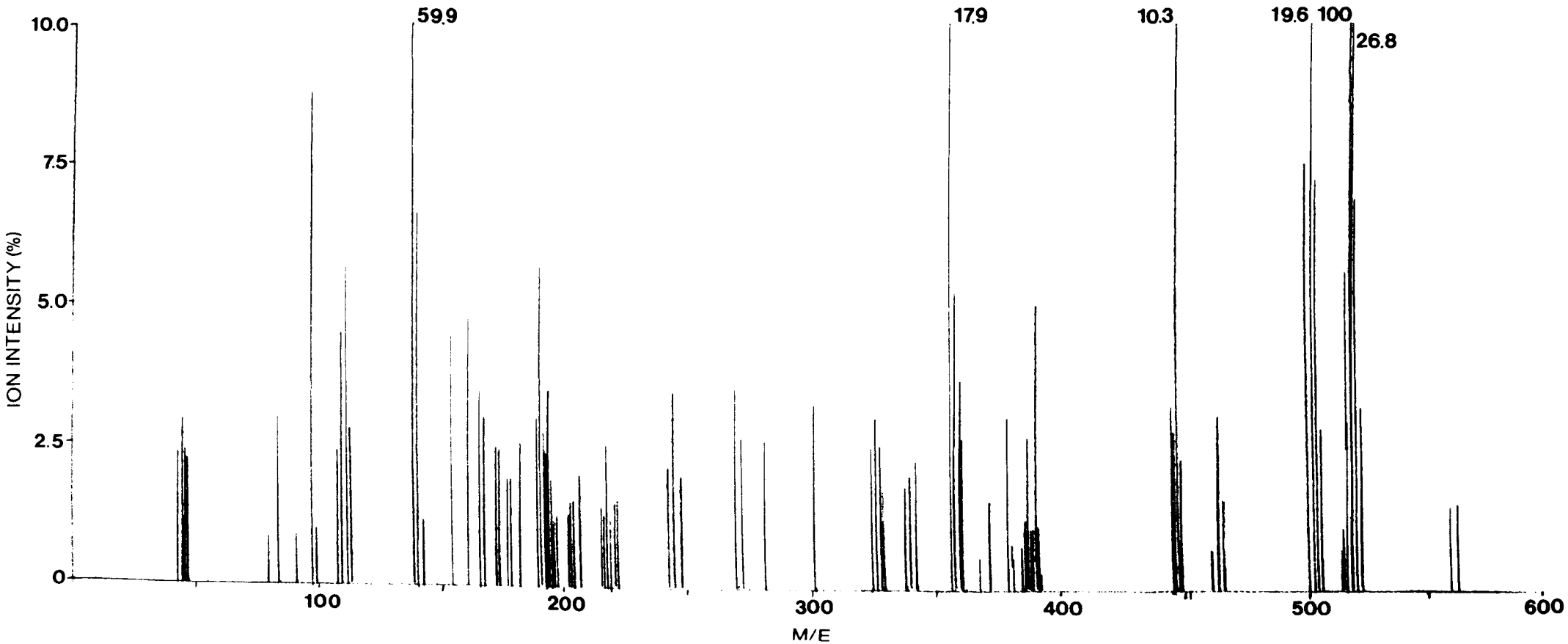


Figure 4.16

Mass spectrum of geldanamycin.

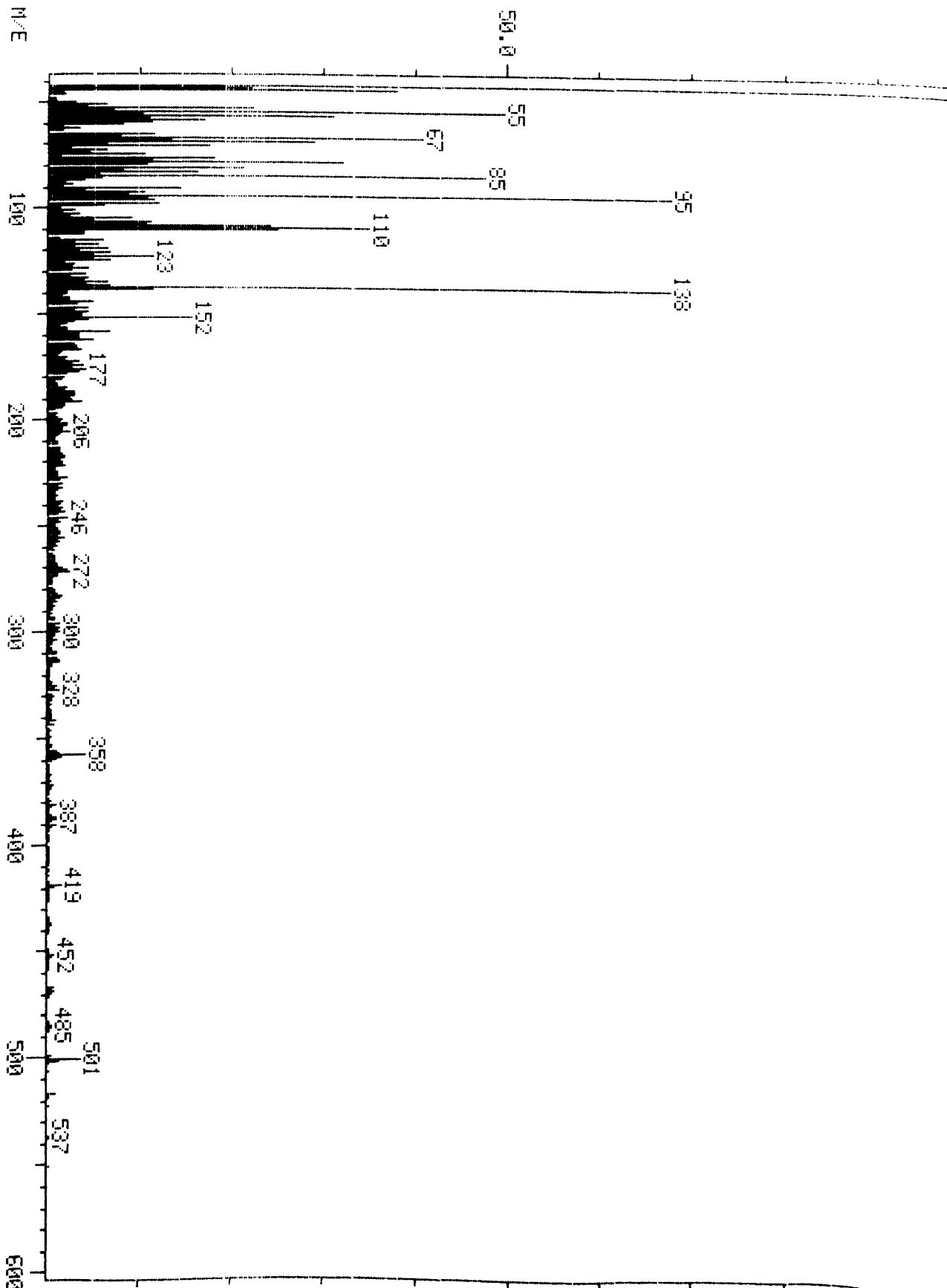


Figure 4.17

Mass spectrum of geldanamycin.

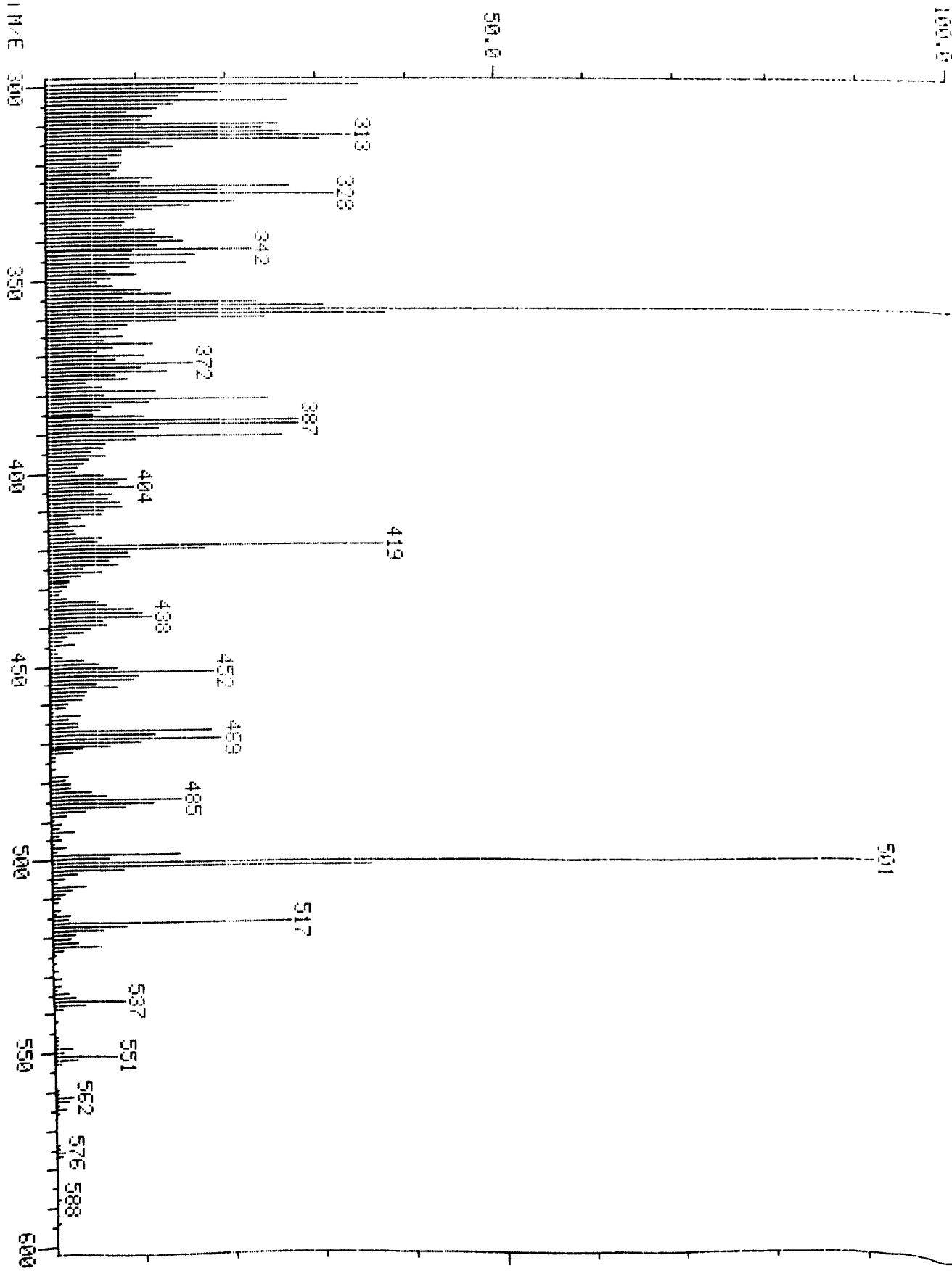
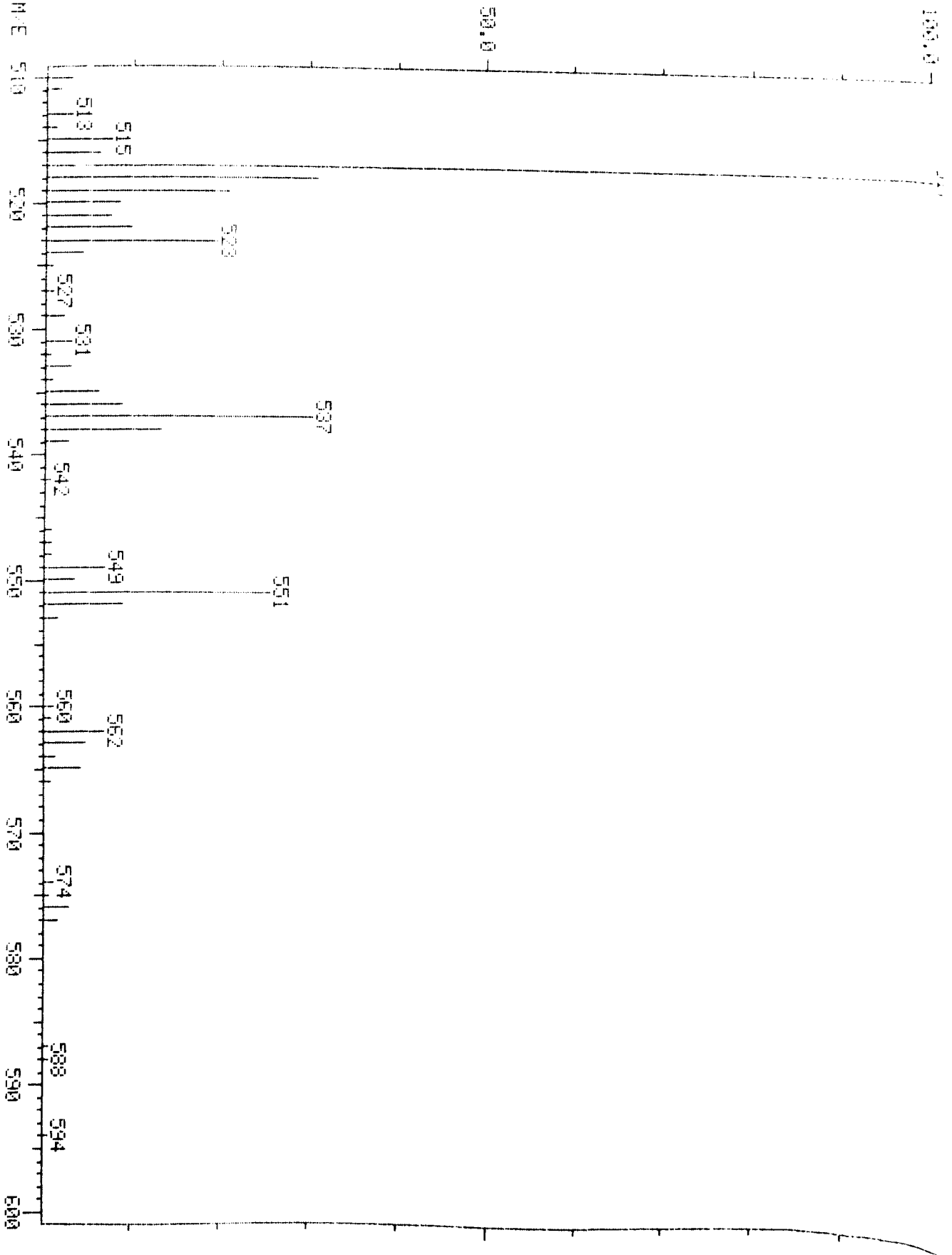


Figure 4.18

Mass spectrum of geldanamycin.



antibiotics were reduced to dryness *in vacuo* and separately applied to the probe of the mass spectrometer. The results can be seen in Figures 4.19 and 4.20. Unfortunately enlargements of these spectra were not made but one can see similarities between these spectra and Figure 4.16 representing our mass spectrum of geldanamycin to the same scale. Initially the large amount of low molecular weight "grass" seen in these spectra was thought to indicate the presence of contaminants in the sample preparations. An alternative explanation may be that the conditions we used to produce the spectra caused more fragmentation of the samples than those used by DeBoer *et al.*, e.g. we may have used a higher ionisation energy and/or a higher probe temperature. DeBoer *et al.* do report that geldanamycin is heat labile and, in the light of this and further data to be presented, it does seem likely that we are simply fragmenting our samples more completely than DeBoer *et al.*

However, at that time, we believed, from the mass spectral data, that our samples were contaminated, we purified them by TLC. Geldanamycin and clone broth extracts of pIA101, pIA102 and pIA103 were applied to TLC plates which were developed in 9:6:6:1 (Figure 4.21). The bands seen to be co-migrating with geldanamycin were scraped from the plate and solvent extracted. These solvent extracts were applied to another TLC plate and this was developed in 9:1 (Figure 4.22).

Figure 4.19

Mass spectrum of clone broth extract containing pIA102.
(corresponding to TLC R_F 0.13)

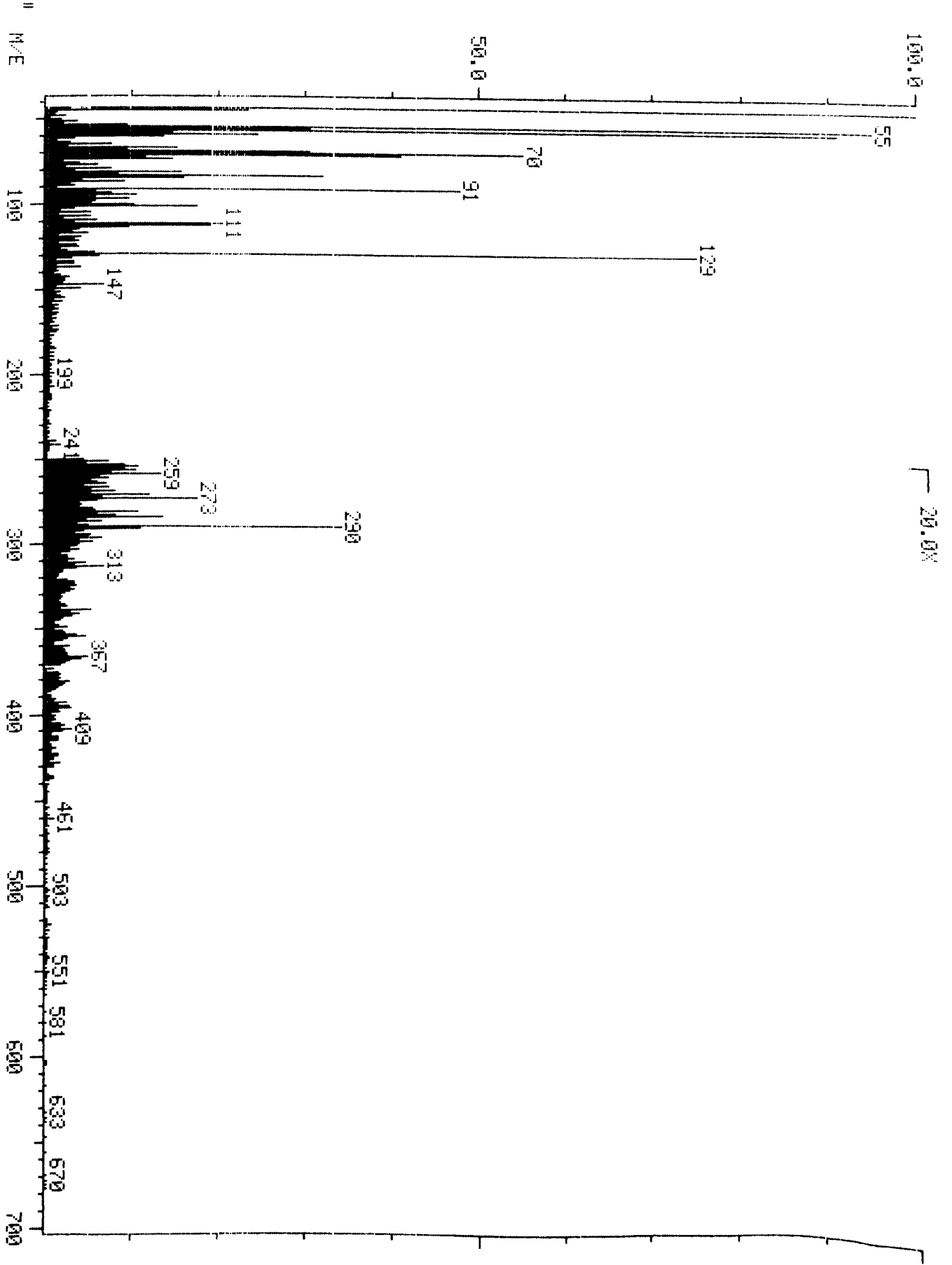
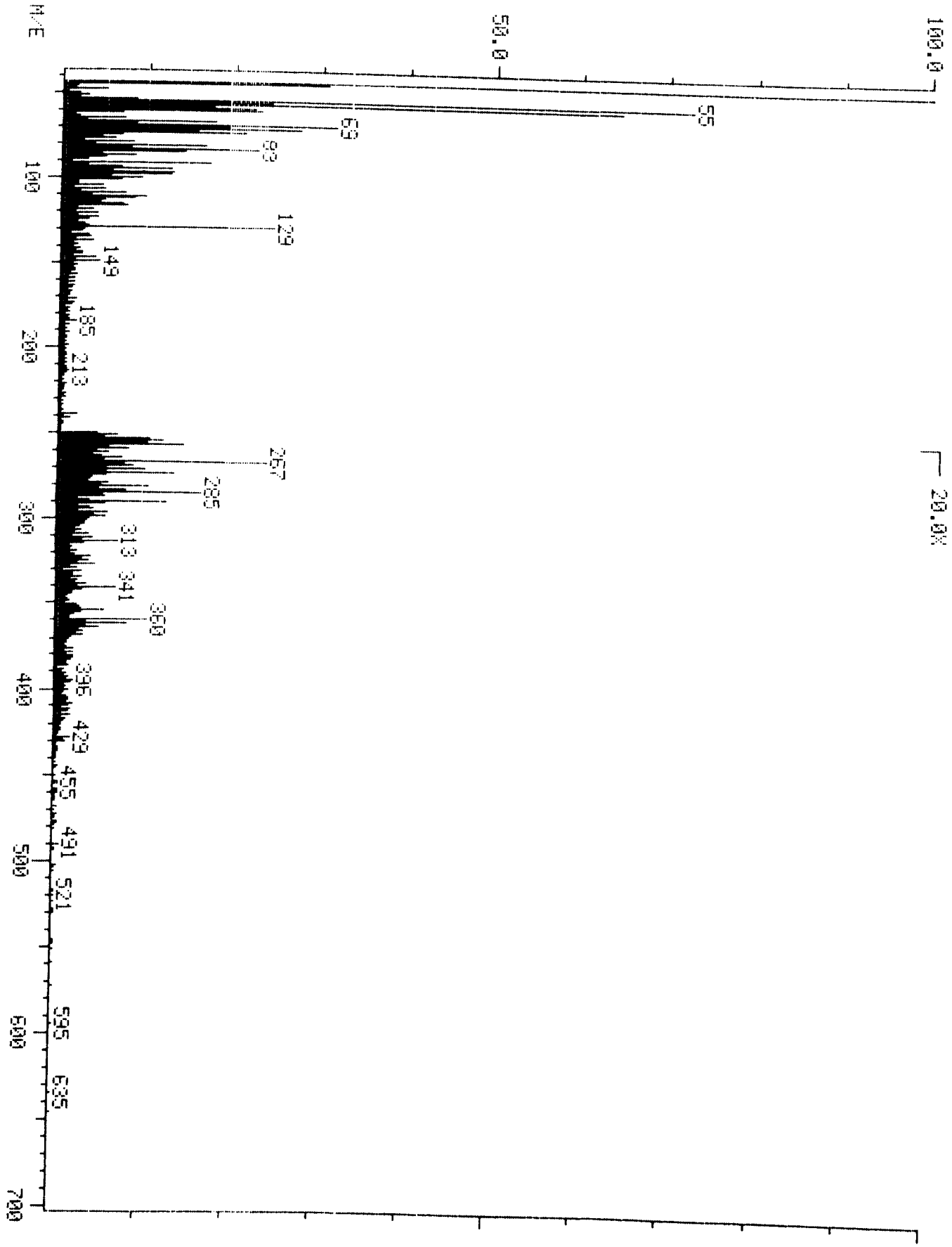


Figure 4.20

Mass spectrum of clone broth extract containing pIA102.
(corresponding to TLC R_F 0.33)

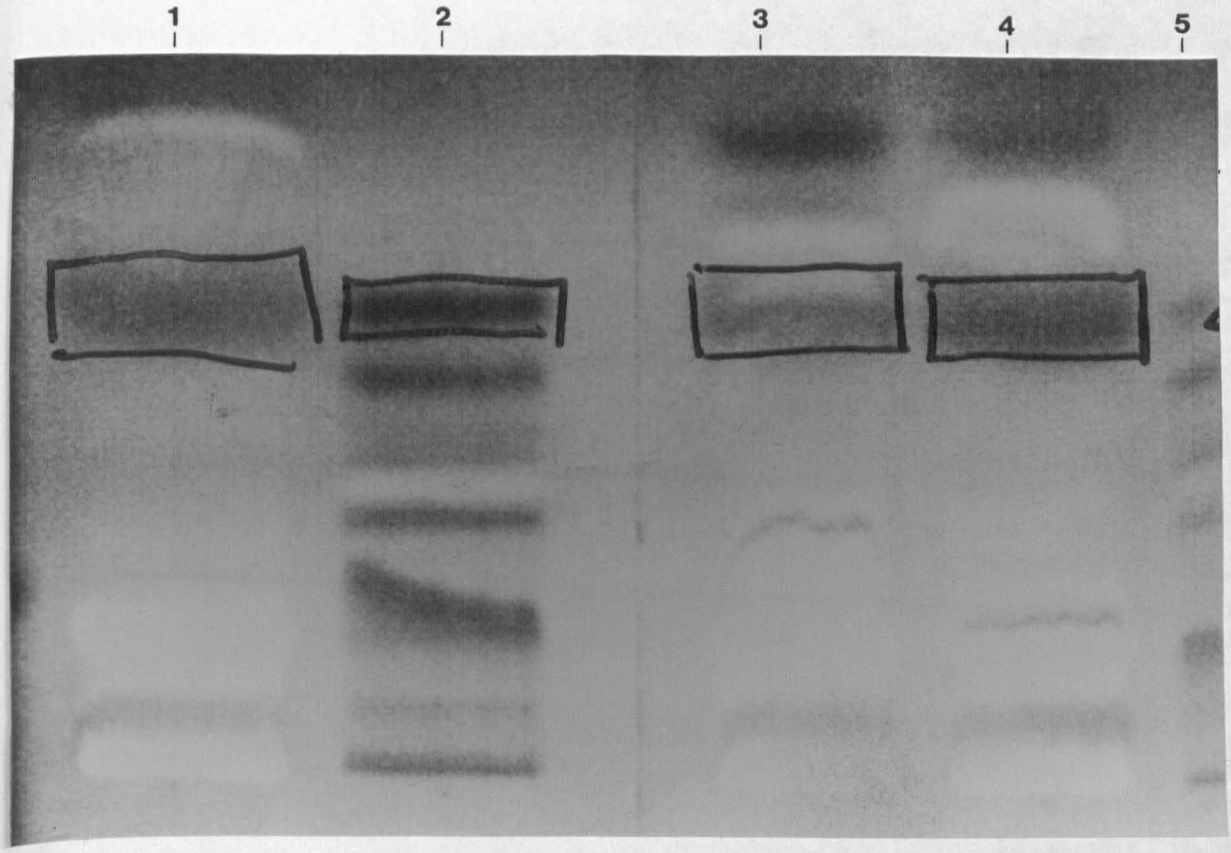


Bands of similar Rf were again scraped from the plate for all of the samples and solvent extracted, filtered and reduced to dryness *in vacuo*. These solid samples were then subjected to mass spectrometry. Figures 4.23 and 4.24 present the results for geldanamycin and Figure 4.25 and Figures 4.26 and 4.27 represent extracts of clones pIA101 and pIA102 respectively.

The mass spectra indicate the presence of an identical set of peaks in the geldanamycin mass spectrum and those of the clone broth extracts and so an identical set of fragments must also be present. The mass spectrum of the clone broth extract of pIA102 would also seem to show the presence of a contaminant and that this contaminant must be closely related to geldanamycin because some of the fragment peaks seen in this spectrum are slightly different in this extract, in that as well as the "geldanamycin" fragment peak being present the other peaks very close to this one in some cases show different relative intensities to those seen in the control sample. The pattern of fragmentation is basically the same but the emphasis is changed slightly within the overall pattern indicating the presence of identical fragments and other closely related fragments differing in mass by only a few units. This could be due, for instance, to the presence of a dihydroxy-substituent of geldanamycin as well as geldanamycin itself in the broth extract of pIA102 (Figure 4.28) which could have formed in the aqueous broth, where there would be an abundant supply of H^+ ions, or to the production of closely related

Figure 4.21

TLC of geldanamycin and clone broth extracts.



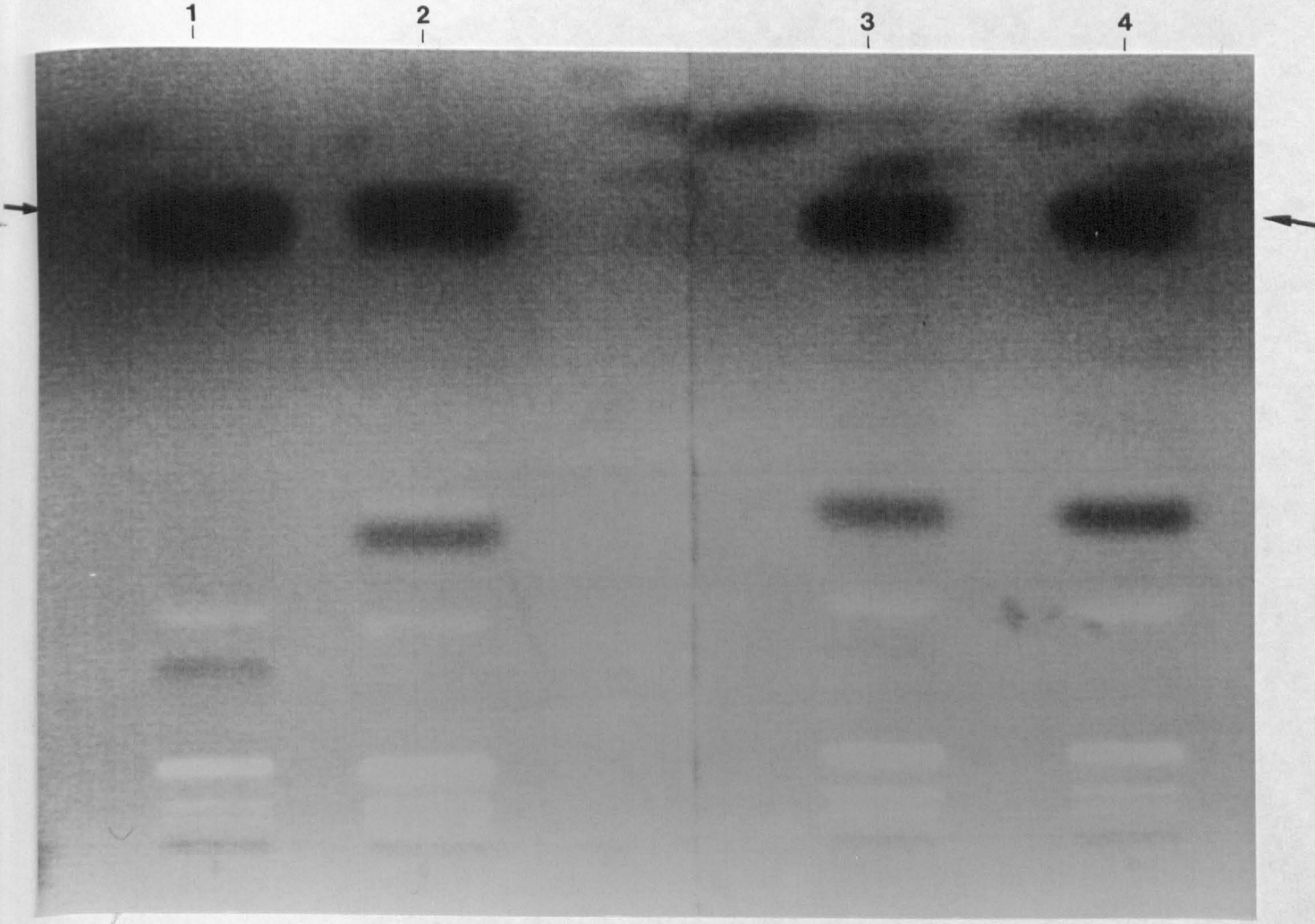
Chromatoplate developed in 9:6:6:1.

1 pIA101 in *S. lividans*.
2 Geldanamycin.
3 pIA103 in *S. lividans*.
4 pIA102 in *S. lividans*.
5 Geldanamycin

The "bands" isolated are indicated.

Figure 4.22

TLC of geldanamycin and clone broth extracts.



Chromatoplate developed in 9:1

1 Geldanamycin.
2 pIA101 in *S. lividans*.
3 pIA102 in *S. lividans*.
4 pIA103 in *S. lividans*.

The "bands" at the R_F indicated were isolated and used for mass spectrometry.

Figure 4.23

Mass spectrum of TLC purified geldanamycin.

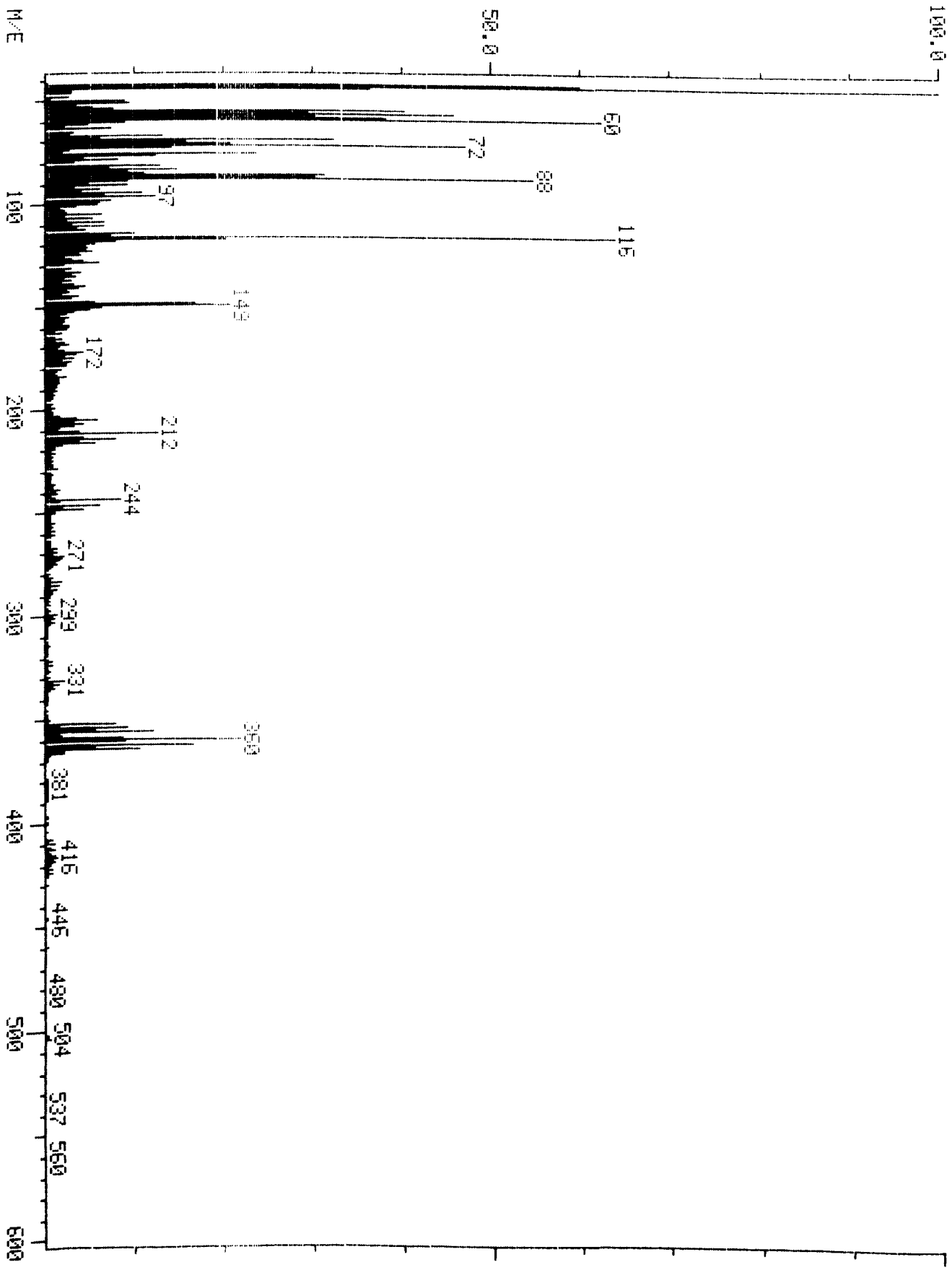


Figure 4.24

Mass spectrum of TLC purified geldanamycin.

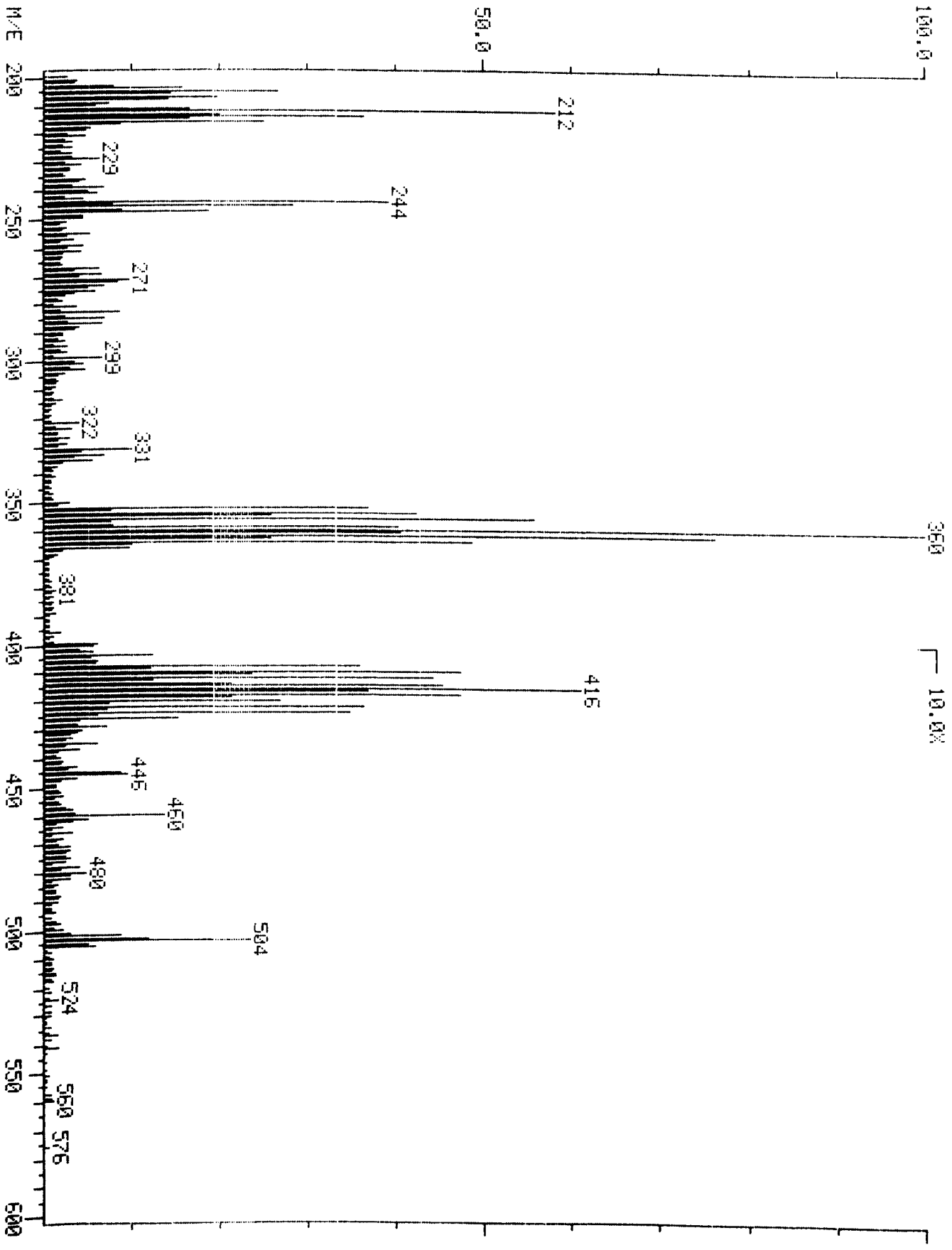


Figure 4.25

Mass spectrum of TLC purified clone broth extract (pIA101).

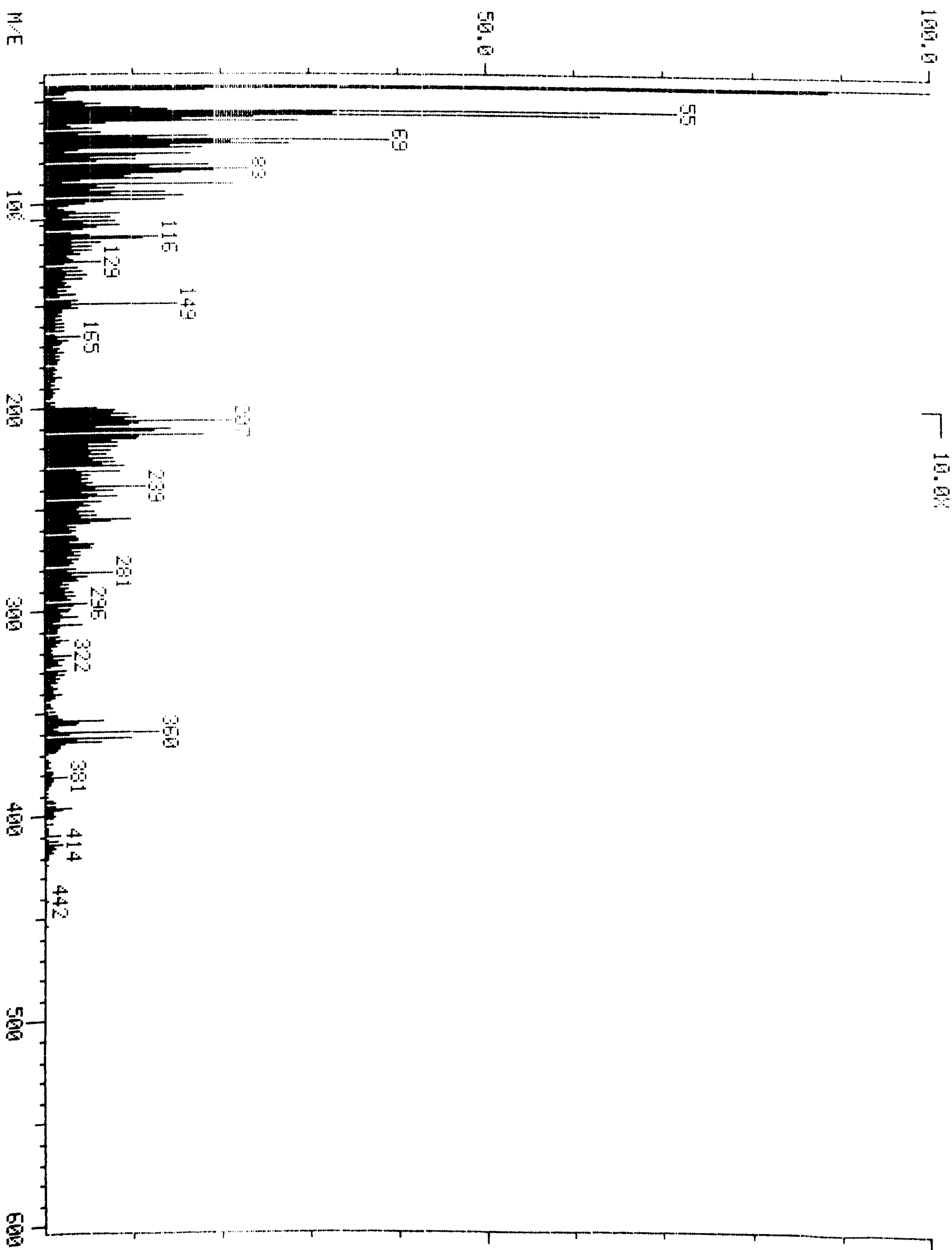


Figure 4.26

Mass spectrum of TLC purified clone broth extract (pIA102).

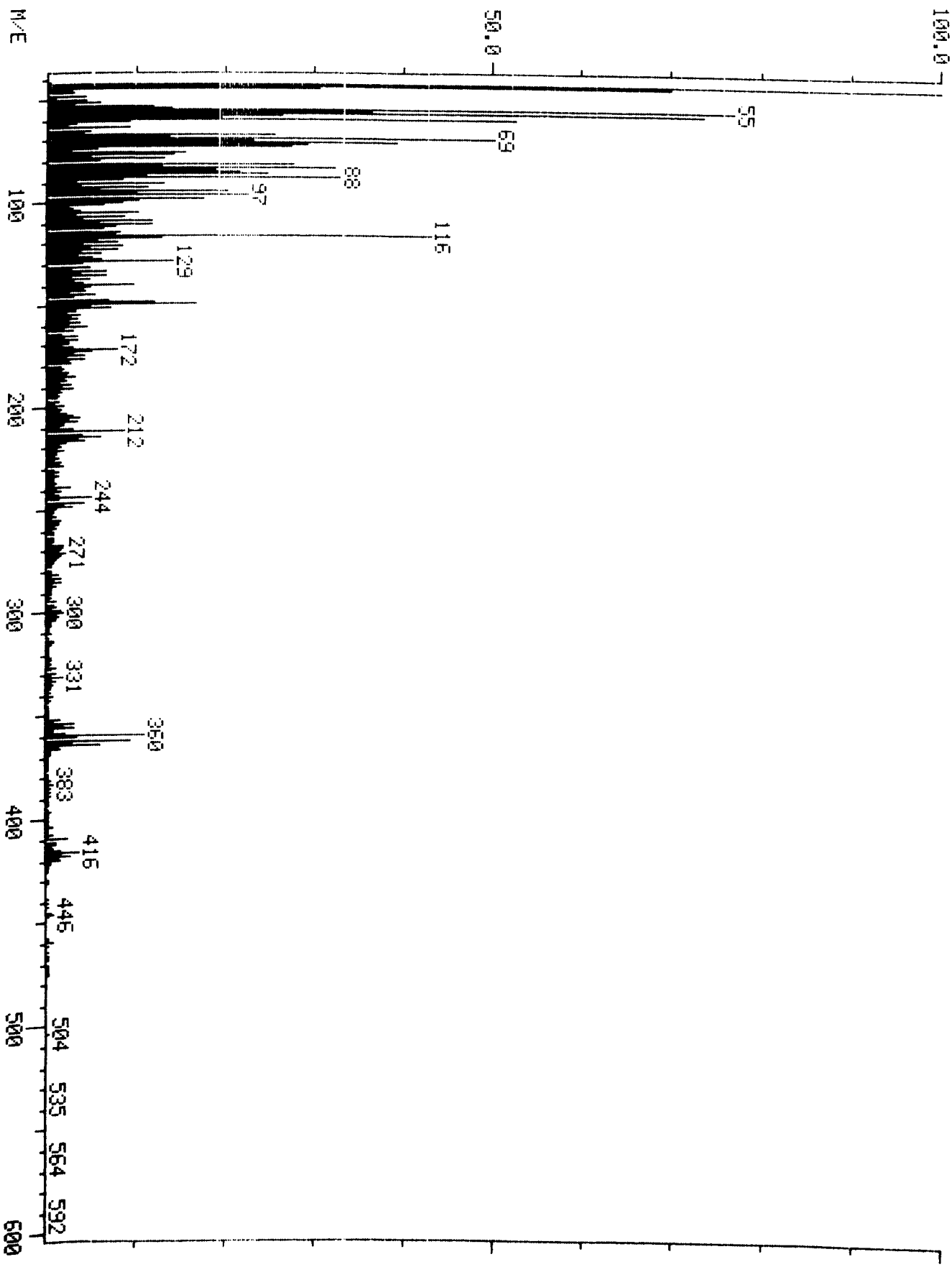
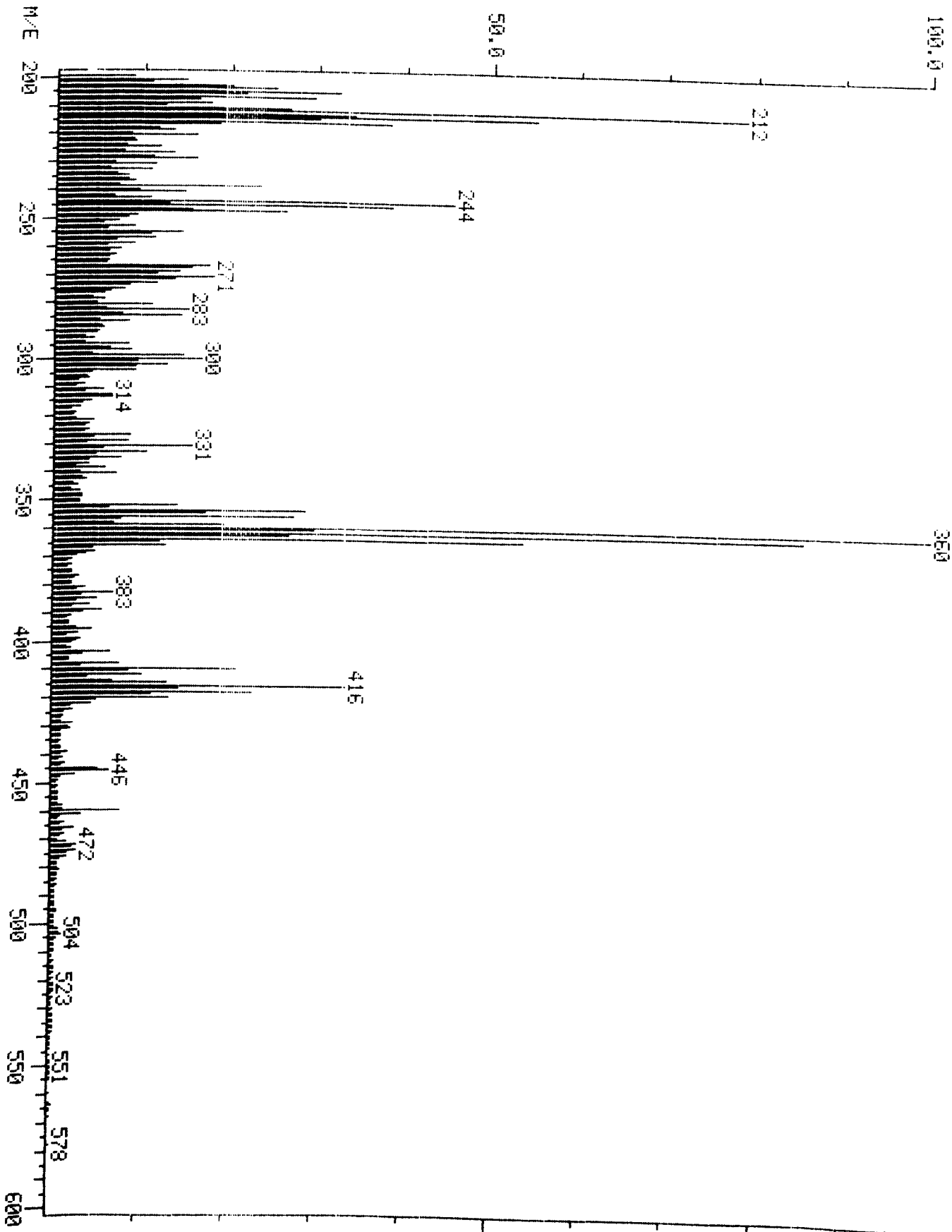


Figure 4.27

Mass spectrum of TLC purified clone broth extract (pIA102).



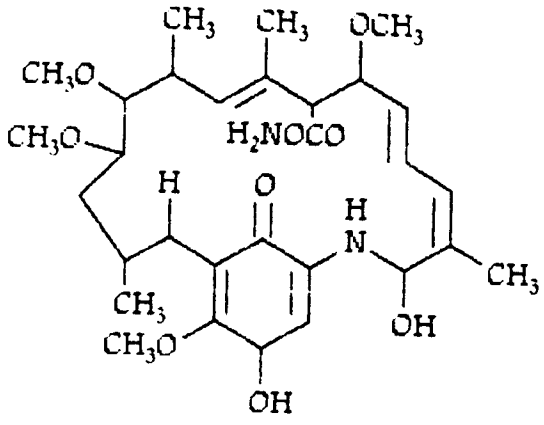
analogues of geldanamycin by variations in the programming of the *pks* responsible for producing this antibiotic.

4.6 Discussion

Although none of the above analyses, with the possible exception of mass spectrometry could prove absolutely the presence of geldanamycin in the clone broth extracts, the weight of evidence from TLC, bio-autography and HPLC suggested that this was indeed the case. The work in this chapter is presented temporally, as it was done, as we sought more and more confirmation that the antibiotic we had detected from these clones was geldanamycin. A lot of the results supported our supposition that the antibiotic was either geldanamycin, or closely related to it, but throughout the analyses we had problems with the levels of "background" in our samples. When confirmation of the presence of geldanamycin in the broth extracts was finally provided by the sensitive tool of mass spectrometry it also indicated the presence of a closely related analogue of geldanamycin in these broth extracts. This analogue itself may have caused the "background" that caused so much confusion in our earlier analyses which were based upon separation techniques to isolate geldanamycin from other compounds in the crude broth extracts.

Figure 4.28

A possible structure for a di-hydroxy- substituent of geldanamycin.



CHAPTER 5

THE PRODUCTION OF GELDANAMYCIN NON-PRODUCING MUTANTS BY "MUTATIONAL CLONING"

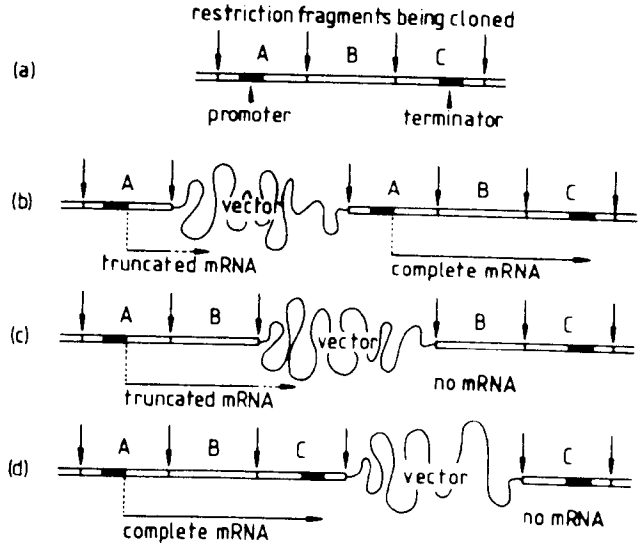
5.1 Introduction

Chater and Bruton (1983) reported the use of an attachment site-deleted derivative, øC31 KC400, of the *Streptomyces* temperate phage øC31 to clone fragments of the genetic determinants for the biosynthesis of methylenomycin A (which are located on the plasmid SCP1). Their protocol involved the shotgun cloning of fragments of total DNA from *S. parvulus* 2296 ($SCP1^+$) of size greater than 2 kb into the phage vector KC400. This gene bank was used to transfect *S. coelicolor* A3(2) and lysogens of this strain were detected by their transduction by the viomycin resistance marker (*vph*) carried by the phage vector. These stable lysogens could not have arisen via the normal site-specific recombination system used by øC31 (Lomovskaya *et al.*, 1980), because the phage is *att*-site deleted, but Chater and Bruton (1983) found that lysogens could be formed by generalised recombination between homologous DNA sequences present both on the host genome and the phage. This homology would be provided by the cloned DNA in the phage and the homologous sequences in the recipient strain.

In principle, the integration of a øC31 derivative into a genome by such a recombinational event will disrupt gene expression at the site of insertion, thus resulting in lysogens with a mutant phenotype. Figure 5.1 indicates that such a mutant phenotype will only

Figure 5.1

The principles of mutational cloning.
(Hopwood *et al.*, 1985)



Mutational cloning. (b), (c) and (d) show the results of recombination through a single crossover between a DNA segment cloned into an attP-deleted ϕC31 derivative (e.g. KC515) and the homologous transcription unit (a) in the wild-type organism from which the cloned DNA originated (with KC515 such recombinants are selected as thiostrepton or viomycin resistant transductants). If, as in segments A and C, the cloned segment contains either the promoter or the last translational termination signal (taken here, for simplicity, to be the transcription terminus), the resulting lysogen retains the wild phenotype. If, as in segment B, the cloned segment contains neither signal, the resulting lysogen will have a mutant phenotype.

result when the cloned DNA fragment is internal to a transcription unit, that is contains neither the translational start nor stop signals. Thus Chater and Bruton examined their *S. coelicolor* lysogens for a reduction in methylenomycin A production from the wild-type levels and from their initial shotgun cloning experiment Chater and Bruton isolated nine such *S. coelicolor* methylenomycin "non-producing" lysogens. (They argue that such mutants are likely to be leaky because the uninterrupted transcription unit can be regenerated by a single crossover and the likelihood of this will depend on the length of the cloned fragment.)

We have already reported that attempts to isolate geldanamycin biosynthetic sequences by shotgun mutational cloning were not successful (Chapter 3). However, Chater and Bruton (1985) suggested this method might be used to analyse previously cloned DNA. They reported the overproduction of methylenomycin A in a producing strain into which large fragments of methylenomycin encoding DNA were used to direct phage integration and from this they deduced the location of a negative regulatory element of the methylenomycin biosynthetic system. These studies also allowed them to deduce the existence of two large transcription units comprising the genes for methylenomycin biosynthesis, resistance and their direction of transcription. These results were of great importance in the field of Streptomycete antibiotic gene cloning providing both a model for the organisation of antibiotic biosynthetic gene clusters and an important new method

for their study.

Malpartida *et al.* (1987) used "mutational cloning" with øC31 derivatives to produce antibiotic non-producing lysogens of *S. violaceoruber* (granaticin) and *S. hygroscopicus* (milbemycin). Mutagenesis was directed by cloned DNA inserts shown by homology with the *act*I and *act*III involved in polyketide antibiotic biosynthesis. This demonstrated that the cloned DNA was involved in the biosynthesis of the antibiotic which it was subsequently used to block the production of. A similar protocol was used by Weber *et al.* (1990) to analyse the erythromycin biosynthetic cluster from *Saccharopolyspora erythraea*, but in this instance integrative plasmid vectors instead of *att*-site deleted phage vectors were used.

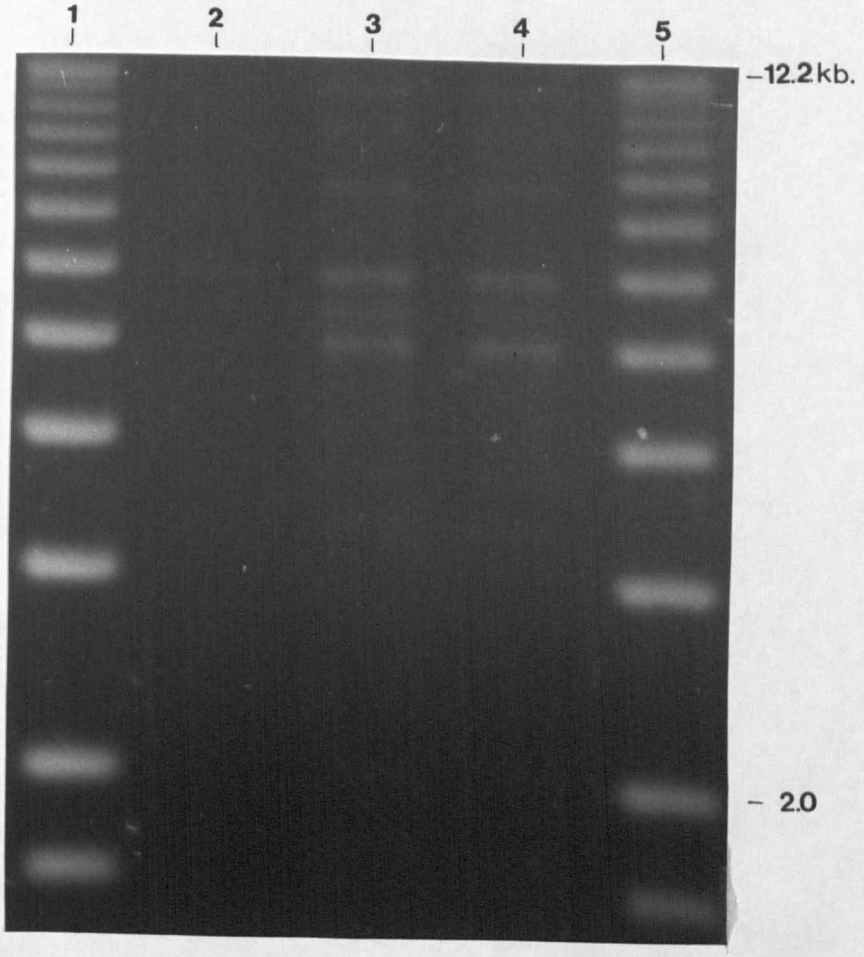
In this chapter we present data which implicates a fragment of the cloned pIA102 DNA in the biosynthesis of geldanamycin by the formation of geldanamycin non-producing lysogens using the *att*-site deleted phage vector øC31 KC515 as a vehicle for insert-directed mutagenesis.

5.2 Producing Geldanamycin Non-Producing Mutants

In Chapter 3 results were presented to show that pIA101, pIA102 and pIA103 restriction digest patterns indicated cloned donor DNA were present in the plasmids. One such digest with *Bam*H1 (Figure 5.2) showed a complicated pattern, including sub-molar bands, which is probably the result of incomplete digestion, but proving

Figure 5.2

*Bam*H1 Digests of pIA101, pIA102 and pIA103



1 1 kb ladder

2 pIA101 *Bam*H1 digest

3 pIA102 "

4 pIA103 "

5 1 kb ladder

the presence of cloned inserted DNA in pIJ61 (which has only a single *Bam*H1 site). A number of DNA bands from pIA102 were isolated from this gel by the "Geneclean" procedure. These fragments were individually sub-cloned into the single *Bam*H1 site of the Streptomycete portion of the *E. coli*/Streptomycete shuttle vector pIJ699. This is a high copy number plasmid and so can be used for the bulk preparation of the cloned fragments which could be simply recovered by a *Bam*H1 digest of the recombinant plasmids.

Two of the smaller DNA sub-clones, approximately 3.5 kb and 4.5 kb were chosen as potential sequences to direct recombination between a geldanamycin-producing host chromosome ("wild-type" *S. hygroscopicus* 3602) and a suitable vector into which they were cloned (øC31 KC515) because they were fragments of cloned DNA from pIA102, a clone possibly expressing geldanamycin production determinants (see Chapters 3 and 4).

Purified øC31 KC515 DNA, carrying tsr^r, was cleaved at its single *Bam*H1 site. The 3.5 kb and 4.5 kb *Bam*H1-*Bam*H1 sub-clones of pIA102 were separately treated with alkaline phosphatase and ligated into the vector to produce øC31 derivatives KC515/3.5 and KC515/4.5 respectively. These recombinant phage molecules were each used to transfect freshly prepared protoplasts of *S. lividans* 1326. Single plaques of both KC515/3.5 and KC515/4.5 were isolated, the phage particles were soaked-out and used to infect a lawn of spores of *S. hygroscopicus* 3602. These lawns were replicated to plates containing thio-

strepton, to select for lysogens and these plates examined for the growth of *S. hygroscopicus* colonies.

Any *S. hygroscopicus* colonies that do grow on thiostrepton must be lysogens, and since this phage is *att*-site deleted the lysogens should have arisen by insert-directed homologous recombination. Twelve such thiostrepton-resistant lysogens were detected with the phage KC515/3.5 and fifteen with KC515/4.5. The KC515/3.5 *S. hygroscopicus* lysogens were all found to be wild-type with respect to geldanamycin biosynthesis when assayed by TLC bioautography. From this we concluded that the 3.5 kb *Bam*H1-*Bam*H1 sub-clone used contains a transcriptional junction (or is not actually part of the geldanamycin biosynthetic pathway), and so though recombination could take place no alteration in phenotype was detectable. No further work was done with these lysogens.

The fifteen KC515/4.5 *S. hygroscopicus* 3602 lysogens, however, were not wild-type with respect to geldanamycin biosynthesis and so were examined in detail.

5.2.1 TLC bioautography of *S. hygroscopicus* 3602 KC515/4.5 lysogens

The lysogens were grown in liquid culture under thiostrepton selection and the broth extracted and subjected to TLC as described in Chapter 4. The chromatoplates were developed in "9:6:6:1" solution and subjected to bioautography using the *B. subtilis* indicator strain. The results are summarised in Table 5.1. The fifteen lysogens tested fell into two distinct groups, A and B,

Table 5.1

Rf. Values from TLC Bioautographs of *S. hygroscopicus* 3602 KC515/4.5.

Geldanamycin	*S.hygroscopicus* 3602, wild-type	*S.hygroscopicus* KC515/4.5 A	*S.hygroscopicus* KC515/4.5 B
Rf.	Rf.	Rf.	Rf.
	0.02	0.02	0.02
0.15	0.19		
	0.24	0.23	0.23
0.33	0.34		
	0.52		
0.68	0.65	0.69	

Group A represents 6 of the 15 KC515/4.5 lysogens and these are producing the Rf 0.69 bioactive spot of geldanamycin.

Group B represents 9 of the 15 KC515/4.5 lysogens and these are producing none of the geldanamycin bioactive spots.

TL chromatogram developed in 9:6:6:1 (ethyl acetate:hexane:dichloromethane:methanol) and overlayed with *B. subtilis* as an antibiotic indicator.

KB515/4.5 A lysogens all exhibiting one of the geldanamycin antibiotic spots which we attribute to geldanamycin. Possible reasons for these differences will be discussed later.

5.2.2 HPLC of *S. hygroscopicus* 3602 KC515/4.5 lysogens.

A number of the KC515/4.5 lysogen broth extracts were also subjected to HPLC analysis using conditions optimised in Chapter 4, - namely:

Mobile phase	0% to 100% methanol (w/v) gradient in 40 mins.
Flow rate	1 ml.min^{-}
Pressure	72 bar.
Detector	U.V. at 254 nm.

Figure 5.3 represents the wild-type geldanamycin producer, Figures 5.4 and 5.5 represent KC515/4.5 lysogens from group A and Figures 5.6 and 5.7 represent KC515/4.5 lysogens from group B. The HPLC chart readings show that: (i) the lysogens of both classes have very little material in their broth extracts compared to that of *S. hygroscopicus* 3602 wild-type, (ii) the broth extracts of the KC515/4.5 lysogens of group A, which do show some antibiotic activity, have a peak at about 15% methanol (w/v) which is also present in the broth extract of the wild-type organism. It therefore seems likely that the TLC bioautography spot (Rf 0.69) which is seen for KC515/4.5 lysogens of group A correlates with this peak on the HPLC trace and that this is one of the bio-active components of the geldanamycin mixture, (iii) the HPLC chart recordings of KC515/4.5 lysogens of group B do not appear to have any peaks at all when compared to a control HPLC gradient to which no sample was applied (data not presented here). We conclude that these

Figure 5.3

HPLC of *S. hygroscopicus* 3602 wild-type.

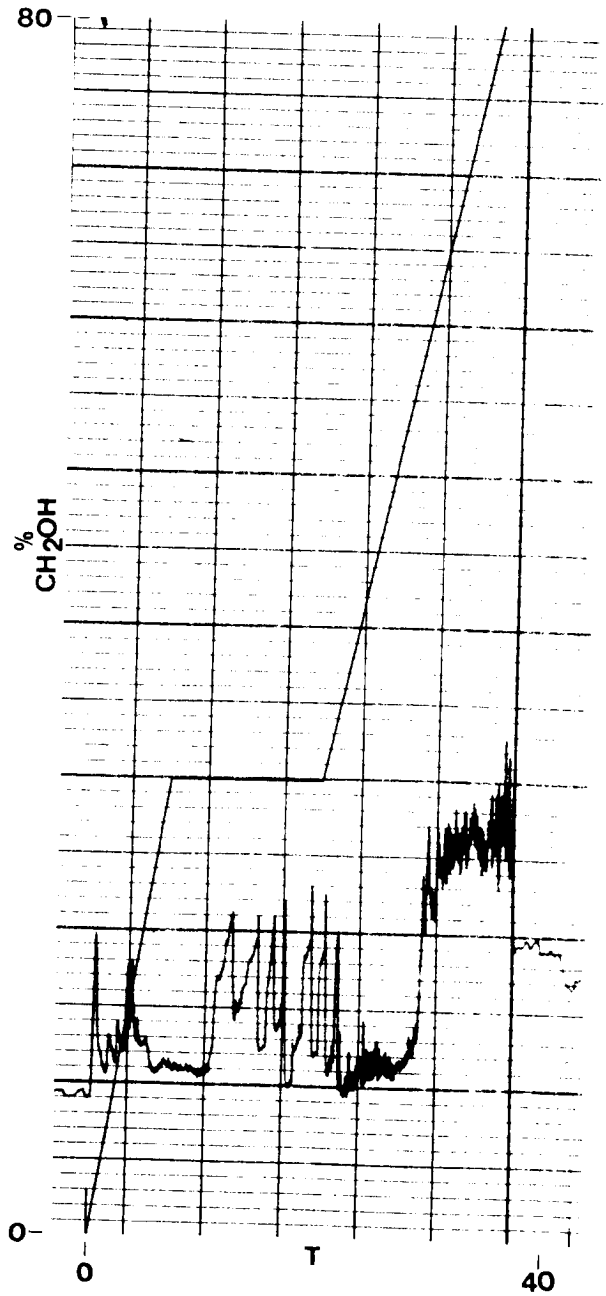


Figure 5.4

HPLC of KC515/4.5 lysogen of group A (Lysogen 6)

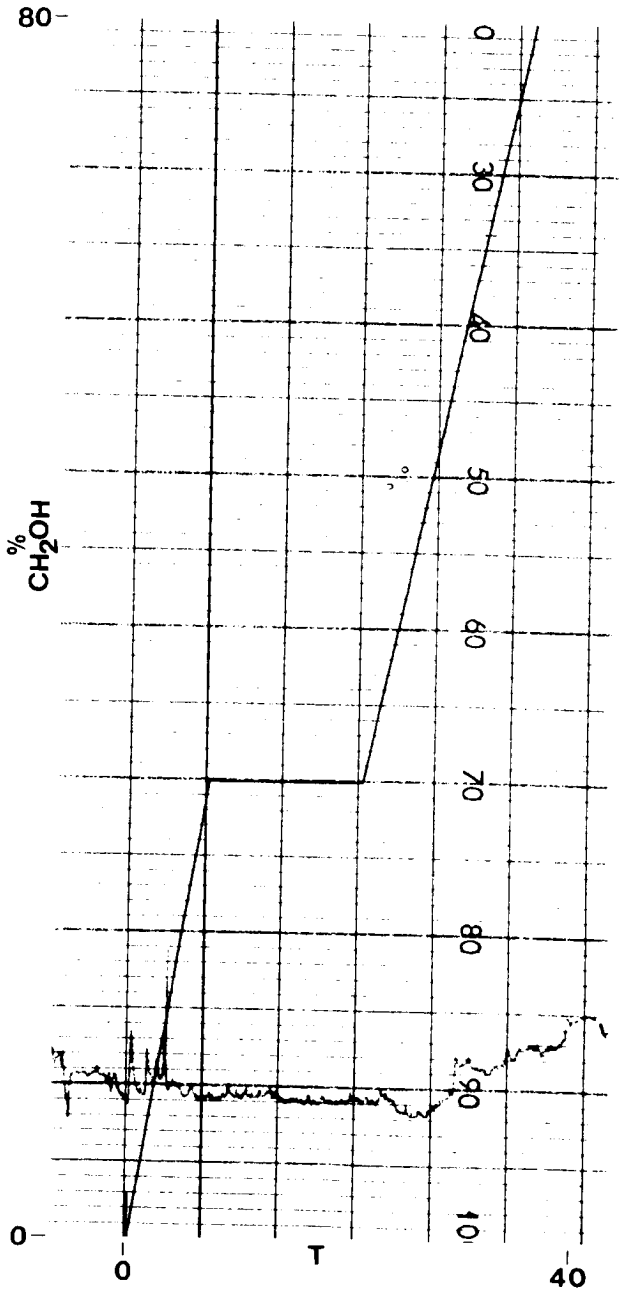


Figure 5.5

HPLC of KC515/4.5 lysogen of group A (Lysogen 9)

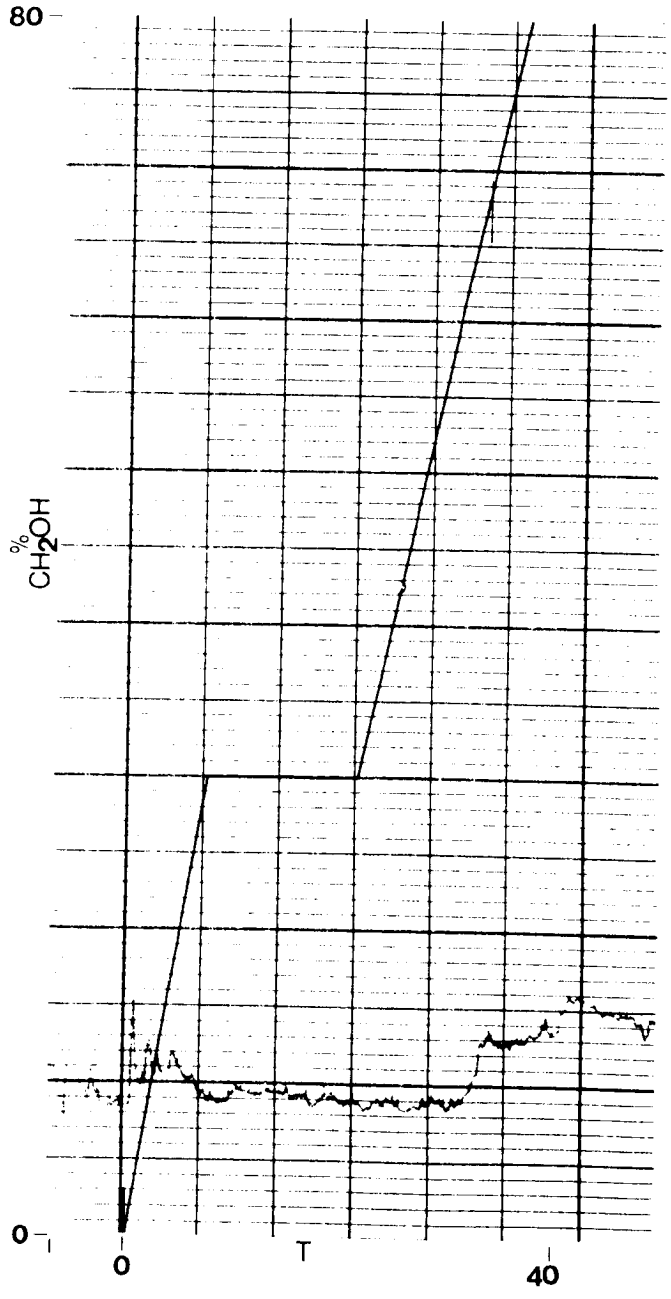


Figure 5.6

HPLC of KC515/4.5 lysogen of group B (Lysogen 3)

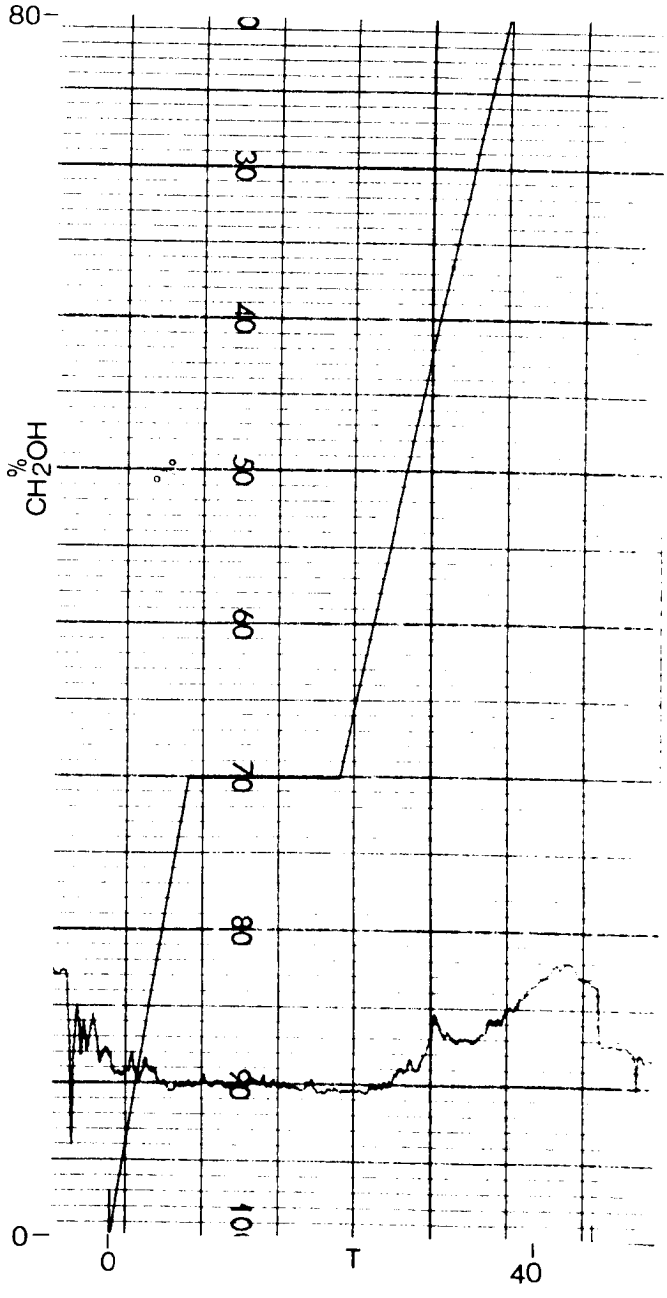
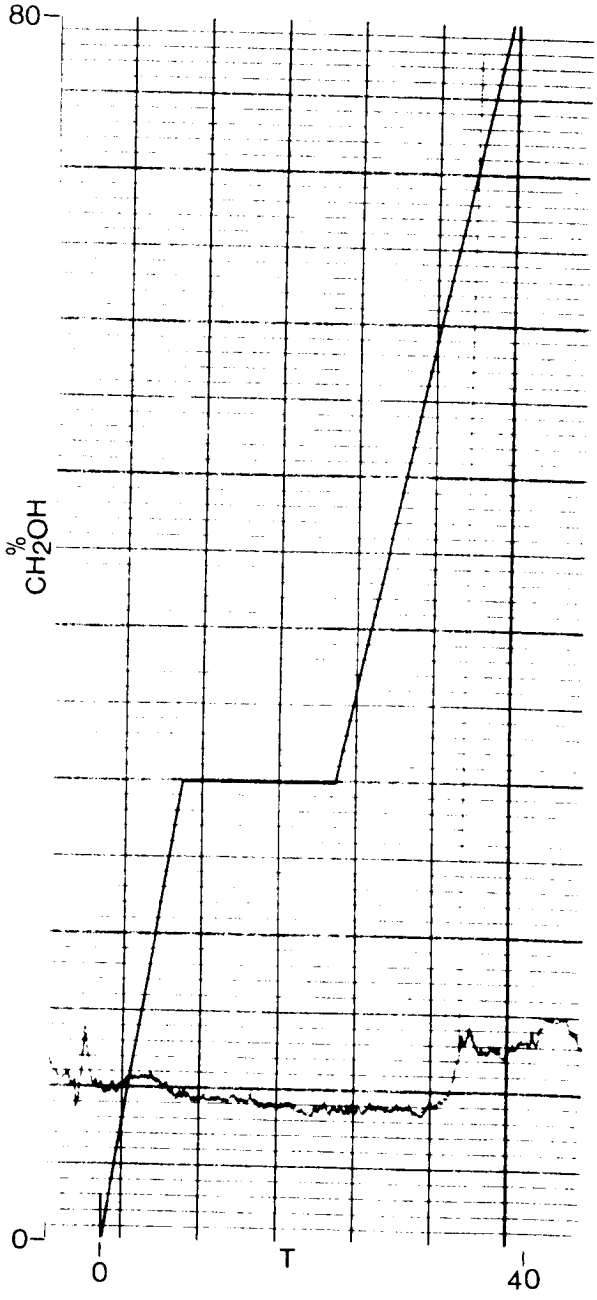


Figure 5.7

HPLC of KC515/4.5 lysogen of group B (Lysogen 5)



lysogens are geldanamycin non-producing.

5.2.3 Edduction of phage øC51 KC515/4.5 from lysogens

It was found that by growing the lysogenic colonies of *S. hygroscopicus* 3602 of both groups A and B on agar without selection, plaques were formed in some cases on replication to lawns of *S. lividans* 1326. Colonies giving rise to plaques in *S. lividans* 1326 were tested and found to be both thiostrepton-sensitive and wild-type with respect to geldanamycin production (as assayed by TLC bioautography).

It was therefore concluded that the loss of the thiostrepton-resistant phenotype was linked to the restoration of the ability to produce geldanamycin and indicated the loss of the integrated phage from the geldanamycin region of the host chromosome by a single cross-over event, which was coupled with the ability of that colony to phage infect the indicator strain, *S. lividans* 1326.

5.3 Discussion

Using a modification of the mutational cloning protocol we were able to isolate a number of *S. hygroscopicus* 3602 colonies which were transduced to thiostrepton resistance by the lysogeny of a øC31 derivative, KC515/4.5. These colonies could be induced to edduct the phage and when this happened a concomitant loss of thiostrepton resistance occurred, as expected.

These lysogens were also identified as being geldanamycin non-producing mutants of *S. hygroscopicus* 3602 and so we conclude that we have achieved successful "mutational cloning". In order for the cloned DNA to direct the homologous recombination of the phage into the host chromosome the cloned DNA must contain part of the geldanamycin biosynthetic pathway (it must also be a fragment internal to a transcription unit within this pathway). Therefore we conclude that at least the clone, pIA102, contains DNA concerned with geldanamycin biosynthesis. Taken in conjunction with the evidence in Chapter 4 we also conclude that we have cloned a large part, if not all, of the geldanamycin biosynthetic pathway.

During the course of this investigation two distinct groups of *S. hygroscopicus* 3602 KC515/4.5 lysogen were detected: (i) Group A capable of producing one of the antibiotic spots seen for geldanamycin on TLC bioautographs, and (ii) Group B which were completely geldanamycin non-producing when similarly assayed. One reason for this could be that the mutations of group A could be leaky and so we are detecting a reduced level of geldanamycin production, certainly the HPLC recordings indicate a reduced level of production. Chater and Bruton (1983), though, indicate that one might expect all mutants produced by insert-directed recombination to be leaky since it requires only a single cross-over to reverse the recombination process and the likelihood of this reversal would be dependent on the length of the cloned insert.

With such a large DNA insert, about 4.5 kb, one would therefore expect all of our mutants to be "leaky" and not just one of the classes and one would also expect them to produce all of the geldanamycin TLC bioautograph spots, albeit in smaller quantities to wild-type *S. hygroscopicus* 3602. This effect is therefore probably not due to "leakiness" of the mutations, which if it is occurring must be doing so resulting in levels of geldanamycin production so low that we cannot detect them, but is due to another reason.

It has been found that the øC31 derivative KC515, when used in mutational cloning procedures can lead to certain spurious-looking results (Hopwood *et al.*, 1990), namely when using fragments that should result in, for example, a non-producing phenotype some of the clones produced are not mutants. They argue that this comes about because of transcriptional read-through into the cloned fragment (previously identified as being promoter-less) from a strong promoter located on the phage DNA, e.g. the strongly promoting sequence associated with the thiostrepton-resistance determinant. We hypothesise that our two classes of geldanamycin "non-producing" mutants result from such a process, where those of group A have the 4.5 kb geldanamycin fragment cloned in the correct orientation and so there is read-through transcription of the genes leading to a reduced level of geldanamycin production, as one might expect this system to be less efficient than a system utilising the wild-type promoter. A loss of promoter efficiency may also be responsible for

the reduced number of antibiotic products seen with lysogen group A compared with the wild-type organism, e.g. by the production of a truncated transcript not specifying the production of some of the "components" of geldanamycin. Lysogens in group B probably have the 4.5 kb fragment cloned into the phage in the incorrect orientation and so no sensible transcription of geldanamycin can take place from a phage determined promoter up-stream resulting in a geldanamycin non-producing phenotype.

CHAPTER 6

DNA-DNA HYBRIDISATION INVOLVING POLYKETIDE SYNTHASE GENES

6.1 Introduction

Malpartida and Hopwood (1984) have argued that if the different Actinomycete polyketide synthases had a common evolutionary origin, as seems most probable, then cloned DNA coding for one synthase might be used as a hybridisation probe for the isolation of others. Indeed, Malpartida *et al.* (1987) proved this to be so in a number of cases. Their study, however, revealed only a partial correlation between polyketide production and the presence of DNA sequences homologous with actinorhodin polyketide synthase probes (*act*I and *act*III) in that only fourteen of the eighteen known polyketide producers showed hybridisation. The four strains (and their respective antibiotics) that failed to hybridise were *S. rimosus* (oxytetracycline), *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus*) (erythromycin), *S. griseus* (candicidin) and *S. curacoi* (aramycin).

Interestingly, the DNA encoding oxytetracycline production has since been cloned from *S. rimosus* and parts of this DNA do hybridise with the *act*I and *act*III polyketide synthase probes (personal communication: Dr. J. Hodgson, SmithKline Beecham, U.K.). This differential hybridisation probably reflects a low level of homology between the actinorhodin and oxytetracycline *pks* genes. The producing organism of erythromycin, *Saccharospolyspora erythraea*, is a bacterium closely related to the

streptomycetes. Although no hybridisation was found between the actinorhodin polyketide probes and total DNA from *S. erythraea* the genes encoding erythromycin biosynthesis were cloned by "chromosome walking" from the resistance determinant, *erm*E. The DNA coding for erythromycin biosynthesis and resistance has also been cloned and extensively studied (Bibb *et al.*, 1985; Stanzak *et al.*, 1986; Weber and Losick, 1988; Dhillon *et al.*, 1989; Weber *et al.*, 1990 and Cortes *et al.*, 1990). The DNA essential for erythromycin synthesis has been partially sequenced and the region encoding part of the polyketide synthase, ORF A, identified. Within ORF A nine separate portions of the predicted amino-acid sequence were found to be very similar to active-site sequences for the constituent catalytic activities of known fatty acid synthases and polyketide synthases, including those for tetracenomycin and granaticin (which do hybridise to the *act*I probe).

Therefore while probing with DNA sequences of known polyketide synthase function may lead to the isolation of further polyketide synthases, the homology at the DNA level between differing *pks*'s may be too low for DNA-DNA hybridisation to occur. Nevertheless, as might be expected, the gene products of non-homologous *pks*'s may be seen to have conserved regions particularly with respect to enzyme active sites.

It is also interesting to note that the polyketide synthases for actinorhodin and erythromycin, which do not hybridise against each other, are of differing types.

The former representing a Type II polyketide synthase and the latter a Type I, or modular, polyketide synthase (see Chapter 1 - General Introduction) and this will be discussed later in this chapter.

As previously stated (Chapter 3) total DNA isolated from the geldanamycin producer *S. hygroscopicus* 3602 showed little, if any, hybridisation with the actinorhodin polyketide synthase probes, *act*I and *act*III. In this chapter we report further DNA-DNA hybridisation experiments involving both known polyketide synthase genes (ORF A from *S. erythraea*) and putative polyketide synthase genes (pIA101; pIA102 and pIA103, derived from *S. hygroscopicus* 3602).

6.2 DNA-DNA Hybridisations Using *ery* ORF A as a Probe

The construct, pBROC397 containing the *ery* A1 open reading frame, Figure 6.1, as a 10.6 kb *Bgl*II fragment cloned into a pAT153 derived plasmid, was purified, digested to completion with *Bgl*II and the resulting fragments separated by agarose gel electrophoresis and a 10.6 kb fragment corresponding to *ery* ORF A1 isolated from the gel using the "Geneclean" procedure.

The 10.6 kb fragment was radioactively labelled with ^{32}P-dCTP using the "Random Primed DNA Labelling Kit" and used as a probe in DNA-DNA hybridisation experiments against pIA101, pIA102, pIA103, *S. hygroscopicus* 3602 "total" DNA, and *S. hygroscopicus* 3672 "total" DNA. The results of this experiment (Figure 6.2) indicate that the *ery* ORF A1 probe hybridised strongly against all of the

Figure 6.1

pBROC 397 : Restriction map of *Bgl*II ended insert.

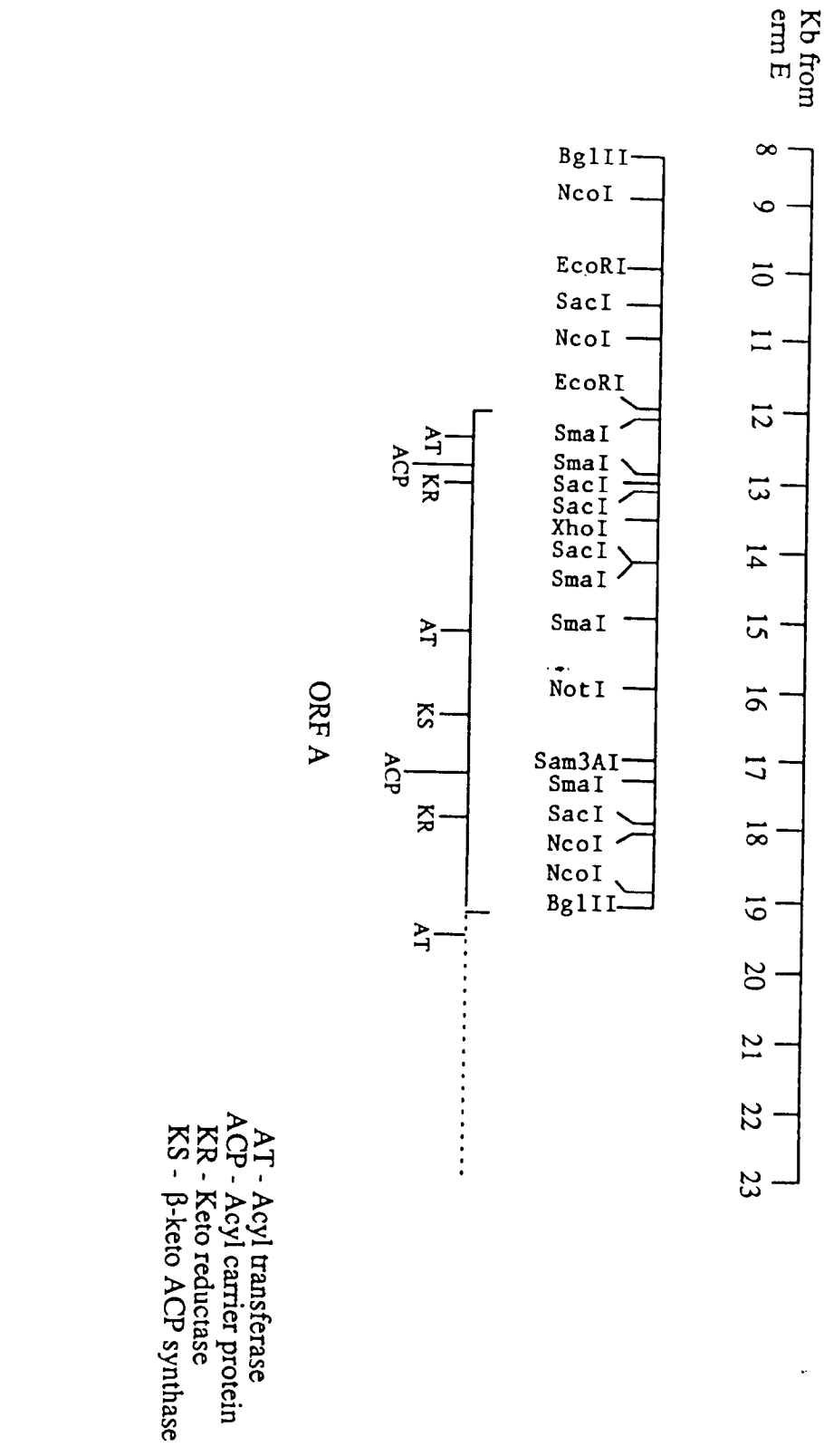
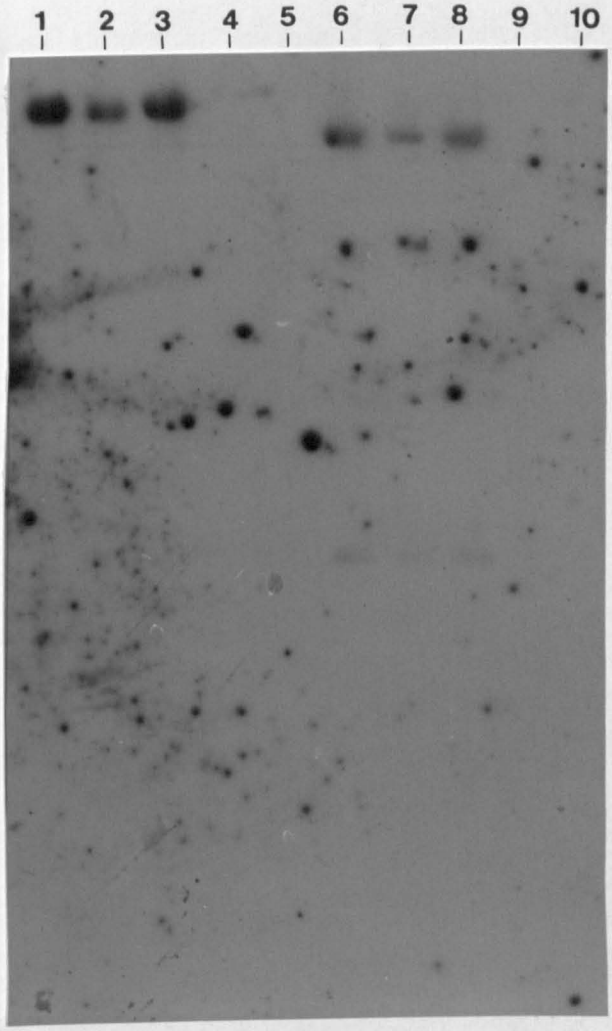


Figure 6.2

Hybridisation of *ery* ORF A1 *vs.* pIA101, pIA102, pIA103 and *S. hygroscropicus* 3602 and *S. hygroscopicus* 3672 "total" DNA.



1	pIA101 (*Bam*HI digest)
2	pIA102 (" ")
3	pIA103 (" ")
4	*S. hygroscopicus* 3602 (*Bam*HI digest)
5	*S. hygroscopicus* 3672 (*Bam*HI digest)
6-10	are repeats of 1-5 digested with *Sst*I.

cloned DNA sequences at both high and low levels of stringency but only hybridised against the *S. hygroscopicus* "total" DNAs slightly at low stringency and even then only at relatively high DNA concentrations.

The fact that the *ery* ORF A1 probe hybridises against cloned DNA but not against "total" DNA is not too surprising and reflects relative concentrations and extent of homology. This was also found when the cloned oxytetracycline *pks* cluster and "total" DNA from its producing organism, *S. rimosus*, were probed with the actinorhodin polyketide synthase probes.

Nevertheless, the probe *ery* ORF A1 does hybridise against pIA101, pIA102 and pIA103 and so there must be homology between these DNA sequences. Since *ery* ORF A1 is known to specify polyketide synthase functions then it is likely that our clones carry at least some of the genes for the synthesis of the polyketide chain of geldanamycin.

6.3 DNA-DNA Hybridisations Using Geldanamycin-Specifying Sequences as a Probe

The fragment of approximately 4.5 kb of DNA used for the gene disruption experiments described in Chapter 5 was labelled with ^{32}P-dCTP using a "Random Primed DNA Labelling Kit" and used as a probe in "dot blot" hybridisations against "total" DNA isolated from *S. lividans* TK24, *S. coelicolor* M145, *Amycolatopsis mediterranaea* (the producer of rifamycin, a naphthalenic ansamycin antibiotic), *S. hygroscopicus* 3672 and *S. hygroscopicus* 3602. The results of the hybridisation

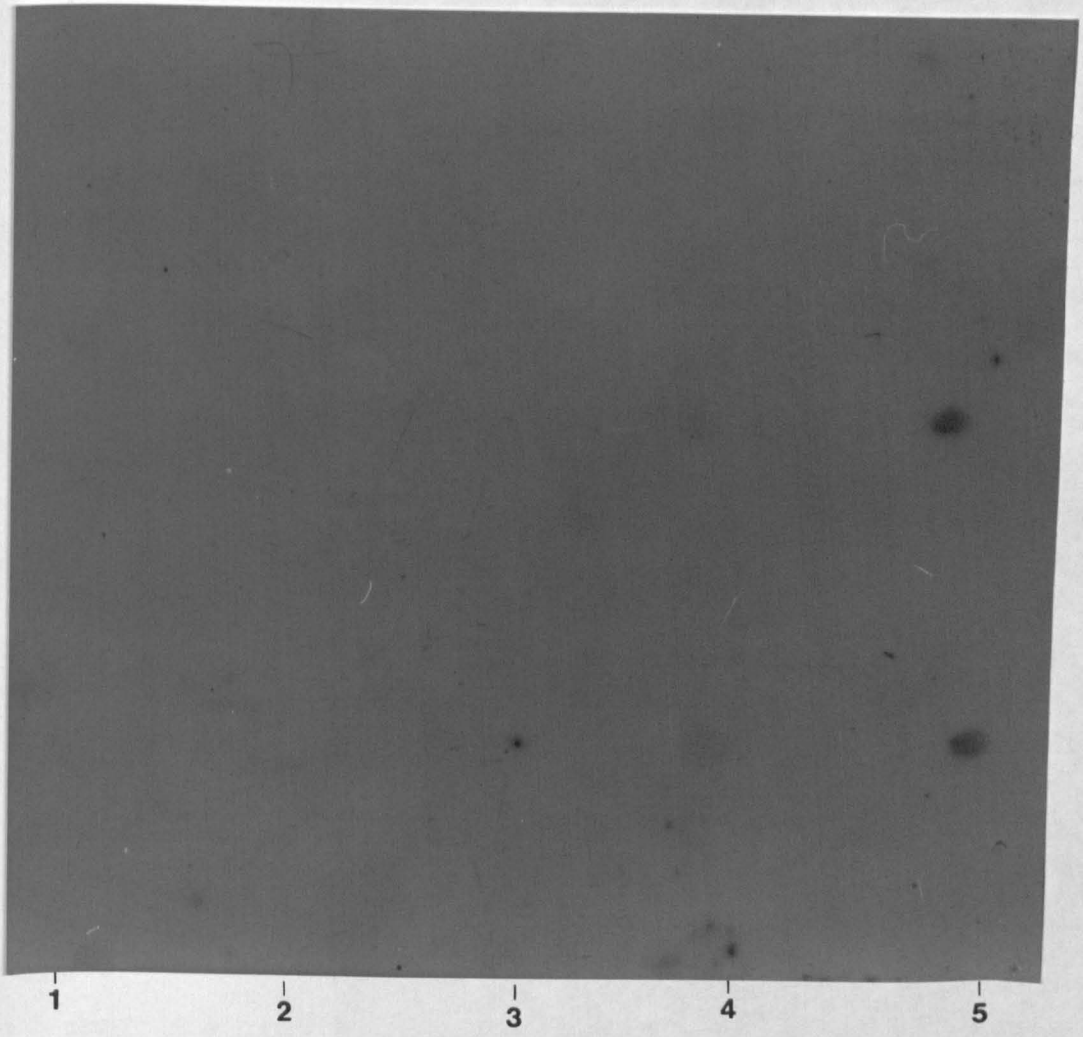
using a high stringency wash show that the geldanamycin specific probe hybridises only with *S. hygroscopicus* 3602, the geldanamycin producer, and rather more weakly with DNA from *S. hygroscopicus* 3672 (Figure 6.3). This probe does not hybridise against DNA from the other strains tested, which are all producers of different polyketides. *S. hygroscopicus* 3672 is the producing organism of herbimycin, an ansamycin polyketide structurally analogous with geldanamycin (Figure 6.4). This DNA sequence would therefore be a useful probe to detect herbimycin specifying DNA sequences from *S. hygroscopicus* 3672. It might be expected that the herbimycin biosynthetic genes are closely related to those for geldanamycin biosynthesis given the structural similarity of their respective polyketide products.

6.4 Discussion

Previous work had shown that the actinorhodin polyketide synthase probes, *act*I and *act*III showed little or no homology with DNA isolated from the geldanamycin-producing *S. hygroscopicus* 3602, which, by direct inference, must contain polyketide synthase specifying DNA sequences. This is also known to be the case for a number of other polyketide producing actinomycetes. One of these *Saccharopolyspora erythraea*, the producer of the macrolide, polyketide antibiotic erythromycin, has been extensively studied and the erythromycin polyketide synthase found to be organised differently to that for actinorhodin. The actinorhodin PKS consists of a multi-

Figure 6.3

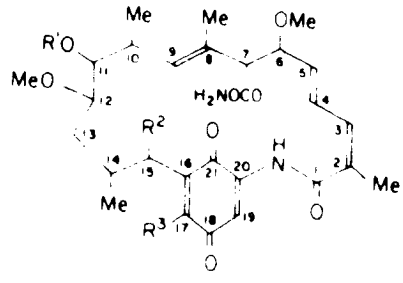
"Dot Blot" of various "total" DNA preparations probed with the *gld* "4.5 kb fragment".



1 *S. lividans* TK24
2 *S. coelicolor* 145
3 *Amycolatopsis mediterranaea*
4 *S. hygroscopicus* 3672
5 *S. hygroscopicus* 3602

Figure 6.4

The structures of geldanamycin and herbimycin.



	R'	R²	R³
Geldanamycin	-Me	-H	-OMe
Herbimycin A	-Me	-OMe	-H
Herbimycin B	-H	-H	-H

enzyme complex with separate polypeptides for each of the different aspects of polyketide chain synthesis. The erythromycin PKS, however, is arranged as three multi-functional enzyme complexes, (one of which is the product of *ery* ORF A1), each of which is responsible for two cycles of polyketide chain extension.

Given that our results indicate homology at the DNA-DNA level between *ery* ORF A1 and the clones pIA101, pIA102 and pIA103 and not to *act*I and *act*III it is very tempting to hypothesise that this reflects similarities between PKSs and that the geldanamycin PKS is organised like that for erythromycin rather than the actinorhodin PKS.

This hypothesis may be supported by the fact that the actinorhodin molecule is structurally simpler than either erythromycin or geldanamycin, involving a shorter polyketide chain, and so PKS organisation may be related to the complexity, or chain length, of the polyketide product. Thus the Type II (actinorhodin) model would represent the simpler polyketides and the Type I (erythromycin) model the more complex polyketides.

Further analysis of the geldanamycin and related ansamycin polyketide synthases is required to substantiate this hypothesis. This will require the isolation of the genes for further ansamycin polyketide synthases.

The use of probes from the erythromycin biosynthetic gene cluster may also allow the isolation of further polyketide synthase genes as an alternative to the actinorhodin polyketide synthase probes. As, for that

matter, may probes derived from pIA101, pIA102 and pIA103, especially as we have already demonstrated hybridisation of a fragment derived from pIA102 with "total" DNA isolated from the herbimycin producer *S. hygroscopicus* 3672.

CHAPTER 7

GENERAL DISCUSSION

7.1 Aims and Achievements

The primary goal of this project was to identify and characterise the biosynthetic genes determining the ansamycin antibiotic geldanamycin. The fact that the geldanamycin molecule is a polyketide suggested that identification could be achieved by probing with DNA known to have polyketide synthase functions. However, probing with the *act*I and *act*III PKS genes from *S. coelicolor* failed to identify the relevant, geldanamycin specifying sequences from chromosomal DNA isolated from *S. hygroscopicus* 3602. As an alternative approach the shotgun cloning of a library of DNA from the producing strain into a non-producing streptomycete host (*S. lividans* TK24) and subsequent screening of the transformants for the acquired ability to synthesise geldanamycin was chosen.

We believe that the data described in this thesis is evidence that this goal has been achieved. However, a number of practical points do bear consideration.

Given that a good screening protocol for geldanamycin production was available, namely selection of the transformants to plasmid-determined thiostrepton resistance; acquisition, by these transformants of antibiotic activity against *B. subtilis* and finally comparison of this antibiotic with purified geldanamycin by TLC, we considered that the isolation of transformant strepto-

mycete colonies carrying plasmid clones specifying geldanamycin would be a potentially successful, if laborious and time-consuming, task.

In fact, clones with the acquired ability to synthesise an antibiotic were isolated relatively quickly, however, the positive identification of this antibiotic was a complicated process. Identification of the antibiotic specified by the clones pIA101, pIA102 and pIA103 required the utilisation of several more techniques of greater complexity than TLC, namely TLC coupled with bioautography, HPLC and mass spectrometry. Only when the results of the last of these techniques were available were we confident that our streptomycete clones were producing geldanamycin.

Most of the problems encountered in identifying geldanamycin, as the product of the streptomycete hosts containing the clones pIA101, pIA102 and pIA103 arose because the identification methods relied upon physical separations of rather crude extracts of spent broths in which the host bacteria were cultured. These crude extracts contained a variety of compounds, most likely *S. lividans* secondary metabolites that created a lot of background in the separations, thereby confusing identifications.

Further confusion was almost certainly caused by the presence in the broth extracts of metabolites that were very closely related to geldanamycin. The existence of these compounds was clearly revealed by mass spectroscopic analysis of TLC-purified broth extracts compared

with similarly prepared, purified geldanamycin. These geldanamycin analogues may arise for several reasons, most likely being by the formation of "dihydroxy-geldanamycin" (Chapter 4, Figure 4.28) in the aqueous solution of the broth, however other substitutions are also possible.

In addition, geldanamycin shows a marked tendency to decompose, especially in solution and on heating. Several of the separation techniques applied to the clone broth extracts, and therefore to the purified geldanamycin control sample, required one or both of these processes. The presence of geldanamycin breakdown products in the analates may well have added to the difficulty in identifying geldanamycin in the clone broth extracts. Difficulty was also experienced in analysing the physical characteristics of the cloned DNA within pIA101, pIA102 and pIA103. Loss of an insert from a plasmid vector is not an uncommon occurrence, with many examples in the literature reporting such DNA instabilities when genetically manipulating streptomycetes. Indeed Dr. S. Foster (Fisons Biochemicals Ltd.) indicated that he had similar problems to ours when attempting to clone the genes required for herbimycin from a *S. hygroscopicus* strain (personal communication).

These DNA instabilities have made mapping the clones impossible so far, so finding some practical way around this barrier is an obvious necessity for their further characterisation.

The reasons for the plasmid instabilities encountered with pIA101, pIA102 and pIA103 are not altogether clear, but in view of our evidence that these clones specify polyketide synthase genes it seems reasonable to assume that some interaction may be taking place between the cloned DNA and genes present on the *S. lividans* host chromosome, for example, PKS/FAS determinants which might be partially homologous to the cloned DNA.

The instability may be exacerbated by the comparatively large size of the inserts contained within the clones, which we estimate to be approximately 15 kb and, if the "modular" hypothesis of *pks* genes holds for geldanamycin, this insert would be expected to encode multiple copies of genes for similar functions, for example seven ß-keto acyl synthase sub-units might be expected. If these genes all exhibited substantial homologies at the DNA sequence level further DNA-DNA interactions could not be ruled out (DNA instability in streptomycetes has been linked with reiterated, repeated sequences. Reviews: Hunter, 1986; Hütter and Eckhardt, 1988). Add to this the consideration that pIJ61, the cloning vector, is of low, but not single, copy number in streptomycete vegetative cells then the possibilities for interactions between homologous DNA are quite numerous and so plasmid instability may not be surprising.

Results described in Chapter 3 show that the DNA sequences cloned in plasmids pIA101, pIA102 and pIA103 exhibited no homology with *act*I and *act*III genes encoding the Type II polyketide synthase of actinorhodin but did

show homology with those for a Type I polyketide synthase, namely ORF A from the erythromycin gene cluster.

One conclusion that might be drawn from this observation is that the geldanamycin biosynthetic gene cluster is arranged as a Type I or modular system. It has already been noted that the gene clusters of several other complex macrolide antibiotics have been found to have a Type I organisation, e.g. tylosin (Leadley, 1991) and that only the relatively simple polyketides are synthesised by a Type II PKS.

However, the estimated size of the cloned inserts specifying geldanamycin synthesis of about 15 kb is small, when compared with the estimated 60 kb of DNA implicated in the biosynthesis of erythromycin. The presumed starter molecule for geldanamycin, amino-hydroxybenzoic acid, would however appear to be a product of the shikimate pathway, the enzymes for which would presumably already exist in *S. lividans* and so would not need to be cloned along with the PKS genes, thus reducing the total amount of DNA that would need to be cloned into *S. lividans* to specify geldanamycin biosynthesis. Whether there is interaction between host and clone sequences in the production of geldanamycin is not clear, but is not an impossibility, e.g. the production of the hybrid antibiotic dihydrogranatirhodin using the cloned genes specifying actinorhodin synthesis to transform the granaticin producer *S. violaceoruber* by Hopwood *et al.* (1985). Further characterisation of the cloned DNA sequences is necessary to clarify the situation.

Using the erythromycin model, it would appear that 15 kb of DNA would be too little to specify all seven condensations required to synthesis the geldanamycin polyketide chain, the subsequent reactions at the ß-carbon residues found in the completed chain (Figure 7.1) and the enzymes required for chain termination and attachment to the benzenic chromophore required to complete the molecule. If this is the case then perhaps the geldanamycin system is intermediate between Types I and II, rather like that for the FAS of *Saccharomyces cerevisiae*.

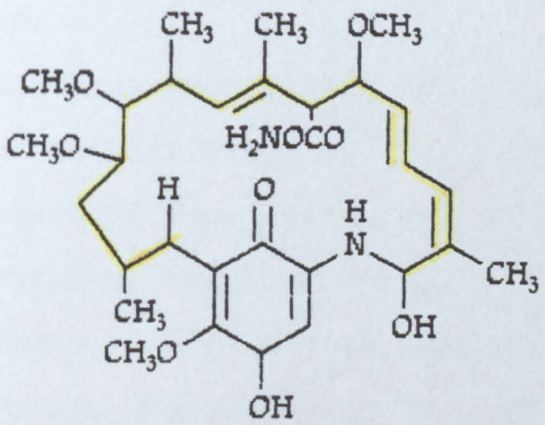
7.2 Future Work

A priority for future work on this project would be to further characterise the cloned DNA specifying geldanamycin biosynthesis. Given the problems previously encountered whilst attempting to do this it may prove easier to use the 4.5 kb sequence of *gld* DNA used to direct the mutational cloning experiments as a probe to detect larger fragments of *S. hygroscopicus* DNA to allow the isolation of the whole of the geldanamycin biosynthetic pathway. Mass spectroscopy could be used to identify DNA sequences specifying geldanamycin production in a suitable host.

Another approach is to use probes for known polyketide synthase functions, e.g. specific regions or domains from *ery* ORF A, against cloned geldanamycin DNA sequences to try to identify similar functions. Short oligonucleotide probes representing known active site

Figure 7.1

The polyketide chain of geldanamycin.



C2 units added		Modifications found at ß-C.
1.	Propionate	fully reduced and dehydrated to alkyl functionality.
2.	Acetate	-
3.	Propionate	reduced and dehydrated to enoyl functionality.
4.	Propionate	-
5.	Acetate	reduced and dehydrated to enoyl functionality.
6.	Acetate (Malonate)	reduced and dehydrated to enoyl functionality.
7.	Propionate	None. Keto functionality intact.

sequences from PKS/FAS domains might also prove suitable.

Information from this approach would be used in conjunction with direct DNA sequencing to provide a clear and detailed map of the geldanamycin specifying DNA sequences to produce a clearer picture of the functioning of PKS systems.

A complete knowledge of the PKS genes for geldanamycin may shed further light on the programming of such systems and, perhaps more importantly may allow us to genetically engineer the system to provide us with alternative end-products. We have already seen how specific mutations in the erythromycin PKS have led to the isolation of predicted shunt-products. An extension of this approach might be to engineer the pathway in such a way as to produce novel therapeutic compounds, for example, by subtly changing the polyketide chain. This might be achieved by effectively deleting some PKS domains, as in the case of erythromycin, but also by substituting one domain for another as for the actinorhodin pathway (Professor D. A. Hopwood, personal communication). It is known that minor changes in antibiotic structures can bring about great changes in antibiotic activities and specificities and so the ability to design drugs has obvious practical repercussions.

Further to this, the more PKS specifying "domains" that are available the easier such work will be, and to this end the cloned *gld* sequences could be used as probes for the detection of DNA fragments specifying other, related polyketides. Two likely candidates would be

herbimycin (from *S. hygroscopicus* 3672) and rifamycin (from *Amycolatopsis mediterraneae*). Herbimycin is structurally very closely related to geldanamycin, in one form actually being an isomer, but exhibits very different biological properties. Comparisons of the geldanamycin and herbimycin PKSs are therefore likely to be most enlightening especially with regard to their "programming". Rifamycin differs from geldanamycin in that it incorporates a naphthalenic rather than a benzenic chromophore in its structure. We have already noted that these two ansamycin groups have widely differing biological activities and so an ability to genetically engineer known polyketide chains onto the different classes of chromophore might lead to the isolation of new antibiotics with interesting properties.

Furthermore, we have noted that the fidelity of the geldanamycin PKS that we have cloned is not as absolute as that found in "wild-type" *S. hygroscopicus* 3602 (mass spectrometry, etc. indicate the presence of analogues of geldanamycin in clone broth extracts). This has interesting implications for the programming of the geldanamycin PKS and its possible future genetic manipulation. To this end it might be beneficial to determine the absolute structures of the geldanamycin analogues produced (possibly by electro-spray mass spectrometry) and their respective biological properties.

REFERENCES

Aszalos, A. and Frost, D. (1975)
Thin-Layer Chromatography of Antibiotics. Method Enzymol. **43**: 172-213.

Bibb, M. J., Schottel, J. L. and Cohen, S. N. (1980)
A DNA cloning system for interspecies gene transfer in antibiotic-producting *Streptomyces*. Nature, **284**: 526-531.

Bibb, M. J., Janssen, G. R. and Ward, J. M. (1985)
Cloning and analysis of the promoter region of the erythromycin resistance gene (*erm*E) of *S. erythraeus*. Gene **38**: 216-226.

Bibb, M. J. and Janssen, G. R. (1986)
Unusual features of transcription and translation of antibiotic resistance genes in antibiotic producing *Streptomyces*. In: Antibiotic Resistance Genes: Ecology, Transfer and Expression. Banbury Report 24: Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. pp.323-334.

Bibb, M. J., Biró, S., Motamedi, H., Collins, J. F. and Hutchinson, C. R. (1989)
Analysis of the nucleotide sequence of the *Streptomyces glaucescens tum*1 genes provides key information about the enzymology of polyketide antibiotic biosynthesis. EMBO J. **8** (9): 2727-2736.

Birch, A. J. (1967)
Biosynthesis of polyketides and related compounds. Science **156**: 202-206.

Bu'Lock, J. D. (1975)
Secondary metabolism in fungi and its relationship to growth and development. In: The Filamentous Fungi, Vol.1, (eds. J. E. Smith and B. R. Berry). E. Arnold, London. pp.33-58.

Buttner, M. J., Smith, A. M. and Bibb, M. J. (1988)
At least three different RNA polymerase holoenzymes direct transcription of the agarose gene (*dag*A) of *Streptomyces coelicolor* A3(2). Cell **52**: 599-607.

Carter, L. and Moran, C. P. (1986)
New RNA polymerase and factor under *spo*0 control in *B. subtilis*. Proc.Natl.Acad.Sci.USA. **83**: 9348-9442.

Chater, K. F. and Merrick, M. J. (1979)
Streptomyces. In: Developmental Biology of Prokaryotes (ed. J. H. Parish). Blackwell Press, London. pp.93-114.

Chater, K. F. (1982)
Streptomyces in the ascendent. Nature **299**: 10-11.

Chater, K. F. and Bruton, C. J. (1983)
Mutational cloning in *Streptomyces* and the isolation of antibiotic production genes. Gene **26**: 67-78.

Chater, K. F. and Hopwood, D. A. (1983)
Streptomyces genetics. Chapter 5 in: The Biology of the Actinomycetes (eds. M. Goodfellow, M. Mordorski and S. T. Williams). Academic Press, London. pp.112-184.

Chater, K. F. (1984)
Morphological and physiological differentiation in *Streptomyces*. In: Microbial Development (eds. R. Losick and L. Shapiro). Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. pp.89-115.

Chater, K. F. and Bruton, C. J. (1985)
Resistance, regulatory and production genes for the antibiotic methylenomycin are clustered. EMBO J. **4** (7): 1893-1897.

Chater, K. F. and Hopwood, D. A. (1988)
Genetics of Bacterial Diversity (eds. D. A. Hopwood and K. F. Chater). Academic Press, London.

Clarke, S., Lopez-Diaz, I. and Mandelstam, J. (1986)
Use of *lacZ* gene fusions to determine the dependence patterns of the sporulation gene *spo*IID in *spo* mutants. J.Gen.Microbiol. **132**: 2987-2994.

Cortes, J., Haydock, S. F., Roberts, G. A., Bevitt, D. J. and Leadley, P. F. (1990)
An unusually large multifunctional polypeptide in the erythromycin polyketide synthase of *Saccharopolyspora erythraea*. Nature **348**: 176-178.

Davis, N. K. and Chater, K. F. (1992)
Spore colour in *Streptomyces coelicolor* A3(2) involves the developmentally regulated synthesis of a compound biosynthetically related to polyketide antibiotics. (In Press).

DeBoer, C., Meolman, P. A., Wnuk, R. J. and Peterson, D. H. (1970)
Geldanamycin, a new antibiotic. J.Antibiotic. **23** (9): 442-447.

Demain, A. L. (1983)
New applications of microbial products. Science **219**: 709-719.

Demain, A. L., Aharonowitz, Y. and Martin, J. F. (1983)
Metabolic control of secondary biosynthetic pathways. In: Biochemistry and Genetic Regulation of Commercially Important Antibiotics (ed. L. C. Vining). Addison-Wesley, Reading, Mass. pp.125-166.

Dhillon, N., Hale, R. S., Cortes, J. and Leadley, P. F. (1989)
Molecular analysis of a gene from *Saccharopolyspora erythraea* which is involved in erythromycin biosynthesis. Mol.Microbiol. **3**: 1405-1414.

Distler, J., Mansouri, K. and Piepersberg, W. (1985)
Streptomycin biosynthesis in *S. griseus*. Adjacent genomic location of biosynthetic genes and one of two streptomycin resistance genes. FEMS Microbiol. Letts. **30**: 151-154.

Donadio, S., Staver, M. J., McAlpine, J. B., Swanson, S. J. and Katz, L. (1991)
Modular organisation of genes required for complex polyketide biosynthesis. Science **252**: 675-679.

Doull, J. L. and Vining, L. C. (1990)
Nutritional control of actinorhodin production by *Streptomyces coelicolor* A3(2): Suppressive effects of nitrogen and phosphate. Appl.Microbiol. Biotechnol. **32**: 449-454.

Drew, S. W. and Demain, A. L. (1977)
Effects of 1^{o} metabolites on 2^{o} metabolism. Ann. Rev.Microbiol. **31**: 343-356.

Dubnau, E., Weir, J., Nair, G., Carter, L. and Moran, C. (1988)
The *Bacillus* sporulation gene Spo6M codes for o^{30} (o^{H}). J.Bacteriol. **169**: 1181-1191.

Enquist, L. W. and Bradley, S. G. (1971)
Characterisation of DNA from *S. genezulae* spores. Dev.Ind.Microbiol. **12**: 225-236.

Errington, J. and Mandelstam, J. (1986)
Use of a *lacZ* fusion to study the dependence pattern of sporulation operon *spo*IIA in wild-type *B. subtilis* and in *spo* mutants. J.Gen.Microbiol. **132**: 2987-2989.

Ghisalba, O., Auden, J. A. L., Schupp, T. and Nüesch, J. (1981)
The rifamycins: properties, biosynthesis and fermentation. Chapter 9 In: Biotechnology of Industrial Antibiotics, (ed. E. J. Vandamire). Marcel Dekker Inc., N.Y. and Basel. pp.281-327.

Gidoh, M., Tsutsumi, S. and Takitani, S. (1981)
Determination of three main anti-leprosy drugs and their main metabolites in serum by HPLC. J.Chromatography **223**: 379-392.

Gil, J. A. and Hopwood, D. A. (1983)
Cloning and expression of a p-aminobenzoic acid synthetase gene of the candicidin producer *Streptomyces griseus*. Gene **25**: 119-132.

Gladek, A. and Zahrzewska, J. (1984)
Genome size of *Streptomyces*. FEMS Microbiol.Letts. **24**: 73-76.

Gramajo, H. C., White, J., Hutchinson, C. R. and Bibb, M. J. (1991)
Overproduction and localisation of components of the polyketide synthase of *Streptomyces glaucescens* involved in the production of the antibiotic tetracenomycin C. J.Bacteriol. **173** (20): 6475-6483.

Gussin, G. N., Johnson, A. D., Pabo, C. O. and Sauer, R. T. (1983)
Repressor and Cro protein structure, function and role in lysogenisation. In: Lambda II (eds. R. W. Hendrix, J. W. Roberts, F. W. Stahl and R. A. Weisberg). Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. pp.93-121.

Guthrie, E. P. and Chater, K. F. (1990)
The level of a transcript required for production of a *Streptomyces coelicolor* antibiotic is conditionally dependent on a tRNA gene. J.Bacteriol. **172** (11): 6189-6193.

Hodgson, D. A. (1982)
Glucose repression of carbon source uptake in *Streptomyces coelicolor* A3(2) and its perturbation in mutants resistant to 2-deoxyglucose. J.Gen. Microbiol. **128**: 2417-2430.

Hooley, P. and Wellington, E. M. H. (1985)
Formation and regeneration of protoplasts in *Streptomyces hygroscopicus*. Letts.Appl.Microbiol. **1**: 77-80.

Hopwood, D. A., Chater, K. F., Dowding, J. E. and Vivian, A. (1973)
Advances in *Streptomyces coelicolor* genetics. Bacteriological Revs. **37** (3): 371-405.

Hopwood, D. A. and Wright, H. M. (1973)
Transfer of a plasmid between *Streptomyces* species. J.Gen.Microbiol. **77**: 187-195.

Hopwood, D. A., Bibb, M. J., Bruton, C. J., Chater, K. F., Feitelson, J. S. and Gil, J. A. (1983)
Cloning *Streptomyces* genes for antibiotic production. Trends in Biotechnol. **1** (2): 42-48.

Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Burton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M. and Schiempf, H. (1985)
Genetic Manipulation of *Streptomyces*: A Laboratory Manual. The John Innes Foundation, Norwich.

Hopwood, D. A., Malpartida, F., Mieser, H. M., Ikeda, H., Duncan, J., Fujii, I., Rudd, B. A. M., Floss, H. G. and Omura, S. (1985)
Production of "hybrid" antibiotics by genetic engineering. Nature **314**: 642-644.

Hopwood, D. A., Bibb, M. J., Chater, K. F., Janssen, G. R., Malpartida, F. and Smith, C. P. (1986)
Regulation of gene expression in antibiotic-producing *Streptomyces*. In: Regulation of Gene Expression: 25 Years On (eds. I. R. Booth and C. F. Higgins). 39th Symposium of the Society of General Microbiology. pp.251-276.

Hopwood, D. A. (1988)
Towards an understanding of gene switching in *Streptomyces*, the basis of sporulation and antibiotic production. Proc.R.Soc.Lond.B. **235**: 121-138.

Hopwood, D. A. and Chater, K. F. (1989)
Antibiotic biosynthesis in *Streptomyces*. In: The Genetics of Bacterial Diversity (eds. D. A. Hopwood and K. F. Chater). Academic Press, London. pp.129-150.

Hopwood, D. A., Sherman, D. H., Khosla, C., Bibb, M. J., Simpson, T. J., Fernandez-Moreno, M. A., Martinez, E. and Malpartida, F. (1990)
"Hybrid" pathways for the production of secondary metabolites. Proc.6th Int.Symp.Gen.Ind.Micro. (GIM90), Strasbourg. pp.

Hopwood, D. A. and Sherman, D. H. (1990)
Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. Ann.Rev.Genet. **24**: 37-66.

Hunter, I. S. (1986)
Gene cloning in *Streptomyces*. Chapter 2 in: DNA Cloning. (ed. D. M. Glover). I.R.L. Press, Oxford. pp.19-45.

Hütter, R. and Eckhardt, T. (1988)
Genetic manipulation. Chapter 4 in: Actinomycetes in Biotechnology (eds. M. Goodfellow, S. T. Williams and M. Mordarski). Academic Press, London. pp.88-184.

Isaacson, D. M. and Kirschbaum, J. (1984)
Assays of antimicrobial substances. Chapter 29 in: Special Topics, 24th Int.C.Ant.AG.Ch. pp.410-435.

Iwai, Y., Nakagawa, A., Sadakare, N., Omura, S., Oiwa, H., Matsumato, S. and Takahoshi, M. (1980)
Herbimycin B, a new benzoquinoid ansamycin with anti-TMV and herbicidal activities. J.Antibiot. **33** (10): 1114-1119.

Kennedy, T. J. and Moran, C. P. E. (1987)
Organisation and regulation of an operon that encodes a sporulation-essential σ-factor in *B. subtilis*. J.Bacteriol. **169**: 3329-3339.

Kieser, T. (1984)
Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid **12**: 19-36.

Kieser, H. M., Henderson, D. J., Chen, C. W. and Hopwood, D. A. (1989)
A mutation of *Streptomyces lividans* which prevents intraplasmid recombination has no effect on chromosomal recombination. Mol.Gen.Genet. **220** (1): 60-64.

Labell, T. L., Trempy, J. E. and Haldenwang, W. G. (1987)
Sporulation-specific σ-factor σ^{29} of *B. subtilis* is synthesised from a precursor protein P^{31}. Proc. Natl.Acad.Sci.USA. **84**: 1784-1788.

Lago, B. P. and Streicher, S. L. (1982)
New genetics of Streptomycetes. A.S.M.News **48** (2): 53-57.

Lancini, G. (1983)
The Ansamycins. In: Biochemistry and Genetic Regulation of Commercially Important Antibiotics (ed. L. C. Vining). Addison-Wesley, Reading, Mass., USA. pp. 231-254.

Lawlor, E. J., Bayliss, H. A. and Chater, K. F. (1987)
Pleiotropic morphological and antibiotic deficiencies result from mutations in a gene encoding a t-RNA-like product in *S. coelicolor* A3(2). Genes and Dev. **1**: 1305-1310.

Leadley, P. (1991)
Hunting the enzymes of polyketide biosynthesis. SERC Bulletin **4** (8): 26-27.

Lechavalier M. P. and Lechavalier, H. (1967)
Biology of the actinomycetes. Ann.Rev.Microbiol. **21**: 71-100.

Lomovskaya, N. D., Chater, K. F. and Mrstumian, N. M. (1980)
Genetics and molecular biology of *Streptomyces* bacteriophages. Microbiol.Revs. **44**: 206-229.

Losick, R., Kroos, L., Errington, J. and Youngman, P. (1989)
Pathways of developmentally regulated gene expression in *B. subtilis*. In: The Genetics of Bacterial Diversity (eds. D. A. Hopwood and K. F. Chater), Academic Press, London. pp.221-242.

Malpartida, F. and Hopwood, D. A. (1984)
Molecular cloning of the whole biosynthetic pathway of a *Streptomyces* antibiotic and its expression in a heterologous host. Nature **309**: 462-464.

Malpartida, F. and Hopwood, D. A. (1986)
Physical and genetic characterisation of the gene cluster for the antibiotic actinorhodin in *S. coelicolor* A3(2). Mol.Gen.Genet. **205**: 66-73.

Malpartida, F., Hallam, S. E., Kieser, H. M., Motamedi, H., Hutchinson, C. R., Butler, M. J., Sugden, D. A., Warren, M., McKillop, C., Bailey, C. R., Humphreys, G. O. and Hopwood, D. A. (1987)
Homology between *Streptomyces* genes coding for synthesis of different polyketides used to clone antibiotic biosynthetic genes. Nature **325**: 818-821.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982)
Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Martin, J. F. (1983)
Biosynthesis of metabolic products with antimicrobial activities. In: Biochemistry and Genetic Regulation of Commercially Important Antibiotics (ed. L. C. Vining). Addison-Wesley, Reading, Mass., USA. p.207.

McCarthy, A. D. and Hardie, R. G. (1984)
Fatty acid synthase - an example of protein evolution by gene fusion. Trends in Biochem.Science **9**: 60-63.

Murakami, T., Arzai, H., Imai, S., Satol, A., Nagaoka, K. and Thompson, C. J. (1986)
The bialaphos biosynthetic genes of *Streptomyces hygroscopicus*: molecular cloning and characterisation of the gene cluster. Mol.Gen.Genet. **205**: 42-50.

Neil, R. (1987)
Structure and Regulation of the Methylenomycin A resistance gene of *S. coelicolor* A3(2). Ph.D. Thesis, University of East Anglia, Norwich.

Neiderwieser, A. and Pataki, G. (1970)
Progress in TLC and Related Methods, Vol.1. Ann-Arbor-Humphrey Sci.Publ., Ann Arbor, Michigan, USA.

Omura, S., Iwai, Y., Takahashi, Y., Sadakane, N., Nakagawa, A., Oiwa, H., Hasegawa, Y. and Ikai, T. (1979)
Herbimycin: a new antibiotic produced by a strain of *Streptomyces*. J.Antibiot. **32** (4): 255-261.

Paulus, T. J., Tuan, J. S., Leubke, V. E., Maime, G. T., de Witt, J. P. and Katz, L. (1990)
Mutation and cloning of *eryG*, the structural gene for O-methyltransferase from *S. erythraea*, and expression of *eryG* in *E. coli*. J.Bacteriol. **172**: 2541-2546.

Ptashne, M. (1986)
A Genetic Switch: Gene Control and Phage Lambda. Cell Press, Cambridge, Mass., and Blackwell, Oxford.

Rinehart, K. L. Jr. and Shield, L. S. (1976)
Chemistry of the ansamycin antibiotics. Forstschr. Chem.Organ. **33**: 231-307.

Rodicio, M. R. and Chater, K. F. (1982)
Small DNA-free liposomes stimulate transfection of *Streptomyces* protoplasts. J.Bacteriol. **151**: 1078-1085.

Rothrock, C. S. and Gottlieb, D. (1984)
Role of antibiosis in antagonism of *Streptomyces hygroscopicus* var. *geldanus* to *Rhizoctonia salani* in soil. Can.J.Microbiol. **30**: 1440-1447.

Rudd, B. A. M. and Hopwood, D. A. (1979)
Genetics of actinorhodin biosynthesis in *Streptomyces coelicolor* A3(2). J.Gen.Microbiol. **114**: 35-43.

Sermonti, G. and Spada-Sermonti, S. (1955)
Genetic recombination in *Streptomyces*. Nature **176**: 121.

Sherman, D. H., Malpartida, F., Bibb, M. J., Bibb, M. J., Kieser, H. M., Hallam, S. E., Robinson, J. A., Bergh, S., Uhlen, M., Simpson, T. J. and Hopwood, D. A. (1988)
Cloning and analysis of genes for the biosynthesis of polyketide antibiotics in *Streptomyces* species. In: Proc.8th Int.Biotechnol.Symp. (eds. G. Durand, L. Bobichon, J. Florent). Soc.Fr.Microbiol., Paris. **1**: 123-137.

Sherman, D. H., Malpartida, F., Bibb, M. J., Kieser, H. M., Bibb, M. J. and Hopwood, D. A. (1989)
Structure and deduced function of the granaticin-producing polyketide synthase gene cluster of *Streptomyces violaceoruber* Tü22. EMBO J. **8** (9): 2717-2725.

Shumard, R. F. and Callender, M. E. (1967)
Monensin: Biological properties and action. Antimicrob.Agents Chemother. **369**: 1968.

Southern, E. M. (1975)
Detection of specific sequences among DNA fragments separated by gel electrophoresis. J.Mol.Biol. **98**: 503-517.

Stanzak, R., Matsushima, P., Baltz, R. H. and Rao, R. N. (1986)
Cloning and expression in *S. lividans* of clustered erythromycin biosynthesis genes. Bio/Technology **4**: 229-232.

Tabor, S. and Richardson, C. C. (1985)
A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc.Natl.Acad.Sci.USA. **82** 1074-1078.

Thompson, C. J., Ward, J. M. and Hopwood, D. A. (1980)
DNA cloning in *Streptomyces*: resistance genes from antibiotic-producing species. Nature **286**: 525-527.

Thompson, C. J., Ward, J. M. and Hopwood, D. A. (1982)
Cloning of antibiotic resistance and nutritional genes in Streptomycetes. J.Bacteriol. **151** (2): 668-677.

Thompson, C. J., Kieser, T., Ward, J. M. and Hopwood, D. A. (1982)
Physical analysis of antibiotic resistance genes from *Streptomyces* and their use in vector construction. Gene **20**: 51-62.

Tsai, J. F-Y. and Chen, C. W. (1987)
Isolation and characterisation of *Streptomyces lividans* mutants deficient in intraplasmid recombination. Mol.Gen.Genet. **208**: 211-218.

Turner, S. M., Errington, J. and Mandelstam, J. (1986)
Use of the *lacZ* gene fusion to determine the dependence pattern of sporulation operon *spo*IIIC in *spo* mutants of *B. subtilis*: a branched pathway of expression of sporulation operons. J.Gen.Microbiol. **132**: 2995-3003.

Vara, J., Lewandowski-Skarbek, M., Wang, Y-G., Donadio S. and Hutchinson, C. R. (1989)
Cloning of genes governing the deoxy sugar portion of the erythromycin biosynthesis pathway. J.Bacteriol. **171**: 5872-

Vögtli, M. and Hütter, R. (1987)
Characterisation of the hydroxystreptomycin phosphotransferase gene (*sph*) of *S. glaucescens*. Mol.Gen. Genet. **208**: 195-203.

Weber, J. M. and Losick, R. (1988)
The use of a chromosome integration vector to map erythromycin resistance and production genes in *S. erythraea*. Gene **68**: 173-180.

Weber, J. M., Schoner, B. and Losick, R. (1989)
Identification of a gene required for the terminal step in erythromycin biosynthesis in *S. erythraea*. Gene **75**: 235-241.

Weber, J. M., Leung, J. O., Maine, G. T., Potenz, R. M. B., Paulus, T. J. and DeWitt, J. P. (1990)
Organisation of a cluster of erythromycin genes in *Saccharopolyspora erythraea*. J.Bacteriol. **172** (5): 2372-2383.

Weber, J. M., Leung, J. O., Swanson, S. J., Idler, K. B. and McAlpine, J. B. (1991)
An erythromycin derivative produced by targeted gene disruption in *Saccharopolyspora erythraea*. Science **252**: 114-117.

Westpheling, J., Ranes, M. and Losick, R. (1985)
RNA polymerase heterogeneity in *Streptomyces coelicolor*. Nature **313**: 22-27.

Williams, S. T. (1978)
Streptomycetes in the soil ecosystem. In: *Nocardia and Streptomyces* (eds. M. Mordarski, *et al.*). Gustavfischer Verlag, N.Y. pp.137-144.

Williams, S. T. and Wellington, E. M. H. (1982)
Actinomycetes. In: Methods of Soil Analysis, Part 2: Chemical and Microbiological Properties (eds. C. Black). Agronomy Monograph No.9, ASA-SSSA, Madison, WI, USA, pp.969-987.

Williams, S. T., Goodfellow, M., Wellington, E. M. H., Vickers, J. L., Alderson, G., Sneath, P. H. A., Sackin, M. J. and Mortimer, A. M. (1983)
A probability matrix for the identification of some streptomycetes. J.Gen.Microbiol. **129**: 1815-1830.

Woese, C. R. and Bleyman, F. (1972)
Genetic code limit organisms - do they exist? J.Mol.Evol. **1**: 223-229.

Woodruff, H. B. (1966)
The physiology of antibiotic production: the role of the producing organism. Soc.Gen.Microbiol.Symposium **16**: 22-46.

Zhou, X., Deng, Z., Firmin, J. L., Hopwood, D. A. and Kieser, T. (1988)
Site-specific degradation of *Streptomyces lividans* DNA during electrophoresis in buffers contaminated with ferous iron. Nuc.Acids Res. **16** (10): 4341-4352.

Zuber, P. and Losick, R. (1987)
Role of AbrB in SpoOA$^-$ and SpoOB$^-$ dependent utilization of a sporulation promoter in *B. subtilis*. J.Bacteriol. **169**: 2223-2230.