THE DISTRIBUTION OF PHENOTYPIC AND GENOTYPIC CHARACTERS WITHIN STREPTOMYCETES AND THEIR RELATIONSHIP TO ANTIBIOTIC PRODUCTION.

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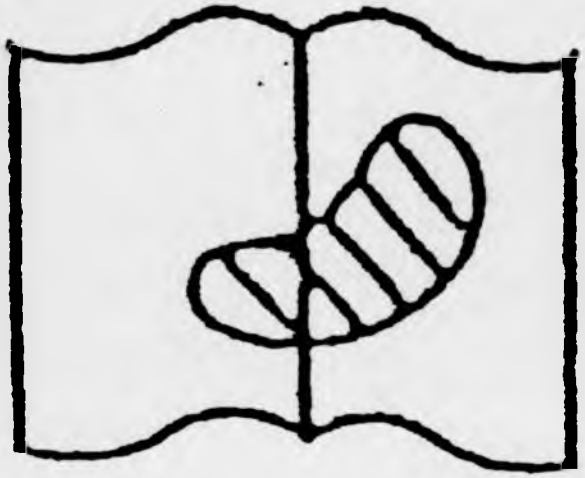

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Declaration

I declare that all of the work contained in this thesis is my own, unless otherwise acknowledged, and has not been used in any previous application for a degree.

Lesley Phillips

I hereby certify that this statement is correct,

Dr E.M.H. Wellington,

(Academic supervisor of this work.)

Abbreviations

AGS	Arginine glycerol salts medium
aph	Neomycin phosphotransferase gene
aphD	Streptomycin phosphotransferase gene
bar	Dimethyl phosphinothricin acetyl tranferase gene
CL	Complete linkage
c.p.m.	counts per minute
DNA	Deoxyribonucleic acid
EDTA	Diaminoethanetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
FAME	Fatty acid methyl ester
GCMS	Gas chromatography mass spectrometry
HPLC	High -pressure (performance) liquid chromatography
HSD	Honestly significant difference
Kb	Kilobase pairs
MIC	Minimum inhibitory concentration
Min.	Minute(s)
MSD	Minimum significant difference
Nb^r	Novobiocin resistance determinant
NMR	Nuclear magnetic resonance
OTU	Operational Taxonomic Unit
PCA	Principle components analysis
Pi	Inorganic phosphorus
RNA	Ribonucleic acid
r.p.m.	Revolutions per minute
Sd	Standard deviation
S_{dice}	Dice coefficient
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
S_j	Jaccards coefficient
SL	Single Linkage
S_{sm}	Simple matching coefficient
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl)aminoethane
tsr	Thiostrepton rRNA ribosomal methylase gene
UPGMA	Unweighted pair-group arithmetic average clustering
UV	Ultraviolet
vph	Viomycin phosphotransferase gene

Summary

A collection of over 300 streptomycetes, comprising both natural isolates and type strains, were assessed for the distribution of fatty acids, antibiotic resistances, compounds in solvent extracts, and sequences that hybridized to antibiotic resistance gene probes. These data were clustered using numerical methods and groups of similar strains were delimited. The production of selected antibiotics by all strains was also determined and the data examined for any correlations with the defined clusters.

Production of bioactive compounds was observed in twenty percent of the natural isolates studied; nigericin and geldanamycin production was common. Geldanamycin was only biosynthesized by strains which also produced nigericin and most strains which were thought to produce a novel antibiotic were also capable of nigericin production. When the sources and concentrations of nutrients in the cultures of antibiotic producers were altered, differentiation responses and the expression of antibiotic production were found to be strain specific. In addition to this, nigericin production could be repressed by oxygen limitation.

In a production medium, fluctuations in fatty acid profiles occurred concomitantly with antibiotic biosynthesis, although this could not be correlated. However, fatty acid profiles did not allow the delimitation of groups of taxonomically related strains, which were cultured in a growth medium. Thin layer chromatography patterns obtained from the solvent extracts of streptomycete cultures showed much variation and were not suitable for use as systematic data.

Antibiotic resistance patterns allowed the delimitation of bioactive strains from certain non-bioactive groups of strains; antibiotic producers showed multiple antibiotic resistance phenotypes, whereas a single selected resistance and a multiple sensitive phenotype was characteristic of non-bioactive strains. Most strains were resistant to penicillin and a large proportion were resistant to nigericin, but strains with resistance to aminoglycosides were rare.

Preliminary evidence showed that DNA from strains with multiple resistance patterns hybridized more often to gene probes (these were internal fragments of *aphD, aph, vph, bar, tsr* Nb^r) than did DNA from strains with multiple sensitive phenotypes. Similarites were observed between strains grouped by their hybridization patterns and those grouped by the chemical class of their product. This work also allowed the selection of phenotypically resistant strains with different resistance genes and possible resistance mechanisms.

Chapter 1.

Introduction

1.1. The Lifecycle and Ecology of the Streptomycetes.

Streptomycetes are commonly found in a variety of terrestrial and aquatic environments, including compost, river mud, sea water (Williams and Wellington, 1982; Williams *et al.*, 1989b) and the intestines of insects (Bignell *et al.*, 1991). They are particularly abundant in the soil (Williams and Wellington, 1982) and are present in both the surface layers and the lower horizons. Soils which are present in countries with warm climates are more conducive to extensive colonisation than are those in cooler areas and streptomycete populations proliferate in grassland and pasture soils rather than those which are waterlogged or acidic (Alexander, 1977; Reed Rodrigues Coelho and Drozdowicz, 1978).

The number and diversity of streptomycetes depends on seasonal variations (Williams, 1978, Huck *et al.*, 1991); although their abundance can vary markedly in different microsites within the same soil sample. For example, Wellington *et al.* (1990) obtained electron micrographs of streptomycete mycelia in the soil showing large uncolonised areas, but with hyphae present in distinct regions within crevices between the soil particles. The size of a streptomycete community also depends on physical characteristics, such as pH and the presence of organic matter.

Streptomycetes play an important role in degrading complex recalcitrant polymers from plant and animal tissue (Williams, 1978). When nutrients have been depleted streptomycetes undergo a series of morphological changes, which begin with the lysis of vegetative mycelia. This provides nutrients for the production of aerial mycelia, which coil and divide from the tip downwards. The walls of the individual compartments formed by this process thicken and round off to give chains of immature, asexual spores which become more spherical and contain pigments when fully mature.

Organisms which sporulate may be well suited to survival in an environment where there are large fluctuations in nutrient availability. Streptomycetes are likely

to exist in the soil predominantly as spores, only becoming active mycelial organisms for brief periods of time when conditions are favourable (Wellington *et al.*, 1990). Germination of spores and the development of mycelium is a rapid process (Wellington *et al.*, 1990) and so shows good adaptation to feast and famine conditions. In addition, the ability to grow in filaments facilitates the search for nutrients over a larger area than would be permitted by unicellular growth.

1.2. The Taxonomy of Streptomycetes.

1.2.1. The Definition of the Genus *Streptomyces*.

Waksman and Henrici proposed the genus *Streptomyces* in 1943, and its taxonomy was developed over the next forty years, by workers such as Silvestri *et al.* (1962) and Williams *et al.* (1983a). *Streptomyces* became well known as a rich source of antibiotics with many new species being described for patent purposes. This was often a subjective procedure, since early attempts at species classification were based on differences in morphology and cultural characteristics. Conditions used in these studies were not standardised and so the overall result was a proliferation in the total number of *Streptomyces* species. The International *Streptomyces* Project was initiated in 1964 to help improve the situation and it resulted in the redescription of over 450 species (Shirling and Gottlieb, 1976).

In 1983, Williams *et al.* (1983a) published a study, which became the foundation of the current *Streptomyces* classification. Correct identification of streptomycetes was viewed as polythetic (Williams *et al.*, 1983a) and was obtained by probabilistic methods (Williams et al., 1983b). The resulting phena contained strains with high overall similarities, but no single character could cause or prevent group membership. The work (Williams *et al.*, 1983a and b) clarified relationships within the genus and led to an extensive revision of the number of genera assigned to the family Streptomycetaceae. It also provided information which was useful for formulating selective isolation procedures (Williams and Wellington, 1982).

Several probabilistic identification matrices have been constructed from the Williams *et al* (1983a) data. The first used forty one distinguishing characters to identify 23 major phena and was a valuable framework for the identification of *Streptomyces* isolates (Williams *et al.*, 1983a). Two further matrices were constructed by Langham *et al.* (1989), one comprised all major clusters from the original classification and the other contained minor and single member clusters. This extended the number of streptomycetes which could be identified probabilistically, whilst utilizing the minimum number of characters required for discriminating taxa.

The great diversity of the genus made it difficult to devise a single workable identification matrix and large numbers of time-consuming tests were therefore required to identify unknown isolates. Several rapid tests have emerged over the last five years, culminating in the introduction of miniaturized numerical identification tests (Kampfer and Kroppenstedt, 1991a). The system was based on physiological characters from which a single probabilistic identification matrix was constructed for all major and minor clusters (Kampfer *et al.*, 1991b). Morphological criteria were not included in this study because they were thought to be too difficult to determine. Many of the groups found by Williams *et al.* (1983a) were confirmed, although some were not detected and the authors concluded that the genus was still overspeciated and suggested the amalgamation of certain species groups and the inclusion of streptoverticillia within the genus *Streptomyces*. *Streptoverticillium* has been formally reduced to synonomy with *Streptomyces* by Witt and Stackebrandt (1990) and the genus now also includes *Kitasatosporia* (Wellington *et al.*, 1992) and the genera *Actinopycnidium*, *Chainia*, *Elytrosporangium* and *Microellobisporia* (Goodfellow *et al.*, 1986; Williams *et al.*, 1989b).

1.2.2. Chemotaxonomic Tests used for Classifying Streptomycetes.

Carbon source oxidation tests have been used in several numerical taxonomic studies (Williams *et al.*; 1983a, Kampfer *et al.*, 1991a) and the more traditional methods relying on detecting pH changes were often biased towards sugar utilization tests. Recently, Bochner (1989) devised an automated microtitre system, which used up to 95 carbon sources for differentiating between Gram-positive organisms. A redox dye indicated increased respiration and the results were automatically stored on a computer database which was equipped with systematic techniques. Other recently developed methods, which have proved useful for detecting enzymatic activity, have used chromogenic and fluorogenic substrates. For example, Goodfellow *et al.* (1987) found that groups, which were defined by Williams *et al.* (1983a), had individual patterns of enzyme activity, whilst Kampfer *et al.* (1991a), who incorporated similar tests in their numerical study, obtained different results from the work of Williams *et al* (1983a).

Pyrolysis mass spectrometry has been used to give whole cell fingerprints of a variety of organisms including streptomycetes (Gutteridge, 1985). Protein profiling has also proved to be a useful taxonomic tool (Kersters, 1985). The latter technique, involves submitting soluble cellular proteins to electrophoresis and extracting the banding pattern obtained on the gel, which can then be compared with the profiles of other strains. For example, Ochi (1989) made ribosomal protein patterns using two-dimensional gels, which showed specific profiles within *Streptomyces* species, whilst Vesselinova and Tsvetkov (personal communication) have shown discrimination between variants of the same strain. Serology has also been applied to *Streptomyces* taxonomy and although it previously relied upon Ouchterlony double diffusion assays, it has now been improved by the use of Enzyme Linked Immunosorbant Assays (Kirby and Rybicki, 1986).

Isoprenoid quinones are present in most bacterial membranes and the menaquinones of streptomycetes, which vary in length and saturation, have proved valuable in *Streptomyces* taxonomy (Alderson *et al.*, 1985). The composition of

isoprenoid quinones changes as the culture ages and so harvesting must take place at specific stages in the growth cycle (Saddler *et al.*, 1986). Fatty acids are also present in bacterial membranes (Kaneda, 1991) and Saddler *et al.* (1986) showed that the fatty acid profiles of *Streptomyces cyaneus* remained relatively constant during both the stationary phase and logarithmic growth. Fatty acid methyl esters (FAMEs) have most frequently been used to differentiate organisms above the genus level (Goodfellow, 1989), although Saddler *et al.* (1987) partially recovered a group of *S.cyaneus* strains from a heterogeneous mixture of related taxa by using FAME profiles.

DNA hybridization studies on streptomycetes have been carried out by various workers, who have then compared their results with species groups which were defined using other methods. Mordarski *et al.* (1985) carried out hybridizations using DNA from members of the *Streptomyces albidoflavus*, *Streptomyces halstedii* and *Streptomyces griseus* clusters, which were previously defined by Williams *et al.* (1983a) and found partial congruence between the two studies. In a similar study, Labeda and Lyons (1991) carried out extensive DNA-DNA hybridizations on the *S.cyaneus* cluster, but found that hybridization values spanned a wide range (40%-60%) and were not consistent with strains belonging to the same species. Another molecular approach has been to obtain fingerprints of bacterial DNA using restriction analysis with pulse field electrophoresis; the patterns produced appear to be highly strain specific and reproducible (K.J. Forbes, personal communication).

Information from RNA sequencing studies has been used to design species specific oligonucleotide probes (Stackebrandt and Charfreitag, 1990; Kemmerling *et al.*, 1989; Witt *et al.*, 1990). Studies to date have concentrated on 16S rRNA sequences in three regions of hypervariability, however Stackebrandt *et al.* (1991) reported that only one third of strains tested could be distinguished. Probes from the gamma region of 16S rRNA, 23S rRNA and the intercistronic spacer remain under

investigation, although the variation in 5S rRNA was considered unlikely to be sufficient for the construction of species-specific probes (Stackebrandt *et al.*, 1991).

1.2.3. Systematic Methods Used in Taxonomic Studies.

Various aspects of the history and development of taxonomic methods have been reviewed by Cain (1962), Cowan (1962) and Sneath (1962), whilst thorough descriptions of techniques that are used are given by Alderson (1985), Sneath and Sokal (1973) and Austin and Priest (1986).

Classifications can be constructed to serve a variety of purposes and phylogenic, genealogical or cladistic classifications (often based on sequence data) attempt to trace evolutionary pathways, whilst taxonomic classifications aim to make generalisations (with respect to a wide range of tests) about their members (Sneath, 1962). Both are equivalent to phenetic classifications, provided there has been no parallel or convergent evolution (Sneath and Sokal, 1973) and they aim to arrange organisms into groups based on high overall similarities and which contain polythetic taxa (Sneath, 1962).

Special purpose classifications are designed for particular disciplines, where traditional taxonomy is inadequate because of its general nature (eg. a classification system for the food industry might only contain spoilage organisms, Heslop-Harrison, 1962). Such methods of grouping strains are artificial and are based on restricted information; they are largely monothetic and a single feature can be sufficient for group membership (Sneath, 1962).

For a good taxonomic classification the number of strains or operational taxonomic units (OTU's) should be greater than sixty to take account of strain variation and sampling error (Sneath and Sokal, 1973). Larger numbers of strains are required for studies which aim to define taxa than for those which examine relationships within species groups (Austin and Priest, 1986). Sokal (1985) stated that stable classifications can result from as few as 60 characters, although the optimum number of tests for a taxonomic study was given as 100 to 150 by Austin

and Priest (1986). The tests, chosen for a numerical study, should be reproducible, standardized, easy to assess and appropriate for all strains (Goodfellow and Dickenson, 1985), whilst the strains should not be biased towards a particular group and should include reference organisms. Suitable statistics can be employed to detect test error (Sneath and Johnson, 1972).

After the tests have been scored the data must be coded; qualitative data is represented in binary form (1 or 0). Quantitative data can also be represented by binary numbers if one arbitrarily divides the scale up into one or more two state characters (Sneath and Sokal, 1973) or alternatively it can be computed in its raw form. Certain forms of qualitative data can be multi-state; for example colours and these can be divided into several two state characters.

The next stage is to calculate similarity and this is achieved using various coefficients, such as those formulated by Jaccard (Sneath, 1957) S_j (a/a+b+c), Dice (1945), S_{dice} (2a/2a+ b+c) and Sokal and Michener (1958) S_{sm} (a+d/ a+b+c+d). The symbols refer to the number of positive and negative matches (a and d respectively) and dissimilar results (c and b). Distance coefficients, such as Euclidian distance (Sokal, 1961) measure geometrical distances between OTUs; this particular coefficient satisfies the Pythagorus theorem by measuring the distance between OTUs in terms of co-ordinates of a right angle. The choice of coefficient can be determined by the nature of the original data matrix and an association coefficient is preferred for binary data, whereas a distance coefficient is suitable for multi-state data (Sokal, 1985). Most taxonomic studies have used Ssm or Sj (Goodfellow and Dickenson, 1985).

Taxonomic structure is found by ordering OTUs into groups of high overall similarity; hierarchical clustering uses an algorithm to search the data matrix for the best matched pairs of OTUs. The couple with the highest similarity value is treated as a single OTU and the computer retrieves the next most similar OTU to form a group. Algorithms differ by their criterion for group admission; single linkage (nearest neighbour clustering, Sneath, 1957) joins an OTU to an established group

at the highest similarity level of any member of that group and complete linkage (furthest neighbour clustering, Sorensen, 1948) clustering joins the OTU at the lowest similarity, whilst average linkage (the unweighted pair group method of arithmetic averages or UPGMA, Sokal and Michener, 1958) joins a new OTU at the average similarity for all group members. The output from these processes is usually represented as a hierarchical taxonomic tree, which summarises the salient points and can be related to taxonomic rank (Sneath 1962). However such trees can distort the distances between major groups (Alderson, 1985). UPGMA is reportedly the algorithm of choice, because it gives high within-group similarity (Sokal, 1985), although single linkage is often used for comparison against UPGMA (Goodfellow and Dickenson, 1985).

All taxonomic relationships are multi-dimensional and ordination methods allow up to three dimensions to be observed and give a more realistic measure of the distances between groups than a dendrogram. They are most useful if the relationship between entities is continuous, but they may distort distances between close neighbours (Alderson, 1985). OTUs are viewed in phenetic or taxonomic space and their arrangement depends to some extent on the position from which they are observed. Examples include principle co-ordinates analysis, which use distance matrices (PCD) and principle components analysis (PCA), which use distance and similarity matrices. Computing the principle components of the resulting matrix involves calculating its eigenvalues and eigenvectors. The eigenvalues are orthogonal and a framework of low dimensionality accounts for a large proportion of the variation in the original data (Sneath and Sokal, 1973, Alderson, 1985); both methods can give similar results, but PCD is less disturbed by missing data than PCA.

Sackin (1985) pointed out that differences between classifications can be due to addition or removal of characters or OTUs and changes in character coding, coefficient and algorithm. Good classifications should remain invariant when different methods are used for measuring similarity and for the clustering data.

They should also remain stable when new tests and strains are added (Sokal, 1985). Strain similarity can be distorted by test error, reproducibility and the statistics used and it has therefore been recommended that duplicate strains amounting to 5% of the total should be included as an internal check (Goodfellow and Dickenson, 1985).

In addition, the taxonomic map should adequately represent the original data matrix (known as goodness of fit) and this can be assessed by calculating cophenetic values. A good classification shows little overlap between phena (Goodfellow and Dickenson, 1985) and Alderson (1985) pointed out that care should be taken when trying to interpret ordination diagrams, since clusters which are distinct in the full hyperspace may overlap in low dimensional plots. However, there may be a tendency to overlook the fact that many organisms from natural habitats do not form tight clusters (Goodfellow and Dickenson, 1985) and hierarchical methods may impose a hierarchy on data which is not warranted.

Identification is the culmination of a taxonomic procedure and has been reviewed by Holmes and Hill (1985). The difference between the frequency of a test in one taxon and that in another can be used to choose good distinguishing characters (Sneath, 1962). Not all members of a taxon give uniform results, but values of 80-85% of organisms is considered positive and 15-20% negative for the overall phenotype of the group (Austin and Priest, 1986). Computer-based identification systems use diagnostic tables and compare results from unknowns with stored data. The likelihood of an isolate belonging to a given taxon is the probability of obtaining the observed test result with a strain of this taxon and is derived by multiplying together the probabilities of individual test results.

1.3. The Biosynthesis, Evolution and Natural Role of Antibiotics in Streptomycetes.

1.3.1. Interactions Between Primary and Secondary Metabolism in Streptomycetes.

Primary metabolism is an interrelated series of enzyme-catalysed reactions, which are ubiquitous to all microorganisms and which function under a very wide range of growth conditions; the physiological processes involved serve known roles and are important for cellular growth. In this type of metabolism, biosynthetic precursors are converted into essential macromolecules (ie. DNA, RNA, protein, lipids and polysaccharides) which are formed as single definite products. Primary metabolites usually have relatively simple constitutions and intermediate products rarely accumulate, because the relevant processes are finely regulated.

Secondary metabolites are produced by certain taxonomically defined groups of organisms, such as the actinomytcetes, and, although they can reflect common molecules present in particular groups (Lechevalier, 1977), they are usually the distinctive products of individual strains (Arai *et al.*, 1976). Well-defined physiological conditions are required for secondary metabolism and it often occurs after growth-associated cellular processes have declined. Secondary metabolites are structurally diverse compounds with intricate molecular frameworks and, although they have no known role in the internal economy of the producer, they often exibit biological activity. In addition, they have low molecular weights and are usually formed by multi-enzyme complexes as mixtures of closely related substances.

Secondary metabolites belong to a large number of different chemical classes (Figure 1.1.), but are formed by a few basic biosynthetic pathways (Turner, 1973) which are related to and use the intermediates of primary metabolism (Figure 1.2.). In primary metabolism, the catabolism of glucose occurs via pyruvate and acetate and then joins the tricarboxylic acid cycle (TCA). Alternatively hexose monophosphate is shunted through the pentose phosphate pathway and returned to glycolysis in the form of triose phosphate. Glucose is eventually converted to carbon dioxide, water and energy (ATP), although

Fig. 1.1. The chemical classification of antibiotics.

The diagram summarizes a method for classifying antibiotics into families and subfamilies, which is based on their chemical structures. It follows the same classification as that used in the Berdy database (1988), where antibiotics are further sub-divided into groups and then individual antibiotics.

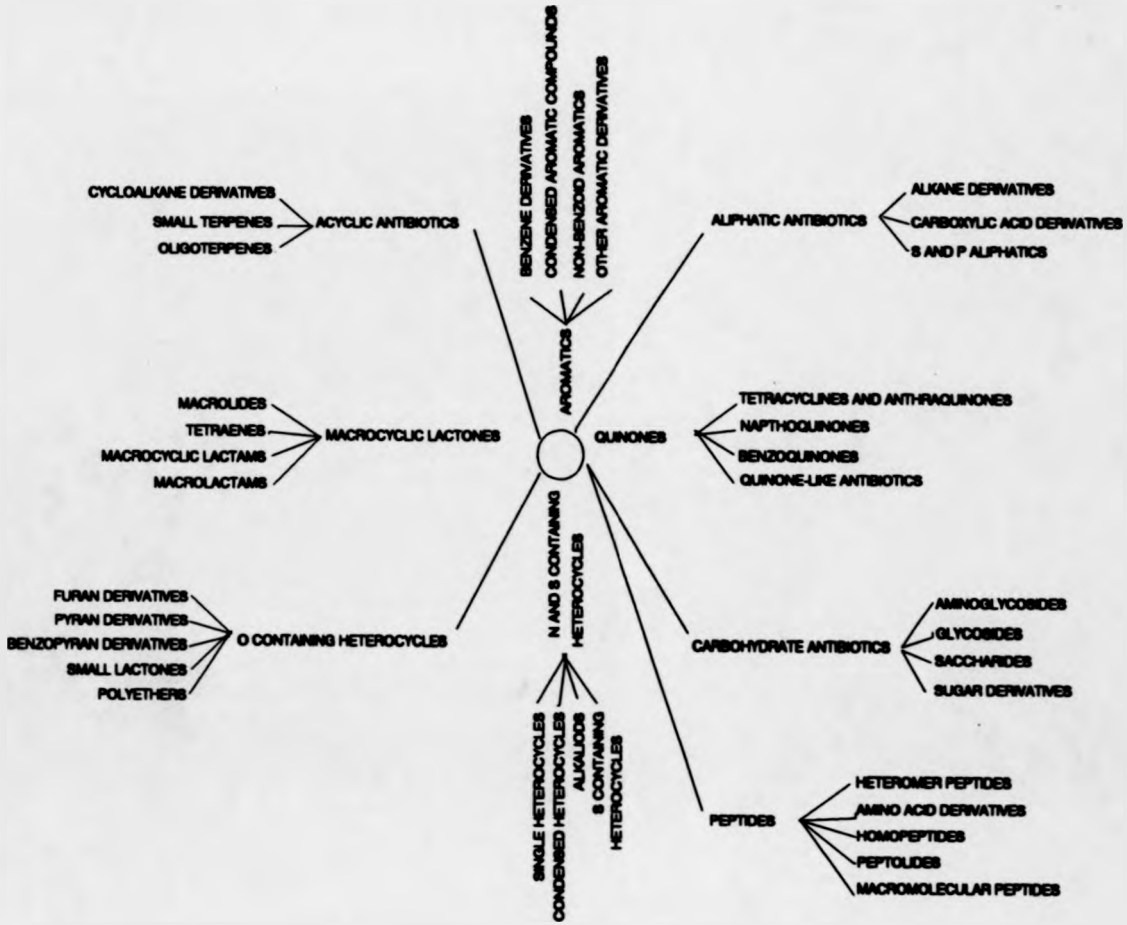
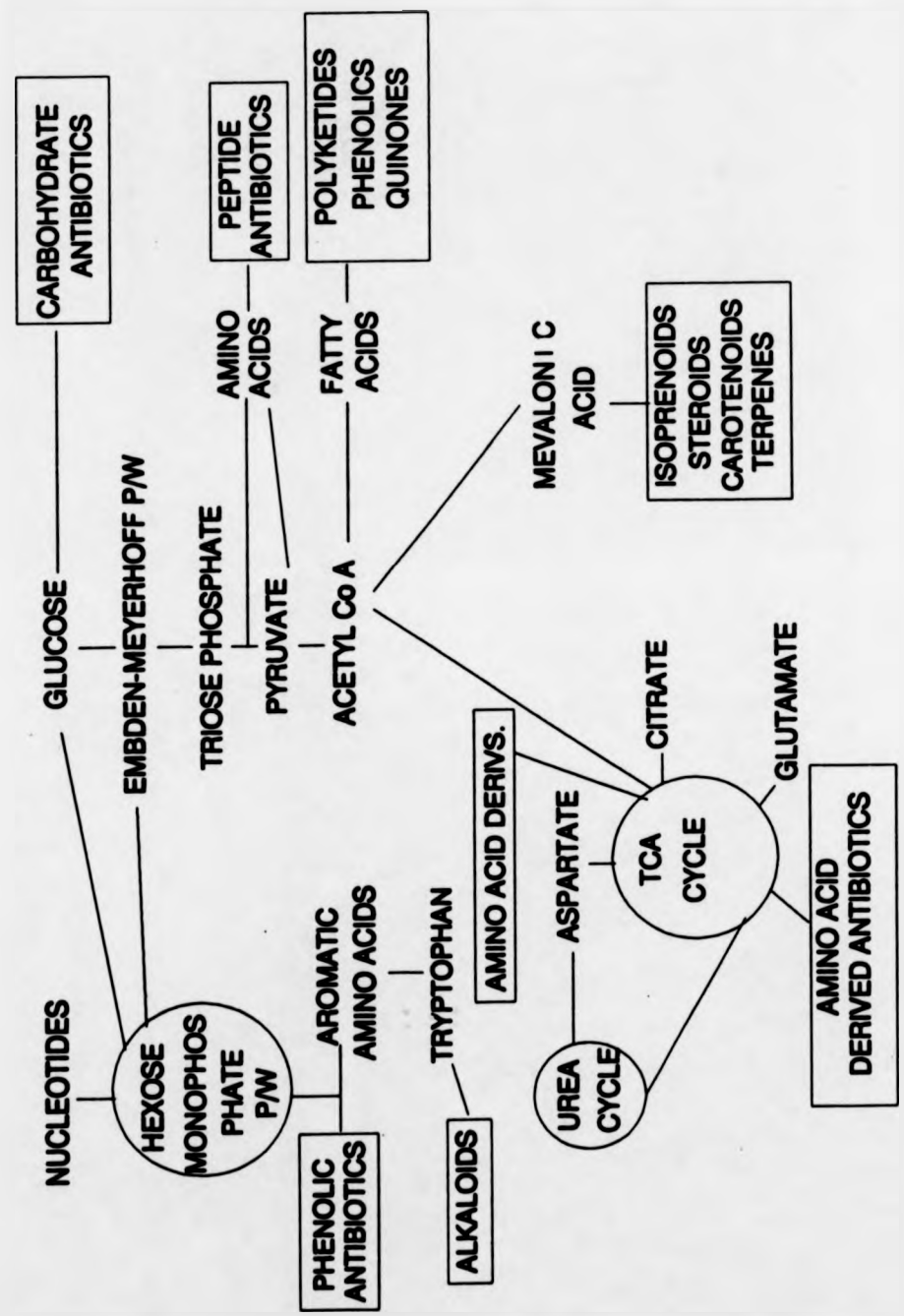

CYCLOALKANE DERIVATIVES
SMALL TERPENES
OLIGOTERPENES
ACYCLIC ANTIBIOTICS
BENZENE DERIVATIVES
CONDENSED AROMATIC COMPOUNDS
NON-BENZOID AROMATICS
OTHER AROMATIC DERIVATIVES
AROMATICS
ALIPHATIC ANTIBIOTICS
ALKANE DERIVATIVES
CARBOXYLIC ACID DERIVATIVES
S AND P ALIPHATICS
MACROLIDES
TETRAENES
MACROCYCLIC LACTAMS
MACROLACTAMS
MACROCYCLIC LACTONES
QUINONES
TETRACYCLINES AND ANTHRAQUINONES
NAPTHOQUINONES
BENZOQUINONES
QUINONE-LIKE ANTIBIOTICS
FURAN DERIVATIVES
PYRAN DERIVATIVES
BENZOPYRAN DERIVATIVES
SMALL LACTONES
POLYETHERS
O CONTAINING HETEROCYCLES
N AND S CONTAINING HETEROCYCLES
CARBOHYDRATE ANTIBIOTICS
AMINOGLYCOSIDES
GLYCOSIDES
SACCHARIDES
SUGAR DERIVATIVES
SINGLE HETEROCYCLES
CONDENSED HETEROCYCLES
ALKALOIDS
S CONTAINING HETEROCYCLES
PEPTIDES
HETEROMER PEPTIDES
AMINO ACID DERIVATIVES
HOMOPEPTIDES
PEPTOLIDES
MACROMOLECULAR PEPTIDES

Fig. 1.2. The interactions of primary and secondary metabolic pathways.

The diagram gives a brief summary which shows how the pathways of secondary metabolism seen in streptomycetes are derived from the catabolic and anabolic pathways of primary metabolism.



intermediates of the glycolytic pathway are used in the anabolism of various primary metabolites, such as amino acids, fatty acids and nucleosides.

The entire carbon skeleton of glucose is incorporated into aminoglycosides (eg. kanamycin, neomycin and streptomycin (Grisebach, 1978), whilst other antibiotics, such as erythromycin and novobiocin contain only a portion in the form of a glycoside moiety (Kominek, 1972; Weber *et al.*, 1985; Stanzak *et al.*,1986). Ribose from the pentose phosphate pathway can be used to form nucleosides or structurally related antibiotics, such as 5-azacytidine, tubercidin and blasticidin S. The biosynthesis of the sugar components in antibiotics has been reviewed in detail by Grisebach (1978).

The shikimic acid pathway of aromatic amino acid synthesis originates from erythrose 4 phosphate in the pentose phosphate pathway and phosphoenolpyruvate from glycolysis. Antibiotics such as lincomycin, actinomycin, thiostrepton and novobiocin are produced directly from aromatic amino acid precursors, whilst ansamycins, such as geldanamycin and herbimycin (Ghisalba *et al.*, 1984) arise from intermediates which lead to shikimate formation. Pathways for the formation of other amino acids, which are involved in antibiotic formation, branch off from triose phosphate (eg. Beta-lactams; Pratt, 1989), pyruvate (eg. bialaphos; Murakami *et al.*, 1986), acetate and the TCA cycle (eg. blasticidin S; Prabhakaran and Gould, 1990). In addition to the standard 18 L-amino acids of primary metabolism microorganisms can use D-amino acids and methyl, dihydro and hydroxy amino acid derivatives to form bioactive cyclic peptides (eg. virgineomycin and cyclosporin A; Monaghan and Tcaks, 1990). Peptide antibiotic formation has been described extensively by Kleinhauf and von Dohren (1987).

Carboxylation of acetate produces malonate, the basic building block of fatty acid biosynthesis, whilst further condensations of these two molecules can lead to the coenzyme-A derivatives of propionate, butyrate, 2-methyl malonate and 2-ethyl malonate, which are involved in polyketide biosynthesis (Floss *et al.*, 1986; Nisbet and Porter, 1989). The substrate specificity of enzyme complexes involved in

polyketide biosynthesis is much lower than that required by enzymes involved in fatty acid anabolism (Kaneda, 1991). Biosynthesis begins with a starter unit (acetate or some other compound) and a highly variable number of malonate, acetate, propionate, butyrate, 2-methyl malonate or 2-ethyl malonate units which become attached together by successive condensation steps. Chain elongation is not dependent on whether the beta-keto group is modified prior to the next condensation step and some of the keto groups are maintained in the final structure, although most are simply reduced to hydroxyl groups. Products of this pathway include actinorhodin, oxytetracycline (Binnie *et al.*, 1989; McDowell *et al.*, 1991) and polyene macrolides, such as erythromycin and polyethers, such as nigericin (Martin, 1977). The isoprenoids, steroids and terpenes are formed via mevalonate from acetate, and geosmin, which gives actinomycetes their characteristic odour, is also formed via this route (Bently and Meganthan, 1981).

1.3.2. The Diversity of Secondary Metabolites from Streptomycetes.

Table 1.1 illustrates the diversity of secondary metabolites with respect to their mode of action, chemical class and spectrum of activity and Fig. 1.1. shows the chemical classification of antibiotic groups and sub-families by their chemical structure and biosynthetic origins. Each sub-family contains a number of groups, for example aminoglycoside antibiotics comprise streptamine, 2-deoxystreptamine and inositol-inoseamine derivatives (eg streptomycin, neomycin and validomycin respectively), other aminocyclitols (fortimycin) and aminohexitols (sorbistin) (Lechevalier *et al.*, 1988). There are over one hundred chemical groups to which the six thousand or more different antibiotics discovered so far (Berdy, 1988) can belong and this constitutes an enormous structural diversity.

The activity spectra shown by antibiotics can differ substantially between compounds belonging to the same group. For example, although the ansamycins geldanamycin and herbimycin are both antifungal compounds, this property is much weaker in herbimycin, whose main activities are against plants and viruses.

Table 1.1. The chemical groups, modes of action and activity spectra of selected antibiotics.

This table shows the diversity of secondary metabolites from streptomycetes (and fungi) and also includes information on antibiotics which were of interest with respect to the research presented later in this thesis; these were bialaphos, herbimycin, geldanamycin, nigericin, erythromycin, neomycin, novobiocin, streptomycin, thiostrepton, viomycin, blasticidin S, kanamycin, oxytetracycline and penicillin G.

Key to activity spectra;

F = fungicidal

G+ = anti-Gram-positive

G- = anti-Gram-negative

H = herbicidal

I = insecticidal

M = anti-mycoplasmal

P = anti-protozoal

T = anti-tumour activity

V = anti-viral

ANTIBIOTIC	CHEMICAL GROUP	PRODUCING STRAIN	MODE OF ACTION	ACTIVITY SPECTRUM
NYSTATIN	TETRAENE	*S.noursei*	Binds to membrane sterols, disturbs permeability.	F,P
ACTINOMYCIN	CHROMOPEPTIDE	*S.antibioticus*	Transcription inhibitor	G+,T
MITOMYCIN	BENZOQUINONE	*S.caespitosus*	Transcription inhibitor	G+,G-,V,T
STREPTOVIRIN	BENZOQUINONE	*S.decaticus*	DNA synthesis inhibitor	G+,G-,M,P,T,(F)
[illegible]	ANTHRACYCLINE	*S.[illegible]*	DNA and RNA synthesis inhibitor	G+,P,T
BIALAPHOS	OLIGOPEPTIDE	*S.hygroscopicus*	Glutamine synthase inhibitor	G+,G-,F,H
HERBIMYCIN	ANSAMYCIN	*S.hygroscopicus*	Protein synthesis inhibitor	V,H (F)
GELDANAMYCIN	ANSAMYCIN	*S.hygroscopicus*	Protein synthesis inhibitor	F,T,V, (G+,G-,P)
NIGERICIN	PYRIMIDINE GLYCOSIDE	*S.hindustanus; S.coeruleorubidus*	Chitin synthesis inhibitor	I,F
MILBEMYCIN	MACROLACTONE	*S.cyanogenus*	Gamma agonist	I
AVERMECTIN	MACROLACTONE	*S.avermitilis*	Gamma-aminobutyric acid agonist	I
POLYOXIN	PYRIMIDINE GLYCOSIDE	*S.cacaoi*	Chitin synthesis inhibitor	F
AMPHOTERICIN B	HEPTAENE	*S.nodosus*	Binds to membrane sterols, disturbs permeability	F
ERYTHROMYCIN	MACROLIDE	*S.erythreus*	Protein synthesis inhibitor	G+,G-,(F)
NEOMYCIN	AMINOGLYCOSIDE	*S.fradiae*	Protein synthesis inhibitor	G+,G-,M
NOVOBIOCIN	Glycosides	*S.niveus*	DNA gyrase inhibitor	G+,G-
STREPTOMYCIN	AMINOGLYCOSIDE	*S.griseus*	Protein synthesis inhibitor	G+,G-,M
THIOSTREPTON	CYCLIC HOMOPEPTIDE	*S.azureus*	Protein synthesis inhibitor	G+,M,F
VIOMYCIN	HOMOPEPTIDE	*S.vinaceus*	Protein synthesis inhibitor	G+,G-,M
BLASTICIDIN S	HETEROCYCLE	*S.griseochromogenes*	Protein synthesis inhibitor	G+,G-,M,F,T
KANAMYCIN	AMINOGLYCOSIDE	*S.kanamyceticus*	Protein synthesis inhibitor	G+,G-,M
NIGERICIN	POLYETHER	*S.hygroscopicus*	Ionophore, interfere with membrane function	G+,M,F,(G-)
OXYTETRACYCLINE	TETRACYCLINE	*S.aureofaciens*	Protein synthesis inhibitor	G+,G-,V
PENICILLIN G	AMINO ACID DER.	*P.chrysogenum*	Cell wall synthesis inhibitor	G+,G-
HERBICIDIN	PURINE GLYCOSIDE	*S.aragonensis*	Nucleoside analogue	T,H,(M)

Geldanamycin has a broader spectrum of activity including antagonism against tumours, protozoa, Gram-negative and Gram-positive bacteria. Other macrolide antibiotics are antiparasitic, antibacterial, antifungal, antiviral and antitumour agents, enzyme inhibitors, immunomodulators and coccidiostats, showing that this family exhibits a wide range of biological activities (Monaghan and Tcaks, 1990).

The modes of action of bioactive secondary metabolites can be categorised into a series of broad groups based on the physiological processes with which they interact and targets include proteases, glucosidases, chitinases and membrane transport; they can also interfere with the biosynthesis of DNA, RNA, proteins and cell wall components. The specific activity of antibiotics can depend on their molecular shape and the distribution of specific functional groups, although compounds with very different structures can act on the same metabolic process (Vining, 1990).

1.3.3. The Evolution of Antibiotics and its Relationship to Antibiotic Function.

Zähner's theory of "elbow room in biochemical evolution" (1982) suggests that secondary metabolic pathways arise in a games room, where a reasonable level of low cost inventive evolution is tolerated. Zähner *et al.* (1982) hypothesized that random combinations of catalytic sequences which gave no advantage to an organism would eventually be eliminated, whilst advantageous combinations would be selected for and maintained. This view of secondary metabolism as evolution in progress was shared by Hütter (1982), who believed that it increased the ability of organisms to cope with a changing environment and Vining (1990), who postulated that new secondary metabolic pathways were acquired when the precursors of primary metabolism became modified by a mutated enzyme. The resulting abnormal product was then modified by random permutations of existing enzymes and advantageous pathways were then be improved by further mutation and selection. Zähner's model also accommodates horizontal gene transfer by "division of labour

in biochemical evolution." Here novel compounds can find a function in an organism other than the one in which they originated. Recent sequence comparisons were consistent with Zähner's gene transfer theory (L.C. Vining, personal communication); for instance, amino acid sequence similarity between fatty acid synthases in animals and polyketide synthases in *Penicillium* was greater than when both enzymes from *Penicillium* were compared. This indicated to Vining that secondary metabolism evolved due to a high rate of gene transfer, rather than coevolution.

Williams *et al.* (1989a) rejected the idea that natural products could have originated from initially neutral mutations, arguing that this was an unlikely route for the formation of the highly ordered gene clusters which give rise to antibiotic production. Instead they reasoned that whilst essential structures and mechanisms evolved early in the evolution of living organisms and are highly conserved, the increased diversification of individual microbial niches led to a later development in genotypic and phenotypic variation. Williams *et al.* (1989a) believed that natural product diversity co-evolved alongside species variety and gave examples of specific compounds, which may have evolved to act in an antagonistic capacity with neighbouring organisms. The sophisticated receptor-antibiotic complementarity of these molecules was seen as evidence for their evolution under the pressures of natural selection specifically as a strategy for survival. Piepersberg *et al.* (1991) took a similar view, believing that secondary metabolism has undergone a long term of evolution, but thought that this may have been in parallel with the metabolism of proteins or RNA with the complete pathways being diversified, degenerated or individualized at a later stage. These workers also suggested that the main period where antibiotics had general and essential functions could have been in precellular times. This is in agreement with recent studies by Julian Davies (personal communication), who has been studying the interactions of certain aminoglycoside and peptide antibiotics with group I introns (which are related to rRNA with respect

to catalytic function). This work has led to the hypothesis that the original function of these molecules could have been to interact with an early form of catalytic RNA.

The gathering of production genes to form clusters could be viewed as a means for conservation under strong selective pressures (the nature of which remains unknown), but this may also allow the transfer of whole biosynthetic pathways (Piepersberg *et al.*, 1991). Piepersberg *et al.* (1991) believe that evolution continued as production genes were acquired, lost and exchanged and that enzyme families involved in secondary metabolism were derived from a few ancient protein groups (not necessarily in producer organisms), whose substrate specificities then evolved divergently and convergently to form various catalytic functions observed today. It is known that genes involved in secondary metabolism are frequently in unstable genomic segments (Altenbuchner and Cullum, 1985; Flett and Cullum, 1987; Birch *et al.*, 1989; Hausler *et al.*, 1989; Leblond *et al.*, 1989) and gene duplication could also be a means of evolution. Genetic instability is often associated with amplification and deletion events in genomic "hotspots" (Demuyter *et al.*, 1991; Schrempf, 1991), which can involve up to 10% of the streptomycete genome (Cullum *et al.*, 1991). Variable streptomycete phenotypes may also be explained by the loss of plasmids (Schrempf, 1982). Hopwood *et al.* (1992) views gene duplication events as a means by which the different forms of polyketide synthase may have evolved.

1.3.4. Theories Proposed for the Function of Secondary Metabolites

One of the leading questions in the study of secondary metabolism is "why are antibiotics biosynthesized?" Even though antibiotics are a highly diverse group of compounds, many of the early hypotheses suggested that they had a universal function. This concept is no longer supported (Vining, 1990) and the present view is that it probably serves a selection of different purposes. There is a great deal of evidence for the functionality of secondary metabolism; firstly the molecules are structurally complex and often show a high degree of complementarity to receptors

at their sites of action. This may suggest a highly sophisticated structure, function relationship (Williams *et al.*, 1989a). Secondly antibiotic biosynthesis is an energy-requiring process and involves a series of complicated reactions, which are are frequently catalysed by unique enzymes and which can respond to environmental factors. Large tracts of DNA are consigned to antibiotic production (Binnie *et al.*, 1989; Stanzack *et al.*, 1990) and the relevant biosynthesis genes are organised into clusters along with regulatory elements and resistance determinants (Chater and Bruton, 1985; Malpartida *et al*,. 1986; Murakami *et al.*, 1988; Binnie *et al.*, 1989; Stanzak *et al.*, 1990).

It may be possible that antibiotics have no function and are an artefact of laboratory cultivation or else they may be produced at such low levels in the natural environment that they remain intracellular. Demain (1981, 1984) has stated several times that antibiotic production in non-autoclaved non-amended soil has been detected and although Williams and Vickers (1986) maintained that convincing evidence was lacking, they believed that production could be possible at low levels within micro-habitats. More recent evidence for antibiotic production in the soil has been provided by Weller and Thomashow (1990), who believe that secondary metabolites are responsible for the success of certain biological control agents.

Some early theories on the function of secondary metabolites considered that they were vehicles for unwanted primary metabolites, which had formed from the metabolism of reactive intermediates and had deleteriously accumulated within the cell (Bu'Lock *et al*, 1965). The fact that many antibiotics are highly toxic themselves was used as evidence against the cellular waste or detoxification theories and Tempest and Neijssel (1975) put forward a modified theory of overflow metabolism. Secondary metabolites were now thought to be adventitious shunt products, which were formed by the action of normal non-specific enzymes on abnormal accumulations of primary intermediates and the biological activity of secondary metabolites was therefore presumed to be accidental. Demain (1984) pointed out that there was an array of regulatory mechanisms to maintain

endogenous products at low levels and that such a system would require the loss of these resulting in competitive disadvantages. The above theories are now discarded by many scientists (Demain 1984; Williams *et al.* 1989a; Vinning 1990), who argue against antibiotic-producing organisms expending large amounts of energy, DNA and enzymes in creating compounds, which at the outset have no use. It has been suggested that the non-specific enzymes involved in the overflow theory may belong to vestigial remnants of a formerly useful pathways. However the existence of dormant pathways is questionable when loss due to mutations and lack of selective pressure is considered.

There are certain cases where shunt products may be a *bona fide* solution to an organism's problem. Hood *et al.* (1992) have shown that proline transport and catabolism are constitutive in a wild type strain of *Streptomyces coelicolor.* Mutant cells, which cannot utilise proline dispose of it in the form of undecylprodigiosin excreted into the medium. These workers suggest that the capacity to produce an antibiotic from an amino acid, creates a sink for excessive amounts of this precursor and that the need to tightly regulate its metabolism is negated; they expect regulation to be tighter in cases where there is no relevant sink compound. Vanek and Mikulik (1978) commented that identical sugar precursors are used in cell wall and aminoglycoside biosynthesis and both processes use the same initial enzymes; consequently cell wall inhibitors can lead to increased aminoglycoside production.

Another early theory of antibiotic function was that they were nutrient reserves and although this theory has been dismissed because antibiotics are usually excreted, it may be true for specific cases. The streptomycin biosynthesis pathway may have developed under positive selection because guanido inositol derivatives served a function as nutrient reserves (Walker 1990). Another hypothesis given by Bu'LocK (1961) was that secondary metabolism maintained enzymes from primary metabolism at a standby level during deleterious growth conditions so that producing organisms could resume normal activity more quickly than dormant species.

Some believe that all secondary metabolites have a biological function and more recently specific functions have been proposed for individual antibiotics and groups of compounds (Draütz and Zähner, 1985). Ionophores are produced by *Nocardia*, *Streptomyces* and *Micromonospora* and can transport specific ions across cell membranes and there is evidence that this plays a useful role in the producer organisms. For example the siderophores and sideramines are involved in the uptake of ferrous and ferric ions (Hütter, 1982) and producers of these compounds have specific membrane proteins for their uptake. Such strains probably have a survival advantage in low iron environments because they are not detrimentally affected by iron depletion. A similar case has recently been cited by Vinning (1990), where a macrotetralide-producing *S. griseus* was able to survive in a high sodium low potassium environment; this was in direct contrast to a non-producing strain. Williams *et al.* (1989a) were convinced that the ionophore lasalocid must have a function because out of all the alternative structural configurations this compound had the best barium-binding capabilities.

Antibiotics have also been proposed as weapons against competitive species. The bleomycins, produced by *Streptomyces verticulus* have been reported to act by binding to guanine containing nucleic acids, especially at sites with alternate purine/pyrimidine bases and sequences with C and T's are cleaved preferentially. Mitomycin C, from *Streptomyces caespitonis*, alkylates DNA and causes cross-linkages to form in helical DNA; it also inhibits DNA polymerase and might inhibit DNA repair. This antibiotic remains inactive until the quinone is enzymatically reduced by the victim, causing activation of the carbamate and azidine moieties. A similar example is given by calichemycin a compound which remains latent until activated by nucleophilic attack, when it develops the ability to cleave double-stranded DNA (specifically after TCCT). Williams et al. (1989a) suggested that the function in all of the above cases was cleavage of non-self DNA.

Functions have also been proposed for certain aminoglycosides and Barabas *et al.* (1976) suggested that streptomycin might be involved in building the cell wall

network in *S. griseus* because streptidine (a component of the streptomycin molecule) was a cell wall constituent. Szabo *et al.*(1985) found streptomycin was bound to the cell walls of hyphae and the spores of *S. griseus*. The antibiotic was liberated mainly in germinating spores and was implicated in the protection of young hyphae. Aminoglycoside antibiotics have also been implicated as regulators of cell wall lysis, since streptomycin has been shown to accelerate the action of autolytic enzymes (Szabo *et al.*, 1990).

Some antibiotics may be agents of metabolic change and may modify gene activity in response to the environment or they could link various activities, which are involved in cytodifferentiation; A-factor, which positively controls sporulation and streptomycin production in *S.griseus* is an example of this (Horinouchi and Beppu, 1986). Intraspecific pheromones which coordinate sporulation within a colony have also been observed in several streptomycetes, namely *Streptomyces viridochromogenes*, *Streptomyces bikiniensis* and *Streptomyces cyaneofusculus* (Professor T. Beppu, personal communication). Antibiotics which affect differentiation include pamamycin, which induced the formation of aerial mycelium and inhibited the uptake of inorganic phosphorus and nucleosides in its producer, *Streptomyces albidoniger* (Pogell, 1984) and methylenomycin A and lincomycin (high concentrations), which inhibited aerial mycelium formation in *S.coelicolor* (Pogell, 1984). However, low concentrations of lincomycin enhance the production of aerial mycelium.

Some antibiotics may confer activity against the activities of organisms from other species and the term ecomone can be used to describe an antibiotic activity which is specifically directed at these organisms in the natural environment. Such an activity could confer a selective advantage on producing organisms and the acceptance that microbial secondary metabolites might be formed in soil microenvironments has helped this idea. For example Rothrock and Gottlieb (1984) described the production of geldanamycin and two other antibiotics by a strain of *Streptomyces hygroscopicus* in sterile soil containing diseased plants.

Martin and Demain (1980) suggested that secondary metabolites could be produced in microenvironments and might confer antimicrobial activities or even reducing predation by protozoa , but to follow the theory through, substances which act on the physiology of higher animals and their immune system must be adequately accounted for; for example B-factor is produced by yeast cells and functions in nocardia by stimulating rifamycin production and it also has a structural similarity to cAMP. Beppu (1985) suggested that some effectors in actinomycetes could be universal signals or messengers for cellular functions in both prokaryotes and eukaryotes. Williams *et al.* (1989a) expanded this idea by suggesting that the molecules which permit specialized glands, neurones and immune blood and tumour cells to communicate (eg. hormones and receptors) were present in unicellular organisms before these larger organs evolved. Some fungi produce sex hormones similar to testosterone, oestradiol, antherdiol and oogoniol and there are also fungal peptide hormones which lead to animal cell proliferation and other compounds (eg. FK506), which can activate T-cells. It has been accepted for a long time that chemical defenses play an important role in the survival of plants and Williams *et al.* (1989a), who pointed out that natural products are most common in organisms which lack an immune system, believe that this is also true for microorganisms.

On the whole the different reputable concepts neither exclude nor contradict one another and each probably plays a part in the true explanation of secondary metabolism, which must encompass mechanisms of evolution, the development of individual functions and nutritional and regulatory aspects.

1.4. The Regulation and Control of Secondary Metabolism.

The course of events associated with the production of antibiotics in the laboratory include a phase of biomass formation (the trophophase) followed by a decline in growth rate and a period of natural product synthesis (the idiophase). Demain (1984, 1985) has suggested that a similar growth and production profile

might be advantageous in the natural environment if the product is either an effector of differentiation or a potent antibiotic which can inhibit the growth of the producer.

Microbial growth is limited in soil ecosystems because nutrients are scarce and so appropriate control systems are required (Mateju, *et al.*, 1985). The various control mechanisms which regulate secondary metabolism in streptomycetes include environmental factors such as carbon, nitrogen, phosphorus, salts, trace metals, oxygen and light and regulation can be through intermediary metabolism or by feedback regulation in secondary metabolic pathways. The negligible expression of antibiotic biosynthesis genes at high growth rates could be due to the repression of inducible catabolic enzymes (Hütter, 1982) or the inhibition of enzyme activity (Iwai and Omura, 1981).

14.1. Carbon Source Regulation.

Glucose exerts carbon catabolite regulation in many fermentations (Iwai and Omura, 1981) and its catabolism accompanies growth. If another substrate is present in the same medium then antibiotic production usually accompanies the utilization of this second compound. The glucose effect interferes with the synthesis of many antibiotics including oleandomycin (Belousova *et al.*, 1985), tylosin (Behal, 1985) and granaticin (James and Edwards, 1988) and is also important for the interconversions of members of an idiolite family. Carbon source regulation can be diminished and antibiotic production stimulated by adding the carbon source periodically as has been shown for the biosynthesis of both candicidin and polyenes (Iwai and Omura, 1981).

When glycerol, sucrose and D-mannose as were used as carbon sources in cultures of *Streptomyces erythraea* they delayed erythromycin production, but increased growth (Sanchez *et al.*, 1984). Erythromycin biosynthesis was only transiently repressed by D-glucose, whilst citrate, propanol and propionate stimulated production of this antibiotic and the effect was thought to be due to induction of propionyl-Co-A carboxylase, one of the first enzymes involved in

erythromycin biosynthesis (Sanchez *et al.*, 1984). Glucose and glycerol are known to completely repress the formation of several enzymes, including tryptophan pyrrolase, kynurenine formamidase II, hydroxykynureninase and phenoxazinone synthase, which are involved in actinomycin D biosynthesis by *Streptomyces parvulus* (Katz *et al.*, 1984, Jones, 1985). Other carbon sources exert a suppressive effect, such as citrate on the production of novobiocin, arabinose on heliomycin biosynthesis (Vinogradova *et al.*, 1985), glycerol on cephalosporin synthesis (Demain, 1985) and mannitol and starch on granaticin production (James and Edwards, 1988).

1.4.2. Nitrogen Source Regulation.

Repression of antibiotic biosynthesis by nitrogen sources such as ammonium ions and certain amino acids is common in streptomycetes (Demain, 1985). Microbial nitrogen metabolism has been reviewed by Gräfe (1982), who concluded that the control of antibiotic formation by nitrogen sources can take three forms. These are (i) positive control, where production is enhanced, (ii) negative control, where antibiotics accumulate after the nitrogen source has been consumed and (iii) control of the product spectrum. Positive nitrogen regulation was thought to be important in rifamycin SV biosynthesis because nitrate induced nitrate and nitrite reductases and enhanced the activity of various dehydrogenases. It also caused a decline in cellular lipid content, suggesting that the presence of nitrate had increased rifamycin biosynthesis at their expense (Chiao *et al.*, 1988).

Nitrogen metabolites can regulate primary metabolism by repressing enzymes that act on nitrogen-containing substrates and changes to both the type and concentration of nitrogen sources can influence antibiotic production (Iwai and Omura, 1981). For example, Sanchez *et al* (1984) found that ammonium strongly repressed erythromycin biosynthesis in *S. erythraea* and that the extent of suppression was proportional to the type of ammonium salt used and its concentration. Differences in erythromycin production were also observed in the

presence of various amino acids and alanine was preferable for both growth and antibiotic formation. Katz *et al.* (1984) reported that other amino acids, namely L-glutamate, aspartate, alanine and D-valine, repressed the synthesis of enzymes which was involved in actinomycin D production.

Rapidly utilised nitrogen sources such as ammonium ion, nitrate and amino acids are good growth substrates, but they sometimes inhibit antibiotic production; examples include oleandomycin biosynthesis by *Streptomyces antibioticus*, and the production of novobiocin by *Streptomyces niveus*. Ammonium ion also exerts a negative effect on the production of streptomycin, cephalosporins, chloramphenicol, leucomycin, tylosin and some rifamycins (Demain, 1985).

1.4.3. Regulation by Inorganic Phosphorus (Pi).

Many idiolites are produced at concentrations of inorganic phosphate (Pi), which are sub-optimal for growth (Demain, 1985) and there are at least two mechanisms responsible for this effect. Pi can repress or inhibit the phosphatases which are involved in antibiotic synthesis, but it can also repress intracellular effectors or possibly other synthetases. Antibiotics with phosphorylated intermediates, such as streptomycin, neomycin, viomycin, turimycin and nourseothricin are subject to the first type of control mechanism (Iwai and Omura, 1981; Muller and Ozegowski, 1985). For example high levels of Pi in the streptomycin fermentation lead to poor antibiotic production and a high concentration of streptomycin phosphate (the last intermediate in the pathway). For antibiotics, which do not have phosphorylated intermediates, but are subject to Pi control, ATP levels decline as Pi decreases and secondary metabolism increases because the metabolic conversions required for antibiotic production are derepressed. As Pi increases, ATP levels increase and so does primary metabolism (Iwai and Omura, 1981).

Candicidin production in *S. griseus* requires the depletion of Pi and extracellular Pi remains low during the entire candicidin idiophase, although

addition of Pi (10mM) at the onset of production, returns the culture to trophophase. During this period ATP levels rise, but protein and RNA synthesis remain constant. This implicates ATP in the control of biosynthesis (Martin and Demain, 1980). High Pi levels repress the synthesis of candicidin synthase (Hütter, 1982) and rifamycin SV production (Chaio *et al.*, 1988). In the latter case growth and ATP levels were enhanced and mycelial lipid levels increased (at 2.5 mM Pi), whilst the enzyme activities involved in rifamycin SV production (methylmalonyl-CoA carboxytransferase and methylmalonyl-CoA mutase) were suppressed. Variations in the adenylate pool were thought to be responsible for these effects. Pi also exerts control over the production of chlortetracycline in *Streptomyces aureofaciens* and an increase in potassium dihydrogen orthophosphate (0.2-0.4mM) accelerates glucose and ammonium consumption, whilst Pi depletion is required before antibiotic production can occur (Martin and Demain 1980). Jechova and coworkers (1985) made similar observations and noted that the intracellular level of adenylates was inversely proportional to the activity of the penultimate enzyme in the chlortetracycline biosynthesis pathway. In *Streptomyces clavuligerus*, another chlortetracycline producer, production increases with increased levels of Pi up to 25mM then declined at higher concentrations (Iwai and Omura, 1981).

1.4.4. Other Bioregulators.

In the cases described above enzyme induction has been caused by the catabolites themselves, but there are also more unusual molecules, which can elicit these effects (reviewed by Beppu, 1985). The best known example of this is the induction of streptomycin biosynthesis by A-factor, which is encoded by the structural gene *afsA* and stimulates transcription of the streptomycin phosphotransferase gene (Horinouch and Beppu, 1986). Demain (1985) stressed that A-factor was most effective when added before growth. The compound is produced by 15% of non-streptomycin-producing streptomycetes and is strain specific. *AfsA*

has an extrachromosomal location in *S.griseus* and this is in contast to the regulatory gene *afsB*, which is located on the *S.coelicolor* chromosome. The latter gene positively regulates the biosynthesis of A-factor in *S. coelicolor* and also plays a role in regulating the production of actinorhodin, undecylprodigiosin and possibly methylenomycin and a calcium-dependent antibiotic. Horinouchoi and Beppu (1986) put forward the hypothesis that the product of this gene, which contains two DNA-protein binding domains, might bind to a regulatory region of a key gene involved in secondary metabolism and either stimulate or decrease its activity. Alternatively the *afsB* protein could bind directly to biosynthetic genes (eg the *act* genes) and stimulate their expression.

Other regulatory substances of actinomycetes include inducing material (IM) in *Streptomyces virginiae*, which effects staphylomycin production and B-factor, which is essential for rifamycin production in *Nocardia* species (Beppu, 1985). The *whiG* gene of *S.coelicolor* encodes an RNA polymerase sigma factor and mutations in this gene block the formation of spores in the aerial mycelium. An increased gene dose of *whiG* gives rise to greater sporulation in *S.coelicolor* and its product is thought to be a positive effector (Mendez and Chater, 1987). Chater *et al.* (1989) sequenced the gene and found it was related to a minor motility sigma factor of *Bacillus subtilis*. Multiple copies of a sigma-dependent promoter from this strain were introduced into *S.coelicolor*, where they caused reduced sporulation. This suggested that the *S.coelicolor* sigma factor recognised and bound to the *Bacillus* promoter and reduced transcription of its normal targets. A range of diverse streptomycetes were found to harbour a *whiG* homologue and this is in direct contrast to the *bldB* gene needed for normal aerial mycelium, which appears to be absent from species other than *S.coelicolor* (Piret *et al.*, 1988).

1.4.5. Genetic Control and Organisation in Secondary Metabolism.

A large number of studies have shown that the genes for the biosynthesis of, resistance to and control of many antibiotics are organised into co-ordinately

regulated clusters (Malpartida and Hopwood, 1984; Murakami *et al.*, 1986; Distler *et al.*, 1987b; Malpartida *et al* 1987, Binnie *et al.*, 1989, Webber *et al.*, 1990). Most appear to be located on the streptomycete chromosome, however the methylenomycin cluster is plasmid-borne (Kirby *et al.*, 1975, Wright and Hopwood, 1976). There is evidence for other production (Kinashi *et al.*, 1987) and resistance (Woodman, personal communication) genes being associated with extrachromosomal elements. The most extensively studied streptomycete genome is that of *S.coelicolor,* a strain which produces several antibiotics including actinorhodin (Rudd and Hopwood, 1979; Malpartida and Hopwood, 1984 and 1986; Malpartida *et al.*, 1987), but much is also known about the genetics of many other antibiotic pathways (Seno and Baltz, 1989).

The streptomycin biosynthesis pathway is fairly well understood (Walker and Walker, 1982) and *S.griseus* has provided a good model to study gene organization and regulation (Distler *et al.*, 1987b) Blocked mutants from this strain have been isolated and analysed for accumulated intermediates (Ohnuki *et al.*, 1985; Distler *et al.*, 1985) and cloned segments of its DNA have been used in complementation studies (Ohnuki *et al.*, 1985). A genetic map of some streptomycin genes is given in Fig.1.3., although currently sequence analysis of the streptomycin gene cluster has revealed at least 14 genes (Piepersberg *et al.*, 1991). The *strB* gene, which encodes an aminidotransferase is linked to *strC* (a dihydrostreptosyl-transferase gene), *strR*, a regulatory gene, which is thought to be a trans-acting positive effector and the resistance gene, which encodes a streptomycin-6-phosphotransferase (*strA*, *aphD*, SPH). A different resistance gene has also been cloned from the biosynthesis gene cluster (Heinzel *et al.*, 1988) and this encodes a streptomycin-3-phosphotransferase. Not all genes involved in streptomycin biosynthesis have been cloned, so evidence for a single gene cluster is incomplete. More is known about the actinorhodin cluster, whose genes are organised into four transcription units, consisting of two polycistronic mRNA's encoding *actI*, *VII*, *IV* and *act V* and *VI* respectively and single small transcripts encoding *actII* (thought to be a positive

Fig. 1.3. Summary of genes, which have been cloned and which are involved in the biosynthesis of streptomycin.

The diagram has been adapted from Seno and Baltz, 1989 and shows the structural organization of the streptomycin gene cluster. DNA segments A to E refer to genes cloned by the following researchers:

A = Ohnuki *et al.*, 1985 (cloned from *S.griseus* ATCC10137).

B = Distler *et al.*, 1985, 1987a and 1987b (cloned from *S.griseus* N2-3-11).

C = Tohyama *et al.*, 1987 (cloned from *S.griseus* ISP 5236).

D = Vallins and Baumberg, 1985 (cloned from *S.griseus* ATCC12475).

E = Kumada *et al.*, 1986 (cloned from *S.bikiniensis* ATCC11062).

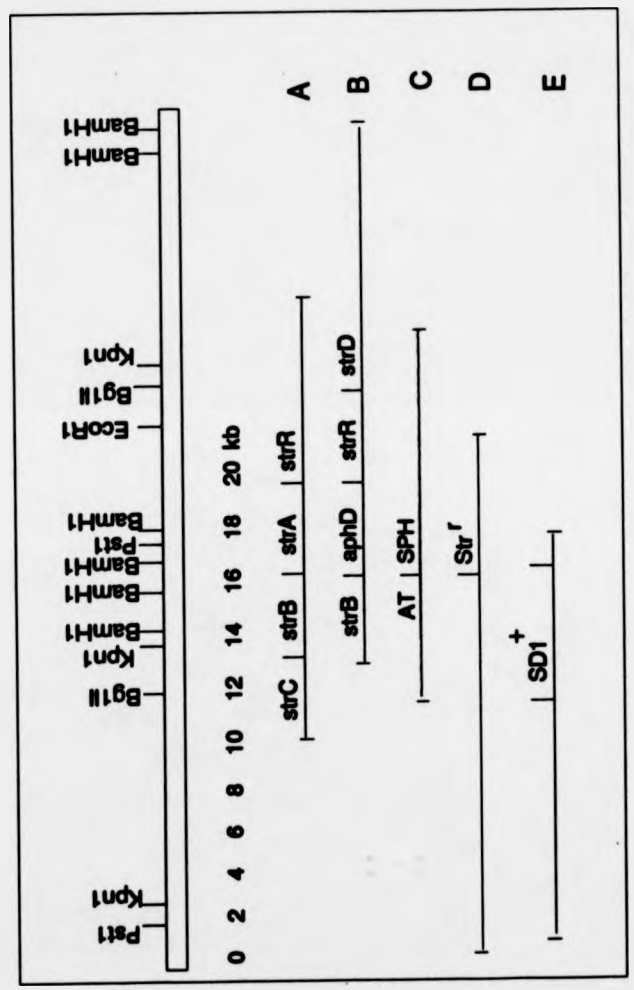


Diagram adapted from Seno and Baltz, 1989.

regulator) and *actII* (Malpartida and Hopwood, 1986). In the case of streptomycin biosynthesis at least 5 partially overlapping transcripts have been detected (Distler *et al.*, 1987b). This type of arrangement might facilitate the co-ordinate or prior activation of resistance genes similar to that demonstrated in *Streptomyces rimosus*, where the *otrA* resistance gene is linked to the early oxytetracycline biosynthesis genes and *otrB* to the late genes (Butler *et al.*, 1989.).

Antibiotic producers may encounter endogenous or foreign antibiotics and might have more than one genetic mechanism specifying resistance to them. Additionally the same resistance mechanism could be under more than one form of regulation (Seno and Baltz, 1989). The streptomycin-6-phosphotransferase gene (Onhuki *et al.*, 1985), which is clustered with streptomycin biosynthesis genes in *S.griseus*, is under both negative regulation by a region downstream of *strA* and positive control by *strR*, which also activates at least one biosynthesis gene. Seno and Baltz (1989) postulated that the negative control responds to exogenous antibiotic, whilst the positive control activates a higher level of resistance prior to antibiotic production.

The promoter regions of several streptomycin biosynthesis and resistance genes have been analysed (*aphD*, *strB* and orf1). The two promoters in *aphD* (P1 and P2) and in orf1 (P1 and P2), the hydroxystreptomycin phosphotransferase gene of *Streptomyces glaucescens* (Vogtli and Hutter, 1987) have two or more transcription start points. This might relate to the need to express the genes in different phases of growth, when different promoter binding specificities are expressed by the predominant forms of RNA polymerase (Seno and Baltz, 1989). One of the *aphD* promoters, located inside the strR gene is constitutive, whilst the other is switched on later in the growth phase (Distler *et al.*, 1987b).

There is thought to be no difference between promoters used in primary and secondary metabolism and base similarities have been observed between different streptomycete promoters. For example, there is a highly conserved 8-10 bp sequence centred around -13 in *aphD*P1, *aphD*P2 and *strB*P, although its

significance is not known. Sequence homology observed within the -10 and -35 regions of promoters does not seem sufficient to explain promoter activity and it is also thought that the high GC content of streptomycete DNA might make the formation of open complexes difficult, necessitating the involvement of transcriptional activators (Seno and Baltz, 1989).

The translational start codon in some streptomycete promoters (eg. *aph*P1), is located at the extreme 5' end of the mRNA. This allows no room for ribosome binding, and so translation might require a "back-tracking" mechanism (Seno and Baltz, 1989).

1.4.6. Antibiotic Resistance Mechanisms in Streptomycetes.

Over the last twenty years the molecular and physiological basis for antibiotic resistance in streptomycetes has received considerable attention and the resistance mechanisms elucidated so far can be classified into three main categories (illustrated by Tables 1.2., 1.3. and 1.4). Many antibiotic producers make enzymes that specifically inactivate secondary metabolites, including the beta-lactamases, which work by opening up covalent bonds in chemical structures. The majority of streptomycetes have been shown to produce these enzymes constitutively (Ogawara, 1981), although the major form of resistance to beta-lactams is the modification of penicillin-binding proteins (Ogawara, 1991). Antibiotics can also be disrupted via the chemical substitution of key residues (Table 1.2.). The position of the substitution is given as a prefix to the enzyme responsible.

Popular forms of self-defence for aminoglycoside producers are acetylation, where acetyl-Co A provides the acetyl group, and phosphorylation, where ATP is the source of phosphate (Cundliffe 1984, 1986); these chemical reactions have also been observed in the producers of peptide antibiotics, but less commonly in the producers of nucleosides and macrolides (Table 1.2). Other enzymes which can confer resistance include hydrolases, such as chloramphenicol hydrolase, which

Tables 1.2., 1.3. and 1.4. give examples of antibiotic resistance mechanisms, which have been classified into three groups; modification by enzyme detoxification, target site modification and exclusion mechanisms.

Key to Tables 1.2. to 1.4.

NP = non-producer of these compounds

M = methylase

AAC = aminoglycosides acetyltransferase

AC = acetyltransferase

APH = aminoglycosides phosphotransferase

PH = phosphotransferase

VPH = viomycin phosphotransferase

GS = glutamine synthetase

Table 1.2. The modification of antibiotics by detoxifying enzymes.

Organism	Antibiotic	Modifying Enzyme	Reference
S.capreolus	capreomycin	PH, AC	Skinner and Cundliffe, 1980, Cundliffe, 1984.
S.coelicolor	macrolide	2' PH's	Marshall *et al.*, 1989
S.fradiae	neomycin	APH(3') AAC(3)	Thompson *et al.*, 1980
S.griseus	streptomycin	APH(6) APH(3')	Distler *et al.*, 1987a. Heinzel *et al.*, 1988
S.griseus	kanamycin (NP)	AAC(3)	Hotta *et al.*, 1988
S.glaucescens	hydroxystrep -tomycin	APH	Hintermann *et al.*, 1984.
S.hygroscop- -icus	bialaphos	AC	Murakami *et al.*, 1986 Sezonov *et al.*, 1990,
S.hygroscop- icus	hygromycin B	APH	Zalacain *et al.*, 1986
S.kanamycet- icus	kanamycin	AAC(6') AAC	Cundliffe, 1986 Murakami *et al.*, 1983.
S.rimosus	paramycin	APH; AAC	Zalacain *et al.*, 1986
S.vendar- -gensis	macrolide	2' glycosylase	Eady *et al.*, 1990
S.venezuelae	chloramphen -icol	hydrolase	Mosher *et al.*, 1990
S.vinaceus	viomycin	VPH	Thompson *et al.*, 1982b
S.virido -chromogenes	bialaphos	AC	Strauch *et al.*, 1988 Wohlleben *et al.*, 1991
Stv.eurocidicus		2 APH's	Zalacain *et al.*, 1986
Stv.spp.	Blasticidin S	AC	Perez-Gonzalez *et al.*, 1990

Table 1.3. Target site modifications, which confer antibiotic resistance.

Organism	Antibiotic	Target Modification	Reference
S.azureus	thiostrepton	rRNA Methylase	Thompson and Cundliffe, 1980
S.fradiae	tylosin	rRNA M	Zalacain and Cundliffe 1991
S.hygros--copicus	bialaphos	2 x GS isoforms	Kumada *et al.*, 1990
S.kanam--yceticus	kanamycin	RNA methylase 23S	Nakano and Ogawara, 1986
S.hygros--copicus	geldanamycin	resistant RNA polymerase	Blanco *et al.*, 1986
S.lividans	macrolide lincosamide	23S rRNA methylase	Jenkins *et al.*, 1989
S.mycofa--ciens	midecamycin	rRNA methylase	Hara and Hutchinson 1990
S.niveus	novobiocin	DNA gyrase +2 unknown mechanisms	Mitchell *et al.*, 1990
S.noursei	nosiheptide	rRNA M	Woodman, 1991 (personal communication)
S.rimosus	oxytetrac -ycline	ribosomal resistance	Ohnuki *et al.*, 1985.
S.spheroides	novobiocin	DNA gyrase	Thiara and Cundliffe, 1989
S.tene--braius	kanamycin (np)	RNA methylase	Skeggs *et al.*, 1987.
S.thermo--tolerans	carbomycin	RNA methylase	Epp *et al.*, 1987
S.tenji--mariensis	apramycin kanamycin (np)	rRNA methylase 16S	Skeggs *et al.*, 1986 and 1987
S.virido--chromogenes		resistant glutamine synthetase	Wohlleben *et al.*, 1991
S.virido-chromogenes		rRNA M	Kamimaya and Weisblum, 1986

Table 1.4. Resistance to antibiotics conferred by drug exclusion or excretion.

Organism	Antibiotic	Exclusion Mechanism	Reference
S.fradiae	tylosin	multi-component ATP-dependant transport system for active secretion	Rosteck *et al.*, 1991
S.griseus	strepto-mycin	transport and permeability mechanisms	Sugiyama *et al.*, 1990.
S.rimosus	oxytetra cycline	drug export	Ohnuki *et al.*, 1985, McDowell *et al.*, 1991

catalyses the removal of a dichloroacetyl moiety from the antibiotic and glycosylases, which introduce a glycoside residue to the relevant compound.

Enzymes, which modify antibiotics may also be involved in antibiotic biosynthesis; for instance the *bar* gene encodes an acetytransferase, which catalyses the acetylation of dimethylphosphinothricin, step 10 in bialaphos production (Murakami *et al.*, 1986). Cundliffe (1984) pointed out that some antibiotic-inactivating enzymes might normally act at an earlier step in the biosynthetic pathway and might not contribute to the mechanism of resistance, which is actually used by the antibiotic-producing organism. Thus the physiological roles of antibiotic-inactivating enzymes should not be assumed, especially when the permeability properties of the cell surface are not known. In addition , certain antibiotics might not have vital groups which are sensitive to modification and it might not be possible to prevent all drugs from binding to their targets by performing chemical modifications (Cundliffe, 1984). This may explain the existence of other resistance mechanisms.

The modification of drug targets in antibiotic-producing streptomycetes is well documented (Table 1.3.). Specific methylation of ribosomal RNA has been frequently cited (Cundliffe, 1986) and is a common means of resistance to macrolide antibiotics. The macrolide-lincosamide-streptogramin type B (MLS) phenotype was first described by Fujisawa and Wiesblum (1981), who found that resistance to subsets of MLS antibiotics, could be induced by a range of these compounds in strains with a variety of diverse phenotypes. Other examples include erythromycin resistance in *S.erythraea* (Skinner *et al.*, 1983), tylosin resistance in *Streptomyces fradiae* (Zalacain and Cundliffe, 1989) and an inducible rRNA methylation in the non-producer *Streptomyces lividans* (Jenkins *et al.*, 1989). Resistance is due to either mono- or di-methylation of adenine residue 2058 (Graham and Weisblum, 1979) and it operates by a mechanism known as translational attenuation (Weisblum 1984). The inducer binds to prokaryotic ribosomes during translation of the *erm* leader region and this destabilizes the

mRNA structure. Then the ribosome binding site for *erm* methylase is free and methylase synthesis begins; thus MLS drugs are prevented from binding to ribosomes. The potency of individual inducers in a given system may be determined by their ability to inhibit the incorporation of different amino acids (Kadam, 1989). Not all producers of MLS antibiotics employ this resistance mechanism, although hybridization analysis has revealed homology between *ermE* and resistance genes in many of these organisms (Stanzack *et al.*, 1990; Epp *et al.*, 1987). Ribosomal RNA methylation has also been reported to confer resistance to the aminoglycoside kanamycin and its derivatives (Skeggs *et al.*, 1986; *Skeggs et al.*, 1987; Nakano and Ogawara, 1986) and to certain peptide antibiotics (Cundliffe *et al.*, 1984).

Target site modification includes enzymatic alterations, such as that found in the RNA polymerase of ansamycin producers (Blanco *et al.*, 1986) and in the DNA gyrase of novobiocin producers (Thiara and Cundliffe, 1989; Mitchell *et al.*, 1990). In the latter case the enzyme is duplicated, one form being resistant and the other sensitive. Similarly protection against bialaphos is conferred by resistant forms of glutamine synthetase (Kumada *et al.*, 1990; Wohlleben *et al.*, 1991) and *S.hygroscopicus* harbours two resistant isoforms of this enzyme. Reduction in the physiological importance of the target is also included in the category of target site modification and the inate bialaphos resistance of streptomycetes with an alternative pathway for ammonium utilization is an example of this. Target site modification confers higher levels of resistance than does antibiotic modification, however if an antibiotic confers a regulatory role during cell growth or differentiation then producers may need to be sensitive to their products at certain times (Cundliffe, 1984).

Resistance by prevention of access to the target can be via a total or partial permeability barrier (ie. is combined with other forms of resistance) or by confining products to discrete sub-cellular compartments. For example, it is thought that in the biosynthesis of aminoglycosides' inert precursors are activated during export from the cell by dephosphorylation (Miller and Walker, 1969). Many antibiotic

producers have been shown to harbour more than one resistance mechanism; for example they might contain more than one substituting enzyme working in conjunction (Table 1.2.), sometimes synergistically (Thompson *et al.*, 1982b). Alternatively, they might contain additional types of resistance mechanisms and effective permeability barriers are thought to exist in many antibiotic producers (Cundliffe, 1984; Sugiyama *et al.*, 1990). Similarly bialaphos and kanamycin resistance are conferred by both enzymic modification and resistant target molecules (Tables 1.2., 1.3. and 1.4.).

1.4.7. The Relationship of Antibiotic Resistance and Biosynthetis to Phenotype and Genotype.

Information on how far phenotypic and genotypic similarities correlate between producers or with one another is still fragmentary. However there has been a remarkable congruence in the resistance phenotype between some producers of similar or identical compounds. For example the rRNA methylases of *Streptomyces sioyaensis*, *Streptomyces laurentii*, *Streptomyces azureus* and *Planomonospora paronyospora* are almost indistinguishable (Thompson and Cundliffe, 1980). These strains produce the highly related peptide antibiotics siomycin, thiostrepton, thiostrepton and sporangiomycin respectively. In addition, the methylases of *Streptomyces actuosus* and *Streptomyces bernenisi* partially resemble that of *S.azureus*. Nosiheptide, the *S.actuosus* product, is similar to thiostrepton, but berninamycin is not structurally related, although it does have a similar mode of action (Thompson *et al.*, 1982b). There is often great symmetry in the organisation and regulation of biosynthetic pathways between different producers of identical compounds (Fig.1.3.) and this may be shown to extend to the pathways of related products, such as streptomycin and bluensomycin (Walker, 1990).

In some cases gene homologies correlate with taxonomy, whilst in others they do not; for example erythromycin biosynthesis and resistance genes from *Saccharopolyspora erythraea* hybridize strongly to macrolide producing

Saccharopolysporas, but only weakly with the DNA of macrolide-producers from other genera (Stanzak *et al.*, 1990). In a different case, rRNA methylases from different genera and from the producers of widely different antibiotics appear related at the DNA level (Skeggs *et al.*, 1987). Reports where genes of secondary metabolism do not correlate in producers of the same compound are rarer, however Mosher *et al.*, (1990) have shown that the chloramphenicol resistance gene of *Streptomyces venezuelae* does not hybridize to the DNA of *Streptomyces phaeochromogenes*, another chloramphenicol producer.

There has been much evidence of genetic homology between families of genes, probably due to their common ancestry. Beta-lactamases may be descendants of or parallel developments to the peptidoglycan-metabolizing peptidases from eubacteria or to the penicillin binding proteins, which are related to a very ancient family of molecules including many esterases and serine proteases (Brenner, 1988). Forseman *et al* (1991) have found significant homology between the beta-lactamase genes from *Streptomyces badius*, *Streptomyces cacaoi*, *Streptomyces fradiae* and *Streptomyces lavendulae* and suggest that the Gram-negative class A beta-lactamases evolved from these. Piepersberg *et al.* (1988 and 1991) illustrated a similar example for the 23S rRNA methylases, stating that they were related to 16S rRNA methyltransferases, whose targets are often very similar. Lim *et al.* (1989) revealed a striking homology between the *aphD* gene of *Streptomyces griseus* with other antibiotic phosphotransferases and protein kinases, suggesting similar structures and a common ancestry; this relationship was also suggested by Distler *et al.*, 1987b, Heinzel *et al.*, 1988 and Piepersberg *et al.*, 1991. Kirby (1990) presented evidence that aminoglycoside phosphotransferase genes originated in an ancestor closely related to actinomycetes and he illustrated this with rooted phylogenetic trees based on protein sequence data. It was also implied that aminoglycoside acetyltransferases, phosphotransferases and nucleotidyltransferases shared a common origin with other kinases and nucleotide-binding proteins because they shared conserved motifs.

Our knowledge on the interplay between antibiotic action, its regulation and the development of resistance is very poor (Piepersberg, 1991). Antibiotic resistance can be acquired by the mutation of resident genes in a given sensitive cell or by taking up additional preformed determinants, as with the resistance genes of clinical bacteria. Piepersberg (1991) regards the hypothesis that most resistance determinants could have originated in producing organisms as proven for many wide-spread mechanisms and DNA sequencing studies between *Streptomyces* and clinical isolates have been used to support the notion of horizontal gene transfer (Trieu-Cout *et al.*, 1987). Kirby (1990) have given further support to this notion by demonstrating (via phylogeny) that aminoglycoside phosphotransferases probably originated in an actinomycete and were transferred to clinical bacteria through transposition. This event was believed to have occurred after the split between Gram-negative and Gram-positive organisms and it was thought likely that several horizontal gene transfer events occurred at different times in the evolutionary past. Evidence against horizontal gene transfer has been given by data base studies at the John Innes Institute (Bibb 1986), where the level of resemblance between *Streptomyces* resistance determinants and those of clinical isolates has been shown to be identical to sequence similarity of *Streptomyces* resistance determinants and those of other proteins. There are barriers to DNA transfer and gene expression, but in view of its diverse nature, soil could provide the relevant conditions for transfer of antibiotic resistance and production genes (Kirby, 1990). The acquisition of genes within the genus *Streptomyces* is a complicated issue, for example O-acetlytransferases, involved in chloramphenicol acetylation, are not found in producers but have been observed in other streptomycetes (Murray *et al.*, 1989). Therefore clinical resistances may originate from a variety of sources, which includes bioactive and non-bioactive actinomycetes, but does not necessarily comprise them.

1.5. The Exploitation of Antibiotics from Streptomycetes.

1.5.1. The History of Natural Product Screening.

The beginning of the antibiotic era was marked by the discovery of penicillin by Alexander Fleming and its extraction twelve years later by Florey, Heatly and Chain. The search for other antibiotic-producing microorganisms accelerated due to the medicinal needs of World War II resulting, in the discover of streptomycin and more broad spectrum compounds such as chloramphenicol and the tetracyclines. To begin with researchers focused on the fungi for sources of new medicinal compounds, but few leads were discovered and major emphasis shifted to the *Streptomyces*. This genus has become the most prolific source of commercially important antibiotics in the history of natural product screening.

The soil is a reservoir of numerous diverse microorganisms and so in the archetypal days of antibiotic discovery little effort was required to isolate novel strains and isolation was designed to match the scale-up procedures of a particular company. Early methods of screening were based on the random screening of high numbers of isolates and included a variety of simple procedures. In one method, plates were inoculated to a density of 300-400 colonies and microbes which were surrounded by a ring of inhibition were selected for further investigation; the drawback of this method was that zones of clearing could form due to pH effects or nutrient depletion. These tests were superceded by screens which overlayed plates (30-200 colonies per plate) with a series of test organisms; antibiosis profiles could also be obtained by overlaying streak plates or plugs of streptomycete growth. Another development was the use of replica plating which provided an easy way to increase the numbers of test organisms (Rhodes *et al.*, 1961;Lechevalier and Corke, 1953).

Secondary screening further tested the capabilities of the organisms under study and often taxonomic classification was used to predict pathogenicity and to give an indication of growth characteristics. The scientific and patent literature was examined to find related organisms which could give an indication of the type of

product being formed and to avoid rediscovering known antibiotics. Other techniques were to scrutinize UV absorption spectra, chromatographs, extraction patterns and bioautographs of the crude extracts. It was during this stage that the feasibility of pharmacological or agrochemical use of a compound was determined. For example some good attributes for the agrochemical products were stability of homogenates in plant tissue, stability to heat, exceptionally high biological activity with a desired spectrum of activity and the ability to move through discs of plant tissue. Following selection the ability to protect plants against target pathogens and the phytotoxicity of the crude extract were determined in small greenhouse tests. If these were successful then large scale trials both in the greenhouse and small plots of land would be undertaken.

1.5.2. Modern Antibiotic Discovery.

Due to the ease of antibiotic discovery described above more sophisticated methodologies were not required until the mid-1960's onwards, when qualitative improvements were brought about at every step of the screening programme. Data feedback systems were initiated and these contained microbiological, biochemical and chemical information about the producing organisms and about the biosynthesis, mode of action and biochemical properties of the antibiotic. A description of new screening methods is presented in the sections below, but the reasons for their development are given in this section.

The probability of finding new antibiotics steadily declined as the number of discovered antibiotics increased and many promising activities were found to be due to known antibiotics (Okami, and Hotta 1987; Ayer *et al*, 1989). Random screening wasted time and resources on the examination of thousands of strains, which lacked desired activities and the use of easily isolatable strains probably meant that highly common strains were screened repeatedly. It is generally accepted that many strains capable of producing useful compounds have been screened under conditions which did not allow expression; for example the tetracenomycins were discovered by

screening the hydroxystreptomycin-producer *Streptomyces glaucescens* under a different set of conditions (Zähner *et al.*, 1982). Another problem is that more than one compound can be present in a culture extract and the activity of many useful highly specific compounds could have been masked by the presence of known antibiotics, which were identified by their spectrum of activity.

1.5.3. The Selection of Organisms for Industrial Screens

At first antibiotics of actinomycete origin were exclusively provided by the *Streptomyces*, but more recently companies have begun to target rarer genera (Williams and Wellington, 1982), consisting of actinomycetes with relatively low isolation frequencies (Okami and Hotta, 1988). Rare genera include actinoplanetes and maduromycetes, *Kitasatosporia* (Omura 1986), *Nocardia*, streptoverticillium and rare forms of streptomycetes. By 1982 one quarter of actinomycete products were provided by non-streptomycetes (Nisbet 1982) and 400 of the 6,000 known antibiotics are produced by actinoplanetes and maduromycetes (Berdy, 1988). The streptomycetes continue to provide larger numbers of diverse antibiotic products than other actinomycete genera (Okami and Hotta, 1988) and there may still be large numbers of *Streptomyces* species or strains with novel antibiotic productivity in nature. Nevertheless, the shift towards rare genera reflects a search for increased variety and aims to reduce the re-isolation of known strains and the rediscovery of known products. A related approach taken by some companies has been to isolate the organisms which they screen from extreme (Williams and Wellington, 1982) or unusual, previously unexplored, environments (N. Porter, personal communication).

The extent to which antibiotic production correlates with taxonomic identification is extremely important when deciding which organisms should be targeted. There is some evidence that selected *Streptomyces* species do have distinctive antibiosis patterns (Wellington and Cross, 1983) and there are also specific taxonomic groups such as *S. hygroscopicus* (Arai *et al.*, 1976) which show

a high incidence of antibiotic production. Generally, however, antibiotic production in actinomycetes is strain specific (Okami and Hotta 1988) and conventional taxonomy cannot be used to predict the type of antibiotic produced, although Okami and Hotta (1988) postulated that there might be some specific genotypes which do correlate with antibiotic production. Classification is still an important part of the drug discovery process because it helps avoid screening duplicate strains and also patents often require a thorough description of the producing organism (O'Donnell, 1988). This type of data is also important because physiological characters which correlate with bioactivity may be discovered and isolation methods can be developed for those species which are useful targets for antibiotic production; for example the actinoplanetes are mainly novobiocin resistant so this compound can be used as a positive selection pressure for their isolation.

1.5.4. The Design of Media for Antibiotic Screening Programs.

In 1981, Iwai and Omura reviewed the culture conditions necessary for secondary metabolite screening. They concluded that different carbon and nitrogen substrates were favoured by different cultures and that generally readily utilized carbon and nitrogen sources were unfavourable for antibiotic production. There are exceptions to this including anticapsin production, which is favoured at 10% glucose (Boeck *et al*, 1971). The use of slowly utilizable sugars and nitrogen compounds, as the sole sources of carbon and nitrogen were advised by Hu and Demain (1979) and Martin and Demain (1980); although the more rapidly utilized nitrogen source, ammonium can be used if ion depressors are also added (Omura and Tanaka, 1984, Omura, 1986).

Large amounts of Pi accelerate carbon and nitrogen utilisation, but tend to reduce antibiotic production. However Imanaka and coworkers (Arima *et al.*, 1965; Miyairi *et al.*, 1970; Miyoshi *et al.*, 1972) discovered three new antibiotics by screening on high (> 180mM) phosphate media. The production of some antibiotics takes place between two threshold concentrations of Pi; for example Hall and

Hassall (1970) described the differential production of two antibiotics by *Streptomyces jamaciensis*. At 0.1mM, monamycin was produced but a different antibiotic was produced at 0.4mM.

Inorganic salts are added to media to increase production and different ones affect different antibiotics; for instance very large amounts of sodium chloride can inhibit antibiotic biosynthesis, or in rarer cases, stimulate production (Okami *et al.*, 1976). Trace elements are needed for enzymes and cofactors and zinc and iron are reported as being the most important for actinomycetes (Weinberg, 1970), with larger amounts being needed for production rather than growth. *S.clavuligerus* required 130 μg/ml ferrous iron for maximum cephamycin C biosynthesis (Rollins *et al.*, 1989). However, there are often thresholds beyond which antibiotic production is negatively or positively affected (Iwai and Omura 1981).

The dilemma of media design is that although specific growth requirements are important for the expression of certain types of secondary metabolites general use media are needed. Researchers have used complex media containing a variety of carbon and nitrogen sources to vary growth rates and physiological states hoping to alter internal metabolite pools and to diversify secondary metabolite production. Nisbet (1982) put forward some guide-lines for the design of media which allow good product formation and he stated that different media should be prepared using a variety of growth-limiting nutrients because secondary metabolite patterns can often vary when same strain is grown on different media (Grabley *et al.*, 1990). For each type of nutrient depletion different forms of the growth sufficient nutrient should be used and readily assimilated forms of carbon and nitrogen ought to be avoided; the recommended C:N ratio was 10 for antibiotic production (Hutter, 1982). Finally use of a polymeric or complexed form of the growth-limiting substrate was thought advisable and an attempt should be made at buffering the medium, which should also include known cofactors.

Increased screening of rare actinomycetes and extremophiles has lead to the development of special ranges of media suitable for their growth (Okami and Hotta,

1988) and selective isolation (Wellington *et al.*, 1987); specific media have also been used to select for growth forms which were more likely to result in antibiotic production (M. Bushell, personal communication). Some companies screen on solid media on the premise that antibiotics decompose more slowly and may diffuse away from destructive enzymes (Iwai and Omura, 1981). Screening for enzyme producers also tends to involve zone clearing assays on solid media, although an advantage of liquid media is that there is a better correlation with scale up procedures. Economic aspects are also of importance and simple growth media form the basis of cheaper commercial processes, although this must be balanced with the fact that this could limit what is discovered.

1.5.5. Extraction Procedures and Chemical Identification Systems.

For the initial stages of screening general use solvents are required. These are usually chosen for the ability to extract a wide range of chemicals, which can then be formulated as required prior to being applied to screens, but they are also selected for safety because large volumes are handled. The solvents which are used include butanol, methanol, ethyl acetate, isopropanol, dichloromethane, diethyl ether and various mixtures.

One of the earliest methods for assessing the probability that an interesting antibiotic was already known was by chromatographic analysis. Aszalos *et al* (1968) presented a rapid TLC method for assessing antibiotics from crude extracts, where eighty four known antibiotics and fourteen solvent systems were used in a two tier system, which assigned major and then sub-group classifications to the antibiotics. The antibiotics could not be identified but the choice was narrowed to a small number, which were then eliminated.

Hamill (1982) described the use of specific chemical reactions on semi-purified preparations to identify various chemical groups. Another innovative method was described by Zähner *et al.* (1982) who selected unique spots from strain extracts run on TLC plates and out of five compounds tested three were novel.

Aszalos (1980) hinted that combinations of many of the new and old techniques (eg. TLC, preparative chromatography, gas chromatography, high performance liquid chromatography, mass spectrometry and infra-red spectroscopy) might increase the number of antibiotics that could be included in a particular identification system.

Techniques, such as high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) spectrometry and diode array detectors meant that the tiniest amounts of active substances could also be isolated and analysed and unstable compounds and minor products could be extracted and purified (Omura, 1986). Many companies now have computer libraries (H.P. Fielder, personal communication), which contain the HPLC and ultraviolet and visible light spectra spectra of hundreds of microbial secondary metabolites, but one of the most useful tools, which many natural product screening companies still use, is the Berdy database (Berdy database, 1988).

1.5.6. Target Directed Screening.

As the rate of discovery of new antibiotics declined screening programmes consisting of differential assays for targeting specific groups of antibiotics were established. For example Zähner *et al.* (1982) explained a novel approach to the detection of narrow spectrum antibiotics, where an unusual bacterium was added to established test organisms (ie. *Staphylococcus aureus* and *B.subtilis*) and then in order to increase the chance of discovering a novel compound, only antibiotics which affected the unusual organism were selected. Many other screens have been developed to detect activity against a variety of human, plant and animal pathogens and target-directed screening may focus on searching for inhibitors of the target sites of known antibiotics or of rationally selected receptor sites. They may also use unusual biological or chemical test procedures to detect rare types of activity or specific structural moieties (Fleming *et al.*, 1982).

1.5.7. Screens used in the Agrochemical Industry.

Highly specific, biodegradable compounds are needed for the control of crop pests because treatment involves the release of large amounts of chemicals into the environment. This often involves choosing a target, which is specific to a certain weed or insect or the selection of a specific bacterial, fungal or viral plant pathogen. For example the conversion of lanosterol to ergosterol (Nisbet and Porter, 1989) has a lethal effect on fungi but is harmless to plants.

Prevention of fungal protoplast regeneration has been used to find chitin and glucan inhibitors because it is difficult to find antifungals with low mammalian toxicity. The fungal membrane is a good target because differential binding to ergosterol rather than cholesterol can ensure the selection of highly specific compounds. Nisbet and Porter (1989) predicted that by cloning genes from *Candida albicans* into defective mutants of *Saccharomyces cerevisiae*, preferential inhibition of recombinants could provide an innovative means of discriminating the most selective compounds.

Some herbicides are inhibitors of glutamine synthetase and cause death by ammonium accumulation. Phosalacine was discovered by use of an assay in which *B.subtilis* was inhibited on minimal medium but not in the presence of glutamine. Other screens for herbicides can involve detecting inhibition of germination and radicle growth from surface-sterilised seeds and foliar or root elongation assays (Heisey *et al.*, 1988).

Some of the early screens detected antifungal activity by the abnormal growth of test organisms or by inhibition of their growth. The nikkomycins were discovered as inhibitors of zygospore formation in *Mucor hiemalis* (Zähner 1986) and were of interest for the development of insecticides because they attack chitin synthesis. A simple assay to detect insecticidal activity is the photoactivity response of insect larvae; healthy mosquito larvae swim away from light and compounds with insecticidal activity prevent this reaction (Heisey *et al.*, 1988).

Highly specific assays were designed for the discovery of avermectins and were based on the fact that these compounds bind to membrane receptors. Avermectins enhance the affinity of these receptors for benzodiazepines and this provides a new incentive to design screens, which may discover second generation avermectins, (Monaghan and Tkacs, 1990)

1.5.8. Screens Used in the Pharmacological Industry.

A range of enzyme inhibitor screens have been used in the pharmaceutical industry and these have been aimed at discovering compounds which inhibit enzymes involved in the synthesis of compounds important in human physiological disorders such as obesity, diabetes, migraines, Parkinsons disease and Senile Dementia. Inhibitors of enzymes involved in adrenalin and lipid synthesis have provided drugs for the treatment of hypertension (eg oudenone, fusaric acid) and cholesterol reduction (monacolin K) and inhibitors of glycosidases are of potential use in the treatment of obesity and diabetes, and protease inhibitors may prove useful for inflammatory, thrombotic and haemorrhagic conditions (Hamill, 1982).

Screens have also been designed to detect inhibitors of essential enzymes involved in bacterial, viral and fungal diseases and such assays must be highly specific for the organism under study. Monaghan and Tkacs (1991) described a screen for inhibitors of HIV reverse transcriptase in their review on bioactive microbial products. The enzyme can now be produced in large quantities in *Escherichia coli*, purified by affinity chromatography and used to test natural products.

Other pharmacological screens have involved the search for beta-lactamase inhibitors, which could be used with beta lactamase sensitive antibiotics such as penicillin; for example novel compounds could be detected by the increased beta-lactamase production of resistant strains in their presence. Mechanism-based screens for beta-lactam antibiotics are reviewed by Okami and Hotta (1988) and Nisbet and Porter (1989), but included a mode of action assay based on peptidase

inhibition, the use of hypersensitive mutants and antimycoplasma activity (Omura, 1986).

Many glycopeptide antibiotics have also been discovered using mechanism based assays, which have exploited their binding with peptides that end in acyl-D-ala-D- ala. Radio-immuno-assay (RIA), enzyme-linked immunosorbant assay (ELISA) and fluorimmunoassay have also been used for glycopeptide detection and have shown little cross reactivity with other antibiotics.

In vitro screens have been developed for the initial stages of antitumour drug discovery (White, 1982), although these drugs must be tested later on the more expensive *in vivo* screens. Initially the drugs were discovered using antimicrobial and cell toxicity assays, but more recently mechanistic approaches have been taken; protozoa have similar multiplication behaviour to tumour cells, and can be affected by the same drugs. For example hitachimycin was discovered on an antiprotozoal screen simultaneously to its discovery as an anticancer compound (Omura, 1986). Recent assays are reviewed by Okami and Hotta (1988) and Nisbet and Porter (1989), who describe the use of mutant cell lines which respond to oncogene expression, antibiotics which intercalate with DNA molecules and the use of human and animal tissue cultures.

1.5.9. Beneficial Products Derived from the Pharmaceutical and Agrochemical Industries.

Demain (1983) listed about 40 different pharmacological activities of microbial secondary metabolites, including anti-inflammatory drugs (eg pyrothrine from *Streptomyces verticillium*), antitumour drugs (eg bleomycins from *S. verticullus*), vasodilators (eg WS1228 A and B from *S. aureofaciens*), inhibitors of the complement cascade (eg complestatin, from *S. lavendulae*) and immunosupressors (eg FK506 from *Streptomyces tsukubaensis*). Drugs which are known to bind to specific receptors include L-156,373, which binds to the oxytocin

receptor and might have a use in delaying premature labour and MY336a from *Streptomyces gabonae*, which is an agonist of the beta-adrenergenic receptor.

The farming industry have benefited from veterinary drugs, produced by *Streptomyces* such as the coccidiostatic agent, lasalocid, and other antibiotics, including monensin and virginiamycin which are marketed as animal growth promoters. The mode of action of virginiamycin is probably as an appetite modifier because it is acts as an antagonist of the brains' gastrin and cholestokinin satiety controlling receptors.

Chitin synthase inhibitors have proved useful as agricultural fungicides and insecticides. For example allosamidin is an insect chitinase inhibitor, which interferes with insect moulting, nikkomycins have been used as acaricides and their relatives, the polyoxins as antifungal compounds. Highly specific insecticides include the avermectins, which work by blocking neuromuscular transmission in arthropods and helminths; their mode of action is to bind to synaptic gamma aminobutyrate receptors and paralyse the victim.

Microbially-produced herbicides include antimetabolites such as the herbicidins, active against dicotyledonous plants, homoalanosine which has a functional resemblance to aspartate and glutamate and the herbimycins, which block angiogenesis and are also active against plant viruses (eg TMV). Enzyme inhibitors also have a place in the herbicide market. For instance, bialaphos is a competitive inhibitor of chloroplast glutamine synthase, causing the accumulation of ammonium and death of plant pests.

1.5.10. Target Directed Isolation and Selection.

If screens are exclusively dependent on target-directed assays the hit rate can still be as low as 1 in 1,000, but this can be improved by including target-directed isolation and selection of organisms. For example Okami and Hotta (1988) isolated aminoglycoside-resistant actinomycetes and obtained aminoglycoside resistance profiles for them. They then preselected strains with many resistances because they

had a higher probability of antibiotic production. In this work novel antibiotic resistance patterns were regarded as markers for new antibiotic producers. The hit rate using this protocol was 1 in 10 and aminoglycosides were not the only type of antibiotic discovered. A similar example is the glycopeptide-directed screening of Rake *et al.* (1986) who isolated actinomycetes on vancomycin-containing media and then cultivated them under conditions suitable for glycopeptide production; the hit rate of these workers was improved from 0.3% to 2.2%. Directed isolation protocols can also involve choosing an environment which is likely to harbour organisms with desired activities and Lievens *et al* (1989) selected plant-associated organisms as a source of antifungals for phytopathogenic diseases. Similarly Verces *et al.* (1990) successfully chose the grapevine carposhere as a source of organisms producing anti-yeast compounds. Biological control agents have also been targetted, because their activity may be due to the production of compounds with biocidal activity (Weller and Thomashow, 1990). The isolation of organisms from dead insect pests is another common approach.

1.5.11. Other Approaches to Antibiotic Discovery.

Actions such as regrowing previously tested organisms on different media or testing them on new screens can lead to novel discoveries about their ability to produce natural products. Previous sections of this introduction contain several examples of compounds which were discovered on a particular screen and were then found to exhibit another useful activity. There have also been examples of strains which produced different compounds under altered conditions of growth.

Modern technology has permitted a more directed manipulation of known antibiotic producers. Okami and Hotta (1988) showed how a biosynthetic pathway could be directed in a different direction by supplying various precursors to the producer organism. Mutasynthesis also uses this approach but with blocked mutants, which require special media for antibiotic production (ie. analogues are incorporated into the biosynthetic pathway of the compound which they cannot synthesize to

produce new antibiotics). Blocked mutants or those with altered biosynthetic pathways are another source of novel antibiotics because they accumulate intermediates which do not build up in the parent strain. There are also reports of foreign antibiotics being converted to new products by the biosynthetic enzymes of other antibiotic producers (Okami and Hotta, 1988).

Protoplast fusion and protoplast regeneration are powerful tools for generating or activating the expression of silent genes in actinomycetes, as in the discovery of indolizamycin (Yamashita *et al.*, 1985a,c). Kurzatowski *et al.*, (1985) have also observed diversification of antibiotic production in cultures from regenerated protoplasts; however protoplast regeneration can result in the loss of antibiotic production as with carriomycin production in *S. hygroscopicus* (Ogura *et al* 1986.,); when this same strain was treated with ethidium bromide and a new antibiotic curromycin was produced.

In a few cases both inter- and intra-specific *in vivo* and *in vitro* genetic recombination have resulted in the formation of new antibiotics. For example the recombination of an auxotrophic non-producing mutant of the turimycin-producing *S. hygroscopicus* with a blocked mutant of a violamycin-producing *S. violaceus* resulted in the discovery of the anthracycline antibiotic, iremycin (Okami and Hotta, 1988).

The development of molecular cloning in *Streptomyces* made the isolation of many biosynthetic gene clusters possible (Chater and Bruton, 1985; Malpartida and Hopwood, 1986; Murakami *et al.*, 1986). Hopwood *et al.*, (1985) and Malpartida *et al.*, (1987) discovered novel antibiotics by carrying out gene transfer experiments between strains which produce different isochromanequinone antibiotics. For example dihydrogranatirhodin was produced when the *act* genes were introduced into the granaticin-producer *Streptomyces violaceoruber*.

1.6. Rationale and Aims for this Research.

Over recent years, much has been learnt about antibiotic biosynthesis, antibiotic resistance and the physiology of antibiotic producers. Many relevant genes have been cloned and thousands of antibiotic producers are deposited in culture collections. This provided the means for the research presented in this thesis, which took a broad approach for studying secondary metabolism. The aims of the work were to investigate the distribution of antibiotic production and antibiotic resistance phenotypes in natural populations of streptomycetes. It was of additional interest to investigate the genetic basis of the observed antibiotic resistances and to examine factors which influenced the expression of antibiotic production in relevant bioactive strains. Another aim was to use any discoveries about the physiological and genetic characters, which are necessary for activity to develop techniques which could predict bioactive and non-bioactive groups of streptomycetes and which would encourage antibiotic gene expression.

A further objective was to search for phenetic and chemotaxonomic characters, which correlated with bioactivity and the characters studied included antibiotic resistance patterns, fatty acid profiles and patterns obtained by thin layer chromatography of solvent extracts from streptomycete cultures. An overall objective was to use the information gathered from the population studies to try to understand the ecological role of secondary metabolism.

Chapter 2.

Materials and Methods.

2.1 Materials

Strains, which were used in this research comprised both type strains and natural isolates. Table 2.1. shows the natural isolates which were taken from the E.M.H. Wellington culture collection and Table 2.2. lists the type strains. Certain other strains were used as a source of plasmid DNA and a list of these is presented in Table 2.3.; the genes which were isolated from the plasmids are given in Table 2.4.

The compositions of media are shown in Table 2.5. and these were autoclaved at 121°C for 20 mins., unless otherwise indicated. Constituents are given in g/l and exceptions to this are also given on the table. Table 2.6. shows the formulations of the buffers and reagents which were used and quantities of components are given in mM, unless otherwise indicated.

Table 2.1. Summary of natural isolates used for this research

Strain Series	Source	Isolation Method	Medium	No. of Strains
A	Italy	Chemostat	Starch	13
B	Warwick	Chemostat	C32	2
C	Warwick	Pour Plate	AGS	98
D	Warwick	Pour Plate	C32	36
E	Italy	Chemostat	C32	11
F	Warwick	Pour Plate	C32	7
MM	Martin Mere	Pour Plate	C32	4
W	Warwick	Pour Plate	C32	4
RB	Italy	Pour Plate	AGS	3
MEL	Brazil	Spread Plate	AGS	4
CAG	Greece	Various	Various	25
JHCC	Various	Various	Various	8
TOTAL				225

Series A to E were isolated by T.N. Whitmore; MM and W by N. Cresswell; RB by R. Bandoni; Mel by M. Walker; CAG by A. Karagouni and JHCC by various workers. Starch and C32 media were developed by T.N. Whitmore.

Key to superscripts on Table 2.2.

[4]Strain was used in Chapter 4 for Fatty Acid Work.

[5]Strain was used in Chapter 5 for Chemical Profiles.

[6]Strain was used in Chapter 6 for Resistance Profiles.

[6-]Strain was used in Chapter 6 for Resistance Profiles, but were not used for cluster analysis.

NB. Superscripts with several numerals (eg. [456]) indicate that the strain was present in more than one study (eg. 4 and 5 and 6) and can therefore be taken to mean [4, 5, 6].

Key to abbreviations used in Table 2.2.

S. refers to .*Streptomyces*.

Stv. refers to *Streptoverticillium*.

The taxonomic identities, which are given on the table were assigned by Williams *et al*. (1983a). All strains apart from ATCC 442, ISP 5233, DSM 40069 and KCC S-0783 were used in Chapter 7. for probing work. In addition to the strains shown in the table, M180 *S.violaceoniger* (C32) was used in Chapter 4 for fatty acid work and Chapter 5 for TLC profiles.

Table 2.2. Summary of type species and reference strains used in this research.

Culture Collection Reference	Name	Tax. ID.	Product
ATCC 27416[6-]	*S. annulatus*	C1	Antibacterial Antifungal
KCC S-0446[6-]	*S. albidoflavus*	C1	
DSM 40260	*S. albofaciens*	C42	Oxytetracycline
DSM 40106[6]	*S. azureus*	C18	Thiostrepton
DSM 40232	*S. baarnensis*	C1	
DSM 40598[6-]	*S. bacillans*	C1	
ATCC 11062[6-]	*S. bikiniensis*	C64	Streptomycin
KCC S-0459[5]	*S. bottropensis*	C19	Bottromycin
DSM 40419[456]	*S. caesius*	C21	
KCC S-0731[456]	*S. caesius*	C21	
DSM 40313[5]	*S. coralus*	C19	
ISP 5442[6-]	*S.coeliatus*	C1	
A3(2)[456]	*S. coelicolor*	C21	Methylenomycin Undecyl-prodigiosin Actinorhodin Polyamines
ISP 5233[4]	*S. coelicolor*	C21	As Above A3(2)
JCM 4220[45]	*S.cyaneus*	C18	
ISP 4213[456]	*S. endus*	C32	Endomycins
ISP 5060	*S. exfoliatus*	C5	
ISP 5022	*S. filamentosus*	C5	Caryomycin
DSM 4112	*S. filipensis*	C30	Filipin
DSM 40323[6-]	*S. flavogriseus*	C1	
ATCC 23907[6-]	*S. flourescens*	C1	Actinomycin-X
KCC S-0133[6]	*S. fradiae*	C68	Neomycin
ISP 5064	*S. gardeneri*	C5	Proactinomycins

Culture Collection Reference	Name	Tax. ID.	Product
DSM 40236[46]	*S. griseus*	C1	Streptomycin
DSM 40068[4]	*S. halstedii*	C1	
ATCC 21705[456]	*S. hygroscopicus*	C32	Bialaphos
ATCC 14607[6-]	*S. hygroscopicus*	C32	Bluensomycin
NRRL 3602[456]	*S. hygroscopicus*	C32	Geldanamycin Nigericin
ATCC 3672[456]	*S. hygroscopicus*	C32	Herbimycins Nigericin
KCC S-0772[46]	*S. hygroscopicus*	C32	Hygromycins
NRRL 5739[456]	*S. hygroscopicus*	C32	Milbemycin
NRRL 3664[46]	*S. hygroscopicus*	C32	Scopofungin
ATCC 31955[4]	*S. hygroscopicus*	C32	L-155-175
ATCC 39447[6]	*S. hygroscopicus*	C32	MA 5000
ATCC 12760[6-]	*S. humidus*	C19	Dihydro-Streptomycin
ISP 5550[6-]	*S. katraie*	C61	Streptothricin Polyenes
DSM 40069[46]	*S. lavendulae*	C61	Streptothricin Polyenes
DSM 40216[6]	*S. lavendulocolor*	C61	
ATCC 3331[6-]	*S. lipmanii*		
KCC S-0783[46]	*S. lividans*	C21	
J.I. 1326[45]	*S.lividans*	C21	
KCC S-0495[456]	*S. melanosporo-faciens*	C32	Melanosporium Elaiophylin
DSM 40091[5]	*S.murinus*	C17	Actinomycin-X
KCC S-0785[456]	*S. lusitanus*	C44	Tetracycline Chlor-tetracycline
DSM 40508[6-]	*S. naraensis*	C1	Naramycins

Culture Collection Reference	Name	Tax. ID.	Product
ISP 5016	*S. narbonensis*	C5	Narbomycin
DSM 40023[6-]	*S. nitrosporeus*	C1	Nitrosporin
NCIMB 9219	*S. niveus*	C1	Novobiocin
ISP 5552	*S. omiyaensis*	C5	Chloramphenicol
ATCC 25481[6-]	*S. ornatus*	C1	Ornamycin
DSM 40268	*S. rimosus*	C42	Oxytetracycline
ISP 5174	*S. roseolus*	C5	Antibacterial
ISP 5122	*S. roseosporus*	C5	Antibacterial
ISP 5413[4]	*S. roseus*	C7	
DSM 40077[6-]	*S. rutgersensis*	C1	Camphomycin
DSM 40445[6-]	*S. subrutilus*	C61	Hydroxy-streptomycin
ISP 5329	*S. termitum*	C5	
KCC S-0519[46]	*S. thermotolerans*	C19	Carbomycin
ISP 5278	*S. umbrinus*	C5	Antibacterial
NCIMB 8852[6]	*S. vinaceus*	C6	Viomycin
DSM 40438[46]	*S. violaceolatus*	C21	
KCC S-0850[456]	*S. violaceoniger*	C32	
ISP 5196	*S. zaomyceticus*	C5	Zaomycin
JHCC 1319[56]	S. spp.		Actin
JHCC 1233[456]	S. spp.		Bialaphos
JHCC 1390[56]	S. spp.		Blasticidin
JHCC 1234[56]	S. spp.		Cyclohexamide
DSM 40049[6-]	S. spp.	C1	
Liv. 463[6-]	S. spp.	C1	
KCC S-0331[6]	*Stv. hachijoensis*	C55	Trichomycin

Culture Collection Reference	Name	Tax. ID.	Product
KCC S-0331[6]	*Stv. hachijoensis*	C55	Trichomycin
ATCC 27441[6-]	*Stv. ladakanum*	SMC	5-Azacytidine
ATCC 23934[6-]	*Stv. mashuensis*	C55	Streptomycin
TOTAL			73 STRAINS

Key to Abbreviations used to denote culture collections

ATCC refers to the American Type Culture Collection.

KCC S refers to strains from the Kaken Chemical Company.

DSM refers to the Deutsche Sammlung von Mikroorganismen.

ISP refers to the International Streptomyces Project.

NRRL refers to strains from the Northern Regional Research Laboratory.

NCIMB refers to the National Culture Collection of Industrial and Marine Bacteria.

J.I. refers to strains from the John Innes Institute.

JHCC refers to strains from the ICI culture collection at Jealotts Hill.

Liv. refers to strains obtained from Liverpool University.

M refers to a type strain, whose origin was unknown.

Table 2.3. shows the strains which provided plasmids for use during this work.

Table 2.3. The plasmid bearing strains used in this research.

Plasmid	Host	Selection
pCKL719[1]	*E.coli* DH1	Ampicillin
pIJ4104[2]	*E.coli* Rec A Delta M15	Ampicillin
pSACJ[3]	*E.coli* (unknown strain)	Ampicillin
pIJ673[4]	*S. lividans* TK24	Viomycin
pIJ680[4]	*S. lividans* TK24	Thiostrepton Neomycin
pGL103[5]	*S. lividans* TK24	Thiostrepton

Key:

The superscripts on the plasmids refer to the suppliers, who were:

1. S. Baumberg, Department of Genetics, University of Leeds.
2. M.J. Bibb, Department of Genetics, John Innes Institute.
3. P. Leadley, Department of Biochemistry, University of Cambridge.
4. John Innes Institute.
5. D. Ritchie, Department of Microbiology, University of Liverpool.

E. refers to *Escherichia*.

S. refers to *Streptomyces*.

Table 2.4. Genes used for preparation of the DNA probes used in this research.

Plasmid	Gene	Source	Enzyme	Restriction Fragment Size (Kb)
pCKL719	*aph*	*S.griseus* DSM 40236	*Pst*I *Stu*I	0.671
pIJ4104	*bar*	*S. hygroscopicus* ATCC 21705	*EcoR*I *Hind*III	0.605
pIJ673	*vph*	*S. vinaceus* NCIMB8852	*Sph*I	0.950
pIJ680	*aph D*	*S. fradiae* KCC 013	*Sst*II	0.953
	tsr	*S. azureus* DSM 40106	*Bcl*I	1.087
pGL103	Nb^R	*S. niveus* NCIB 9219	*Bgl*II	1.100

Explanation of genes qouted in Table 2.4.

aph = streptomycin phosphotransferase gene (Vallins and Baumberg, 1985).

bar = modified bialaphos resistance determinant, dimethyl phosphinothricin acetyltransferase gene (Murakami *et al.*, 1983).

vph = viomycin phosphotransferase gene (Thompson *et al.*, 1982a).

aphD = neomycin phosphotransferase gene (Thompson *et al.*, 1982a).

tsr = thiostrepton rRNA methylase (Thompson *et al.*, 1982a).

Nb^r = undetermined novobiocin resistance determinant (Mitchell *et al.*, 1990).

S. refers to *Streptomyces*.

Table 2.5. Media used in this research.

Key to Superscripts in Table 2.5..

[1]. The amount of constituents in the medium is given as g/l unless otherwise indicated

[2]. Added after autoclaving from filter sterilized stocks.

[3]. Amounts are given as grams per 800ml.

All media were autoclaved at 121°C for 20 mins.

pH was adjusted to the values shown prior to autoclaving.

Table 2.5. Media used in this research.

Medium	Ingredients	[1]Amount g/l
A37 Broth	Glucose	5.0
	Starch	15.0
pH 7.0	NaCl	5.0
	Corn Steep Liquor	10.0
	Soya Bean Meal	10.0
	$CaCO_3$	32.0
Arginine	Glycerol	12.5
Glycerol	Arginine	1.0
Salts	KH2PO4	1.0
(AGS)	K_2SO_4	0.5
	NaCl	1.0
pH 8.0	Agar	15.0
[2]Trace Elements	$MgSO_4.7H_2O$ (10g/l)	1ml
Solution for	$ZnSO_4.7H_2O$ (1g/l)	1ml
AGS.	$FeSO_4.7H_2O$ (10g/l)	1ml
	$CuSO_4.5H_2O$ (1g/l)	1ml
	$MnSO_4.4H_2O$ (1g/l)	1ml

Medium	Ingredients	[1]Amount g/l
Glucose	KH_2PO_4	7.0
Mineral	K_2HPO_4	3.0
Salts	KNO_3	4.0
	$MgSO_4.7H_2O$	1.0
	NaCl	1.0
	[2]Add 133ml filter sterilized glucose (30%) after autoclaving.	
GYM	Yeast Extract	4.0
	Malt Extract	10.0
	Agar	15.0
ISP7	Glycerol	15.0
	Tyrosine	0.5
pH 7.3.	Asparagine	1.0
	K_2HPO_4	0.5
	TES	1.0ml
[2]Trace	$MgSO_4.7H_20$	0.5
Element	$FeSO_4.7H_20$	1.0
Solution (TES)	$MnCl_2.4H_2O$	1.0
for ISP7	$ZnSO_4.7H_20$	1.0

Medium	Ingredients	[1]Amount g/l
L Broth	Bacto Tryptone	10.0
	Yeast Extract	5.0
	NaCl	5.0
	Glucose	1.0
Malt Extract	Broth	20.0
Agar (Oxoid)	Agar	15.0
Nutrient Agar (Oxoid)		28.0
Nutrient Broth (Difco)		13.0
Oatmeal Agar	Oatmeal	20.0
	Yeast Extract	5.0
	Agar	15.0
	pH 7.2	
	Steamed before autoclaving.	
R5	Sucrose	103.0
	K_2SO_4	0.25
	$MgCl_2.7H_2O$	10.12
	Glucose	10.0

Medium	Ingredients	[1]Amount g/l
R5-Continued	Casaminoacids	0.1
	Yeast Extract	5.0
	TES Buffer	5.73
	[2]Trace Element Solution	2.0ml
	Agar	22.0
[2]Trace	ZnCl	20.04
Element	$FeCl_2.7H_2O$	0.2
Solution	$CuCl_2.2H_2O$	0.01
for R5 and R2.	$MnCl_2.4H_2O$	0.01
	$Na_2B_4O_7.10H_2O$	0.01
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.01
[2]After autoclaving and	0.5% KH_2PO_4	10.0ml
immediately prior to use	5M $CaCl_2.2H_2O$	4.0ml
these ingredients (opposite)	20% L-proline	15.0ml
were added per litre.	1N NaOH	7.0ml
[3]R2	Sucrose	103.0
	K_2SO_4	0.25
	$MgCl_2.7H_2O$	10.12
	Glucose	10.0
	Casamino acids	0.1
	Distilled Water	800ml
	Agar	22.0

Medium	Ingredients	[1]Amount g/l
R2 (continued)	0.5% KH_2PO_4	10.0ml
After autoclaving and	3.68% $CaCl_2.2H_2O$	80.0 ml
immediately prior to use	20% L-proline	15.0 ml
the opposite ingredients	5.73% TES Buffer	100.0 ml
were added per 800 ml.	(pH7.2)	
	[2]Trace Element Solution	2.0ml
	1N NaOH	5.0ml
Sautons Medium	Glucose	15.0
(Modified)	Asparagine	5.0
pH 7.2	Casein Hydrolysate	2.0
	Sodium Citrate	1.5
	KH_2PO_4	5.0
	$MgSO_4.7H_2O$	0.5
	Ferric Ammonium Citrate	0.1
	[2]Glucose is filter sterilized and added after autoclaving.	
Yeast Extract	Yeast Extract	3.0
Malt Extract	Bacto-Peptone	5.0
(YEME)	Malt Extract	3.0
	Glucose	10.0
	Sucrose	340.0
	2.5M Magnesium Chloride (hexahydrate) 2.0 ml/l added after autoclaving.	

Media constituents were supplied by Oxoid, Difco, BDH or the Sigma chemical company.

Table 2.6. Buffers and reagents used in probing work.

Table 2.6. gives the buffers and reagents, which were used during this research. They were made up using double distilled water and were sterilized by autoclaving at 121°C, when appropriate.

Key to superscripts used in this research:

[1] The quantity of constituents is given as mM unless otherwise stated.

[2] The DNA was dissolved to give 10 mg/ml in sterile distilled water. Following this it was syringed through a narrow bore needle, boiled for 10 minutes, chilled rapidly on ice and stored at -20°C.

Table 2.6. Buffers and reagents used in probing work.

Reagent	Ingredients	[1]Quantity (mM)
Acid Phenol	Phenol (analar)	5.00 (g)
Chloroform	Chloroform	5.00 (ml)
	Water	1.00 (ml)
	8-hydroxyquinoline	5.00 (mg)
Alkaline SDS	NaOH	300.00
Solution	Sodium Dodecyl Sulphate	2%
Denaturing	NaOH	1000.00
Solution	NaCl	1500.00
Denhards Solution	Ficoll (MW400,000 Sigma)	2%(w/v)
	Bovine Serum Albumin (Fraction 5 Sigma)	2%(w/v)
	Polyvinyl Pyrrolidone (MW360,000, Sigma)	2%(w/v)
[2]Non-homologous DNA	Salmon sperm DNA	10.00 (mg/ml)
GTE	Glucose	50.00
	Sodium-EDTA (pH 8)	10.00
	Tris-HCl	25.00
TBE	Tris-HCl	89.00
Buffer	Boric acid	89.00
	Sodium-EDTA (pH 8)	2.00

Reagent	Ingredients	[1]Quantity (mM)
TE	Tris-HCl (pH8)	10.00
Buffer	Sodium-EDTA (pH8)	1.00
Lysozyme	Lysozyme	2.00 (mg/ml)
Solution	Heat Treated RNAse	50.00 (mg/ml)
	Tris-HCl (pH8)	0.30
	Sodium-EDTA (pH8)	25.00
5 x Loading	Sucrose	6.00 (g/l)
Buffer	Sodium-EDTA (pH 8)	0.37 (g/l)
pH 8.00	Sterile Distilled	10.00(ml)
	Water	
	Bromophenol Blue	25.00%
Neutralizing	NaOH	1500.00
Solution	Tris (pH7.4)	1000.00
[2]Non-Homologous	Salmon Sperm DNA	10.00 (mg)
DNA	Sterile Distilled	10.00 (ml)
	Water	
Phenol	Phenol (HPLC grade)	100.00 (g)
Chloroform	Chloroform	100.00 (ml)
Mix	Tris-HCl (pH8) to equilibrate.	

Reagent	Ingredients	[1]Quantity (mM)
Phenol Solution	Phenol (HPLC grade)	500.00 (g)
	8-hydroxyquinoline	0.50 (g)
	TE Buffer (+ 0.1M NaOH)	65.00 (ml)
Prehybridization	3 x SSC	15.00 (ml)
Solution	4 X Denhards solution	4.00 (ml)
	Non-homologous DNA (Salmon Sperm DNA)	100.00 (μg)
	Make up to 100ml with sterile deionzed distilled water.	
RNAse	RNAse	50.00 (μl)
Solution	Sterile distilled water	950.00 (μl)
20 x SSC	NaCl	175.32 (g/l)
	Sodium citrate	88.23 (g/l)

2.2. Strain Maintenance.

2.2.1. Routine Maintenance of Strains.

(See Table 2.4 for media)

Streptomycete strains were maintained on slopes in sterile universals of both of GYM and oatmeal agar, at 30° C. *B. subtilis* was maintained on nutrient agar and cultured for use in bioassays in nutrient broth at 30°C. *Aspergillus niger* was maintained on malt extract agar at 30° C, but for bioassay *A. niger* spores were suspended in glycerol salts medium. *E. coli* strains were maintained on nutrient agar containing a concentration of 35 μg per ml of ampicillin. *S. lividans* (TK24) was maintained on R5 medium. Alternatively the plasmid bearing mycelium was stored as a frozen paste until required.

2.2.2. Culture Storage.

Short-term storage was on the above slopes at 4° C. Long term storage was in 20% glycerol solution at -20° C. *Streptomyces* strains were grown up on oatmeal agar slopes until they sporulated. 5 ml of 20% glycerol solution was added to each universal with a sterile wide bore pipette. The tip of the pipette was used to scrape the spores and mycelium from the surface of the culture. The resulting suspension was dispensed into a sterile bijoux and stored at -20° C. Log phase *E.coli* and *B.subtilis* were pelleted in a centrifuge (14,000g at 10° C for 5 mins.) and were also stored in 20% glycerol solution at -20° C.

2.2.3. Strain Resuscitation.

Ampoules-containing freeze dried mycelium were heated and dipped in cold water to break the glass. Dried down streptomycetes were then resuspended in 500 μl YEME. Aliquots of this were used to inoculate Oatmeal plates and YEME broth. These were incubated at 28° C until the organisms had grown.

2.2.4. Standardization of Streptomycete Inocula.

Whenever it was necessary to standardize inoculum size between strains, spore and mycelial suspensions were counted using a haemocytometer slide (0.02 mm Thoma). Four fields of view were counted for each sample. These were then averaged and used to calculate the number of spores and mycelia per ml. Suspensions were then standardized at 1×10^7 spores and mycelial fragments per ml.

2.3. Characterization of Streptomycetes

2.3.1. Extraction of Bioactive Compounds.

For the extraction of non-polar (lipophilic) compounds, Ehrlenmeyer flasks (250 ml) containing 30 ml A37 medium were inoculated with a loopful of streptomycete spores from a slope. These were incubated at 28° C in an orbital shaker at 200 r.p.m.. After 6 days, 10 ml ethyl acetate was added to each culture. The flasks were stoppered with rubber bungs to prevent the solvent from evaporating and were shaken (200 r.p.m) for 1 hour at room temperature. The flask contents were transferred to centrifuge tubes and spun at 14,000g at 10° C for 15 mins. The solvent phase was removed, placed in a universal and vacuum evaporated to dryness in a desiccator. The dried extracts were resuspended in 200 μl ethyl acetate and analysed by thin layer chromatography.

For the extraction of streptomycin, a 6 day old culture was spun down at 14,000g at 10° C for 5 mins. The supernatant was collected into a 100ml glass pot and dried down in an oven at 80° C. The residue was resuspended in 200 μl methanol (in which >20 mg/ml of streptomycin was soluble) and used for TLC.

2.3.2. Thin Layer Chromatography (TLC).

TLC was carried out on 0.25 mm silica gel with fluorescent indicator (UV254). The baseline was 2 cm from the bottom of each plate. 20 μl of each

sample was loaded as a 1 cm line along the baseline with a 1cm interval between each sample and a 1.5 cm margin at the edges of the plate. Plates were run until the solvent front was 2 cm from the top of the plate. The following three solvent systems were routinely used: (1) ethyl acetate : n-hexane : dichloromethane : methanol (v/v 9:6:1:1); (2) dichloromethane : methanol (v/v 9:1) and (3) butanol : acetic acid : water (v/v 3:1:1). Table 2.7 shows the approximate Rf values of commonly used antibiotics in these solvent systems. Pure forms of relevant antibiotics were used as standards.

Table 2.7. Rf values of antibiotics screened for TLC.

Antibiotic	Solvent System 1 (Rf)	2 (Rf)	3 (Rf)
Streptomycin	0.0	0.0	0.2
Novobiocin	0.22, 0.31, 0.4.	N.T.	N.T.
Thiostrepton	0.00	0.68	1.73
Nigericin	0.72	0.84	N.T.
Herbimycin A	0.37	0.75	N.T.
Herbimycin C	0.31	0.86	N.T.
Geldanamycin	0.14, 0.41, 0.62.	0.84 (top spot)	N.T.

Key to Table 2.7.: N.T. = Not Tested.

Rf values were calculated by dividing the distance travelled by the compound by the distance of the solvent front from the base line. Replicate samples were used to give a mean Rf value for each compound.

TLC plates were removed from the solvent tanks and air dried. A number of methods were used to detect antibiotics, but colouration under visible light and UV fluorescence at 254 nm were used routinely. Plates were also routinely sprayed with vanillin reagent (vanillin, 3.0 g; ethanol, 100.0 ml; Conc. H_2SO_4, 0.5 ml) and then heated at 100° C for 10 mins. Other methods included covering the plates in a fine spray of 10% H_2SO_4 or with 0.2% ninhydrin in acetone. Plates treated in this way were also heated for 10 mins at 100° C. Table 2.8. shows the various reactions of commonly used antibiotics with these reagents.

2.3.3. Bioautography.

Antibacterial bioautograms involved inverting pre-run TLC plates were on a 200 ml base layer of nutrient agar in a Nunc bioassay dish. These were left for 30 minutes to allow the metabolites to diffuse into the medium. The plates were removed and seeded overlays containing 200 μl log phase *B. subtilis* cells ($1x10^6$ per ml) was poured evenly onto the base layer. These dishes were incubated at 28^oC overnight.

For antifungal bioautograms pre-run TLC plates were sprayed with *A. niger* spores ($1x10^5$ per ml) in glycerol salts solution. Plates were then placed in bioassay dishes, supported on sterile bottle tops for 3-7 days at 30° C. They were suspended above wet filter paper to provide a humid environment. Zones of inhibition for both types of bioautography were correlated with spots on replicate uninoculated TLC plates.

2.3.4. *In vivo* Agrochemical Screens.

Data relating to the bioactivity of the strains on *in vivo* agrochemical screens (herbicidal, insecticidal, fungicidal and plant growth regulator) was made available by Sarah Rees (ICI Agrochemicals). Information consisted of activities detected within each screening department and whether the activity was

Table 2.8. Visualization of antibiotics.

Anti-biotic	Visible Light	UV Light	Ninhy-drin	10% H_2SO_4	Vanillin
Strepto -mycin	-	-	-	Brown	Brown
Neomycin	-	+	Plum	Green	Beige
Viomycin	-	+	Violet	-	Lemon
Kanamycin	-	-	Purple	Brown	Yellow-Green
Blast-icidin	+	+	Green	Purple	Green
Oxytet -racycline	Yellow	-	Yellow	Rust	Yellow-Green
Erythro- -mycin	-	+	-	Brown	-
Novo- -biocin	-	+	Brown	Beige	-
Thio- -strepton	Yellow-	+	Brown	Rust-Brown	Orange-Brown
Nigericin	-	-	ND	ND	Scarlet
Herbi- -mycin	Yellow	+	ND	ND	Green
Gelda- -namycin	Orange-yellow	+	ND	ND	Green

ND = Not Done

- = Not Visible

+ = Visible

reproducible. However each screen comprised a range of assays to detect activity against a variety of agricultural pests. For example a typical plant pathology screen might comprise *Rhizoctonia*, *Botrytis* and *Pythium* species.

2.3.5. Taxonomic Identification.

Full taxonomic identifications were carried out on a random selection of the natural isolates in the study. This work was done by Dorothy A.Sanders, Department of Biological Sciences, University of Warwick, with the exception of the scanning electron microscopy data.

The taxonomical identities of streptomycete strains was done according to the probability matrix of Williams *et al.* (1983b).Good identifications were assessed as follows:

1. The Wilcox coefficient was greater than 0.85.
2. The taxonomic distance was equal to or less than 0.4.
3. The standard deviation was less than 2.
4. There was a low number of characters scored against the most suitable identification (ie the first numeral in the characters against and the identification columns respectively).

Ten strains gave good identifications whilst a further seventeen fell slightly short of the requirements. These identifications were used when a method was being assessed for use in taxonomy. This left 35 strains which did not identify closely with any species group on the Williams *et al.* (1983a) probability matrix. Although the identities of these strains remains unknown, much morphological, physiological and phenotypic information was generated about them. They may belong to minor clusters of the genus (Williams et al., 1983a) and could be identified using the probabilistic identification matrix of Langham *et al.* (1985), or else they represent rare streptomycetes.

Table 2.9. Taxonomic data on natural isolates.

Strain	Wilcox Coeff.	Tax. Dist.	SD	Characters Against	Identity x>y>z
$C13^{56}$	0.57	0.38	0.49	3:4:3	19:1:3
$C24^{567}$	0.5	0.399	0.92	3:5:5	19:15:12
$C28^{45}$	0.96	0.38	1.23	4:4:5	1:3:19
$C33^{456}$	0.45	0.47	3.52	7:10:5	1:15:3
$C40^{56}$	0.71	0.42	2.35	6:3:61	5:12:19
$C53^{5}$	0.94	0.403	1.75	4:5:5	12:19:15
$C70^{45}$	0.91	0.44	2.7	5:10:10	12:15:18
$C77^{456}$	0.52	0.45	2.19	7:5:7	19:3:5
$C98^{456}$	0.91	0.39	0.69	4:7:6	19:21:15
$C108^{456}$	0.46	0.43	1.73	-	19:1:3
$C109^{456}$	0.95	0.35	0.28	3:4:6	1:19:15
$C121^{456}$	0.73	0.37	0.19	3:3:4	19:3:1
$C129^{4567}$	1.0	0.4	1.9	6:6:7	15:12:19
$C141^{456}$	0.66	0.42	2.13	4:5:5	6:1:3
C174	0.57	0.396	2.41	4:6:7	3:1:19
$C177^{456}$	0.97	0.42	2.1	6:6:7	1:15:19
$C184^{4567}$	0.99	0.46	2.34	6:8:11	19:12:15
$C208^{456}$	0.8	0.36	0.0001	2:5:4	19:15:1
$C212^{4567}$	0.98	0.41	1.17	4:6:7	19:18:1
$C222^{4567}$	0.68	0.42	2.06	5:8:6	1:15:12
$C229^{467}$	0.0	0.4	2.3	5:5:6	3:1:15
$C245^{4567}$	0.8	0.46	3.3	6:9:10	12:15:29
$D2^{456}$	0.76	0.49	3.1	8:7:8	12:18:15
$D3^{456}$	0.42	0.42	1.48	5:5:5	19:15:3
$D5^{567}$	0.67	0.42	2.1	5:4:7	1:3:19

Strain	Wilcox Coeff.	Tax. Dist	SD	Characters Against	Identity x>y>z
D9	0.89	0.42	2.35	7:5:5	15:1:9
D61^{456}	0.93	0.4	1.06	3:5:7	19:1:5
D79^{56}	0.75	0.37	0.19	2:4:9	19:1:15
D98^{56}	0.96	0.38	1.23	4:4:9	1:19:15
D125^{56}	0.66	0.35	1.04	3:6:5	3:15:19
D140^{456}	0.76	0.4	1.97	5:6:7	15:19:1
D179^{45}	0.8	0.38	1.16	4:7;4	1:15:19
E1^{467}	0.88	0.34	0.84	3:5:5	3:1:19
E31^{4567}	0.79	0.42	3.57	5:6:7	32:15:12
E46^{4}	0.78	0.44	2.02	6:7:9	19:12:-
E55^{4}	0.95	0.48	3.77	7:10:9	12:19:18
E57^{4}	0.87	0.4	1.7	6:4:5	15:12:19
F28^{4}	0.55	0.41	1.99	6:6:9	1:19:15
F53^{567}	0.94	0.33	0.15	4:5:5	12:15:19
F66^{4}	0.87	0.44	2.76	6:7:6	12:19:15
CAG1$^{6-7}$	0.7	0.43	2.5	5:7:7	12:18:19
CAG2$^{6-7}$	0.7	0.51	3.7	10:8:10	19:18:12
CAG3$^{6-7}$	0.93	0.54	4.35	12:10:9	19:29:18
CAG4$^{6-7}$	0.63	0.44	1.69	5:6:4	18:19:6
CAG5$^{6-7}$	0.967	0.47	2.77	8:8:6	19:37:6
CAG6$^{6-7}$	0.97	0.42	1.4	15:6:7	18:15:12
CAG7$^{6-7}$	0.57	0.51	3.76	9:9:6	19:61:6
CAG8^{6-}	0.99	0.38	1.22	4:5:6	15:18:19
CAG9^{6-}	0.98	0.37	0.09	3:6:5	18:15:19
CAG10$^{6-7}$	0.79	0.47	2.58	7:8:6	19:18:15
CAG11$^{6-7}$	0.91	0.47	3.64	7:9:10	15:18:19

Strain	Wilcox Coeff.	Tax. Dist	SD	Characters Against	Identity x>y>z
CAG12[6-7]	0.61	0.51	3.74	9:9:9	19:1:37
CAG13[6-7]	0.94	0.43	0.47	7:7:8	37:12:19
CAG16[6-7]	0.55	0.53	4.18	9:10:12	19:18:15
CAG17[6-]	0.94	0.39	1.38	3:4:6	1:19:12
CAG18[6-7]	0.87	0.51	4.41	10:7:10	5:61:1
CAG19[6-7]	0.62	0.44	1.84	6:6:5	12:19:15
CAG21[6-]	0.67	0.44	2.8	7:7:6	15:12:19
CAG23[6-7]	0.65	0.5	4.3	8:10:10	1:15:18
CAG24[6-7]	0.97	0.4	0.97	4:6:7	19:18:12
CAG25[6-7]	-	0.47	2.9	8:7:9	19:1:5
CAG26[6-7]	0.76	0.47	2.9	8:8:9	19:12:18

The strains in this table were selected arbitrarily for identification. Various other strains had been identified prior to the work presented in this thesis. They were A10, A19, A39 and B4, which identified to cluster 19 and A26 and E44, which identified to cluster 12.

Key to superscripts and abbreviations used in Table 2.9.:

Wilcox Coeff. = Wilcox Coefficient.

Tax. Dist. = Taxonomic Distance.

x>y>z = x is the most probable identity; y is the next most likely identity; z is the third most likely identity.

[4]Used in Chapter 4; Fatty Acid Study.

[5]Used in Chapter 5; Chemical Profile Study.

[6]Used in Chapter 6; Antibiotic Resistance Study.

[6-]Used in Chapter 6, but not for clustering.

[7]Used in Chapter 7; Gene Probing Study.

(NB. where several superscripts have been used as in [456], this mean [4, 5] and [6].)

2.3.6. Scanning Electron Microscopy.

Strains were grown on sterile glass coverslips, inserted into plates of oatmeal agar at an angle of 45°. 10 μl spore suspension was streaked between the interface of the coverslip and the medium. The plates were incubated at 30° C. When the streptomycete had sporulated the coverslips were removed (the mycelium and spores having adhered to them) and placed in an atmosphere of formaldehyde overnight. These were used for observation under the Scanning Electron Microscope. The glass coverslips were mounted onto electron microscope stubs using electrodag 915 high conductivity paint (Agar Scientific Ltd.). A fine layer of gold was deposited onto each sample for 80 secs. using a Biorad-E5200 sputter-coater (20 mA, 10 lb/square " Argon). Coated samples were observed under vacuum using a JEOL T330A scanning electron microscope.

Partial identifications were carried out on a large number of strains by examining their spore surface morphology in the scanning electron microscope. 73 strains were randomly selected from the C, D, E and F series (Table 2.1). This was done to establish the proportion of strains isolated on C32 medium (D,E and F series, Table 2.1.), which were *S.viridochromogenes*. The species is easily recognised by its characteristic rugose ornamentation and tightly spiralled spore chains. The exercise also provided an opportunity to form a library of photographs (Fig.2.1.), which help to reidentify strains and eliminate duplicates. Table 2.10. shows the results from the exercise. In addition, all strains in the MM and W series were preselected for spiral, rugose spore chain morphologies by examination under SEM by N. Cresswell (Department of Biological Sciences, Warwick University).

Strain	Wilcox Coeff.	Tax. Dist	SD	Characters Against	Identity x>y>z
CAG12[6-7]	0.61	0.51	3.74	9:9:9	19:1:37
CAG13[6-7]	0.94	0.43	0.47	7:7:8	37:12:19
CAG16[6-7]	0.55	0.53	4.18	9:10:12	19:18:15
CAG17[6-]	0.94	0.39	1.38	3:4:6	1:19:12
CAG18[6-7]	0.87	0.51	4.41	10:7:10	5:61:1
CAG19[6-7]	0.62	0.44	1.84	6:6:5	12:19:15
CAG21[6-]	0.67	0.44	2.8	7:7:6	15:12:19
CAG23[6-7]	0.65	0.5	4.3	8:10:10	1:15:18
CAG24[6-7]	0.97	0.4	0.97	4:6:7	19:18:12
CAG25[6-7]	-	0.47	2.9	8:7:9	19:1:5
CAG26[6-7]	0.76	0.47	2.9	8:8:9	19:12:18

The strains in this table were selected arbitrarily for identification. Various other strains had been identified prior to the work presented in this thesis. They were A10, A19, A39 and B4, which identified to cluster 19 and A26 and E44, which identified to cluster 12.

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Partial identifications were carried out on a large number of strains by examining their spore surface morphology in the scanning electron microscope. 73 strains were randomly selected from the C, D, E and F series (Table 2.1). This was done to establish the proportion of strains isolated on C32 medium (D,E and F series, Table 2.1.), which were *S.viridochromogenes*. The species is easily recognised by its characteristic rugose ornamentation and tightly spiralled spore chains. The exercise also provided an opportunity to form a library of photographs (Fig.2.1.), which help to reidentify strains and eliminate duplicates. Table 2.10. shows the results from the exercise. In addition, all strains in the MM and W series were preselected for spiral, rugose spore chain morphologies by examination under SEM by N. Cresswell (Department of Biological Sciences, Warwick University).

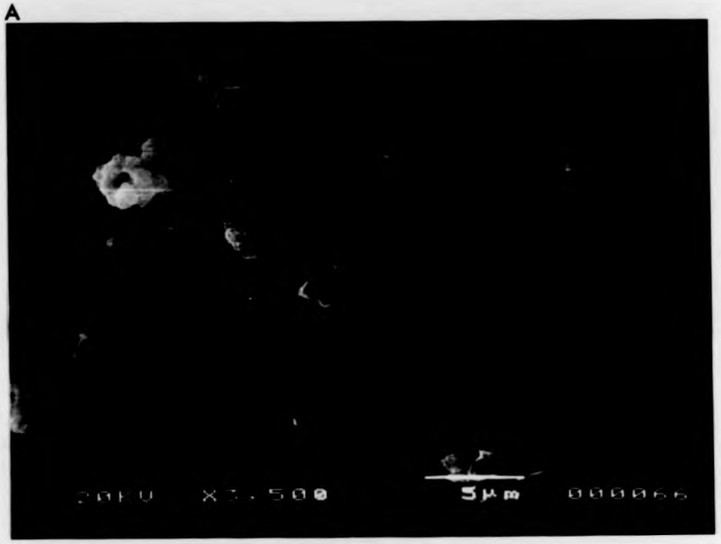
Figure 2.1. Scanning electron micrographs of streptomycete spore chains

Photograph.

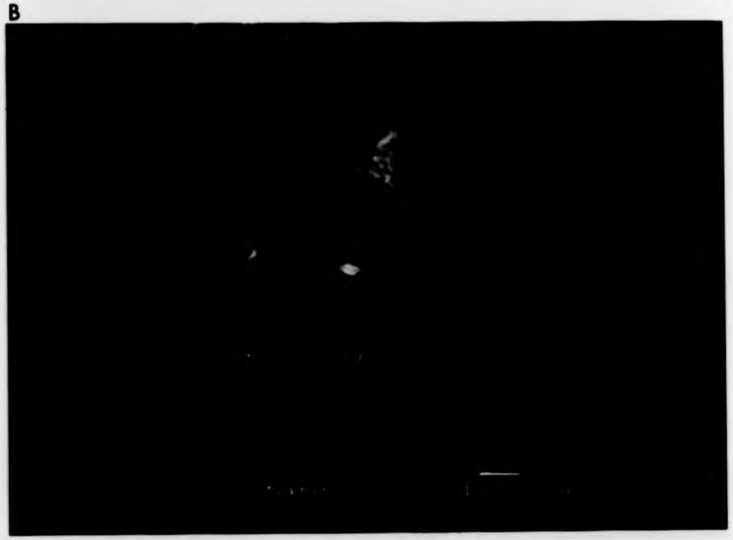
A and B = Rugose spore chains

C and D = Smooth and straight spore chains

A



B



C

D

Table 2.10. Spore morphology of a selection of natural isolates.

Chain Type	Spore Ornamentation	C Series (No. strains)	D,E,F Series (No.strains)
Spiral	Rugose	2	6
Spiral	Smooth	2	2
Straight	Smooth	34	17
Hooked	Smooth	4	2
Straight	Warty	1	3
Total No. Strains		43	30

Six strains out of the thirty isolated on C32 medium were of the desired identity (16.7%). This compared with two out of forty-three strains isolated on AGS medium. From these results C32 is 3.8 times more successful for the isolation of *S.violaceoniger* strains, although the vast majority of strains isolated by both methods do not belong to this species group.

2.4. Fermentation Work.

2.4.1. Comparison of Antibiotic Production on Different Media.

A comparison of antibiotic production in Sautons medium with and without casein hydrolysate, R5 with and without casamino acids, MSG and ISP7 was made. Thirty ml medium in a 250 ml Erlenmeyer flask was incubated for six days at 28° C. After incubation antibiotics were extracted as in section 2.3.

2.4.2. Timecourses of Antibiotic Production in Strain D153.

Sixty ml ISP7 (500 ml flask) was inoculated with 60 μl spore suspension (1 x 10^7 per ml). Incubation was at 28° C and 200 r.p.m. for 7 days. Samples (5 ml) were removed at 24 hourly intervals. After the samples were collected they were

frozen until required. Biomass (dry weight) was measured for each sample and the presence of geldanamycin and nigericin were determined by TLC.

2.4.3. Oxygen Limitation Experiment.

Fifteen flasks containing ISP7 medium (30 ml) were inoculated with 60 ul spore suspension (1 x 10^7 per ml). These were incubated for 76 hours at 28° C. Then 5 control flasks were prepared as follows: 3 were left as they were and 2 were subasealed. The remaining flasks were also sealed with subaseals, but were flushed with nitrogen gas. A set volume of nitrogen was removed from the flask and an equivalent volume of oxygen was syringed in to make concentrations of 0, 5, 10, 15 and 21% oxygen. Flasks containing 21% O_2 were also controls. The volume of each flask was measured, prior to the experiment, by filling them with water up to the subaseal level and then weighing them. The precise volume of gas required for each oxygen concentration was then calculated. Samples (2 ml) were taken every twelve hours. Biomass was measured (dry weight) and the presence of antibiotics was detected using methods 1 and 2 in section 2.3.

2.4.4. Dry Weight Measurements.

A known volume of filtered mycelium from a culture broth was dried down to constant weight in pre-weighed glass universals at 100^oC.

2.4.5. Nutrient Gradients.

The defined medium was based on ISP7 with variation of either the nitrogen, carbon or phosphorus source. The carbon sources used were glucose, sucrose and glycerol,at concentrations between 0 and 20 g/l. Nitrogen sources were tyrosine asparagine and sodium nitrate, at concentrations between 0 and 4 g/l. Gradients of dipotassium hydrogen orthophosphate were examined between 0 to1 g/l, 0 to 5 g/l and 0 to 10 g/l. The gradients were prepared by omitting the nutrient under scrutiny from the 50 ml base layer poured on a slant in a 10 x 10 cm bioassay

dish. The 50 ml top layer was made at the maximum concentration for the test nutrient. This was poured onto the base layer to give a flat surface. Diffusion from the top layer to the bottom layer resulted in a plate with a continuous gradient from 0 to maximum g/l for each nutrient. 35 μl streptomycete spore and mycelium suspension ($1x10^7$ per ml) was dispensed in an even line across the gradient and the plates were incubated at 30° C for up to 2 weeks. The growth on the plates was observed every day for diffusible pigments and to observe the growth and development of each organism.

Antibiosis for selected strains was observed after 90 hours growth. Streptomycete cultures were killed by exposing them to 5 mins. shortwave (254 nm) UV light. Then the plates were overlaid with test organisms. For antifungal activity 50 μl *A.niger* spores (1×10^6 per ml) were inoculated into 15 ml YEME medium and used to overlay gradient plates. They were grown at 30^oC for 3 days. The position and size of zones, where there was no fungal growth were measured. For activity against *B.subtilis* 50 μl log phase ($1x10^6$ per ml) cells were used to inoculate 15 ml nutrient agar. Gradient plates were overlaid and incubated at 28° C overnight. Clear zones were recorded with respect to their size and position on the gradient

2.5 Fatty Acid Analysis.

2.5.1. Extraction Procedure.

The following reagents were used during the analysis of whole cell fatty acids:

Reagent 1.; Saponification reagent: 45g NaOH, Fisher certified ACS grade; 150 ml methyl alcohol HPLC or spectroanalysis grade; 150 ml deionized, distilled water.

Reagent 2.; Methylation reagent: 325 ml 6 N hydrochloric acid (HCl); 275 ml methyl alcohol (as above).

Reagent 3.; Extraction solvent: 200 ml hexane, Fisher HPLC grade; 200 ml ethyl ether, anhydrous Fisher certified spectroanalysed.

Reagent 4.; Base wash: 10.8g sodium hydroxide (as above); 900 ml deionized distilled water.

Streptomycetes were grown in 50 ml modified Sautons medium at 28° C for three days. Cells were harvested by filtration through Whatman 3 mm filter paper in a Buchner filter and washed with distilled water. The wet biomass was dried at 100° C to a constant weight. 50 mg dried biomass was transferred to a Teflon stoppered "Pierce" reactivial and saponified in 1ml Reagent 1 at 100° C for 30 mins.The tube was vortexed for 5-10 secs. at times 0 and 5 mins. A 2 ml amount of Reagent 2 was added to the tube, which was then vortexed for 5-10 secs. This was placed in an 80 +/- 1° C water bath for 10 mins. to carry out the methylation reaction. The Fatty Acid Methyl Esters (FAMES) were extracted into 1.25 ml Reagent 3 by rotating the tube end-over-end for 10 mins. The aqueous lower phase was discarded and 3ml Reagent 4 was added. The tube was rotated for 5 mins. Then 2/3 of the organic extract was added to a Gas Chromatography (GC) sample vial after drying over anhydrous Na_2SO_4.

2.5.2. Combined Capillary Gas Chromatography Mass Spectroscopy (GCMS).

GCMS was carried out by Sue Slade, Department of Biological Sciences, University of Warwick.

A Kratos MS25 RFA Mass Spectrometer in conjunction with a Carloerba MFC 500 Gas Chromatograph was used. The column was a Chrompack CP Sil5 (non-polar) of length 25m, inner diameter 0.33 mm and film thickness 0.12 μm. The carrier gas was helium, the temperature ramp 100° C for 8 mins. then 4° C per min. to 250°C and the flow rate 2 cubic cm /min. A 1.5 μl aliquot (50% split ratio) was injected into the GCMS at 240° C. The source temperature was 200° C and an ionization an electron impact of 70 eV was used. A qualitative standard mix of methyl esters, supplied by Supelco (Catalogue No. 4-7080) was used in order to

detect the presence of specific compounds. For the timecourse experiments a quantified internal standard (methyl undecanoate) was used so that the amount of each methyl ester relative to this standard could be calculated using the area under each peak.

2.5.3. Timecourses.

Fatty Acid Analysis Procedure and GCMS Protocol were as above, except cells were harvested daily for 7 days. Biomass samples from each timecouse were frozen at -20° C until they had all been collected then the FAMES were isolated and analysed. Dry weight measurements were taken and antibiotic production was analysed in timecourse 2.

2.6. Metabolite Profiles.

These were based on the results from extraction method 1, TLC system 1 and visualization under ultra-violet light. For each strain the presence or absence of 29 compounds was scored using an external standard to aid between plate comparisons. Method development is discussed in Chapter 5.

2.7. Resistance Profiles.

2.7.1. The Use of Antibiotic Gradient Plates to Determine Phenetic Resistance in *Streptomyces* Strains.

Resistance was defined by reference to sensitivity of a large population of streptomycetes (193 strains). This was achieved by testing the population using gradient plates composed of AGS medium. To make antibiotic gradient plates, 150 mls AGS medium was poured as a 0-5 mm slant in a bioassay dish (22 x 22 cm). A further 150 mls AGS, containing the prescribed antibiotic was poured on top of the base layer to give a flat surface. The plates were poured immediately prior to use and then 75 μl spore and mycelial suspension (1×10^7 spores and mycelial fragments

per ml) was inoculated in an even line using a Finn pipette across gradient plates (containing one of the following antibiotics) and incubated for 6 days at 30° C. The antibiotics tested were thiostrepton (0 to 50 ug/ml), neomycin (0 to 10 ug/ml), novobiocin (0 to 100 ug/ml), viomycin (0 to 30 ug/ml), streptomycin (0 to 10 ug/ml), erythromycin (0 to 100 ug/ml), oxytetracycline (0 to 100 ug/ml), kanamycin (0 to 100 ug/ml), nigericin (0 to 17 ug/ml), blasticidin S (0 to 100 ug/ml) and penicillin G (0 to 100 ug/ml). Growth across the gradient was measured in mm's and since the strains did not grow where an antibiotic had reached an inhibitory level, the measurement was related to the minimum inhibitory concentration. An extrapolated value was taken from these measurements using the following equation:

$$\frac{\text{LENGTH OF LINE OF GROWTH (mm)}}{\text{TOTAL LENGTH OF GRADIENT (mm)}} \times \text{MAXIMUM ANTIBIOTIC CONC. } (\mu g/ml) = \text{LEVEL OF ANTIBIOTIC RESISTANCE } (\mu g/ml).$$

These tests were done in duplicate for each strain and the results were only scored when control plates containing no antibiotic showed growth. the distances (and extrapolated antibiotic concentrations) at which the population became constant were as follows; thiostrepton, 2.25 cm (5 μg/ml); neomycin, 4.5 cm (2 μg/ml); novobiocin 4.5 cm (20 μg/ml); viomycin 4.5 cm (2 μg/ml); streptomycin, 7.88 cm (3.5 μg/ml); erythromycin, 9 cm (40 μg/ml); oxytetracycline, 10.13 cm (45 μg/ml); nigericin, 19.95 (16 μg/ml); blasticidin S 0.5 cm (2 μg/ml); penicillin G 21.38 cm (95 μg/ml).

Antibiotics were obtained from the Sigma Chemical Company Ltd., except for viomycin, which was supplied by the Upjohn Company and blasticidin, which was a gift from ICI Agrochemicals plc.

2.7.2. Determination of the Relationship Between Antibiotic Concentrations Extrapolated from Gradient Plates and Corresponding Concentrations from Pour Plates.

AGS gradient plates and pour plates with the same depth of agar were poured and incubated at 28° C for 5 days. Pour plates contained the concentration of the relevant antibiotic at its cut-off concentration (Section 6.2.1.). Plugs of agar were removed from duplicate AGS gradients at the position of the estimated cut-off point for thiostrepton, neomycin, kanamycin and streptomycin (6.2.1). These were placed, antibiotic side uppermost on nutrient agar seeded with *E.coli* (711) for streptomycin and *B.subtilis* (supplied by D. Sanders, Department of Biology, University of Warwick) for the other antibiotics (10 ul overnight culture per ml of medium). Controls were plugs, of the same dimensions as above, from the corresponding pour plates. Bioassays were carried out at 37° C overnight and the mean zone size of triplicate samples from duplicate plates were compared.

2.8. DNA Isolation and Probing.

2.8.1. Small Scale Alkaline Lysis for Plasmid Preparation of *E.coli*.

A single colony of *E.coli* was inoculated into 5 ml L-broth which contained 35 μg/ml ampicillin. After overnight incubation at 37° C the cells were pelleted and resuspended in 1 ml TE buffer. This suspension was pelleted in the microfuge and then resuspended by vortexing in 150 μl ice cold GTE. The sample was incubated at room temperature for 5 mins., after which 200 μl of 0.2N sodium hydroxide and 1% SDS was added. The tube was inverted 2-3 times to mix the solution, which was kept on ice until it was clear (5 mins.). Ice cold potassium acetate (150 ul at pH 4.8) was added and the tube inverted immediately (5 x) and then kept on ice for 5 mins. After centrifuging at high speed in the microfuge for 5 mins the supernatant

was transferred to a fresh eppendorf. This was treated with phenol-chloroform and chloroform-isoamyl alcohol. The supernatant was ethanol precipitated and resuspended in TE buffer.

2.8.2. Maxi Preparation of Plasmid from *E.coli.*

A single colony of plasmid-bearing *E. coli* was used to inoculate 10 ml L. Broth containing 35 μg/ml ampicillin. This was incubated at 37° C overnight and used to inoculate 1l of the same medium. After a further overnight incubation the cells were pelleted by centrifugation in 4 x 250 ml pots (4000g, 4° C, 10 mins).

8mls GTE was added to each pair of pellets. The pooled volumes were placed into oakridge tubes and a spatula tip of lysozyme was added to each. These were incubated for 10 mins. at room temperature. 15 ml of 0.2N NaOH with 1% SDS was added and the tubes were gently inverted for 1 min. Then they were left for a further 10 mins. on ice. Vigorous sharp jerks were administered to the tubes upon addition of 12 ml of 5M sodium acetate (pH4.8), which were again left on ice for 10 mins. The tubes were then centrifuged at 39,000g at 4° C for 30 mins. The supernatant was isopropanol precipitated, the supernatant was discarded and the pellet was dried by inverting on a tissue for 30 mins. It was then resuspended at 37° C in 31mls TE buffer. After the presence of plasmid DNA was confirmed by running some of the sample on a minigel, density gradient centrifugation was carried out.

2.8.3. Density Gradient Centrifugation.

The volume of the sample was measured and 1.05g caesium chloride was added per ml of sample. 50 μl ethidium bromide (10 mg/ml) was added. This mixture was pipetted into a centrifuge tube (Beckman T053OE), weighed and balanced with another tube. The tubes were heat sealed and spun at 55,000g for 18 hours.

The plasmid band was visualised by using a UV transilluminator. A sterile needle was used as a vent while another needle and a syringe facilitated the removal of the band. One volume of TE saturated with butan-1-ol (1:1) was repeatedly added, shaken and the solvent phase removed until the lower phase was clear. The remaining volume was measured and mixed with two volumes of water and six volumes of ice-cold ethanol. This was placed at -20° C overnight. the solution was centrifuged at 39,000g and 4° C for 15 mins. The ethanol was discarded and the tube vacuum desiccated to dryness. The DNA was then resuspended in an appropriate volume of TE buffer.

2.8.4. Large Scale Isolation of Plasmid DNA from Streptomycetes.

500 ml of *S.lividans* TK24 containing either pIJ673 or pIJ680 was grown for 2-3 days in shake flasks of TSB supplemented with ampicillin (35 μg/ml) were spun down at 4,000g for 20 mins. These were resuspended in 5ml of lysozyme solution and incubated for 30 mins. at 37° C. The cells were gently mixed and 2.5 ml alkaline SDS solution added. This was thoroughly mixed by pipetting up and down, incubated for 20 mins. at 70° C with slightly unscrewed caps and then cooled to room temperature. Phenol chloroform (acid) extraction was then carried out on the sample followed by isopropanol precipitation and caesium chloride centrifugation.

2.8.5. Isolation of *Streptomyces* " Total" DNA.

Streptomycete spore suspensions (200 μl) were inoculated into YEME, supplemented with 3% glycine. After 2-3 days the mycelium was harvested by filtration using a Buchner filter, through two sheets of Whatman No 1 filter paper. The mycelium was washed with 10% glycerol and stored as a paste at - 20° C.

50 mg of mycelium was resuspended in 500 μl lysozyme solution and incubated at 37° C for 30 mins. in an eppendorf tube. Then 250ul 2% SDS was added and the solution vortexed for 1 min. This solution was then extracted with neutral phenol-chloroform and isopropanol precipitated.

2.8.6. Phenol Extraction.

An equal volume of neutral phenol-chloroform was added to the sample. The solution was vortexed for 1 min. and centrifuged at 20,000g for 15 mins. The upper phase was transferred to another container leaving the white interface behind. This was repeated until no interface was seen. Then half the volume of chloroform : iso-amyl-alcohol (25 : 1) was added and the solution was again vortexed for 1 min, but centrifuged at 20,000g for 2 mins.

2.8.7. Quantitation of DNA

Readings were taken at 260 nm and 280 nm. An OD_{260} of 1 was taken to be equivalent to 50 μg/ml DNA and an OD_{260}/OD_{280} ratio of 1.8 taken to be pure DNA.

2.8.8. Restriction Endonuclease Digestion of DNA.

Ingredients	Volume(μl)
10 x Enzyme buffer	2.0
DNA in TE buffer	2.0
Restriction endonuclease	2.0
Sterile distilled water to	20.0

The above mixture was incubated at 37° C for 1-3 hours. Double digests were made up as above, but made up to 19 μl. They were incubated for an appropriate time and then 2 μl of the second enzyme was added and the incubation was continued for another 1-3 hours.

2.8.9. Dot Blots.

Streptomyces chromosomal DNA was denatured by adding an equal volume of denaturing solution and incubated at room temperature for 10 mins. The reaction was neutralized with four volumes of 0.5M HCl, 0.5M Tris (pH 7.4) and 1.5M NaCl, 20 x SSC, mixed together as a 1:1:2 ratio.

Hybond nylon filters were prewetted in 10 x SSC and placed in a commercial dot blotting apparatus (Hybri-Dot Manifold-1050MM. Bethesda Research Laboratories, Life technologies Incorporated PO Box 6009, Gaithersberg, MD 20877 U.S.A). Samples, containing 1 μg DNA were ejected rapidly into the sample wells and transferred under vacuum onto the nylon filter. The filter was removed from the manifold and dried. The DNA was fixed by UV cross-linking on a longwave transilluminator for 4 mins. Filters were heat sealed in plastic bags until use.

2.8.12. Agarose Gel Electrophoresis.

A 1% agarose solution was prepared by boiling in 1 x TBE. Ethidium bromide was added to make a final concentration of 0.5 μg/ml. The cooled agarose was then poured into the gel apparatus(BRL minigel 1060 or BRL 1087 model H5); this having been prepared by taping the ends and inserting a comb to make the wells. The set gel was submerged in TBE and DNA samples containing 0.2 volumes of loading buffer were injected into the wells. Electrophoresis was carried out at 75V for 3 hours or at 20V overnight. Restriction digested lambda bacteriophage DNA was used as molecular weight markers of 23.31, 9.416, 6.557,4.361, 2.322, 2.027, 0.564 and 0.125Kb. (Hopwood *et al.*, 1985)

2.8.13 Ethanol Precipitation.

DNA was precipitated by mixing with 0.1 volume of 3M sodium acetate and 2 volumes of ice cold ethanol. After chilling for 3 hours at -20° C the precipitate

was centrifuged at 14,000g for 15 mins., washed in ice cold 70% ethanol and recentrifuged at 14,000g for a further 5 mins. The DNA pellet was dried under vacuum and resuspended in TE buffer.

2.8.14. Isopropanol Precipitation.

One tenth of a volume of 3M sodium acetate (pH4.8) was added and mixed into the sample, followed by 1 volume of isopropanol. This was incubated at room temperature for 5-15 mins and then spun at 15,000g for 2 mins. The supernatant was discarded and the pellet dried. The DNA pellet was then resuspended in an appropriate volume of TE buffer.

2.8.15. Electroelution.

The required band was excised from an agarose gel using a sharp scalpel, having been visualised under long wave ultra-violet light (300-360 nm) to avoid damage to the DNA. The band was placed in a well in the IBA electroeluter with TBE with the valve in the "up-position". The channels were filled with 7.5M ammonium acetate, using a syringe and making sure that there were no air bubbles. The apparatus was run at 100V for 20 mins. The absence of the DNA from the band of agarose was checked under the transilluminator. The ammonium acetate and DNA was removed from the appropriate channel using a needle and syringe and placed in an eppendorf. The DNA was then precipitated in 2 volumes of ice cold ethanol for 3 hours at -20° C. The precipitate was centrifuged at 10K for 15 mins., washed in 70% ethanol and recentrifuged at 10K for a further 5 mins. The DNA pellet was then dried under vacuum and resuspended in 10 μl TE buffer. 3 μl was then run on an agarose gel to check for the presence of the DNA.

2.8.16. Prehybridization.

Filters were sandwiched between layers of nylon mesh (no more than 4 filters per stack). Any air bubbles between the layers were removed and the stack

was rolled up and placed in a Hybaid tube, along with a small amount of 2 x SSC The roll was unravelled until it stuck to the walls of the tube. Any air bubbles between the wall and the filters were removed. The liquid in the tube was exchanged for 15 ml prehybridization solution. The sealed tube was placed in the Hybaid Oven overnight at 70° C.

2.8.17. Preparation of the Probe.

Random primed labelling was carried out using the following mixture.

Ingredients	µl
Probe DNA (preboiled for 10 mins)	5.0
dATP	1.0
dTTP	1.0
dCTP	1.0
Reaction Buffer	2.0
Sterile distiled water	7.0
Klenow	1.0
$dCT^{32}P$	2.0

Ingredients were added in the order shown to keep handling of the radioactive nucleotide to a minimum. During the preparation the mixture was kept on ice. Following addition of $dCT^{32}P$ the mixture was incubated at 30° C for 30 mins. The reaction was quenched using 160 ul TE buffer containing 0.1% SDS.

2.8.18. Removal of Unincorporated Nucleotides.

A Sephadex G50 column was prepared in a 150 mm Pasteur pipette with a glass wool filter. The 180 µl reaction mixture was run through the column, using 180 µl amounts of TE buffer with 0.1% SDS to wash it through. Each 180 ul

elution was collected in an eppendorf. The first peak was incorporated DNA and could be followed down the column using a giegercounter. Eluent was collected up to the beginning of the second peak (unincorporated nucleotide). Samples belonging to the first peak were pooled and used in the hybridization reaction.

2.8.19. Hybridization.

The radioactively labelled DNA was boiled for 10 mins. with 500 ul salmon sperm DNA (10 mg/ml) and then snap cooled on ice. 10μl of this was removed for Cherenkov counting. The rest was added to the prehybridized filters after 10 ml of prehybridization solution had been removed, leaving 5 ml for the hybridization reaction. Hybridization was at 70° C overnight in the Hybaid oven.

2.8.20. Stringency.

For the purposes of dot blotting filters were washed twice for 30 mins., in a volume of 50 ml, at each of three levels of stringency. These were 3 x SSC and 0.1% SDS, 1 x SSC and 0.1% SDS and 0.2 x SSC and 0.1% SDS, all at 70° C. Autoradiography was carried out at each stringency. For southern blotting filters were washed twice for 30 mins. in 2 x SSC; 0.1% SDS and twice for 30 mins in 0.2 x SSC; 0.1% SDS, again at 70° C. Autoradiography was carried out after the highest stringency wash.

2.8.21 Cerenkov Counting.

This was carried out in a Beckman LS700 scintillation counter, using program 10 (1 min.). 10 μl radioactive sample in an eppendorf tube was placed inside a scintillation vial. Disintegrations per min., per 700 μl sample were calculated. This was multiplied by 2.5 to approximate to Cerenkov counts per min.

2.8.22. Detection of Radioactivity.

Depending on the specific activity of each probe varying times of exposure were used, but in general these were one week after the first wash, two weeks after the second and four weeks after the third. For autoradiography, X-ray film (Fuji X-Ray) was exposed to hot filters in Harmer autoradiography cassettes containing intensifying screens. The time of exposure was variable. X-ray film was developed by placing in developing solution (Kodak LX-24) for 5 mins. and washing in water. The developed film was fixed (Kodak FX-40) for 5 mins. and rinsed in water again. Alternatively a Beta-Scope (Betagen, 100 Beauer Street, Walton, MA 02154, U.S.A) or Phosphorimager (Molecular Dynamics Ltd., 4, Chaucer Buisiness Park, Kemsina, Sevenoaks, Kent, TN15 6PL, U.K.) were used and so exposure times were greatly reduced by direct counting. Positive hybridizations were scored on filters where the negative controls did not hybridize and positive controls (1 ng DNA from the plasmid containing the gene and 1 μg DNA from the source organism).

2.9. Cluster Analysis. (Fig.2.1).

Data was stored in Foxbase (Fox Software Inc., 118 W.South Boundary, Perrysberg, Ohio, 43551.) and in Paradox Relational Database version 3.5 (Borland International, 1800, Greenhills Road, PO Box 660001, Scotts Valley CA 95067-0001). Data matrices, taken from the database provided input for NTSYS-pc (Exeter Publishing, Ltd, 100 North Country Road, Building B, Setauket, New York 11733), where either the SIMINT (interval data) or SIMQUAL (binary data) programs were used to give similarity and dissimilarity matrices. In SIMINT either the Manhatten distance (Lance and Williams, 1967) or euclidean distances were calculated, whilst the simple matching coefficient (Sokal and Michener, 1958), Jaccard's coefficient (Jaccard, 1908) and the Dice coefficient (Dice, 1945) were used in SIMQUAL.

Hierarchical clustering was performed in SAHN, using algorithms for Single Linkage (Florek *et al.*, 1951a and b), Complete Linkage (Lance and Williams, 1967b) and the Unweighted Pair Group Method with Arithmetic Averages (Sokal and Michener, 1958). Graphics hardcopy of phenograms were provided by the treeG program. Cophenetic matrices were obtained using COPH and cophenetic correlations with MXCOMP and MXCOMPG. Multivariate analysis in the form of principle components analysis (PCA) was performed either by using MACSPIN on an Apple Mackintosh or by using the EIGEN and PROJ programs in NTSYS. MXPLOT and MXPLOTG were used to obtain plots of the data.

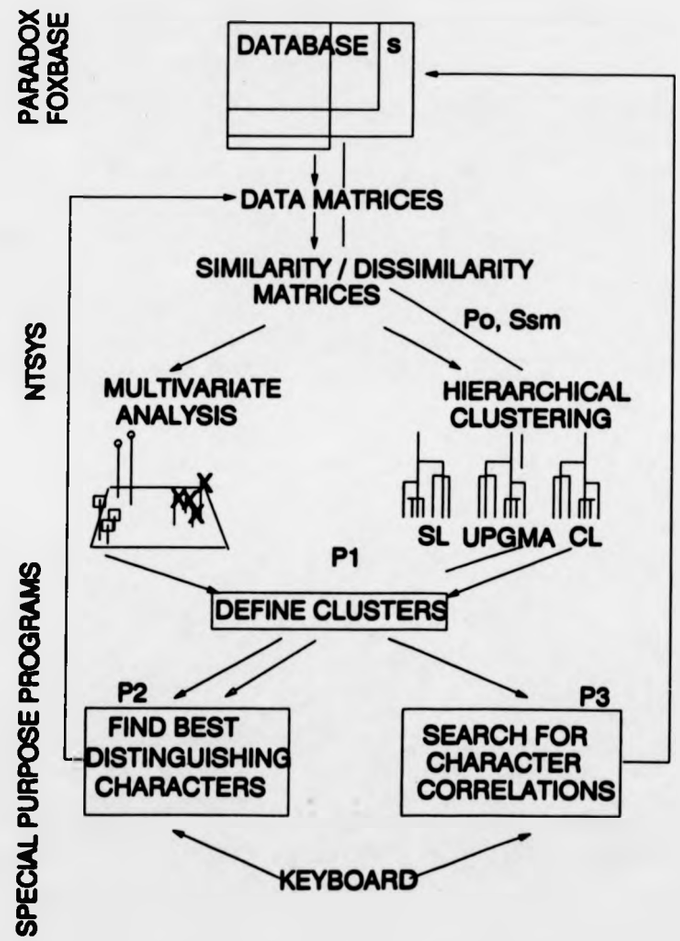
Clusters were defined by eye when PCA was used, however special purpose computer program was used in conjunction with hierarchical clustering (P1, Fig. 2). The P1 program was able to list all alternative similarity levels, at which clusters could be defined. After the computer operator had chosen one of these levels the relevant clusters of strains were defined and the strains in each group listed. A second program (P2) established the percentage of strains in each group with various characters from the original data matrix. These were then listed in descending order within each cluster. Each possible position for a character was given a value, which increased the further a value was from the central row of the table. All values for a given character were added to give a final score. High final scores, corresponding with high standard deviations were regarded as good distinguishing characters and of value for numerical taxonomy. In order that correlations between different data sets could be established (for example, do clusters based on antibiotic resistances correlate with bioactivity?) a third program (P3) was written. The percentage of strains in each group, as defined by P1, with any type of data stored in the database(s) could be calculated. A fourth program (P0) was designed to run within paradox. This used Ssm in SIMQUAL and UPGMA from SAHN as defaults and automatically fed into P1 and P2.

Computer programs were written by G Barry Joyce and Steve Greensides (Department of Mathematics, University of Warwick).

Fig. 2.2. Summary of data handling procedures used in this research.

The diagram shows the stages used when carrying out both heirarchical clustering and ordination. P0, P1, P2 and P3 refer to special purpose programs, which were written by Greg King, Barry Joyce and Steve Greensides.

Two databases (Paradox and Foxbase) were used, whilst NTSYS was used for cluster analysis and special purpose programs allowed some additional datahandling.



Chapter 3.

The Distribution and Expression of Antibiotic Production in Natural *Streptomyces* Isolates.

3.1. Introduction.

Streptomycetes are usually cultivated in nutritionally rich media and antibiotic production normally begins when the culture conditions become limiting (Hütter, 1982); this shows that antibiotic biosynthesis is regulated. The major factors involved in the control of secondary metabolism are carbon and nitrogen catabolites, phosphorus, inorganic salts, trace metals (Vanek and Mikulik, 1978; Martin and Demain, 1980; Iwai and Omura, 1981) and other bioregulators, such as A-factor (Beppu, 1985; Horinouchi and Beppu, 1986). Cultural conditions such as oxygen tension (Yegneswaran *et al.*, 1988) are also known to be important influences on the biosynthesis of bioactive metabolites.

Carbon catabolite regulation inhibits the synthesis or activity of enzymes involved in antibiotic production (Katz *et al.*, 1984; Jones, 1985). A variety of carbon sources gives rise to catabolite repression including glucose (Belousova *et al.*, 1985; Behal, 1985; James and Edwards, 1988) plus glycerol and sucrose (Sanchez, 1984; Demain, 1985). Similarly, nitrogen catabolites have been shown to inhibit enzymes which are important for secondary metabolism (Katz *et al.*, 1984); for example, rapidly utilized nitrogen sources such as nitrogen, ammonium and amino acids inhibit the production of a variety of antibiotics (Sanchez *et al.*, 1984; Omura, 1984a and b; Demain, 1985). Many idiolites are produced at sub-optimal phosphorus concentrations (Demain, 1985) and this is due to the control of either phosphatase enzymes (Iwai and Omura, 1981, Muller and Ozegowski, 1985) or antibiotic synthases (Hütter, 1982).

One aim of the research presented in this thesis was to investigate the distribution of antibiotic production in natural *Streptomyces* isolates and to discover phenotypic and genotypic characters which correlated with it. Bioactivity was

measured for the strains concerned by use of a variety of complex and defined media, extraction procedures and screens (Sections 2.3.1 - 2.3.4.). However, the proposed study was limited by the screening methods used and the literature indicated that unconventional media often yielded interesting observations about antibiotic production. Imanaka and co-workers (Arima *et al.*, 1965; Miyairi *et al.*, 1970; Miyoshi *et al*, 1972) discovered new antibiotics on high phosphate media and high glucose concentrations have been shown to favour the production of some antibiotics (Boeck *et al.*, 1971). A smaller study, which involved testing the expression of antibiotic production with fewer strains, but under a wider set of conditions enabled the larger bioactivity study to be assessed in an objective manner (Section 3.3).

Results

3.2. Bioactivity as Measured Under Standard Conditions.

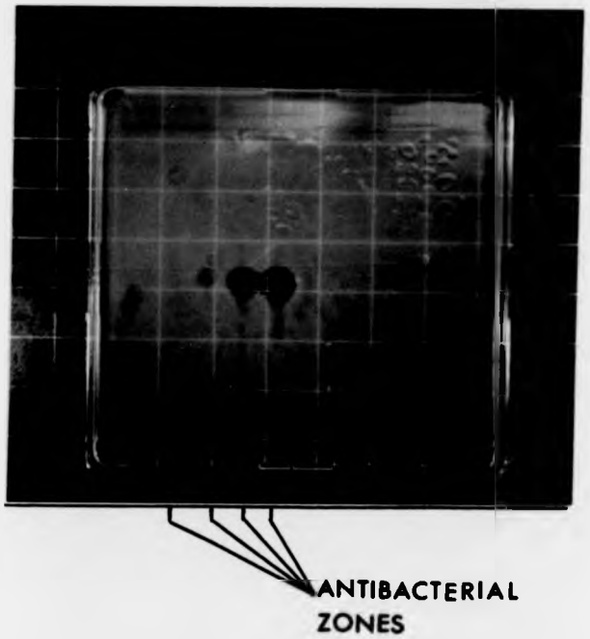
Anti-*B.subtilis* and anti-*A.niger* bioautograms were used to measure antibacterial and antifungal activity and this was supplemented with data from *in vivo* agrochemical screens, which measured activity against a wide range of organisms. Typical activity spectra for nucleoside, polyether, nonactin and cycloheximide producers were detected on the *in vivo* screens, whilst nigericin, geldanamycin and herbimycins A and C were detected using TLC. An example of an antibacterial bioautogram is shown in Fig. 3.1.

3.2.1. Antibacterial Activities.

Twenty-three strains showed activities against *B.subtilis* (Fig. 3.2.), which were not due to any of the specific compounds tested for. Eight of these strains were replicated, but, although each replicate was bioactive, the antibiotic profiles were variable, suggesting that the range of substances with biological activity for some producers might be diverse.

Fig 3.1. Example of an antibacterial bioautogram.

The diagram shows a bioassay dish, containing a base layer of nutrient agar, onto which a TLC plate, containing extracts from streptomycetes was inverted for 30 mins. This was then removed and the base layer was overlayed with nutrient agar seeded with *B.subtilis*. The plate was photographed after overnight growth at 28°C and shows zones of clearing where antibacterial compounds have diffused from the TLC plate onto the bioautogram.



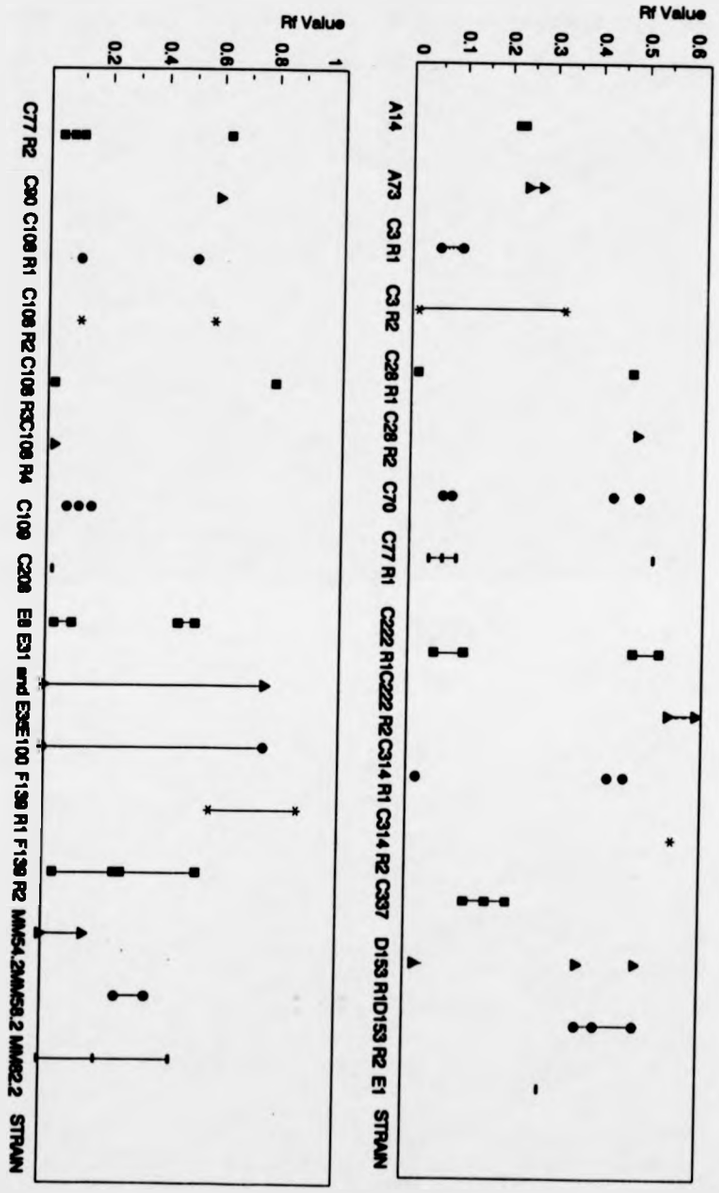


Fig. 3.2. Summary of activity against *B.subtilis*, which was shown by various streptomycete strains during this research.

The diagram represents a stylized thin layer chromatography plate. Each strain has been given a symbol, which is positioned at Rf values where the extract from the relevant strain gave an antibacterial zone on a bioautogram. Sometimes several zones were merged and this is represented on the diagram by an adjoining line. Certain strain labels are followed by R1 or R2 and this indicates that the strain was replicated.

Standard conditions were used throughout this work, except for a variable inoculum density (a loopful of spores) which may have caused changes in the rates of substrate utilization and oxygen demand and may have had an adverse effect on antibiotic production. Factors which affect the reproducibility of TLC are discussed in Chapter 5.

3.2.2. Antifungal Activities.

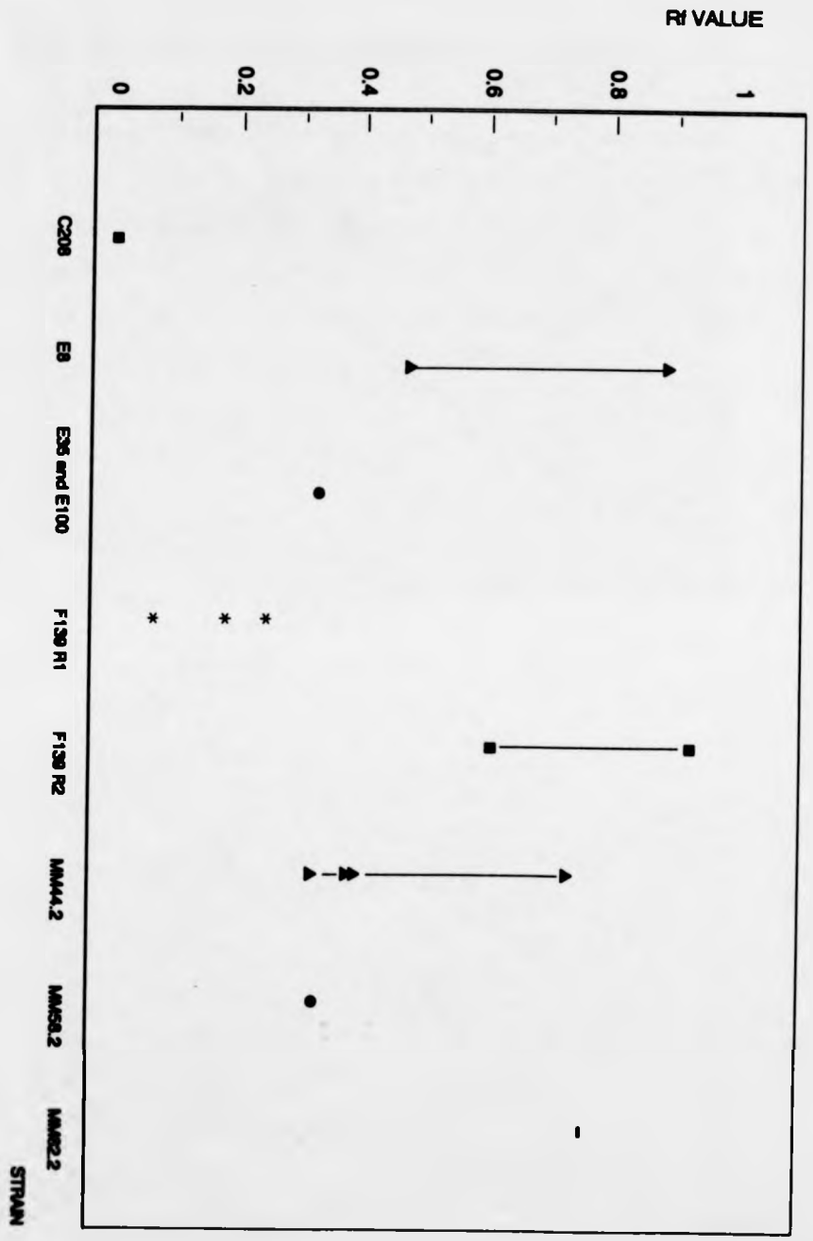
Of eight strains which were active against *A.niger* (Fig 3.3), four produced compounds that were possibly novel antibiotics. Three others belonged to the MM series of isolates, which were not assessed on agrochemical screens. However, they showed additional activity against *S.endus* (091) during plug assays, which were part of a series of tests aimed at distinguishing between duplicate strains within the MM and W series isolates (data not shown). The remaining strain was a nonactin producer (C208).

Apart from MM44.2, strains which showed antifungal activity were also active against *B.subtilis* (Fig 3.2.), however different solvent systems were used for the different types of bioautogram, so it is difficult to comment on whether this was caused by the same compounds. Strains E35 and E100 appeared to have identical bioactivities because they gave the same profile on both types of bioautogram, but their profiles on agrochemical screens differed. F139 (R2) and E8 also appeared similar, although F139 produced geldanamycin and nigericin, whilst E8 did not.

All strains with antifungal activity (apart from C208) were identified by SEM as *S.violaceoniger* (C32) strains. This species group contains many strains which produce bioactive secondary metabolites (Arai *et al.*,1976) and includes the *S.hygroscopicus* sub-species group, whose products have frequently been patented as novel antibiotics (DeBoer *et al.*, 1970; Iwai *et al.*, 1980). Strains of *S.hygroscopicus* autolyse during short term storage and it was noted that F139 and E35

Fig. 3.3. Summary of strains showing activity against *Aspergillus niger*.

The diagram is a stylized bioautogram, showing the Rf values at which specific strains showed antifungal activity. Each strain has been given a symbol, which is positioned at the Rf value corresponding to the centre of the zone of clearing. Sometimes zones were merged and this is indicated by an adjoining line. Strain names are sometimes followed by R1 or R2 and this indicates that they have been replicated. E35 and E100 gave a similar profile and are represented by the same symbol.



also possessed this trait.

3.2.3. Specific Biological Activities.

Table 3.1 shows profiles of the natural isolates in which bioactivity was confirmed. Many of these strains were used in other work in this thesis and are therefore marked with a superscript to indicate which strain was present in which study. All strains were used in Chapter 5 for chemical profiles and so the superscript identifying these strains has been omitted. Strains marked[4] were used in Chapter 4 for fatty acid analysis, strains denoted[6] were used in Chapter 6 for Resistance work and strains indicated by[7] were used in Chapter 7 for gene probing.

3.2.4. Agrochemical Activities.

A large number of strains were given to ICI when the original isolation work was done, including most of the random subset of strains chosen for this work. ICI provided summaries of agrochemical activities for all of the strains they had been given, enabling bioactivity in many of the strains presented in Table 3.1. to be confirmed and indicating additional bioactive strains (also placed in Table 3.1.). Additional strains (C47, D1, E92, E53, F150) which produced compounds that might be novel were selected from the larger culture collection and were used to confirm trends observed during this research.

C47 was later confirmed to produce cycloheximide and borrelidin and D1 produced nigericin and nonactin. In addition to their novel activities E92 produced nigericin and geldanamycin, and E53 and F150 produced nigericin. E92, E53 and F139 were thought to produce the same new compound, whilst E35 (Table 3.1.) produced 2-deoxycytosine in addition to the profile shown.

Table 3.1. Bioactive *Streptomyces* isolates discovered and used during this research.

Strain	Type of Activity Detected								
	Geld	Nig	HA	HC	Cyc	Act	Nuc	Ab	Af
A10[67]	-	+	-	-	-	-	-	-	-
A14	-	-	-	-	-	-	-	+	-
A46[46]	-	+	-	-	-	-	-	-	-
A73=67	-	-	-	-	-	-	-	+	-
C3	-	-	-	-	-	-	-	+	-
C28[4]	-	-	-	-	?	-	?	-	-
C38[67]	-	+	-	-	-	-	-	-	-
C70[45]	-	-	-	-	-	-	-	+	-
C77[456]	-	-	-	-	-	-	-	+	-
C90	-	+	-	-	-	-	-	-	-
C108[46]	-	-	-	-	-	-	-	+	-
C109[46]	-	-	-	-	-	-	-	+	-
C208[46]	-	-	-	-	-	+	-	-	-
C222[4]	-	-	-	-	-	-	-	+	-
C314[4]	-	-	-	-	-	-	-	+	-
C337	-	-	-	-	-	-	-	+	-
C338[6]	-	+	-	-	-	-	-	-	-
C402[67]	-	-	+	+	-	-	-	-	-
D5[67]	-	-	+	+	-	-	-	-	-
D125[6]	-	+	-	-	-	-	-	-	-
D153[467]	+	+	-	-	-	-	-	-	-
E1[467]	-	+	-	-	-	-	-	-	-
E8[467]	-	-	-	-	-	-	-	+	+
E31[467]	-	+	-	-	-	-	-	+	-
E35[467]	+	+	-	-	-	-	-	+	+

Strain	Type of Activity Detected								
	Geld	Nig	HA	HC	Cyc	Act	Nuc	Ab	Af
E100[467]	+	+	-	-	-	-	-	+	+
F53[67]	-	?	-	-	?	-	?	-	-
F139[467]	+	+	-	-	-	-	-	+	+
RB259[6]	-	+	-	-	-	-	-	-	-
RB286[6]	-	+	-	-	-	-	-	-	-
MM44.2[6]	+	+	-	-	-	-	-	+	+
MM58.2[67]	+	+	-	-	-	-	-	+	+
MM82.2[67]	+	+	-	-	-	-	-	+	+
2813	+	+	-	-	-	-	-	-	-
2817[467]	+	+	-	-	-	-	-	-	-
2818[467]	+	+	-	-	-	-	-	-	-

Key to Table 3.1.

+ = Present

- = Absent

? = one of the compounds marked by ? can be produced by the relevant strain but which one has not been confirmed.

Geld = Geldanamycin

Nig = Nigericin

HA = Herbimycin A

HC = Herbimycin C

Cyc = Cycloheximide

Act = Nonactin

Nuc = Nucleoside

Ab = Antibacterial

Af = Antifungal

3.3. Factors Affecting the Expression of Bioactivity.

A selection of streptomycetes which produced different combinations of nigericin, geldanamycin and other activities were chosen to investigate the expression of these antibiotics in a more detailed manner. These were A10, A46, C38, C338, C402, D5 , D125, D153, E1, E31, E35, E100, F139, 2813, 2818, MM44.2, MM58.2, MM82.2, RB259, NR3602. The strains were streaked across carbon, nitrogen and phosphorus gradients and examined for changes in differentiation and antibiotic production.

3.3.1. Choice of Medium.

The original bioactivity data (excluding that done at ICI) were generated on a complex medium (A37), but in order to examine the effects of various media components on the secondary metabolism of the strains, it was necessary to find a defined medium on which antibiotic production could occur. The liquid form of the medium was used in antibiotic production timecourses, which could also be related to alterations of fatty acids in the culture (Chapter 4), whilst the solid form was used for nutrient gradients.

Table 3.2 shows how different media initiated differences in the expression of antibiotic production. The three strains gave similar profiles, apart from F139, which did not produce geldanamycin on R2 and which did not grow on either R^- or S^- media. Geldanamycin production was only observed on media, which contained trace elements (eg. ISP7). Production of both antibiotics was also greatly enhanced on ISP7 and so this medium was chosen for work on the expression and repression of antibiotic production in the above sub-set of strains.

Table 3.2. The expression of geldanamycin and nigericin production on different media.

Medium	Growth			Antibiotic Production		
	F139	D153	E100	F139	D153	E100
R2	+	+	+	-	G	G
R2-	-	+	+	-	G	G
S	+	+	+	-	-	-
S-	-	+	+	-	-	-
ISP7	+	+	+	G+N*	G+N*	G+N*
MSG	+	+	+	N	N	N

* = Large amounts of metabolites

\- = Absent

\+ = Present

N = Nigericin production

G = Geldanamycin production

R2 = R2 medium

R2- = R2 medium without casaminoacids.

S = Sautons medium

S- = Sautons medium without casein hydrolysate

MSG = Mineral salts and glucose

3.3.2. Effects of Nutrient Gradients on Differentiation.

Tables 3.3. and 3.4 summarize observations made about the differentiation responses observed within geldanamycin and nigericin producers when they were grown on nutrient gradients. Plating out was in triplicate and the positions of one strain relative to the others was changed for each replication in order to minimize the effect of temperature and oxygen gradients and to prevent any diffusible products from one strain affecting another more than once. Plates with strains grown only on ISP7 were used as a comparison, but the results were not judged relative to this medium since the carbon and nitrogen sources had been altered.

The trends summarized in Table 3.3 and 3.4 were first observed after 3 days and remained stable throughout the experiment (14 days), apart from those seen on the sucrose gradients which disappeared with time. Mycelium progressed through the streptomycete lifecycle at a different rate depending on its position along the gradient and some strains remained immature for 2 weeks at specific positions on the gradients.

Ten strains were affected by Pi gradients and most had differentiated mycelium from mid-plate to the highest concentrations, which were above the normal phosphorus concentration of ISP7 (i.e. 0.5 g/l).

KEY to Tables 3.3 and 3.4

The tables illustrate where fully differentiated mycelia were observed amongst the strains and gradients studied. The figures given refer to the position of sporulating mycelia along the gradient, unless a superscript has been given. ^{a} indicates that aerial mycelium was present over the range given and ^{v} indicates that only substrate mycelium was present. - denotes that the mycelium was at the same stage of development along the whole gradient.

The nutrients are expressed in grams per litre on these tables. These values were extrapolated from measurements of distance along gradient plates.

Table 3.3. Summary of the effects of nitrogen and phosphorus gradients on differentiation.

Strain	Pi	Tyrosine	$NaNO_3$	Asparagine
D5	5.5-10	-[v]	0-2.6	0-1.8
C402	6-10	-[v]	0-2.5	0-0.8
2818	-[a]	0-1.2[a]	0-3[a]	2.3-4[a]
2813	6-10[a]	-[v]	-[v]	2.8-4[a]
MM82.2	7-10[a]	1-2.4[a]	0-1.6[a]	2-4[a]
MM58.2	0-7.5[a]	-[v]	0-2.4[a]	-[a]
MM44.2	7.5-9.5[a]	-[v]	2.6-4[a]	0-1
D153	-	2.4-4[a]	0-2.4	0-2[a]
A10	-[a]	-[v]	0-2.2	-[v]
A46	-[v]	0-1.8[a]	-[v]	-[v]
D125	-	-[a]	-	-[v]
C38	-	0-1.2	-	0-2
C338	3-10	-[a]	-	2-4
RB259	-[v]	0-1.4	0-2.4	0.8-1.8 3.6-4
E1	5-10	-	1.2-4	0-1 2-4
E31	-[a]	-[a]	0-2.4	0-2
E35	-[a]	2.8-4	0-2.8	0-1.6
E100	6-10	2.2-4[a]	0-2.8	0-1.6 2.4-4
F139	3-10	0-1.4[a]	2.8-4	0-1.2 2.4-4

Table 3.4. Summary of the effects of carbon source gradients on differentiation.

Strain	Glucose	Sucrose	Glycerol
D5	0-10.5	-	0-8
C402	0-10	-	0-5
2818	0-14	0-6[a]	-[a]
2813	0-10	0-12[a]	0-4[a]
MM82.2	0-12	0-8[a]	0-13.3
MM58.2	-	0-4[a]	-
MM44.2	0-10	-	0-10
D153	0-10	-	-
A10	0-8.5	-[a]	0-10
A46	0-7.5	0-8[a]	0-10
D125	0-9	-	-
C38	0-5.3	-	0-9
C338	0-10	-	-
RB259	0-10	0-8	-
E1	0-5	-	-[a]
E31	0-6	0-16	-
E35	13-20	-	0-8
E100	0-8	-	0-8
F139	12-20	-	-[a]

When carbon source gradients exhibited an effect, differentiation was usually most advanced at concentrations below 15 g/l, whereas higher concentrations seemed to inhibit morphological differentiation. Strains E35 and F139 had immature mycelia at concentrations below 12 g/l on glucose, and the mature forms occurred up to the most concentrated point on the gradient. Depending on the strain, high and low concentrations of the nitrogen sources could encourage differentiation, although low to mid levels were predominant and asparagine sometimes encouraged differentiation at two separate ranges of concentrations within a single replicate strain. E100 caused some adjacent strains (E31, E35 and 2813) to mimic its differentiation pattern on the nitrogen gradients (not shown in Table 3.3), but this was not observed when these organisms were grown on plates without E100, suggesting that a diffusible substance was responsible. All strains affected were thought to belong to the *S. violaceoniger* species group, suggesting an intra-species regulator. There are many examples of inter-species (Landau *et al.*, 1984; Barabas and Szabo, 1977) and strain-specific effectors, including both antibiotic and non-antibiotic substances (Beppu, 1985; Pogell, 1984; Horinouchi and Beppu, 1986; Chater *et al.*, 1989).

3.3.3. The Effects of Nutrient Gradients on Antibiotic Production in a Selection of Streptomycetes.

A smaller selection of isolates, which had varied bioactivity spectra were selected to examine how nutrient gradients affected the production of antibacterial (Tables 3.5. and 3.6) and antifungal compounds (Tables 3.7 and 3.8). A herbimycin producer was not selected because no simple assay procedure was available for detecting the compound directly on agar plates.

Table 3.5. Antifungal activity on nitrogen gradients.

Strain	Tyrosine	Nutrient g/l $NaNO_3$	Asparagine
C338	NA	0-0.2	NA
D153	CA	CA	CA^{+}
MM82.2	0-1.2^{-}	CA^{-}	CA^{+}
E100	CA^{+}	CA	0-3.4^{-}
D125	NA	NA	NA
F139	0.4-3.8^{-}	CA	CA^{+}

Table 3.6. Antifungal activity on carbon source gradients.

Strain	Glucose	Nutrient g/l Sucrose	Glycerol
C338	NA	NA	NA
D153	8-20	NA	9-20
MM82.2	0-8	0-14^{-}	0-18^{-}
E100	0-16^{m}	NA	5-13^{m}
D125	NA	NA	NA
F139	12-20	NA	10-20

Key to Tables 3.5. and 3.6.

NA = No activity.

CA = Activity over total gradient length.

$^{+}$ = Larger zone diameter at higher nutrient level.

$^{-}$ = Larger zone diameter at lower nutrient level

^{m} = Larger zone diameter in the centre of the range.

Table 3.7. Antibacterial activity on nitrogen gradients.

Strain	Tyrosine	Nutrient g/l $NaNO_3$	Asparagine
C338	CA	0-3$^-$	2.5-3.2 3.6-4.0
D153	CA$^-$	CA$^-$	CA
MM82.2	0-2.2	0-3.4$^+$	0-2
E100	0-3.2$^-$	CA$^+$	0-2.6$^-$
D125	NA	NA	NA
F139	CA$^-$	CA	NA

Table 3.8. Antibacterial activity on carbon source gradients.

Strain	Glucose	Nutrient g/l Sucrose	Glycerol
C338	NA	NA	CA
D153	6-13$^+$	NA	CA$^+$
MM82.2	CA	NA	CA$^-$
E100	10-20$^+$	NA	5-20$^+$
D125	NA	NA	NA
F139	NA	NA	CA$^+$

Key to Tables 3.7. and 3.8.

NA = No activity.

CA = Activity over total gradient length.

$^+$ = Larger zone diameter at higher nutrient level.

$^-$ = Larger zone diameter at lower nutrient level

Even when strains were thought to produce the same compounds, nutrient gradients affected antibiotic production in a strain-specific manner. For example, two strains were thought to produce nigericin as a single product and because this compound has both antibacterial and antifungal activity, such a strain might be expected to show the same trends on both type of assay. However, C338 showed correlating antibacterial and antifungal activity only on very low levels of $NaNO_3$. Antibacterial activity without antifungal activity was observed on glycerol, asparagine and tyrosine gradients, suggesting that this strain might be capable of producing an additional compound. No antibiosis was detected for D125, indicating that the conditions required for antibiotic production in this strain had not been met.

MM82.2 and D153 were previously (Table 3.1.) reported to produce nigericin and geldanamycin, but they gave different responses on some gradients. C338 produced an antifungal compound, which inhibited *A.niger* at low sucrose concentrations, but there was no corresponding antibacterial activity, suggesting that a different antibiotic was being produced. Wherever C338 or D153 showed corresponding antifungal and antibacterial activity, it overlapped rather than was exactly equivalent. Interestingly, bioautography of D153 grown in ISP7 later showed three additional antifungal zones, which were not due to nigericin.

F139 and E100 had already been shown to produce additional antibacterial and antifungal activities to geldanamycin and nigericin. The results from this study confirmed the additional antifungal activity in both cases, although geldanamycin could not be distinguished from any other antibacterial activity by this assay.

3.4. The Characterization of Antibiotic Production in D153.

3.4.1. Timecourses.

Timecourses of D153 cultures showed the onset of geldanamycin production at 82-106 hrs (82-94 hrs., rep. 2; 94-106 hrs.,rep. 1) and of nigericin biosynthesis at 106 hours (Fig.3.4). The intensity of both types of bands became progressively

Fig. 3.4. Timecourse of geldanamycin production by D153.

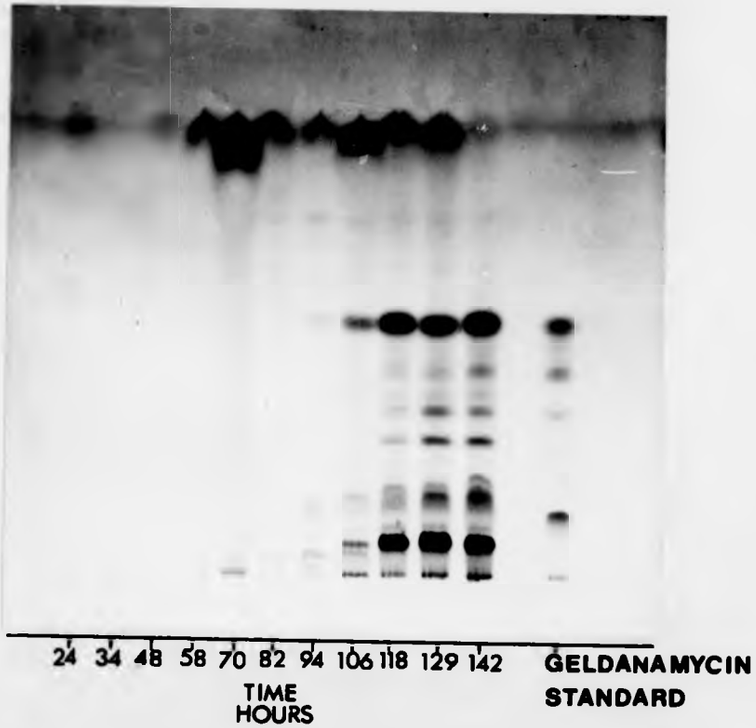
The photograph illustrates the results of an experiment, which determined the profile of geldanamycin production on ISP7 medium (28°C; 200 rpm) over a period of 6 days.

Geldanamycin can be recognized by comparing the pure antibiotic with corresponding bands within the strain extracts.

Fig. 3.4. Timecourse of geldanamycin production by D153.

The photograph illustrates the results of an experiment, which determined the profile of geldanamycin production on ISP7 medium (28°C; 200 rpm) over a period of 6 days.

Geldanamycin can be recognized by comparing the pure antibiotic with corresponding bands within the strain extracts.



stronger with time. This information was used to design an experiment which would investigate the impact of reduced % O_2 on antibiotic production in this strain.

3.4.2. Repression of Nigericin Biosynthesis.

Nigericin can prevent the discovery of narrow spectrum compounds on agrochemical screens because it has a very potent broad spectrum of activity and can therefore mask the presence of narrow spectrum compounds within the same extract. Certain strains (eg. A10, A46, C90, D72) only produced nigericin on solid, but not in liquid media, whilst other strains (eg E1, E31, E35) produced higher levels of this compound on solid media (Data supplied by ICI). It was of interest to try to discover the reason for this and differences in oxygenation were hypothesized as a possible cause. The cultures were treated as described in section 2.4.3. and the atmosphere inside the flasks was altered at 76 hrs, because this was assumed to be prior to the onset of antibiotic production.

Geldanamycin biosynthesis in D153 was unaffected by the experimental conditions and the onset of production in the batch culture control was similar to that in the previous timecourse (section 3.4.1). However, nigericin was not observed in any of the cultures grown in reduced atmospheres of oxygen, whilst the subasealed control cultures were delayed in the onset of biosynthesis. However, nigericin production occurred earlier than previously observed in the 21% O_2 control, and presumably more O_2 was present here than in flasks which were subasealed without disturbing the internal atmosphere.

Table 3.9. The effect of oxygen on geldanamycin and nigericin production in D153.

	Onset of Antibiotic Production (hrs)	
$\%O_2$	Nigericin	Geldanamycin
0	absent	present at 94
5	absent	present at 94
10	absent	present at 94
15	absent	present at 94
Controls		
Normal atmosphere	94	76 to 94
Normal atmosphere	94	present at 94
21% O_2	[1]94/103	present at 94
Subasealed	[1]117/129	present at 94

Key to superscript

[1]refers to the first instance at which production was detected within each duplicate.

3.5. Discussion

3.5.1. The Distribution of Antibiotic Production in *Streptomyces* Isolates.

The compound found most frequently in natural *Streptomyces* isolates was the ionophoric polyether nigericin. This compound perturbs ion gradients across cell membranes by complexing specific cations (eg. Na^+) and encasing them in an ampiphilic shell, which can then passively diffuse through membranes. There is evidence that some polyethers carry out specific functions for their producers, therefore the high incidence of producers might relate to nigericin serving a useful function in *Streptomyces*. Lasalocid and monensin are closely related to nigericin and have been shown to bind to spore surfaces in a manner similar to the germination inhibitor of *S. viridochromogenes* (Grafe *et al.*, 1986; Ensign, 1976).

Interestingly, these compounds are often found in streptomycete extracts from solid surface cultures (J. Benner, personal communication). Other evidence for the natural role of polyethers has been presented by Williams *et al.* (1989a) who argue that the highly sophisticated receptor-antibiotic complementarity, as seen for example in the lasalocid-Barium complex, implies active evolution towards a survival strategy. Grabley *et al.* (1990) provided similar evidence for nigericin, in that the antiviral and antibacterial properties and the binding of Na^+ and K^+ to this polyether were not improved when its C1 carboxyl group (which participates directly in ligand binding and is implicated in the biological effect) was chemically altered.

Production of the ansamycin antibiotic, geldanamycin was also common. This is an inhibitor of bacterial and eukaryotic RNA polymerase and blocks initiation during the early stages of transcription. Rothrock and Gottlieb (1984) demonstrated geldanamycin production by *S.hygroscopicus* var *geldanus* in sterilized soil and showed that the compound controlled the level of *Rhizoctonia solani* without harming the host plant. If production can occur under natural conditions then geldanamycin producers might have a selective advantage when competing with other soil microflora. Interestingly, *S.hygroscopicus* var *geldanus* was observed to produce nigericin during this research.

The co-production of related idiolites, as observed for the herbimycins is not unusual (Tanida *et al.*, 1980; Lazar *et al.*, 1983,) and the co-synthesis of herbimycins A and B has been already been reported (Iwai *et al.*, 1980). Reasons for the frequent association between production of geldanamycin and nigericin may lie in the fact that both antibiotics have portions of their structure that are biosynthesised via the polyketide pathway. Geldanamycin is derived from 3-amino-5-hydroxybenzoate, but also has a polyketide-derived handle stretched between adjacent benzenic portions. The aromatic moiety comprises acetate and propionate units, with O-methyl groups derived from methionine, and two C2 units from glycolate (Ghisalba, 1985).

Nigericin is a fairly lipophilic molecule made up of cyclic ethers and is formed by the condensation of C2, 3 and 4 units into a polyene precursor via a triepoxide intermediate (Cane *et al.*, 1983). The discovery of the biosynthetic route of polyethers lead to the proposition that there was a common genetic basis influencing the synthesis of both polyenes and polyethers, O'Hagan (1988) indicated that there were similarities between the structures of certain macrolides and polyethers, suggesting that both polyether and macrolide antibiotics also had a common genetic source and that the stereochemical similarities between them were dictated by a common enzyme system. It is interesting to hypothesize that geldanamycin production could share biosynthetic enzymes with the nigericin pathway, especially in view of the fact that genes, involved in the early stages of polyketide biosynthesis, have been shown to hybridize strongly with one another (Malpartida *et al.*, 1987; Kakinuma *et al.*, 1991). Alternatively, the two antibiotics may have simply co-evolved in the same progenitor strain. Strains which do not express geldanamycin production may contain silent genes or represent a second line of descendants which retained only the nigericin pathway. A gene transfer event could also have caused the two lines of descent.

The data presented in Table 3.1. suggest that 20% of strains from a random selection of streptomycetes could produce a known broad spectrum antibiotic (mainly nigericin). Three of these were also considered to be potential producers of novel agrochemicals (E35, E100 and F139). In addition, 5 new strains with potentially novel activities (section 3.2.4.) produced broad spectrum compounds and there are similar cases cited in the literature where novel compounds have been discovered in nigericin producers (Grabley *et al.*, 1990a). Both nigericin and geldanamycin are broad spectrum antibiotics and a novel metabolite might only be discovered if part of its spectrum of activity was different. This led to the hypothesis that other potentially novel antibiotics might be found by close examination of strains, presumed to produce only unwanted broad spectrum metabolites.

3.5.2. Factors Effecting Differentiation and Antibiotic Production.

It is well known that nutrient depletion induces sporulation and secondary metabolite biosynthesis (Iwai and Omura, 1981; Demain, 1984 and 1985). The metabolism of glucose and other carbon sources usually accompanies growth, with differentiation occurring when they become growth limiting. Where low nutrient levels illicited positive differentiation the cause was probably nutrient limitation. The trend of high Pi levels to permit a positive differentiation response seems unusual and may be related to pH effects rather than Pi concentration. The pH of the gradient should be 7.3 at the time of inoculation, but since a culture might metabolize at different rates along the gradient, a pH gradient might also form. It is also possible that pH effects occurred in other gradients, for instance the fermentation of sugars tends to decrease the pH, whilst the fermentation of amino acids and nitrate increase pH. Changes in pH may trigger differentiation before nutrient depletion occurs or might provide an additional inhibitory factor. The concentration of H^+ and OH^- ions are known to cause changes in membrane properties and cell wall formation (Bader, 1986) and these are both important factors in the morphological development of streptomycetes.

The different response profiles of individual strains indicates that differentiation in streptomycetes is strain specific and may reflect differences in rates of substrate utilization, thresholds for responses to nutrient limitation, or the presence of special mechanisms for the uptake of nutrients, which are in short supply.

A wide variety of pigment effects accompanied growth on nutrient gradients, including differences in the colouration of vegetative mycelium, aerial mycelium and spores, diffusible pigments and the presence of pigment droplets on culture surfaces. This tendency for polymorphism may be related to an evolutionary strategy, which helps streptomycetes to cope with a changing environment, and since pigments are often secondary metabolites, a close association between

cytodifferentiation and secondary metabolism was suggested. For instance, the two herbimycin producers showed similar morphologies on different gradients.

The antibiosis profile of an organism seemed to depend on its nutrient environment; for example, bioactive strains did not biosynthesize their products under some conditions and new activities were found in certain strains, indicating that they showed a more varied product spectrum when observed under more diverse conditions. Differences in the detection limit of the antibacterial and antifungal assay could have influenced results and it is possible that non-bioactive strains could contain hidden activities if they were grown under the correct conditions. It is also relevant that high levels of ions can reverse the biocidal activity of ionophores and this may help to explain specific cases where zone sizes were smallest at high nutrient concentrations (eg. on the $NaNO_3$ gradients).

Alterations in differentiation are often accompanied by changes in secondary metabolism. During this work, antibiotic production was observed in both differentiated and non-differentiated mycelia on nutrient gradients and this may suggest that whilst the production of some antibiotics is closely associated with cytodifferentiation, others provide other functions within the cell, which may or may not be related to the availability of nutrients.

Certain strains produced nigericin on solid but not liquid media, and it was hypothesized that the circulation of air in a bioassay dish would be slower than in a shake flask, causing diffusion gradients of oxygen. The best aeration might be expected to occur at the edges of the plates and that this might be responsible for the predominance of nigericin biosynthesis on solid media. However, the above results indicated that reduced oxygenation prevented nigericin biosynthesis.

A major difference between solid and liquid cultures is agitation; fragmentation has been observed in the shake flask cultures of several nigericin-producing strains and it is thought that this might be a negative influence on antibiotic production. Small mycelial fragments may be incapable of antibiotic production due to insufficient capacity for oxygen transfer (Personal communication, M.E. Bushell).

Other theories include speculation that diffusible products capable of inducing secondary metabolism might reach higher concentrations at localized areas on solid media than if they were subjected to rapid distribution during batch fermentations. Nutrient limitation is a less favoured theory because the effect is observed on a variety of media, some of which are highly complex.

Chapter 4

The Use of Fatty Acid Profiles for Grouping *Streptomyces* Species.

4.1.Introduction

Fatty acids are building blocks of bacterial membrane constituents (eg. the acyl components of cellular lipids) and they are synthesized by a specific pathway, in which a single starter unit and a small number of malonate units are joined together by condensation. A beta-keto group is formed at each of these steps and it is subsequently modified by reduction, dehydration and by hydrogenation prior to the next chain extension. The biosynthesis of fatty acids was reviewed by Martin (1977) and Kaneda (1991) who described two major families of fatty acids, which were derived from different precursors. The branched chain fatty acids include iso-, anteiso- and acyclic forms. Odd chain iso-branched fatty acids use isovaleryl-CoA as a precursor, whilst even chain iso-branched forms use isobutyryl CoA. Anteiso-branched fatty acids have 2-methylbutyryl-CoA as the initiator and acyclic forms originate from cyclic carboxylic acid primers. (ii) The straight chain fatty acids are synthesized from acetyl-CoA (gives an even number of C atoms) or propionyl-CoA (gives an odd number of C-atoms) as the primer molecule.

Fatty acid analysis has frequently been used to distinguish between bacterial genera (Lechevalier, 1977; Goodfellow, 1989; Kaneda, 1991), but they have also proved useful in the delimitation of species groups in *Bacteriodes*, (Brondz *et al.*, 1991) and for strain determination in a variety of bacteria (Gudmestad *et al.*, 1988; Cacciapouti *et al.*, 1991). Members of the genus *Streptomyces* have fatty acid profiles which comprise straight chain, iso- and anteiso-branched chain fatty acids with a carbon chain length between 14 and 18 atoms (Hofheinz and Grisebach, 1965; Lechevalier, 1977; Popisil *et al.*, 1985; Saddler *et al.* 1986 and 1987). Hydroxylated methyl esters have been observed in some *Streptomyces* species

(Kroppenstedt, 1985), but there is little information about the distribution of cyclopropane fatty acids in streptomycetes, apart from the presence of Me. cis-9,10-methylenehexadecanoate in *S.cinnamonensis* (Popisil *et al.*, 1985).

One approach to the discovery of new microbial metabolites is to target taxonomic groups, which have a high incidence of antibiotic producers; the *S.violaceoniger* group fits into this category (Williams *et al.*, 1983 a and b; Arai *et al.*, 1976). These organisms have a characteristic spore chain morphology, which distinguishes them from other streptomycetes and it was of interest to know if they differed in any other respects. In 1987, Saddler *et al.* demonstrated that fatty acid profiles could be used to cluster *S.cyaneus* strains away from other members of the genus and this success indicated that it might be interesting to determine the fatty acid profiles of *S.violaceoniger*. However, the evidence that Saddler *et al.* (1987) used to suggest that fatty acid profiles in streptomycetes remained stable during batch culture growth (Saddler, 1986) was weak because it was based on the reproducible profile of only one strain, NCIB 9616. Quantifying fatty acid levels might accentuate variations due to differences in growth rates between strains under standard conditions. Therefore a study which examined qualitative differences between strains was designed to find any major differences between the fatty acid profiles of *S.violaceoniger* strains compared with other streptomycetes.

There is a close relationship between fatty acid metabolism and the polyketide pathway of secondary metabolite biosynthesis (Martin, 1977, Hopwood and Sherman, 1990, Fig.1.1). Intermediates of fatty acid biosynthesis and the fatty acids themselves have been shown to be precursors of polyketides and related antibiotics and certain studies have indicated that polyketide producers might contain more iso-branched fatty acids (Popisil *et al.*, 1985; Hafner *et al.*, 1991). Two common products of natural *Streptomyces* isolates (nigericin and geldanamycin) were derived from polyketide pathways and so a second study was undertaken, using an antibiotic production medium to examine the relationship of geldanamycin and nigericin production with fatty acid profiles.

4.1.2. Choice of Strains and Test Materials.

Twenty-six type strains were used for this study (Tables 2.2 and 4.1.), including eleven *S.violaceoniger* strains and seventeen *S.griseoruber* strains, which have been taxonomically well studied. Both groups represented tight, clearly defined clusters and were therefore good marker strains. A small selection of other type strains was included to give a broader view of the genus.

A selection of natural isolates was chosen arbitrarily from several of the series shown in Table 2.1. (Table 4.1). They comprised, 1 A, 25 C, 9 D, 8 E, 7 F and 11 MM groups of strains. Thirty-five were isolated by specific procedures for cluster 32 streptomycetes (series D, E, F and MM) whilst twenty-six were not. Twenty-eight natural isolates were fully identified (Table 2.9); nine gave high Wilcox probability identification scores and five gave acceptable values (Table 4.2). A further twelve strains were identified as *S.violaceoniger* (C32's) by their rugose spore chains (Table 2.10). The remaining strains were unidentified, but were not cluster 32's, since scanning electron microscopy indicated a non-rugose spore chain morphology. Three type strains and five natural isolates were duplicated. This is 10.6% of the total sample set and is twice the number of replicates recommended for use in cluster analysis by Sackin (1985).

Fig.4.1 shows the mixture of FAMES, used in this study. Twenty-five compounds were observed, including branched, straight chain, hydroxy- and cyclopropane fatty acid methyl esters.

Fig. 4.1. Fatty acid methyl ester profile of the qualitative standard used for this research.

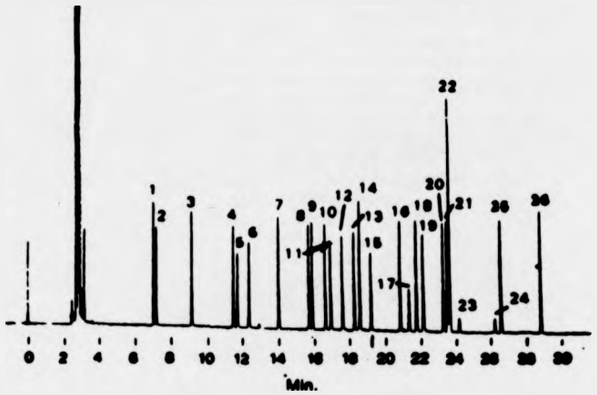
The diagram is a GCMS profile of the qualitative FAME profile (supplied by Supelco) which was used to identify fatty acids present in strain extracts.

FIG. 4.1. **Bacterial Acid Methyl Esters CP™ Mix**

Catalog No. 4-7080

This mixture contains a total of 10mg/ml methyl esters in methyl caproate

For qualitative identification only. Relative peak sizes may vary from lot to lot.



SP®™-1 fused silica capillary column, 30m x 0.25mm ID, Film Thickness: 0.25 μm, Linear Velocity 20cm/sec., He, set at 150°C, Col. Temp.: hold 4 min. at 150°C, then to 250°C at 4°C/min., Inj. Temp.: 250°C, Det. Temp.: 280°C, Det.: FID, Sens.: 4 x 10^{-11} AFS, Sample: 1 μl of Cat. No. 4-7080 (10 μg total esters), Split Ratio 100:1.

FORM NO. 8883F

1.	11:0	Me. undecanoate
2.	2-OH 10:0	Me. 2-hydroxydecanoate
3.	12:0	Me. dodecanoate
4.	13:0	Me. tridecanoate
5.	2-OH 12:0	Me. 2-hydroxydodecanoate
6.	3-OH 12:0	Me. 3-hydroxydodecanoate
7.	14:0	Me. tetradecanoate
8.	i-15:0	Me. 13-methyltetradecanoate
9.	a-15:0	Me. 12-methyltetradecanoate
10.	15:0	Me. pentadecanoate
11.	2-OH 14:0	Me. 2-hydroxytetradecanoate
12.	3-OH 14:0	Me. 3-hydroxytetradecanoate
13.	i-16:0	Me. 14-methylpentadecanoate
14.	$16:1^{9}$	Me. cis-9-hexadecenoate
15.	16:0	Me. hexadecanoate
16.	i-17:0	Me. 15-methylhexadecanoate
17.	17:0Δ	Me. cis-9,10-methylenehexadecanoate
18.	17:0	Me. heptadecanoate
19.	2-OH 16:0	Me. 2-hydroxyhexadecanoate
20.	$18:2^{9,12}$	Me. cis-9,12-octadecadienoate
21.	$18:1^{9}$	Me. cis-9-octadecenoate
22.	$18:1^{9}$	Me. trans-9-octadecenoate &
	$18:1^{11}$	Me. cis-11-octadecenoate
23.	18:0	Me. octadecanoate
24.	19:0Δ	Me. cis-9,10-methyleneoctadecanoate
25.	19:0	Me. nonadecanoate
26.	20:0	Me. eicosanoate

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Table 4.1. Type strains and Natural Isolates in fatty acid study.

Species Group	Cluster Number	Number of Type Strains	Number of Isolates
S. albidoflavus	C1	2	3
S. atroolivaceus	C3	0	1
S. roseus	C7	1	0
S. rochei	C12	0	2
S. viridosporus	C15	0	2
S. cyaneus	C18	1	0
S. diastatochromogenes	C19	1	6
S. griseoruber	C21	7	0
S. violaceoniger	C32	11	13
S. pactum	C44	1	0
S. lavendulae	C61	1	0
S. spp.		0	32
Total		26	59

Key to Abbreviations

S. refers to *Streptomyces*.

C refers to cluster group, as defined by Williams *et al.*, 1983a.

4.2.-4.5. Results.

4.2. The Distributions of Fatty Acid Methyl Esters Within Streptomycetes.

4.2.1. The Distribution of Individual FAMEs and FAME Classes Within Representatives of the Genus *Streptomyces*.

Fifteen methyl esters, comprising FAMEs with 14-19 carbon atoms were found in the population of streptomycetes studied (Table 4.2), although more than 17 carbon atoms was rare. Variations of octadecanoate were found in three *S.violaceoniger* strains (*S.hygroscopicus* var *geldanus*, NR3602, *S.endus*, ISP4213 and a natural isolate, MM 2817) and a *S.griseoruber* strain (*S.violaceolatus*, DSM40438), which also contained nonadecanoate. This was in agreement with the 14 and 18 carbon atoms quoted in the literature as typical for the FAMEs of streptomycetes (Lechevalier, 1977; Saddler *et al.*1986 and 1987; Popisil *et al.*, 1985; Hofheinz and Grisebach, 1965), apart from nonadecanoate, which might indicate that the strain was a member of the genus *Actinomyces* (Kroppenstedt, 1985). However, *S.violaceolatus* was a type species and was unlikely to have been misidentified. Pentadecanoate was the only ubiquitous fatty acid, but six other fatty acids were common (Table 4.2.).

All organisms contained both branched and straight chain FAMEs and this was in accordance with the work of Saddler *et al.* (1987), Popisil *et al* (1985) and Kroppenstedt (1985). A cyclopropane FAME was found in approximately 30% strains. The majority of streptomycetes (93%) had at least one hydroxylated methyl ester, which was in contrast to previous work and might be explained by the instability of hydroxy fatty acids (Kroppenstedt, 1985).

All three strains containing cis-9, 12-octadecanoate also had cis 9,10-methylenehexadecanoate, which was only present in twenty-six strains. The latter fatty acid was associated less frequently than average with all forms of tetradecanoate.

Table 4.2. The distribution of FAMEs in streptomycetes.

No.	FAME	NAME	No. Strains.
7.	14:0	Me.tetradecanoate	81
8.	i-15:0	Me.13-methyltetradecanoate	23
9.	a-15:0	Me.12-methyltetradecanoate	80
10.	15:0	Me.pentadecanoate	85
11.	2-OH 14:0	Me.hydroxytetradecanoate	81
13.	i-16:0	Me.14-methylpentadecanoate	44
14.	16:1	Me.cis-9-hexadecanoate	84
15.	16:0	Me.hexadecanoate	60
16.	i-17:0	Me.15-methylhexadecanoate	84
17.	17:0 >	Me.cis-9,10-methylene hexadecanoate	26
18.	17:0	Me.heptadecanoate	83
19.	2-OH 16:0	Me.hydroxyhexadecanoate	22
20.	18:2	Me.cis-9,12-octadecanoate	3
23.	18:0	Me.octadecanoate	1
25.	19:0	Me.nondecanoate	1

N.B. The total number of strains in the study is 85, but including duplicates there are 93 OTUs.

Key to abbreviations used in Table 4.2.

Me. denotes Methyl.

4.2.2. The Diversity of FAME Pattern Types Within Streptomycetes.

The strains in the study differed by the number of FAMEs, which ranged from 6-11 and the diversity of types, which could include hydroxy-, cyclopropane- and long-chain FAMEs. Table 4.3. illustrates how this diversity was distributed and shows how unique fatty acid profiles were observed for seven type strains and ten natural isolates, whilst sixteen further patterns were replicated amongst the remaining strains.

Table 4.3. Fatty acid profiles observed within streptomycetes.

PATTERN TYPE	14:0	i-15:0	a-15:0	15:0	2-OH 14:0	i-16:0	16:1	16:0
1	1	0	1	1	1	0	1	0
2	1	0	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1
4	1	1	1	1	1	0	1	1
5	1	0	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1
7	1	0	1	1	1	1	1	1
8	1	0	1	1	1	1	1	1
9	1	0	1	1	1	0	1	1
10	1	0	0	1	1	0	1	1
11	1	1	1	1	1	0	1	1
12	1	0	1	1	0	1	1	1
13	1	0	1	1	0	1	1	1
14	1	1	1	1	1	1	1	1
15	1	0	1	1	1	0	1	0
16	1	0	1	1	1	0	1	1
17-33			Various					

Table 4.3. Fatty acid profiles observed within streptomycetes.

PATTERN TYPE	i-17:0	17:0 >	17:0	2-OH 16:0	18:2	18:0	19:0	No.
1	1	0	1	0	0	0	0	18
2	1	0	1	0	0	0	0	11
3	1	0	1	1	0	0	0	5
4	1	0	1	0	0	0	0	4
5	1	0	1	1	0	0	0	4
6	1	0	1	0	0	0	0	4
7	1	1	1	0	0	0	0	4
8	1	1	1	1	0	0	0	3
9	1	0	1	0	0	0	0	2
10	1	1	1	1	0	0	0	2
11	1	1	1	0	0	0	0	2
12	1	1	1	0	0	0	0	2
13	1	1	1	0	0	0	0	2
14	1	1	1	0	0	0	0	2
15	1	0	1	1	0	0	0	2
16	1	1	1	0	0	0	0	2
17-33			Various					1

Key to Table 4.3.

1 = FAME present

0 = FAME absent

4.3. Hierarchical Clustering to Determine Inter-relationships Between Streptomycetes Based on their Fatty Acid Profiles.

The binary data, discussed in section 4.2., was used for conventional taxonomic clustering and resulting groups were tested by comparing the topologies of 2 phenograms (Figs. 4.2 and 4.3.). Fig. 4.2. was created using the Dice coefficient (S_{dice}) and the UPGMA algorithm, whilst Fig. 4.3. was obtained by the same method, but using S_{sm}. Good group formation was characterized by clearly well separated tight clusters at a similarity level of 83.6% for Fig. 4.2. and 89% on Fig. 4.3., where two sets of seven identical groups were defined. Three strains proved to be exceptions and were in different clusters for each phenogram.

The point of major change on the dendrograms was where there were many short stems, above and below which groups merged or separated. This occurred at a similarity level of 89 % for Fig. 4.2. and 93% for Fig. 4.3. where sixteen and seventeen groups, respectively, were defined. Comparison of the two sets showed that 9 groups remained intact apart from 4 strains and groups 10 and 11 (Ssm) merged to form group 6 (Sd) and groups 5 and 9 (Ssm) merged to form group 5 (Sd), whilst the three remaining groups were partially disrupted between the two phenograms. This coupled with the fact that short stems can often be highly insignificant indicated that the data was stable and the cophenetic correlation supported this, giving a mantle coefficient of 0.802 for Fig.2.

The groupings were subsequently examined for correlations with taxonomic data and bioactivity. Figs. 4.4 and 4.5 illustrate the distribution of these two factors across the 7 groups of the phenogram shown in Fig.4.2. No correlations between fatty acid profile and either antibiotic production or *Streptomyces* phena (Williams *et al.*, 1983a and b) were found, nor did the distribution contain particular strain series grouped together. Most of the *S. violaceoniger* strains, which were thought to produce nigericin clustered to group 2, whilst the nigericin producers found in group 1 also produced geldanamycin. All the strains, which produced compounds

Fig. 4.2. Phenogram based on fatty acid profiles, created using S_{dice} and UPGMA.

The diagram illustrates the groups which formed at two similarity levels (83.6% and 89%) on a phenogram, created using fatty acid profiles. Similarity was calculated using the Dice coefficient and UPGMA was used to form the phenogram.

FIG.4.2.

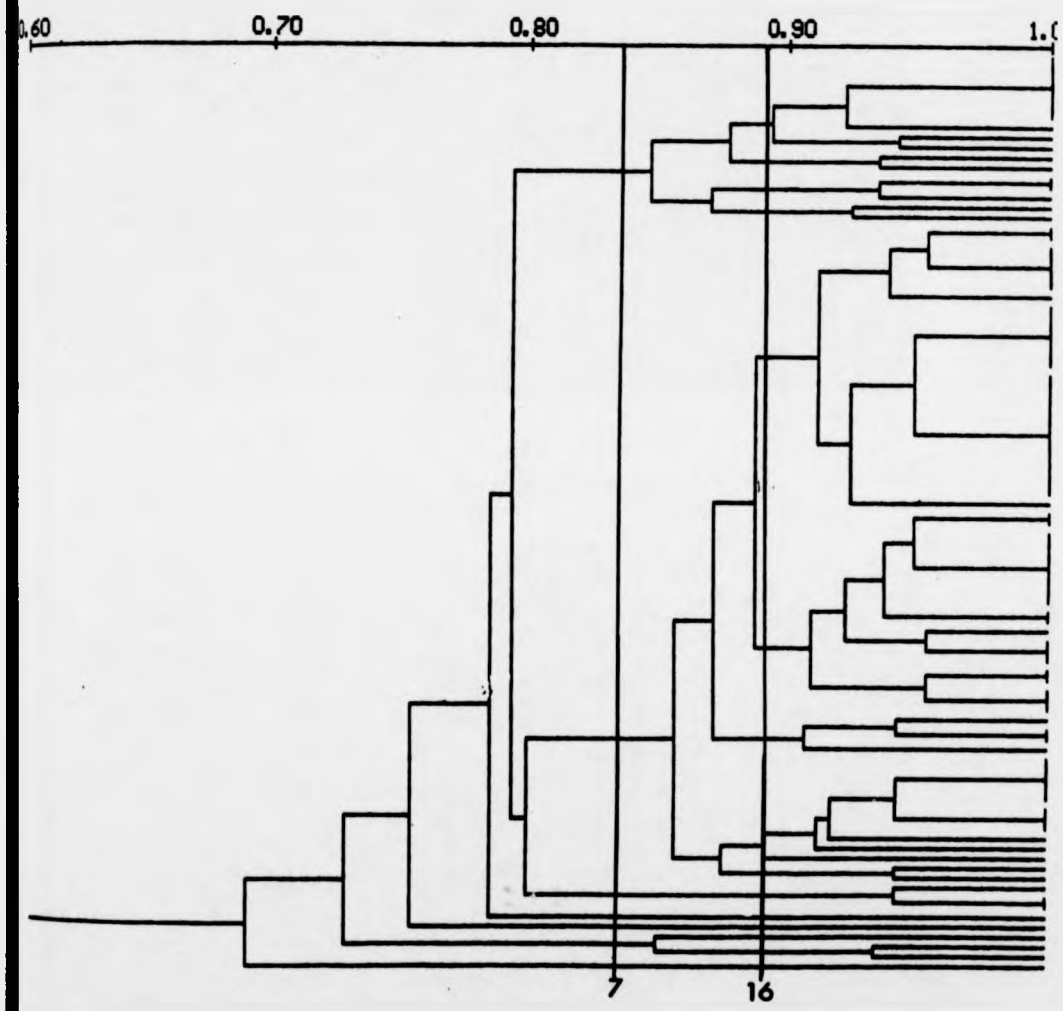


Fig. 4.2. Phenogram based on fatty acid profiles, created using S_{dice} and UPGMA.

The diagram illustrates the groups which formed at two similarity levels (83.6% and 89%) on a phenogram, created using fatty acid profiles. Similarity was calculated using the Dice coefficient and UPGMA was used to form the phenogram.

FIG.4.2.

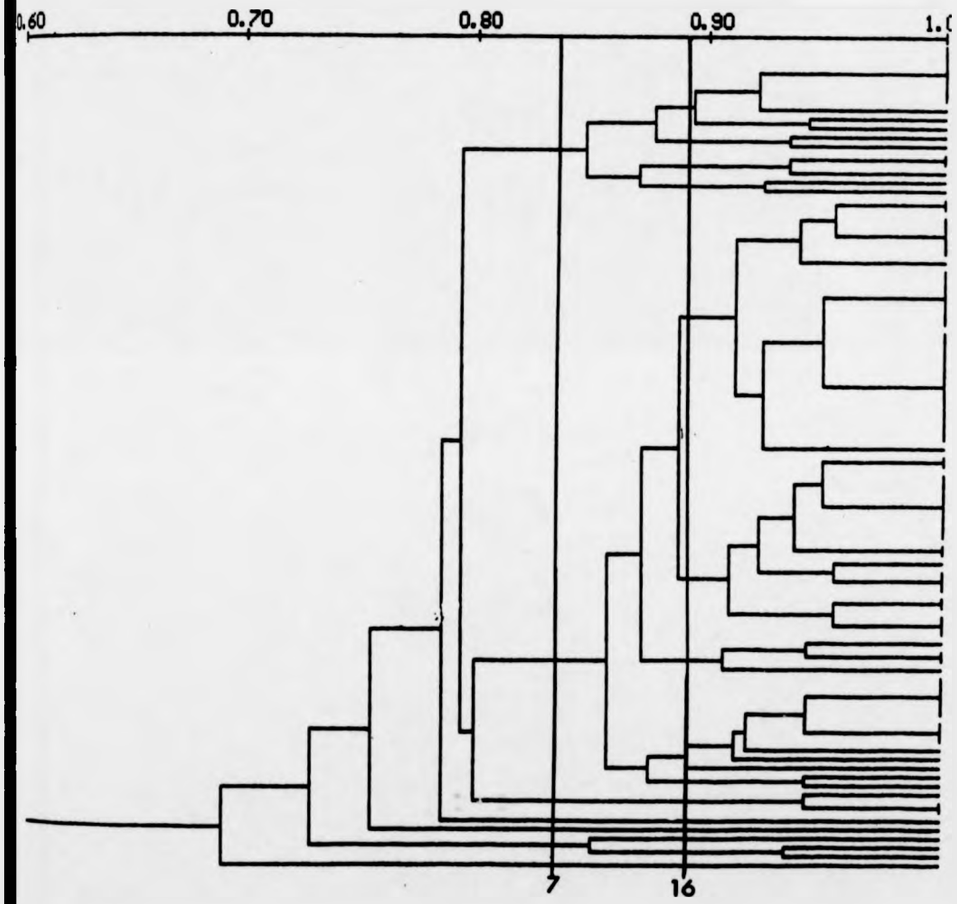


Fig.4.3. Phenogram based on fatty acid profiles, created using S_{sm} and UPGMA.

The diagram illustrates the groups, which were chosen at two similarity levels (89% and 93%) on a phenogram, created using fatty acid profiles. Similarity was calculated using the simple matching coefficient and UPGMA was used to form the phenogram.

FIG.4.3.

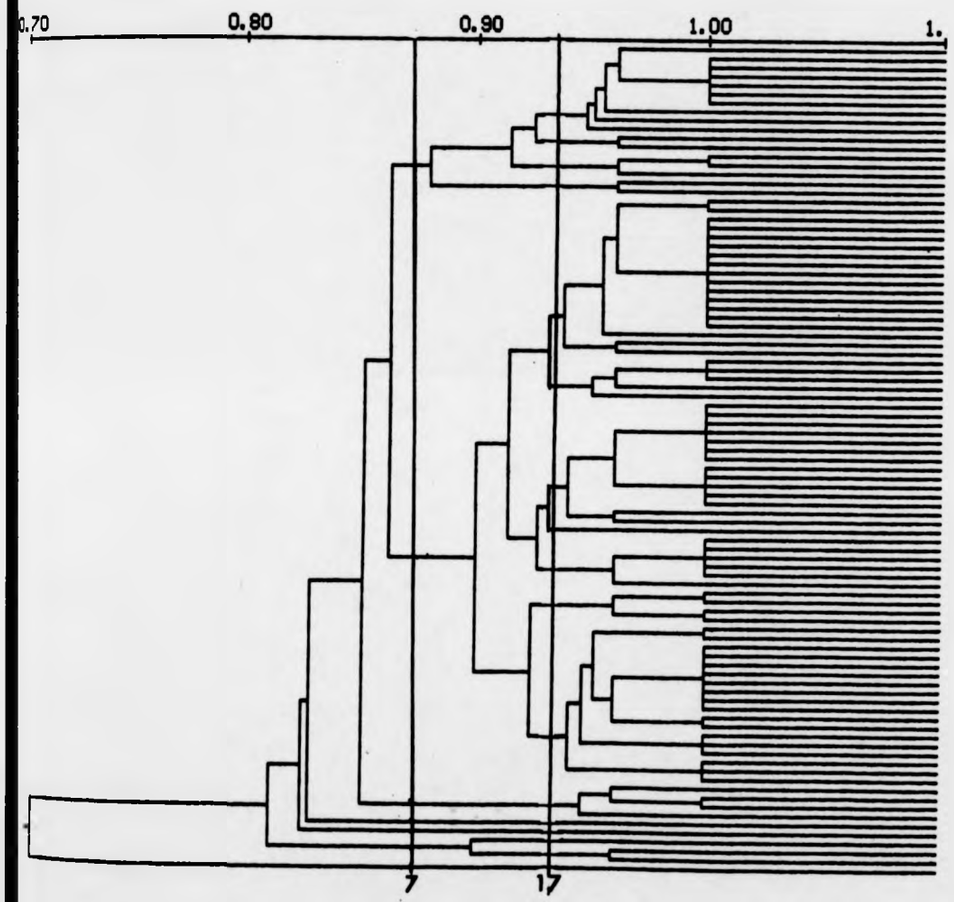


Fig.4.4. The distribution of *Streptomyces* species across a phenogram (Fig. 4.2.) based on fatty acid profiles.

The diagram indicates where various streptomycete taxa clustered on a phenogram created using fatty acid profiles, whose similarities were assessed using the dice coefficient. These relationships were presented as a tree by use of UPGMA, whilst the streptomycete taxa which are shown were defined by Williams *et al.* (1983a) The group number is given from the top of the phenogram downwards at a similarity level of 83.6.

NB. Duplicate strains are included in this diagram.

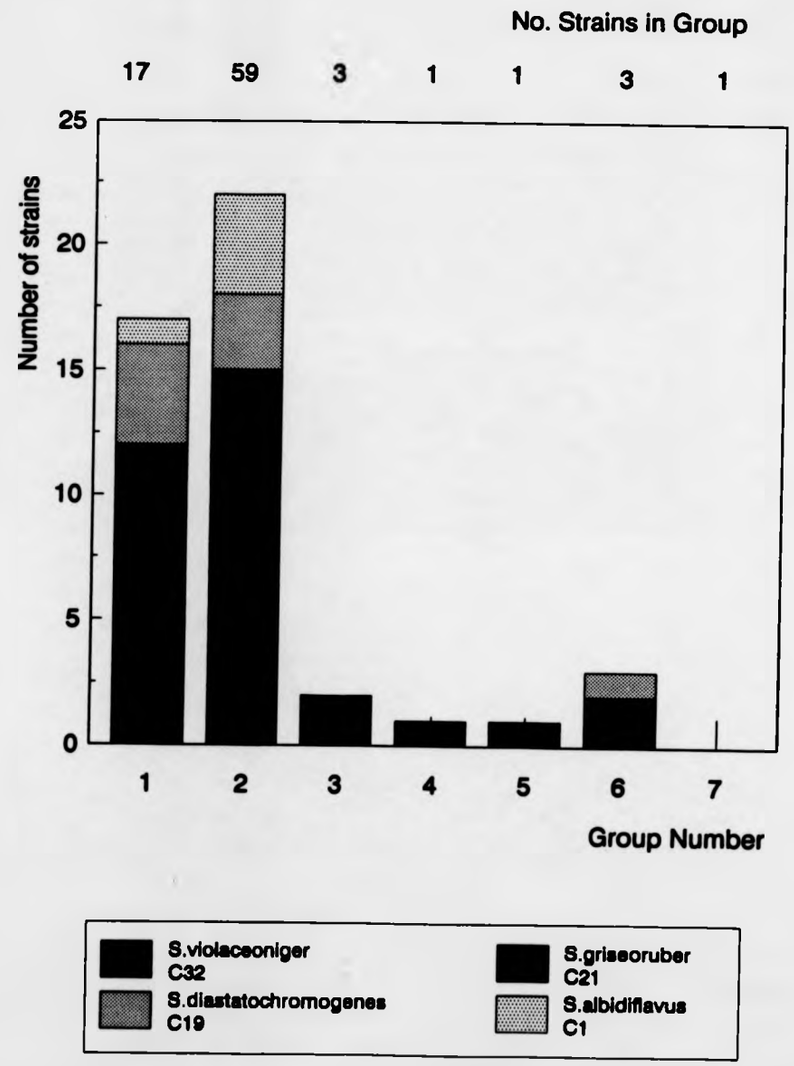
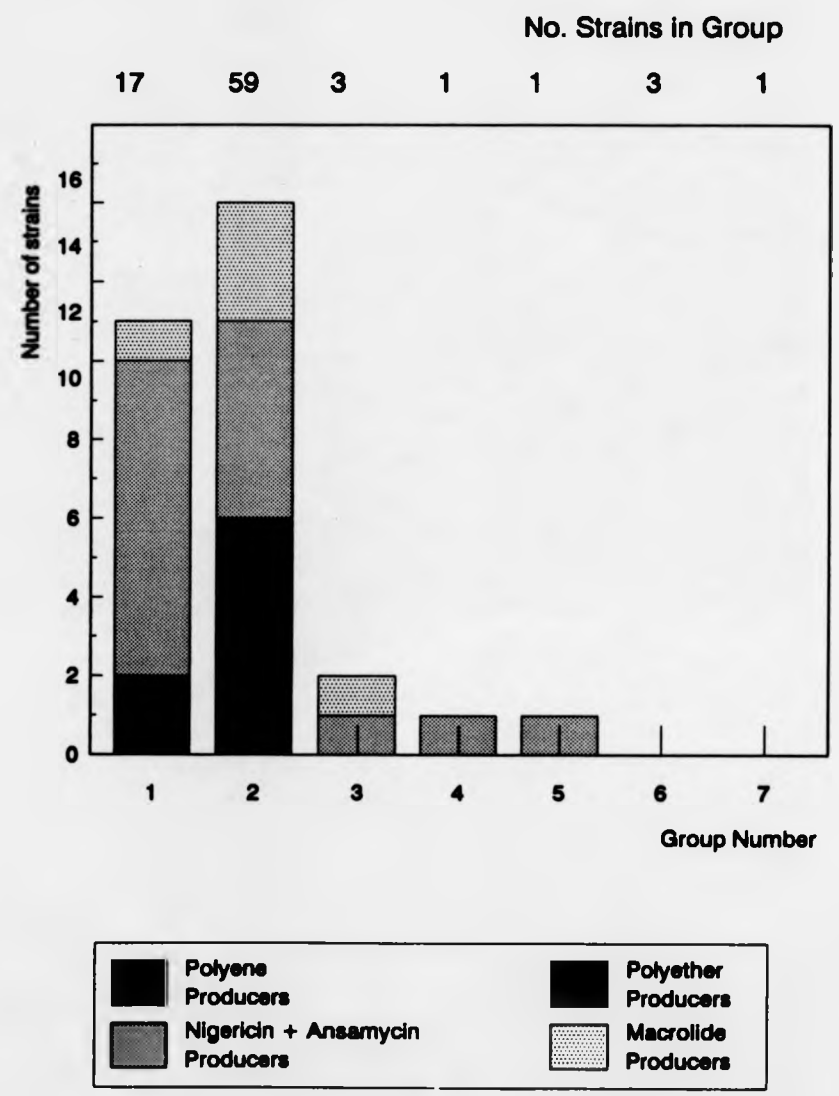


Fig. 4.5. The distribution of antibiotic producers across a phenogram (Fig.4.2.) based on fatty acid profiles.

The diagram shows where groups of strains, which were classified by the chemical structure of the antibiotics which they produced, clustered on a phenogram created using S_{dice} and UPGMA and based on fatty acid profiles.

NB. duplicate strains are included in this diagram.



that might be novel agrochemicals were in group 2, apart from one E100 duplicate, which formed group 5. Six out of eight duplicated strains clustered to group 2, but 2 duplicates were split. E100 was in group 2 and group 5, whilst A1002 had two replicates in group 2 and one in group 1.

4.4. Distributions of FAMEs Within Sub-groups of the Population.

No general relationship between fatty acid profiles and taxonomic status or antibiotic production was found in section 4.3.2. Closer examination of the raw data was made to see if any specific relationships existed between individual FAMEs and particular groups of strains.

4.4.1. The Distribution of FAMEs in *Streptomyces* Species Groups.

Specific FAMEs were assessed for their ability to distinguish between *Streptomyces* species groups. A FAME was evaluated as a good distinguishing character if it had a high standard deviation and was present in different groups at < 20 and > 80% strains. In addition 2 x Sd from the mean was used to assess significant differences, although the small size of the sample sets was taken into account when making observations. All *S.diastaochromogenes* (C19) strains lacked Me.heptadecanoate. These strains could also be differentiated from the remaining population, by being less likely to have Me.14-methylpentadecanoate (present in one strain) and Me.hexadecanoate (present in 2 strains), although the desired split of < 20 and > 80% was not observed. Significantly fewer *S.violaceoniger* (C32) strains contained Me.13-methyltetradecanoate when compared with the mean for the entire population. The distributions of Me.2-hydroxytetradecanoate in *S.diastatochomogenes* (C19), Me.2-hydroxyhexadecanoate in *S.albidoflavus* (C1) and Me.octadecanoate in *S.griseoruber* (C21) showed significant differences from the mean. However, the small sample sizes of some of the above groups meant that these latter observations were not statistically valid. More strains would need to be

Table 4.4. The distribution of FAMEs within *Streptomyces* Species.

FAME	C1	C19	% Strains C21	C32	Other	Mean	Sd
14:0	100.0	85.7	100.0	95.8	95.2	95.4	2.9
i-15:0	20.0	42.9	42.9	4.2	35.7	27.1	15.2
a-15:0	100.0	100.0	10.0	91.7	92.9	94.2	3.2
15:0	100.0	100.0	100.0	100.0	100.0	100.0	0.0
2-OH 14:0	80.0	100.0	85.7	87.5	90.5	89.4	4.2
i-16:0	40.0	14.3	42.9	29.2	73.8	51.8	22.8
16:1	100.0	100.0	100.0	100.0	97.6	98.8	1.2
16:0	60.0	28.6	57.1	41.7	97.6	70.6	27.8
i-17:0	100.0	100.0	100.0	100.0	97.6	98.8	1.2
17:0	20.0	0.0	42.9	29.2	35.7	30.6	10.5
17:0>	100.0	100.0	100.0	95.8	97.6	97.6	31.5
2-OH 16:0	40.0	28.6	28.6	25.0	23.8	25.9	3.9
18:2	0.0	0.0	0.0	12.5	0.0	3.5	5.7
18:0	0.0	0.0	14.3	0.0	0.0	1.2	4.0
19:0	0.0	0.0	14.3	0.0	0.0	1.2	4.0
Number Strains.	5	7	7	24	42	85	85

This table shows the distribution of specific fatty acids amongst strains which have been grouped by their taxonomic identity (Williams *et al.*, 1983a).

examined before further comments could be made.

4.4.2. The Distribution of FAMEs Within Producers of Specific Classes of Antibiotics.

It was interesting to see if information could be gleaned by comparing the general FAME profiles of producers of specific classes of antibiotics (Table 4.5). The same rules as in section 4.2.2 were used to assess this data.

The FAME profiles of all groups of strains were fairly similar and there were no FAMEs which could be classed as good distinguishing characters. This explained why these strains did not emerge as related groups during the cluster analysis. However, some interesting observations were made from Table 4.5. None of the twenty polyether producers contained the iso-branched fatty acid Me.13-methyltetradecanoate and the rare Me.cis-9,12-octadienoate was only found in polyene or polyether and ansamycin producers. Unfortunately there were only 5 producers of macrolides and macrolide-like antibiotics in the study, but preliminary observations suggested that the fatty acid profiles of these strains could be different in several respects. They could be more likely to contain the iso branched Me.13-methyltetradecanoate and the cyclopropane FAME, Me.cis-9,10-methylenehexadecanoate and they may be less likely to have anteiso-branched Me.12-methyltetradecanoate, Me.heptadecanoate and Me.hydroxytetradecanoate.

4.4.3. Distribution of FAMEs Amongst Isolates from Specific Geographical Locations and Isolation Procedures.

The different strain series were examined for a predisposition towards any specific fatty acids which could reflect differences in the environment from, which they were isolated or even in the isolation procedure used. The results are shown in Table 4.6., which indicates that there were no instances, where differences in specific FAMEs related to strain series.

Table 4.5. The distribution of FAME's within specific antibiotic producers.

The table shows the distribution of fatty acids amongst strains which have been grouped by the chemical class of their antibiotic product. The ansamycin producers were geldanamycin apart from 3672 the herbimycin producer and nigericin belongs to the polyether class of antibiotics.

Key to superscripts used in Table 4.5.

[1]There were 57 other strains but 2 of them produced fatty acid derived metabolites. These were A3(2) *S.coelicolor* and KCC S-0785 *S.lusitanus* the tetracycline producer. These were included in the column for all fatty acid derivatives.

[2]The mean and standard deviation refer to columns 1-5.

Table 4.5. The distribution of FAMEs within specific antibiotic producers.

FAME	Strains with Specific Classes of Antibiotic Product (% strains) Polyene	Niger-icin	Nig+ Ansa	Macrolide	Others	[2]Mean	[2]Sd	All Ab. Prods.
14:0	100.0	100.0	93.3	100.0	94.6	96.4	3.4	96.7
i-15:0	33.3	0.0	0.0	40.0	29.1	10.7	17.3	16.7
a-15:0	100.0	100.0	93.3	80.0	96.4	92.8	6.8	93.3
15:0	100.0	100.0	100.0	100.0	100.0	100.0	0.0	100.0
2-OH 14:0	100.0	100.0	100.0	80.0	90.9	96.4	7.8	96.7
i-16:0	0.0	60.0	26.7	20.0	54.6	28.6	17.0	33.3
16:1	100.0	100.0	100.0	100.0	98.2	98.8	8.6	100.0
16:0	66.7	60.0	100.0	60.0	50.9	82.5	19.6	83.3
i-17:0	100.0	100.0	100.0	100.0	96.4	97.6	1.7	100.0
17:0>	33.3	40.0	20.0	60.0	23.6	32.1	15.4	33.3
17:0	100.0	100.0	100	80.0	100.0	96.4	7.8	96.7
2-OH 16:0	0.0	40.0	33.3	20.0	20.0	28.6	11.9	30.0
18:2	33.3	0.0	13.3	0.0	.00	10.7	10.1	10.0
18:0	0.0	0.0	0.0	0.0	1.8	1.2	0.9	0.0
19:0	0.0	0.0	0.0	0.0	1.8	1.2	0.9	0.0
No Strains	3	5	15	5	[1]55	83	83	30

Table 4.6. The distribution of FAMEs amongst various strain series.

FAME	MM	C	D	E	F	Mean	Sd
14:0	90.1	91.3	100.0	100.0	100.0	94.7	4.5
i-15:0	100.0	30.4	44.4	12.5	28.6	24.11	4.7
a-15:0	100.0	87.0	88.9	100.0	100.0	93.1	6.3
15:0	100.0	100.0	100.0	100.0	100.0	100.0	0.0
2-OH. 14:0	100.0	95.7	100.0	87.5	100.0	96.6	4.2
i-16:0	18.2	65.2	44.4	75.0	100.0	58.6	24.8
16:1	100.0	100.0	88.9	100.0	100.0	98.3	4.1
16:0	18.2	78.3	66.7	87.5	100.0	69.0	26.5
i-17:0	100.0	95.7	100.0	100.0	100.0	98.3	2.1
17:0 >	18.2	21.7	11.1	37.5	42.9	24.1	10.2
17:0	100.0	100.0	100.0	100.0	100.0	100.0	0.0
16:0 2-OH	36.4	21.7	22.2	25.0	28.6	25.9	5.6
18:2	9.1	0.0	0.0	0.0	0.0	1.7	3.4
18:0	0.0	4.4	0.0	0.0	0.0	1.7	2.2
19:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total No. strains	11.0	23.0	9.0	8.0	7.0	58	

The table shows the distribution of fatty acid profiles amongst strains which have been grouped by their isolation series. The MM series were all *S. violaceoniger* and therefore would interfere with any observations made in this section.

4.5. Alterations in the FAME Profile of D153 During Batch Culture and its Relationship to Secondary Metabolite Biosynthesis.

Qualitative fatty acid data was not related to secondary metabolism in a general way and could not be used to predict bioactivity. It was hypothesized that changes in the levels of fatty acids through the growth cycle might be more indicative of a strains bioactive capabilities. A feasibility study was carried out to test this hypothesis. The common occurence of geldanamycin and nigericin in streptomycetes has been discussed in Chapter 3 where it was mentioned that both antibiotics have portions manufactured by the polyketide route, therefore a strain (D153), which produced these compounds was chosen for this work. D153 was not included in the initial population study, but was chosen because it was a good canidate for chemostat work. Consequently this strain contained some additional FAMEs, which can be found listed in the key (Fig. 4.1.).

4.5.1. Choice of Medium.

Sautons medium was used to generate fatty acid profiles, but it was discovered that antibiotic production for a number of strains was not expressed on this medium and so ISP7 was chosen for this study, after it was shown to be compatible with GCMS procedures (Table 4.7).

Fatty acid profiles of D153 were similar for the two media, although Me.tridecanoate, Me.trans-9-octadecanoate and Me.octadecanoate were not observed using Sautons medium and the cyclopropane FAME Me.cis-9,10-methylenoctadecanoate was not observed on ISP7 cultures (NB. all of these compounds were only observed in single replicates). Five other peaks were detected which were not present in the standard mix but which were consistent for all replicates on both types of media. An example of a GCMS trace is given in Fig.4.5.

Table 4.7. The effect of Sautons medium and ISP7 on the FAME profile of D153.

	Fatty Acid						
13:0	14:0	i-15:0	a-15:0	2-OH 14:0	i-16:0	16:1	16:0
Sautons Rep1							
0	1	1	1	1	0	1	1
Sautons Rep2							
0	0	1	1	1	0	1	1
ISP7 Rep1							
1	1	1	1	1	0	1	1
ISP7 Rep2							
0	0	1	1	1	0	1	1

i-17:0	17:0>	17:0	2-OH 16:0	18:2	18:1	18:0	19:0>
Sautons Rep1							
1	1	1	1	0	0	0	1
Sautons Rep1							
1	1	0	0	0	0	0	0
ISP7 Rep 1							
1	1	1	1	0	1	1	0
ISP7 Rep 2							
1	1	1	1	0	0	0	0

The table shows the qualitative FAME profiles of strain D153 in replicate on Sautons and ISP7 media. The data was taken after 3 days growth.

Fig. 4.6. Example of a GCMS trace from strain D153.

This diagram shows a GCMS trace from a fatty acid extract of D153, which was taken after 3 days growth at 28°C, in ISP7 medium.

Key to Peak Numbers

8 = Me. 13-methyltetradecanoate

9 = Me. 12-methyltetradecanoate

10 = Me. pentadecanoate

13 = Me. 14-methylpentadecanoate

14 = Me. cis-9-hexadecanoate

15 = Me. hexadecanoate

16 = Me. 15-methylhexadecanoate

16a = Me. cis-9, 10-methylenehexadecanoate

17 = Me. heptadecanoate

18 = Me. 2-hydroxyhexadecanoate

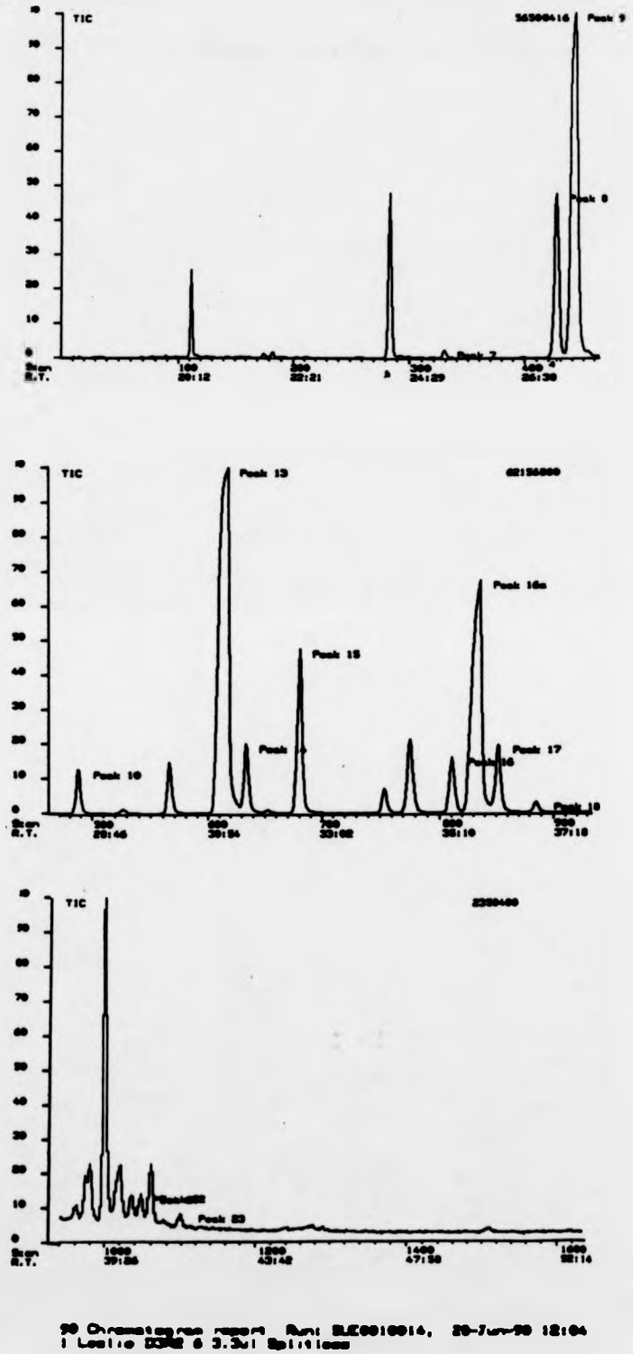
21 = Me. trans-9-octadecanoate

Me cis-11-octadecanoate

22 = Me. octadecanoate

23 = Me. cis-9, 10-methyleneoctadecanoate

FIG.4.6.



4.5.2. Timecourse of Fatty Acid Biosynthesis Versus Antibiotic Production in D153.

Quantitative and qualitative changes in the fatty acid profile of a selected strain were examined over 7 days, with the aim of detecting shifts in the nature and levels of fatty acids and to try to correlate them with onset of antibiotic production.

Table 4.8. shows the results of the qualitative experiment where FAMES with chain lengths of between C14 and C18 carbon atoms were first detected at day 2. On day 3, this range was extended to include Me. tetradecanoate, Me. octadecanoate and Me. cis 9-10 methyleneoctadecanoate. FAMEs with a chain length of 18C appeared transiently.

Fig. 4.6. shows part of a separate experiment where an internal standard which allowed relative quantifications was included in the fatty acid samples. Fig.4.7. shows the corresponding antibiotic profile for D153, with respect to batch culture growth. Geldanamycin production began at day 3, peaked between days 4-5 and then remained constant up to day 7, whilst nigericin production peaked at day 7, although small traces began to appear at day 3. Fatty acids which appeared transiently in the above experiment were not detected this time and this could be due to these compounds being produced at very low levels, which fluctuated about the detection limit of the GCMS.

All fatty acid levels increased up to day 2 and this was in line with a logarithmic increase in biomass. Subsequently all fatty acids, apart from Me pentadecanoate, Me. heptadecanoate, Me. cis-9-hexadecanoate and Me. 15-methylhexadecanoate, followed a similar profile (ie decreased until day 3, then began to rise until day 6, when levels fell with the onset of cell death). However from day 2 Me. pentadecanoate and Me. heptadecanoate levels continued to increase, until the onset of stationary phase at day 3 (this was also the onset of antibiotic production). The levels of these fatty acids and Me. cis-9-hexadecanoate fell until day 4, when they began to rise, but then followed the pattern of the other

Table 4.8. Qualitative differences in the fatty acids of D153 with time.

FAME	(Day + rep) Blank	Blank	D1R1	D1R2	D2R1	D2R2	D3R1	D3R2
14:0	0	0	0	0	0	0	1	1
i-15:0	0	0	0	0	1	1	1	1
a-15:0	0	0	0	0	1	1	1	1
2-OH 14:0	0	0	0	0	1	1	1	1
i-16:0	0	0	0	0	1	1	1	1
16:1	0	0	0	0	1	1	1	1
16:0	0	0	0	0	1	1	1	1
i-17:0	0	0	0	0	1	1	1	1
17:0>	0	0	0	0	1	1	1	1
17:0	0	0	0	0	1	1	1	1
2-OH 16:0	0	0	0	0	1	0	0	1
$18:1^{9}$	0	0	0	0	1	0	0	0
$18:1^{11}$	0	0	0	0	1	0	0	1
18:0	0	0	0	0	0	0	0	1
19:0>	0	0	0	0	0	0	0	1

1 denotes the presence and 0 the absence of a fatty acid.

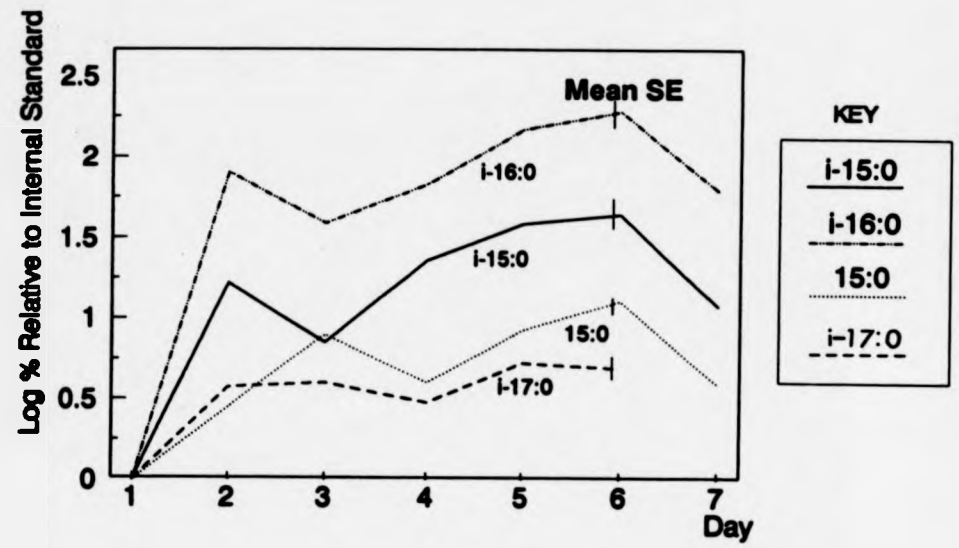
fatty acid graphs. The level of Me. 15-methylhexadecanoate showed less fluctuation than other fatty acids in the study.

Table 4.8. Qualitative differences in the fatty acids of D153 with time.

FAME	D4R1	D4R2	D5R1	D5R2	D6R1	D6R2	D7R1	D7R2
14:0	1	1	1	1	1	1	1	1
i-15:0	1	1	1	1	1	1	1	1
a-15:0	1	1	1	1	1	1	1	1
2-OH 14:0	1	1	1	1	1	1	1	1
i-16:0	1	1	1	1	1	1	1	1
16:1	1	1	1	1	1	1	1	1
16:0	1	1	1	1	1	1	1	1
i-17:0	1	1	1	1	1	1	1	1
17:0>	1	1	1	1	1	1	1	1
17:0	1	1	1	1	1	1	1	1
2-OH 16:0	1	1	1	1	0	1	0	0
$18:1^{9}$	0	0	0	0	0	0	0	0
$18:1^{11}$	0	0	0	0	0	0	0	0
18:0	0	0	0	0	0	0	0	0
19:0>	1	0	0	1	0	0	0	0

Fig. 4.6. Changes in the fatty acid profile of D153 through a cycle of batch culture growth.

The graphs show how the levels of FAMEs, which were present within D153 during batch culture growth on ISP7, changed relative to the concentration of an internal standard (a known amount of which was introduced into each sample prior to GCMS).



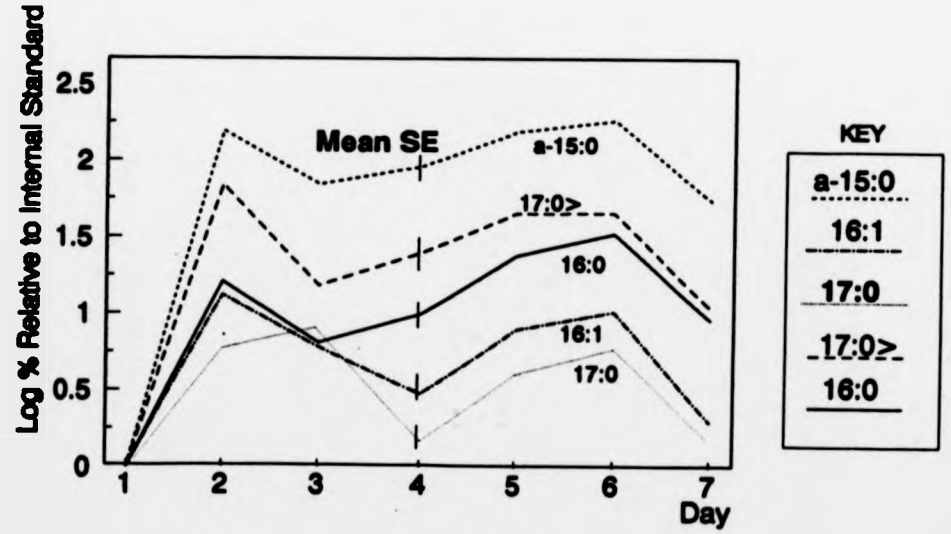
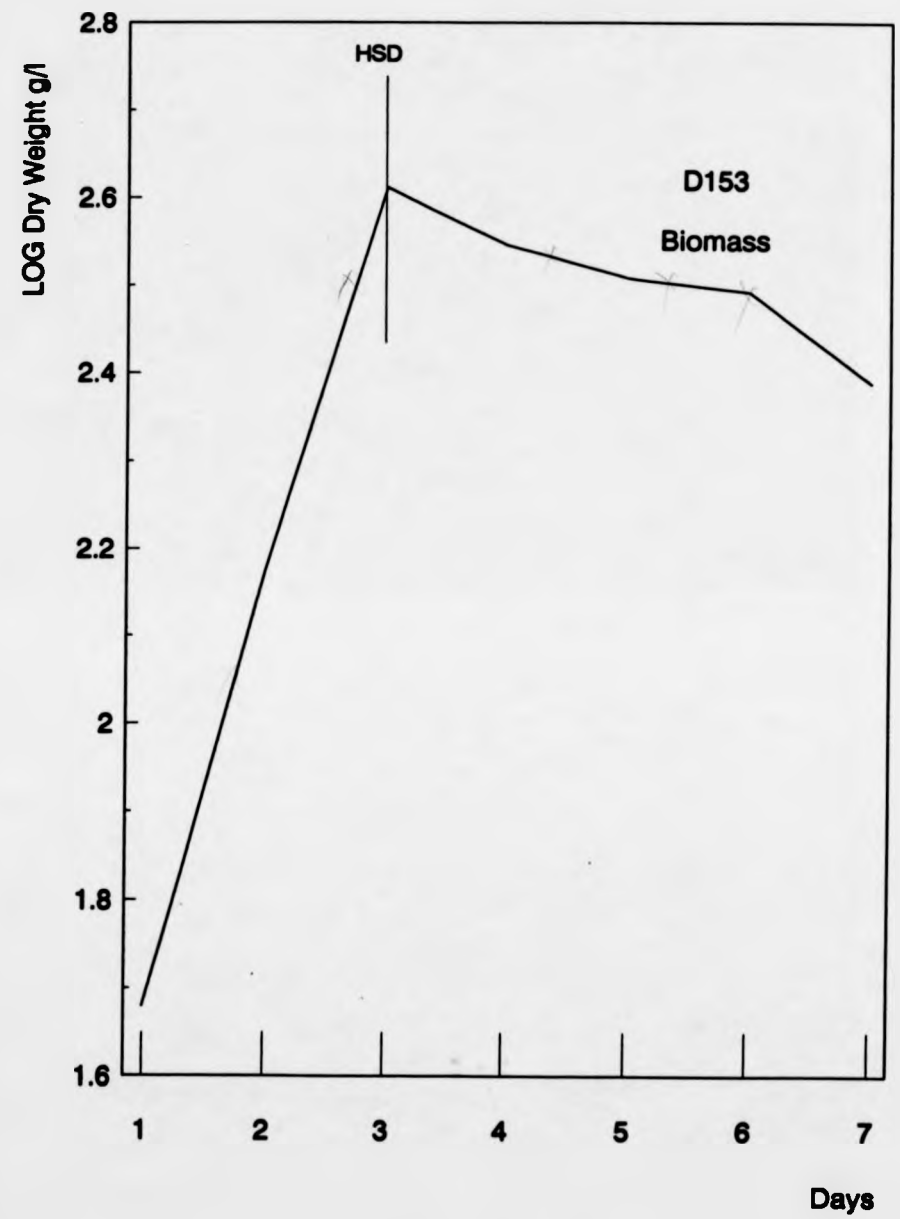


Fig. 4.7. Profile of antibiotic production by strain D153.

The photograph shows a TLC plate which contains ethyl acetate extracts from a culture of D153 which were taken at different times during batch culture growth. The green compounds were geldanamycin, whilst the scarlet red compound was nigericin; the pure antibiotics are present in the centre of the TLC plate and helped to identify the compounds in the extract.

Fig. 4.8. Growth curve of D153 through batch culture growth.

The graph shows changes in the levels of biomass in D153 during batch culture growth. (Biomass is given as the log of the dry weight).



Me. 15 methylhexadecanoate showed no significant difference in amount between days 2 and 4, but followed the same trend as the others after day 4.

It is not possible to say from this data whether the observed fluctuations in fatty acid levels related to geldanamycin and nigericin biosynthesis, but fluctuations in cellular fatty acids in D153 did not follow the same profile as cellular biomass. The ratio of fatty acid to biomass increased during stationary phase, whilst antibiotic production was occurring. Prior to the onset of stationary phase and secondary metabolism there were major changes in the levels of fatty acids. More experiments would be required to establish exactly how or if these events are interconnected.

4.6. Discussion.

New information on the presence of certain fatty acids in streptomyetes was uncovered during this work; hydroxylated fatty acids were much more common than has previously been thought (Kroppenstedt, 1985). These fatty acids are fairly unstable and their absence in other studies may be due to their degradation rather than an inability of the organisms under study to produce them. R. Kroppenstedt (personal communication) has found that hydroxy fatty acids are highly diagnostic for some streptomycete species, including *S.violaceoniger*, although a similar observation was not made during this work. There is no information in the literature about the distribution of cyclopropane fatty acids in populations of streptomycetes and this work has shown that 30% of the genus might produce at least one cyclopropane fatty acid.

Although, there were 33 FAME patterns in 85 strains, the attempt to delimit *S.violaceoniger* (Williams *et al.*, 1983a and b) from other streptomycetes failed. Reasons for this may lie in differences between this study and the successful study of Saddler *et al.* (1987). Both studies used type strains which represented tight clusters, and markers from other groupings were also included. However, natural isolates were pre-selected by spore morphology in the study of Saddler *et al.* (1987) compared to an arbitrarily chosen selection that comprised a variety of species and

unidentified strains (in this study). Work presented in Chapter 2 has shown that many of these isolates did not conform to described streptomycete taxa and if this was reflected by their fatty acid profiles they may have prevented the delimitation of tight groups for more typical strains.

Streptomyces species delimitation may require quantitative data, as opposed to the qualitative data used in this study. Saddler (1987) based an assumption that fatty acid profiles remained constant during logarithmic growth and stationary phase on a study based on one strain, grown in Sautons medium, with three day intervals between readings (Saddler, 1986). However, fluctuations in fatty acid levels occurred on a daily basis for D153 in ISP7 medium (section 4.5). Kroppenstedt (personal communication) suggested that changes in fatty acid composition at different growth states were responsible for his failure to cluster *S. violaceoniger* amongst a tree comprising 12 different *Streptomyces* taxa; clustering was however, achieved by removing three C32 strains (Williams *et al.*, 1983a), two of which were present in this study. Kroppenstedt's work comprised entirely type strains which may be biased towards strains, which have been patented for antibiotic production. They would differ from many of the antibiotic producers included here, since industrial strains are selected for the production of either one or a group of related idiolites. Antibiotic-producing isolates often produce several different compounds (Chapter 3), which may or may not relate to fatty acid profiles. Work carried out with D153, a producer of nigericin and geldanamycin, suggested that this was possible. The levels of fatty acids in this strain fluctuated in line with changes in secondary metabolism, although this was not known to be correlated in any way. However this study was carried out in a production medium (ISP7), compared to Sautons medium, which aims to optimize biomass for taxonomic studies. D153 did not produce antibiotics in Sautons medium (Chapter3).

CHAPTER 5

The Relationship Between Chemical Profiles, Taxonomic Status and Biological Activity.

5.1. Introduction.

The classification and identification of streptomycetes has generally relied on numerical methods which use a wide variety of tests to obtain polythetic taxa (Williams *et al.*, 1983a and b; Langham *et al.*, 1989; Kampfer *et al.*, 1991a and b), although studies comprising large numbers of substrate utilization tests have also given good data for classification studies (Bochner, 1989; Goodfellow *et al.*, 1987). In the above studies the nature of what is being scored is fairly clear (eg. the presence of an enzyme), but other work has generated patterns which have been used to classify strains and species, when the precise nature of the components involved are not known. Protein profiling is an example of this, where unidentified cellular proteins are submitted to electrophoretic separation and the banding patterns of different strains or species are compared (Kersters, 1985, Vesselinova and Tsvelkov, personal communication).

Pattern recognition has also been used during the drug discovery process to predict the likely chemical nature of specific bioactive products and a variety of schemes have been developed to reduce the time spent analysing unknown chemical compounds (Aszalos, 1980). For example, Aszalos *et al.* (1965) described how they used 14 solvent systems to assign sub-group classifications to 84 known antibiotics, unknown compounds were then processed by TLC and placed in a sub-group and although they were not identified, the list of possible chemicals was shortened.

Thin layer chromatography was used to determine the bioactivity of isolates used for this thesis (Chapter 3); during that work, a banding pattern comprising a

variety of unknown metabolites was observed within each strain extract and it was interesting to see if these profiles correlated with phenetic relationships.

The profiles were also assessed for their use in forecasting bioactive natural isolates. This was done in two ways, firstly with respect to the whole profile and secondly by following the hypothesis of Zähner *et al.* (1982), who described how TLC could be used to predict novel compounds by selecting unique spots for further examination.

5.1.1. Characteristics of Strains Chosen for this Study.

A random selection of streptomycetes, comprising members of various isolation series (Tables 2.1., 2.9., 3.1. and 5.1) were selected.

Table 5.1. Origins of strains used in this study.

Strains	No.
A	7
B	1
C	79
D	32
E	6
F	5
MM	14
W	3
RB	2
Other	5
Types	19
Total	173

Taxonomic characterisation resulted in identification of 41 natural isolates to the species level. This is illustrated in Table 5.2., which also summarizes the identities of the type strains. Characterization of the strains with respect to their bioactivity and or antibiotic activity is given in Table 3.1.

Table 5.2. *Streptomyces* species involved in this study.

Spp.	No.Natural Isolates	No.Type Strains.
C1	5	0
C3	1	0
C12	3	0
C15	2	0
C17	0	1
C19	9	2
C21	0	4
C32	21	7
C44	0	1
S.spp.	123	4
Total	164	19

C refers to taxonomic cluster as defined by Williams *et al.*, 1983a.

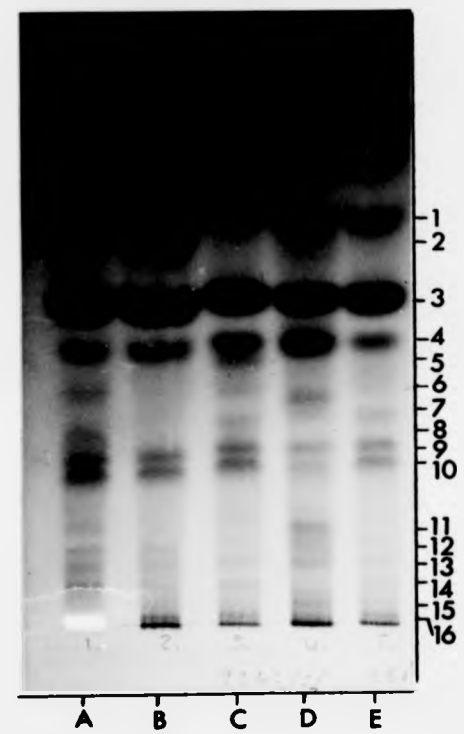
5.2. The Development of Methodology.

5.2.1. Scheme for the Numbering and Scoring of TLC Spots.

TLC profiles, which were generated by ethyl acetate extraction and run on TLC, using solvent system 1 were visualized under UV light (sections 2.3 and 2.6). When these were examined for all of the strains studied, 16 regularly observed spots and 13 rare compounds were revealed. Each of these spots was assigned a number from 1-29 (Fig. 5.1.).

Fig 5.1. Diagram to show the numbering system used to score spots on TLC plates.

The photograph shows the TLC profiles of 5 strains, which are denoted A to E; these strains were morphologically very similar and were thought to be replicates and so their extracts were run alongside one another. The profiles provided a good example of the way in which spots were scored because the sixteen most frequently observed spots were present (marked 1-16).



The plates were examined by eye and scored for the presence and absence of these metabolites. The negative control comprised uninoculated, incubated and extracted medium, whilst positive controls were provided by running a standardized strain extract on every plate. The negative control gave 4 spots, which had similar Rf values to test spots, but development of plates with vanillin always showed that they were different from those being scored.

5.3. Results.

5.3. The Frequency of Occurrence of Different Spots.

Metabolites in the study were not identified, except if they corresponded to an antibiotic standard. A detailed discussion on the distribution of these unknown compounds is therefore unnecessary, except to discuss briefly what types of compound they might be.

5.3.1. Distribution of Metabolites.

The silica adsorbent on the TLC plates was hydrophilic and bound to and immobilized polar compounds. Conversely, the extraction solvent (ethyl acetate) and the solvent system (No.1, Chapter 2.3.2.) were non-polar and compounds which were extracted and separated the most successfully were lipophilic. The closer a compound was to the bottom of the plate, the "more polar" it was. Spots near the baseline were as common as the "more lipophilic" spots but were usually fainter (Table 5.3.).

Table 5.3. The frequency of the spots scored by TLC.

Spot	No. Strains with Spot
1	182
2	116
3	171
4	173
5	177
6	86
7	63
8	83
9	75
10	176
11	182
12	61
13	95
14	119
15	126
16	132

This table shows the total number of strains which were examined and includes replicate strains.

Other factors which could have affected the mobility of compounds in the extracts included the presence of double bonds and certain functional groups on molecules, which raise their adsorption affinity to the silica. For example, saturated hydrocarbons are highly mobile in lipophilic solvents, methyl groups have little effect and carbonyls adsorb less strongly than amino or hydroxy groups. The

possession of several functional groups are only partially additive because steric effects are also important.

5.4. The Estimation of Test Error Involved in Scoring Metabolite Patterns.

5.4.1. The Effect of Operator Error in Scoring Metabolite Patterns.

Ten plates containing 64 strain profiles were chosen to rescore several months after they had first been assessed. Both sets of results were then compared and the number of different answers noted down; 6.4% of the 16 most common spots were recorded differently between the two sets of observations. This value was above the desired figure of 5%, but below the 10% level of acceptability set down by Sneath and Johnson (1972). 90.63% of the profiles examined were affected by this error, with an average of 1.9 discrepant spots for every strain (Sd = 1.47). The spread of the discrepancies is shown in Table 5.4.

Table 5.4. The number of spots in a subset of 64 strains affected by operator error.

No. Discreps.	No. Strains	Tot. No. Discreps.
0	6	0
1	28	28
2	12	24
3	9	27
4	4	16
5	3	15
6	2	12
Total	64	122

Discreps. = discrepencies; Tot. = total.

Certain spots were prone to more error than others (Table 5.5.).

The two compounds nearest the base line accounted for 35% of the total errors made, whilst another 30% was contained in spots 11-13 and the remainder (35% error)

Table 5.5. The distribution of operator error amongst the 16 most common spots scored.

Spot	No.Errors.	P erroneous result (Sneath and Johnson, 1972)
1	5	0.041
2	3	0.024
3	1	0.008
4	4	0.032
5	5	0.041
6	6	0.049
7	7	0.058
8	7	0.058
9	3	0.024
10	1	0.008
11	10	0.085
12	13	0.145
13	4	0.032
14	10	0.085
15	20	0.194
16	23	0.245

The total number of errors was 122 and this gave an overall test error of 0.064.

was dispersed amongst the first 10 spots. Interestingly the operator error for spots 1-10 was 3.4%, which is acceptable for taxonomic data (Sneath and Johnson, 1972). The increase in error towards the base line may be related to poor resolution between weakly eluted compounds. Spots 11-16 were confined to the bottom quarter of the developing distance (Fig.5.1.) and were closer together than the other spots. Due to the proximity of these compounds, a large quantity of one might result in difficulty in scoring neighbouring metabolites and also the amount of a substance can sometimes affect the Rf value if very low and high concentrations are compared.

The Rf value of a pure compound can be affected by the presence of impurities and because the samples in this study were mixtures of metabolites and the Rf values of individual compounds may have varied depending on the total composition of the extract. Variation between and within TLC plates might also exert an influence on operator error, but the presence of an external standard meant that these should not be major factors. Purified antibiotics which were used to identify specific antibiotics allowed between plate variation to be measured. The Rf of a geldanamycin standard from 10 different TLC plates gave a standard error of 0.013. (The mean Rf was likely to lie +/- 0.0257 from the measured spot in 95% of cases and +/- 0.034 in 99% cases).

The humidity at the time of application and during development in the tank can cause variations in Rf and this is especially true for lipophilic substances. A multi-component solvent system was used and therefore the development distance was important because the solvent system would partially separate along the chromatograph, making the Rf value dependent upon the developing distance in relationship to the starting point. TLC plates were therefore developed to 1cm below the top of the plate and the tank atmosphere was kept saturated to prevent the solvent from evaporating off the layer during chromatography.

The measurement of within plate variation required an internal standard to be present within each extract; this was difficult because it had to be distinguishable

from other spots in the study (Fig 5.1) and also the RF of any standard might be affected by the other compounds in the extracts. It was possible to take various precautions, which might prevent plate effects; the tank atmosphere was kept saturated and TLC plates did not touch each other or the filter paper (used to saturate the atmosphere) and so the solvents could not rise by capillary action. (A non-saturated atmosphere would allow more volatile constituents of the multi-component solvent to evaporate from the TLC plates in a preferential manner; the rate of evaporation would decrease from the edge to the middle of the plate, causing Rf values to increase from the centre to the edge of the plate.)

5.4.2. Reproducibility of Samples.

Forty sets of strains, which were duplicated from growth in a shake flask up to TLC, were included in the study. The mean probability of erroneous spots between duplicate strains was 10.6% (Sneath and Johnson, 1972) and 97.5% duplicates contained erroneous results. The error between replicated strains was probably partially due to operator error, which was 6.4% (Table 5.5) and so an additional 4.2% error was due to inconsistency between replicates. Table 5.6. shows the spread of errors for each of the most common spots.

Table 5.6. The distribution of errors for different spots.

Spot	No.Errors	P1	P2	P3
1	12	0.184	0.041	0.143
2	15	0.25	0.024	0.226
3	12	0.184	0.008	0.176
4	10	0.147	0.032	0.115
5	16	0.276	0.041	0.235
6	17	0.306	0.049	0.257
7	16	0.276	0.058	0.218
8	18	0.342	0.058	0.284
9	10	0.147	0.024	0.123
10	10	0.147	0.008	0.139
11	16	0.276	0.085	0.191
12	15	0.25	0.145	0.105
13	18	0.342	0.032	0.31
14	21	0.388	0.085	0.303
15	18	0.342	0.194	0.148
16	17	0.306	0.245	0.061

The total number of errors was 241 and this gave an overall test error of 10.6%.

Key:

P1 = P erroneous result (between duplicate strains).

P2 = P operator error (taken from Table 5.5.).

P3 = P1-P2: Strain error.

The "No. Errors" column refers to the number of errors, which were observed between duplicate strains (P1).

The reproducibility of two standard producing strains was examined in detail. These were NRRL 3602 and AM 3672, producers of geldanamycin and herbimycin respectively. Extracts that had been taken from different cultures of these strains, which were produced on different dates were run alongside one another (Fig. 5.2.) and were found to be highly variable. A second series of extracts, which had been produced in different flasks, but at the same time (Fig 5.3.) were also highly variable. This problem was overcome by making large extractions, freezing them and using the same samples as external strain standards on all subsequent plates.

Variations in inoculum size might have been an important factor in determining the metabolic status of a culture at the time of extraction and because a complex medium (A37) was used the probability of creating alterations in gene expression might have been increased. Variation of physical factors, such as opening and closing incubators, switching the shaker and lights off and on could have caused transient changes in temperature, oxygenation, shear factors and illumination. All these variables could happen at random at any point along the six day incubation time; the data was accumulated over several seasons and duplicates were done randomly within this time period. These variables coupled with the many possible thresholds of nutrients and cofactors might be capable of initiating many different metabolic effects. In addition, certain metabolic triggers in streptomycetes may be intrinsically variable.

Figs. 5.2. (A; top photograph) and 5.3. (B: bottom photograph)

Fig. 5.2. Extracts of AM 3672 and ATCC 3602, which were produced at different times.

Key

1 NRRL 3602; extract produced on 1/11/88

2 NRRL 3602; extract produced on 2/11/88

3 NRRL 3602; extract produced on 4/10/88

4 NRRL 3602; extract produced on 10/11/88

5 NRRL 3602; extract produced on 20/10/88

6 ATCC 3602; extract produced on 1/11/88

7 ATCC 3602; extract produced on 2/11/88

8 ATCC 3602; extract produced on 4/10/88

9 ATCC 3602; extract produced on 10/11/88

10 ATCC 3602; extract produced on 20/10/88

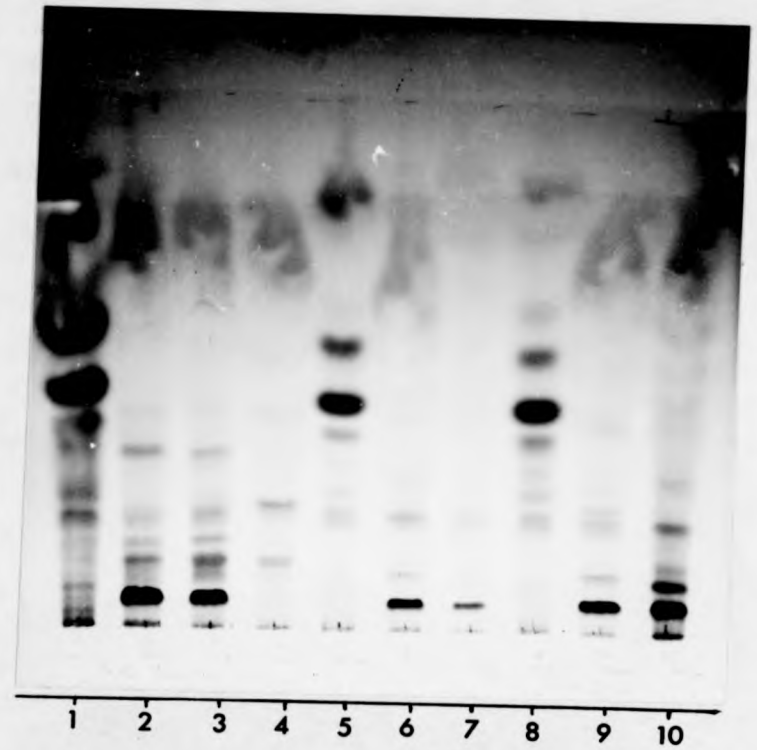
Fig. 5.3. Extracts of different cultures of AM 3672 and ATCC 3602, which were produced at the same time.

1 to 4 = ATCC 3672 (AM = ATCC)

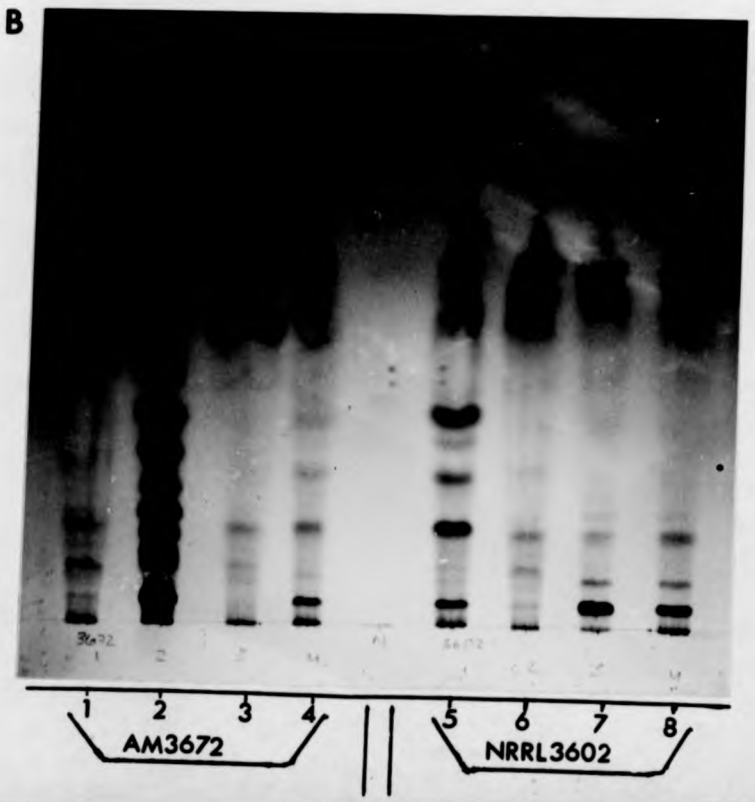
5 to 8 = NRRL 3602

The extracts of all of these samples were produced on the same day, but from different cultures, which had been incubated alongside each other.

A



B



5.5. Cluster Analysis.

Although the estimates of error showed that the data were not suited to clustering, the aim of the study was to assess chemical profiles as both a taxonomic tool and a means of predicting bioactivity. An example of clustered data is therefore presented in this section. In addition to the sources of error discussed above there were probably additional errors which affected the calculation of similarity between strains. For example, different compounds could have had the same RF value and identical compounds might have been biosynthesised by different biochemical routes. This meant that observed similarity did not necessarily equal true phenetic or genetic similarity.

5.5.1. The Distribution of Streptomycetes Based on Chemical Profiles Using Hierarchical Methods.

Figures 5.4 and 5.5 show phenograms created using the chemical profiles of natural isolates and type strains. Both diagrams were produced using the Dice coefficient and UPGMA. Comparison of fig. 5.5. with a phenogram clustered with the simple matching coefficient and UPGMA, showed fragmentary resemblance. For example, the phenogram based on natural isolates, single member groups were intact, but larger groups were separated via groupings of 4-8 strains.

Neither of the phenograms showed a correlation with taxonomic identity or with bioactivity. Figs 5.6. and 5.7. illustrate this for 54 groups, taken at 70% similarity on the phenogram comprising natural isolates (Fig.5.5). In addition, most replicate strains were placed distantly from one another on the dendrograms. Nine of the bioactive strains with rare spots (section 5.6) and five other bioactive strains were placed in single member groups. However three out of the four producers of antibiotics with possible novel activities were in the two groups with the largest numbers of members.

Fig. 5.4. Phenogram showing relationships between type strains, based upon TLC profiles.

Binary data based on TLC profiles were used to create the phenogram; similarity values were calculated using the Dice coefficient and the UPGMA algorithm was used to form the diagram.

The names of the type strains used are given on the diagram.

FIG.5.5.

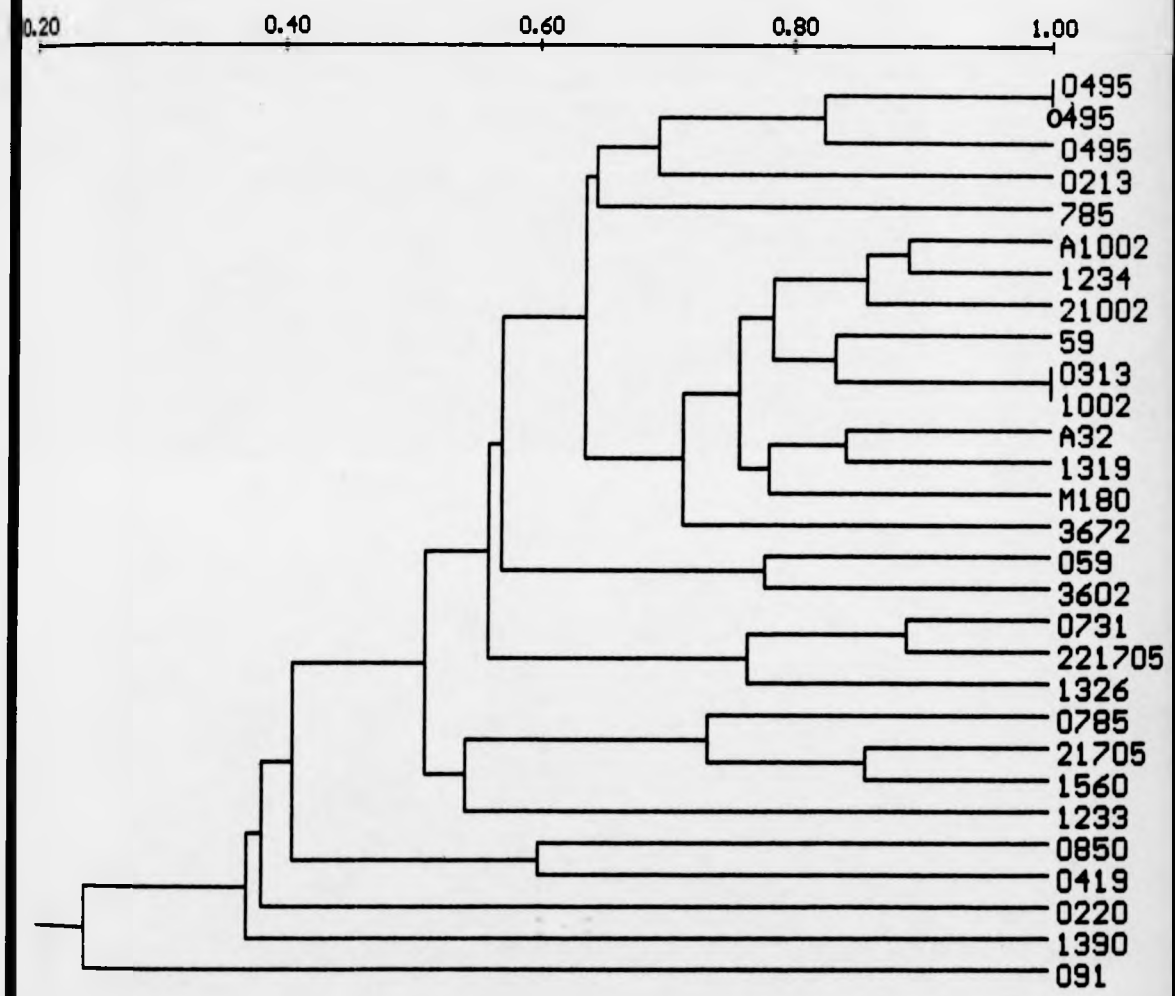


Fig. 5.5. Phenogram showing relationships between natural isolates, based upon TLC profiles.

Binary data based on TLC profiles were used to create form the phenogram; similarity values were calculated using the Dice coefficient and the UPGMA algorithm was used to form the diagram.

Binary data based on TLC profiles were used to create form the phenogram; similarity values were calculated using the Dice coefficient and the UPGMA algorithm was used to form the diagram.

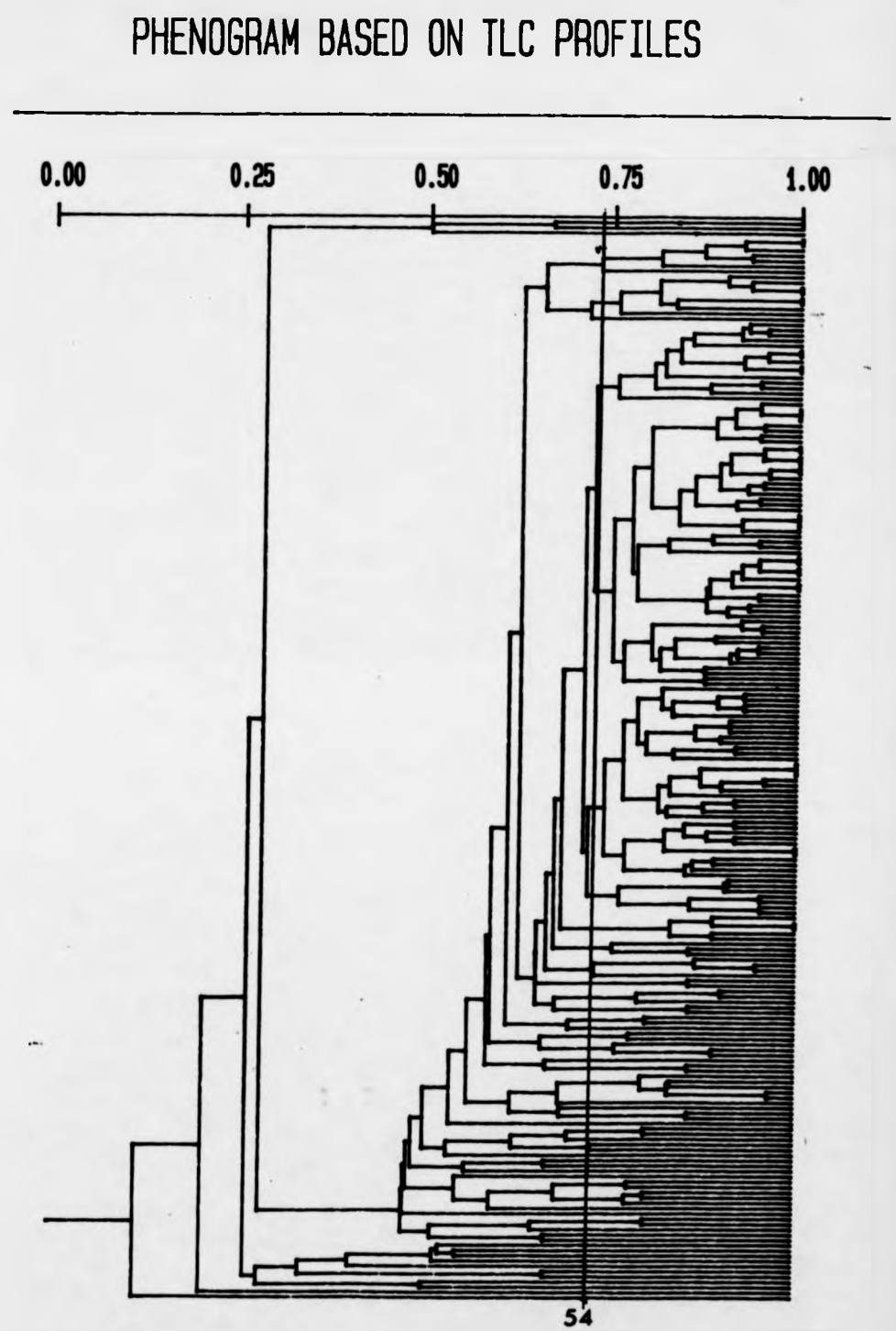


Fig. 5.6. The distribution of *Streptomyces* species across the phenogram (Fig. 5.4.).

The diagram is a stacked bar chart showing the proportion of strains in each of 54 groups (as shown in Fig. 5.4.), which identified to strain clusters as defined by Williams *et al.* (1983a).

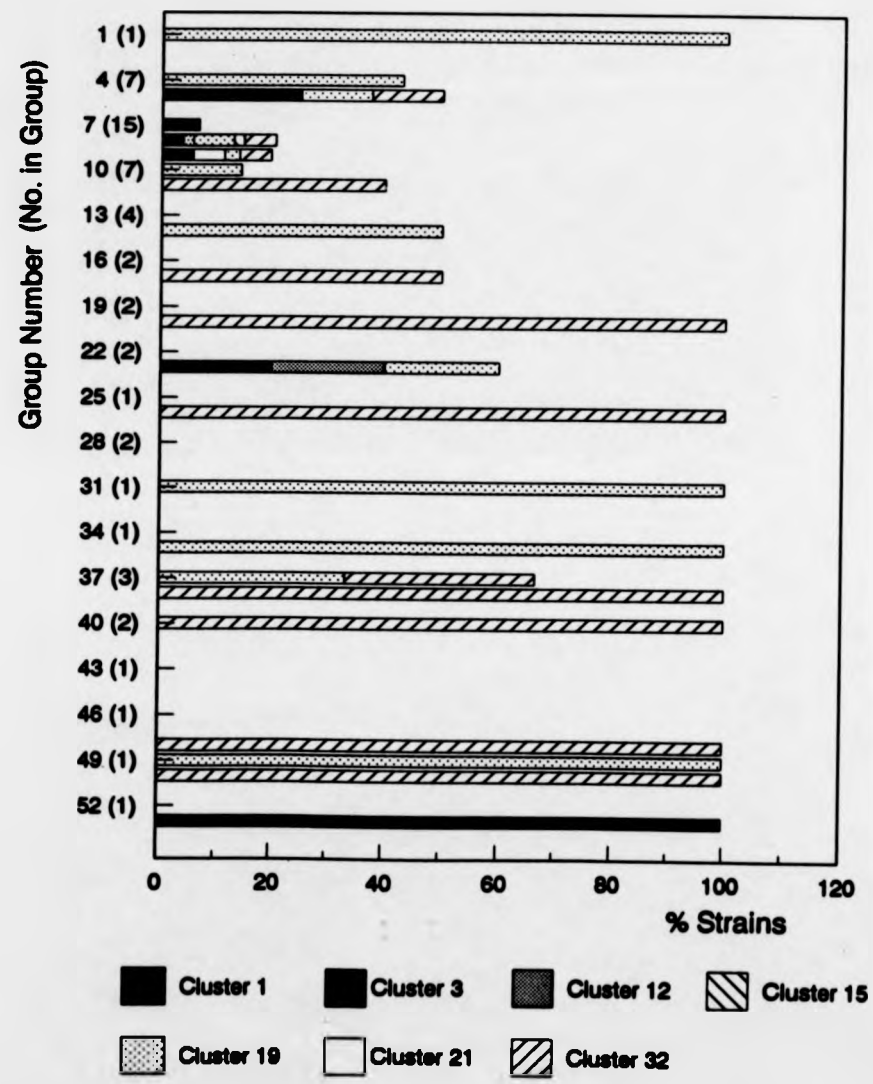
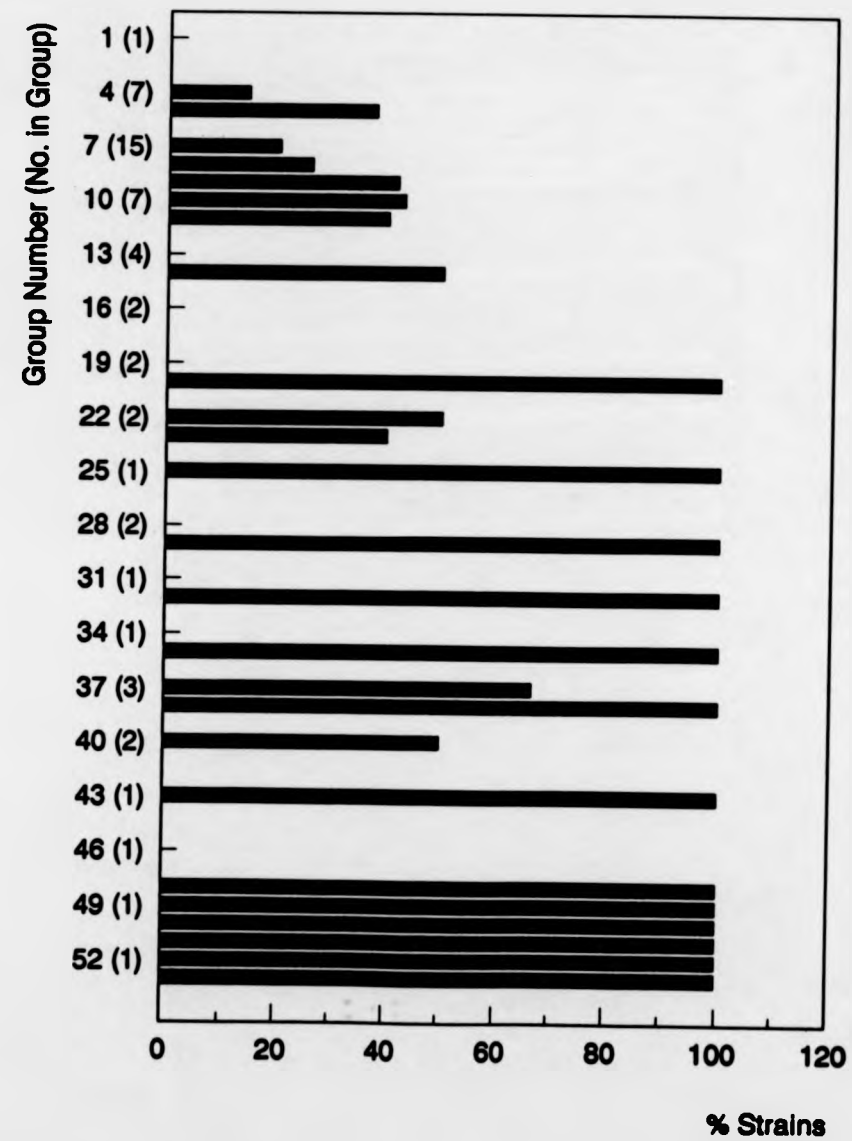


Fig. 5.7. The distribution of bioactive natural isolates across the phenogram (Fig. 5.4.).

The diagram illustrates how bioactive natural isolates were distributed with respect to their TLC profiles and shows that no discrimination was made between antibiotic producers and non-producers. In addition (not illustrated by the diagram) no pattern was observed with respect to the distribution of producers of chemically related antibiotics.



5.5.2. Ordination of Streptomycete Chemical Profiles.

Principle components analysis was carried out on the binary data from this study where cluster 32 strains tended to be located either side of the y axis (Fig.5.8.). A small group of strains which produced both nigericin and geldanamycin remained closely associated as the ordination diagram was rotated (Figs. 5.9. and 5.10.); one of these strains (present in replicate) also produced a compound which might have novel agrochemical activity. The formation of this group may be explained by the fact that geldanamycin (and possibly spots correlating with the production of nigericin and other antibiotics) would be present within the profile. Apart from these two observations, no other correlations were observed amongst these data.

Fig. 5.8. Principle components analysis of TLC profiles to show the position of members of the *S. violaceoniger* group.

The diagram illustates how most *S. violaceoniger* strains clustered to an area, which surrounded the y-axis.

S. violaceoniger strains are shown by circles as opposed to spots, which denote other strains.

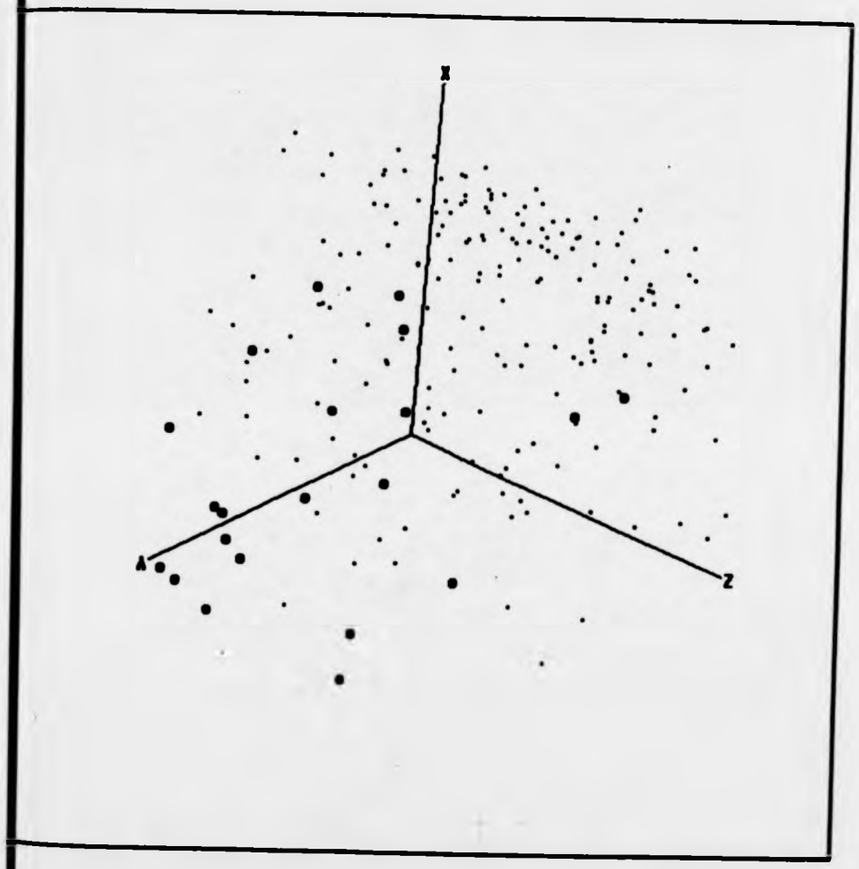


Fig. 5.9. Principle components analysis of TLC profiles, to show the position of nigericin producers.

The diagram shows how nigericin producers did not form a cluster, apart from a small group of strains, which also produced geldanamycin.

The nigericin and geldanamycin producers are shown by a circle and a number, which refers to their position within the original data matrix. Other strains are represented by a spot.

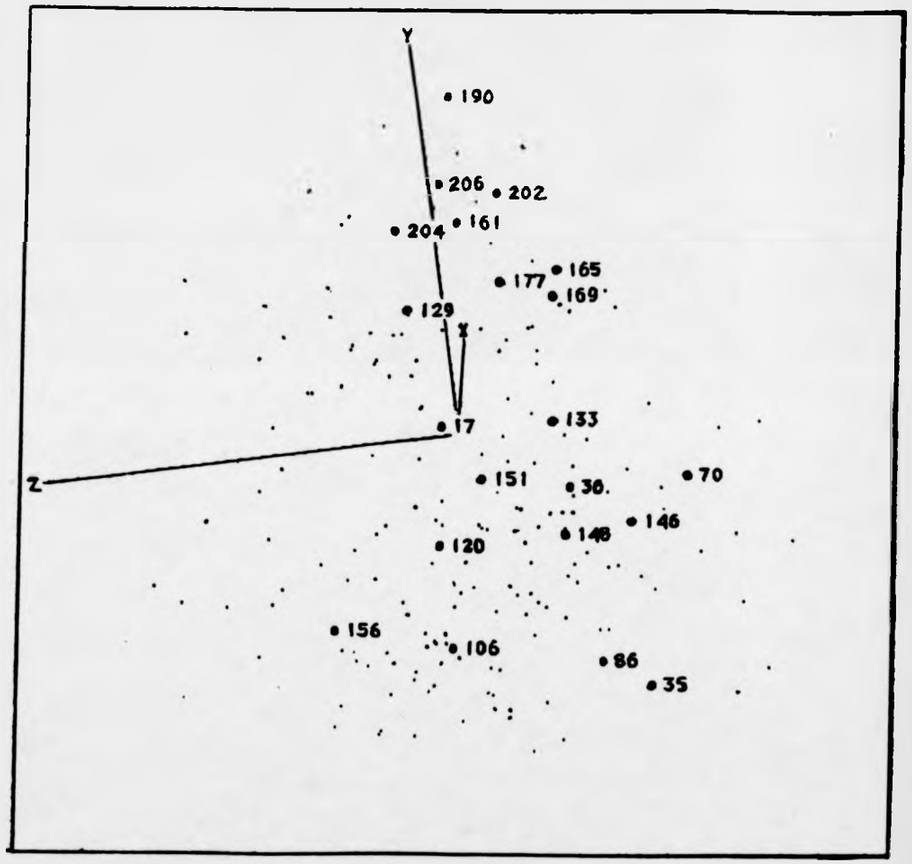
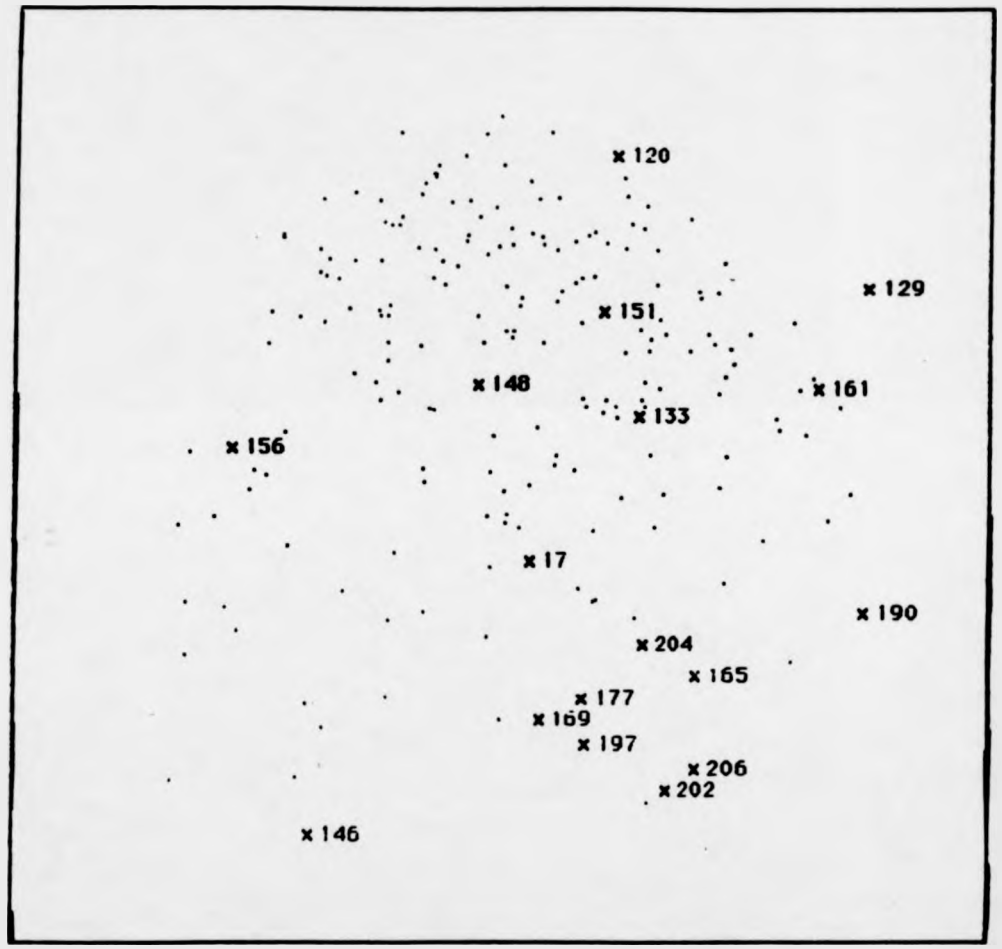


Fig. 5.10. Principle components analysis of TLC profiles, to show the position of nigericin producers.

The diagram is the same as Fig. 5.9., but is presented in a different orientation. It shows the position of a small group of strains, which produced both nigericin and geldanamycin.

The nigericin and geldanamycin producers are shown by x and a number, which refers to their position within the original data matrix.

FIG.5.10.



5.7. Recognising Interesting Strains Using TLC.

Table 5.7. Strains with rare spots.

Spot	No. Strains	Strain Names
17	4	C70 C129 D5 F89
18	6	C6 C11 C15 C33 C212 D153
19	10	C3 C4 C52 C70(2) C77(4) C108(2) C206(2) C314 C341 E8
20	6	C11 C141 C212 C314 C341 W98.1
21	3	C52 C203 F53
22	4	C141 D2 F53 F139
23	2	C77 C141
24	2	C70 D52
25	3	C11 C32 F89
26	1	C15
27	2	C208 C212
28	2	C208 C314
29	2	C222 F89
Total	47	29

The rare spots are numbered from 17 to 29 and the strains, whose extracts contained each of the rare spots is given in the last column with the number of replicates, which gave a spot in parentheses, if it was more than one.

Twenty-nine spots were scored in all, but thirteen of these were very rare (Table 5.7.)and were only observed in twenty-nine strains (NB. six of these strains

contained two rare spots, whilst a further six had three). Approximately 25% of the strains examined in Chapter 3 proved to be bioactive, compared with 45% of strains which could be selected because their extracts contained an unusual spot. There were two strains in this study, which produced compounds which might be novel agrochemicals (E8 and F139), although it is not known whether the rare spots comprised bioactive compounds. Zahner *et al* (1982) carried out a similar study and tested unique spots for biological activity. He found that 3 out of 5 compounds comprised novel antibiotics with slight antibacterial properties. The antibacterial activities observed in this work were not followed up, but six strains showed unidentified activities against *B.subtilis*.

5.7. Conclusions.

The errors observed in this study meant that the data collected were poorly reproducible and therefore were unsuitable for taxonomy. Hierarchical and non-hierarchical cluster analysis did not allow the delimitation of species-groups or the prediction of bioactivity. However, TLC was of possible use for predicting bioactive strains by choosing those with unusual spots.

CHAPTER 6

Relationship Between Antibiotic Resistance Phenotype and Biological Activities.

6.1.1. Introduction.

Selected *Streptomyces* species have distinctive antibiosis and resistance patterns, therefore resistance to antibiotics has been used for both classification (Wellington *et al.*, 1987) and selective isolation (Wellington *et al.*, 1990), but in general, antibiotic production and resistance is strain specific (Hotta *et al.*, 1983a and b, Okami and Hotta, 1988). Characterization by resistance to a wide variety of macrolide and aminoglycoside antibiotics has shown that many streptomycetes have individual patterns of multiple resistance. Fujizawa and Weisblum (1981) reported finding a diverse range of resistance phenotypes to macrolide, lincosamide and streptogramin antibiotics and Hotta *et al.* (1983a) showed a wide variety of aminoglycoside-resistance patterns in type strains and natural isolates.

A number of studies have reported that biosynthesis and resistance genes are genetically linked (Chater and Bruton, 1985; Murakami *et al.*, 1986; Skeggs *et al.*, 1987), but few studies have been concerned with the distribution of antibiotic production (Chapter 3) and resistance phenotypes or their corresponding genotypes in natural populations of streptomycetes. It is known that producers of auto-toxic compounds require self-defence mechanisms to secure survival against their own products (Cundliffe, 1986, 1989), but resistance mechanisms are not limited to such strains (Fujizawa and Wiesblum, 1981). For instance, resistance to kanamycin is exhibited by the producer, *S.kanamyceticus* (Murakami *et al.*, 1983, Cundliffe, 1986) and *S.griseus* (Hotta, 1988), *S.tenebrarius* (*Skeggs et al.*, 1987) and *S.tenjimariensis* (Skeggs *et al.*, 1986 and 1987), which are also aminoglycoside producers, but do not produce kanamycin. More strikingly, the non-producer *S.lividans* has an inducible rRNA methylase (Jenkins *et al.*, 1989) and a variety of

strains, which do not biosynthesize chloramphenicol are known to produce O-acetyltransferases, which detoxify this drug (Murray *et al.*, 1989).

The requirement for self-resistance determinants has been used in certain studies to demonstrate that strains which produce identical compounds can be grouped together on the basis of resistance to antibiotics of the same chemical family (Fujizawa and Weisblum, 1981; Hotta *et al.*, 1983a; Bibikova *et al.*, 1990). There is also evidence that novel compounds can be found by selecting strains with unusual resistance profiles and indolizamycin was discovered during protoplast fusion experiments because the resistance patterns of the producing strain differed from those of the two parental strains (Yamashita *et al.*, 1985b).

There may be a link between unusual resistance profiles and the production of specific secondary metabolites. If so, antibiotic-resistance profiles could be used to select strains capable of producing certain classes of biologically active compounds from natural populations. This work was aimed at testing this hypothesis and at evaluating the phenotypic diversity of streptomycete populations based on antibiotic-resistance profiles. Such information is also of ecological interest, since the survival of soil microorganisms depends upon their ability to find suitable niches in which they can grow and survive. Antibiotic production and resistance are specializations, which have been attributed with survival functions (Williams *et al.*, 1989). Expression of antibiotic resistance in soil isolates could have implications for the production of antibiotics in the natural environment and may reflect selection pressures, which influence *Streptomyces* species *in situ*. Other implications of the work concern the evolution of secondary metabolism and the dissemination of relevant genes. The distribution of a variety of antibiotic-resistance phenotypes was therefore determined within a selection of soil isolates.

6.1.2. Choice of Strains.

145 strains were chosen to represent a heterogeneous group of streptomycetes from different geographical locations and which were obtained using a variety of isolation methods (Tables 2.1 and 6.1).

Table 6.1. Natural isolates selected for the study of antibiotic resistance patterns.

Strain Series	No.Strains
A	7
B	1
C	58
D	26
E	6
F	2
MM	5
MEL	6*
CAG	22*
RB	3
JHCC	9*
Total	145

* The CAG, MEL and 5 of the JHCC strains were not used in the clustering described in section 6.4. This leaves 112 strains which were used in the cluster analysis.

A variety of type strains were chosen to represent a selection of known antibiotic producers and these are indicated in Table 2.2. Table 6.2. summarizes the

cluster groups (Williams *et al.*, 1983a) to which the type strains and any identified isolates belong. More details about identified isolates are given in Table 2.9.

Table 6.2. Taxonomic identity of strains used for analysis of antibiotic resistance.

Cluster Group	No. Type Strains	No. Isolates
C1	14 (1)	4 (3)
C6	1 (1)	0
C12	0	2 (2)
C15	0	2 (1)
C18	1 (1)	2 (0)
C19	2 (1)	8 (5)
C21	5 (5)	0
C32	9 (9)	10 (10)
C37	0	1 (0)
C44	1 (1)	0
C61	4 (1)	0
C64	1 (0)	0
C68	1 (1)	0
*S.*spp.	6 (4)	116 (91)
*Stv.*spp.	3 (1)	0
Total	48 (26)	145 (112)

Numbers in parenthesis refer to the number of strains used in the cluster analysis (Section 6.4). Details on individual strains are presented in Table 2.9. and the products of the type strains are given in Table 2.2., whilst the bioactivity of the natural isolates included in this study is presented in Table 3.1.

Results.

6.2. The Distribution of Antibiotic Resistance in Streptomycetes.

6.2.1. The Distribution of Antibiotic Resistance in Natural Isolates.

Figures 6.1 and 6.2. show the distribution of ten antibiotic resistances within a random population of *Streptomyces* isolates and type strains used for cluster analysis (Section 6.4.). There were two forms of distribution for antibiotic resistance within the population; increasing concentrations of some antibiotics elicited an exponential decrease, followed by a major change in gradient, where the resistant population became more constant and this identified populations of resistant and sensitive strains. The second type of resistance distribution occurred when antibiotics had a linear graph, showing a more gradual variation of phenotype. It was difficult to define resistant and sensitive populations for this type of distribution and so for the purposes of clustering an arbitrary value was assigned. Consequentially these data had a greater probability of giving an erroneous result (Table 6.4.). The most commonly occurring resistances within the population of isolates was also illustrated by Figs.6.1. and 6.2. Resistance to aminoglycosides was rare and only seen at low levels of the antibiotics, whilst penicillin, nigericin and oxytetracycline resistances were common.

The quantitative data were converted to qualitative data, using the distribution of each antibiotic resistance as a guideline. Strains, which showed a minimum inhibitory concentration before the elbow of a curve were designated as sensitive and those showing growth beyond it were called resistant. The extrapolated cut-off values were 2 μg/ml for neomycin, blasticidin, kanamycin and viomycin, 3.5 μg/ml for streptomycin, 5 ug/ml for thiostrepton, 15 μg/ml for nigericin, 20 μg/ml for novobiocin, 40 μg/ml for erythromycin, 45 μg/ml for oxytetracycline and

Fig. 6.1. The distribution of antibiotic resistance phenotypes within a population of *Streptomyces* natural isolates.

The graphs show the distribution of resistance to 8 antibiotics, within the random population of streptomycetes isolates, studied. The y axis shows the proportion of the population that were resistant, whilst the x-axis shows a range of antibiotic concentrations, which were extrapolated from the distance of mycelial growth, which occurred along antibiotic gradient plates.

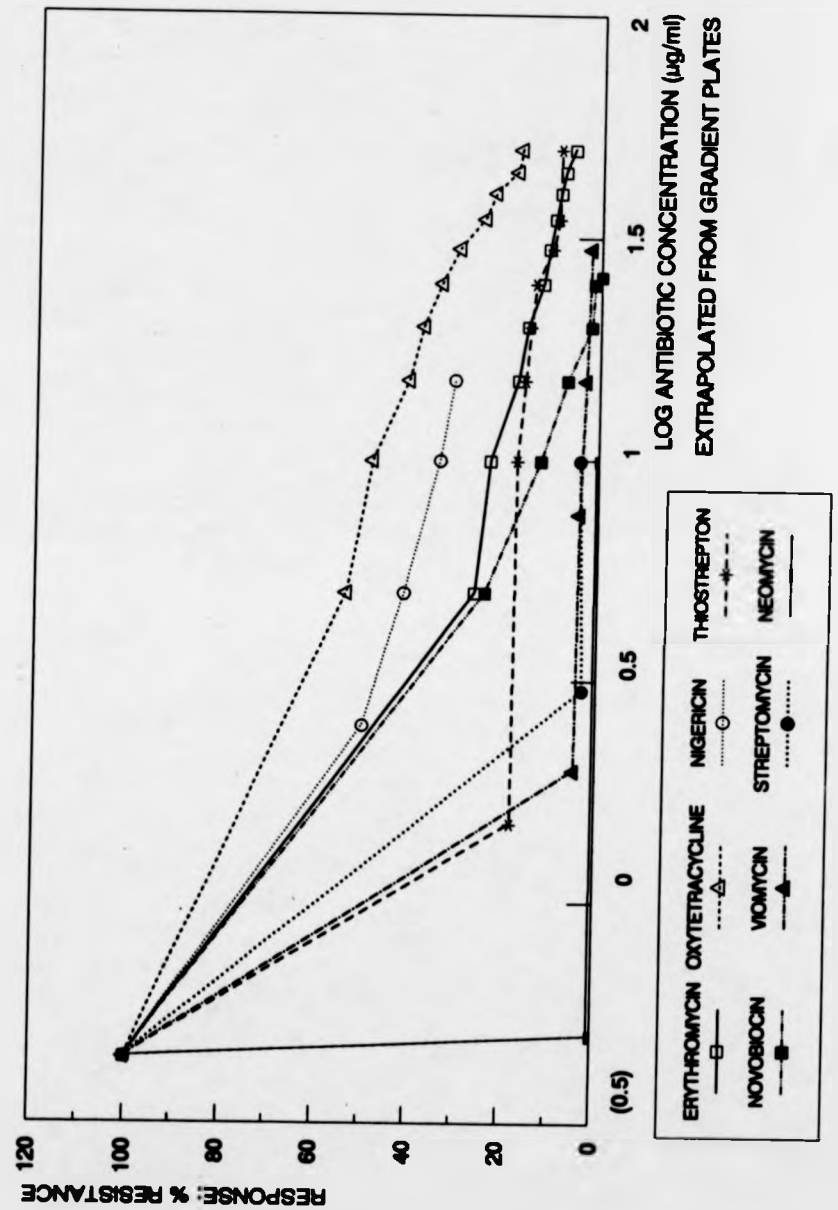
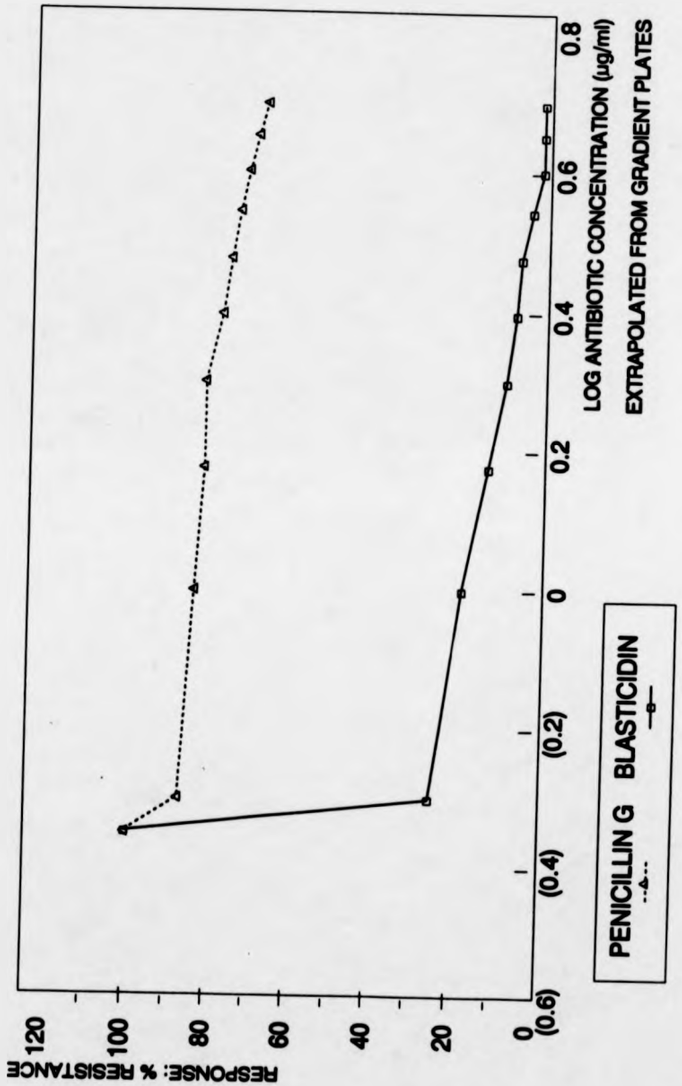


Fig. 6.2. The distribution of antibiotic resistance phenotypes within a population of *Streptomyces* natural isolates.

The graphs show the distribution of resistance to 2 antibiotics, within the random population of streptomycetes isolates, studied. The y axis shows the proportion of the population that were resistant, whilst the x-axis shows a range of antibiotic concentrations, which were extrapolated from the distance of mycelial growth, which occurred along antibiotic gradient plates.

NB. Kanamycin resistance is omitted from the diagram because it was only observed at a low level for a single replicate of one natural isolate.



95 μg/ml for penicillin, but these could also be given as distances of the lines of growth along the gradient (Section 2.7.1). Test error calculations, pattern analysis and clustering were carried out on these binary data.

Some of the extrapolated cut-off values determined for this work appeared to be at low antibiotic concentrations, but they reflected the distribution of antibiotic resistance and sensitivity within the population studied. A similar study to the above was carried out by Shaw *et al.* (1991), who correlated antibiotic-resistance patterns in clinical isolates with hybridization to resistance gene probes. In that study a cut-off-point between resistance and sensitive strains was determined by using known concentrations of antibiotics in disc susceptibility assays, where a minimum zone size was used as a guideline for delimiting the population.

6.2.2. The Diversity of Antibiotic Resistance Profiles.

Table 6.3. gives the profiles of resistance, which were observed for 193 different strains. The duplicates of each strain were merged into one profile by assigning a mismatch as positive arbitrarily (this was not done for the cluster analysis, where duplicated data were included). Although there are 4×10^7 (11!) theoretical antibiotic-resistance profiles, 83 strains exhibited unique patterns. whilst, a further 21 patterns were distributed amongst 110 different strains. One hundred and ninety strains contained at least one of the three most common antibiotic resistances and 25% isolates had a sensitive phenotype apart from showing resistance to penicillin.

Table 6.4. The distribution of antibiotic resistance patterns within a population of *Streptomyces* isolates.

The table shows 21 resistance profiles, which were present in more than one strain each, but there were also a wide variety of unique antibiotic resistance patterns.

Key:

+ means that resistance was shown at the cut-off concentration

- means that sensitivity was shown at the cut off concentration

Antibiotic Resistance	Pattern Type 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22 - 108
Blasticidin	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	Various
Erythromycin	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	+	+	-	+	-
Kanamycin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Neomycin	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-
Nigericin	-	+	-	+	-	-	+	+	-	-	-	-	-	+	-	+	+	+	+	+	+	+
Novobiocin	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
Oxytetracycline	-	-	-	-	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+
Penicillin G	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptomycin	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+
Thiostrepton	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	+	+
Viomycin	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	+	+	+	+	+	+
Number of strains	51	7	5	5	5	4	3	3	3	2	2	2	2	2	2	2	2	2	2	2	2	83

NB Total No. strains = 193

Total No. combinations, which are possible with 11 antibiotics = 4×10^7

6.2.3. A Comparison Between Resistance Phenotypes Observed on Complex and Defined Media.

Resistance was determined on antibiotic gradient plates made from AGS, which is a defined medium and is different to the complex medium A37, which was used to generate bioactivity. It was of interest to know how the expression of resistance would differ on a complex medium and therefore a solid form of A37 (15g/l agar) containing the antibiotic viomycin (in the gradients) was examined with the use of 64 duplicate strains from the above study. Results showed 4 resistant strains on A37; two of these had not shown resistance on AGS, whilst the others had grown to a greater distance along the A37 gradient. The latter might reflect differences in expression levels or differences in diffusion effects along the two types of gradient (see also section 6.3.3.)

6.3. Examination of Reproducibility and Test Error.

The data were assessed for suitability for use in cluster analysis, by determining error found within tests and duplicate OTUs.

6.3.1. Error Within Tests.

Table 6.4. shows the error found in each resistance test, calculated according to Sneath and Johnston (1972), from a total of 150 duplicates (138 had been used for the cluster analysis, whilst the additional 12 pairs comprised replicates of some of these strains). The mean test error fell below the recommended value of 5%. Although the test for penicillin resistance showed error above the 10% level of acceptability.

Table 6.4. Table to show the presence of error in antibiotic resistance tests.

Resistance	No. Discrepancies	Test Error %
Penicillin G	33	12.583
Nigericin	26	9.586
Blasticidin	19	6.795
Erythromycin	19	6.795
Oxytetracycline	19	6.795
Thiostrepton	11	3.812
Novobiocin	9	3.096
Viomycin	6	2.042
Streptomycin	5	1.695
Neomycin	4	1.352
Kanamycin	1	0.335

The error for the whole series of tests was 4.84

Error increased as the occurrence of an antibiotic resistance increased and there was also a high probability of error when there had been difficulty in assigning a cut-off concentration, which distinguished between resistant and sensitive populations (penicillin, nigericin, oxytetracycline). The strains responsible for the erroneous results were examined in detail (section 6.3.2.).

6.3.2. Examination of the Stability of Resistance in Strains Causing Erroneous Results in Antibiotic-Resistance Profiles.

Two types of discrepancy could occur. Either a strain showed resistance in both replicates of the test, but one of the duplicates fell below the level at which a strain was called resistant (section 6.2.1) or a strain was completely resistant in one

test and completely sensitive in another. Table 6.5. shows the distribution of discrepant results in 150 pairs of OTU's and gives the mean probability of error for the two types of discrepancy.

Table 6.5. The distribution of two types of discrepancy for antibiotic resistance profiles.

Resistance	Total Number	Discrepancy Around Cut-Off Point	Resistant Versus Sensitive
Penicillin	33	22	11
Nigericin	26	11	15
Blasticidin	19	9	10
Oxytetracycline	19	14	5
Erythromycin	19	11	8
Novobiocin	9	8	1
Thiostrepton	11	4	7
Neomycin	4	0	4
Streptomycin	5	1	4
Viomycin	6	0	6
Kanamycin	1	0	1
No Errors	152	80	72
P error	4.84	2.49	2.23

In an attempt to understand errors between duplicates further, the lowest level of resistance, for a discrepant pair, was subtracted from the highest resistance and the resulting value was divided by the highest concentration on the gradient plate. The resulting figure (the fraction of the gel, involved in the discrepancy) was used for a comparison of all discrepancies from all antibiotics. An average of 40.1% of the

gradient was involved in strains with a discrepancy about the cut-off point (Sd = 0.249). The mean % gradient involved in the second type of discrepancy was 51.3% (Sd = 0.326). The distribution of discrepancies amongst the strains was also examined and 17 strains with 3 or 4 anomalous results each, were highly variable with respect to expressing antibiotic resistance and they contained 35.5% of all errors. A further 20 strains, with 2 errors each, had 26.2% of the error. Minor discrepancies could be due to error in pouring gradient plates and differences in test conditions affecting antibiotic diffusion in the plates, whilst other anomalous results may have been due to differences in resistance and may have been caused by differential gene expression.

6.3.3. The Estimation of Minimum Inhibitory Concentrations from Antibiotic Gradient Plates.

Antibiotic in the top layer of a gradient plate might be expected to diffuse to the base layer in a complex manner, therefore some work was carried out to see how closely antibiotic concentrations estimated from gradient plates reflected antibiotic levels found in pour plates at the corresponding concentration. The results are shown in Table 6.7.

A log-dose response curve was produced for streptomycin and found to be linear from 1 μg to 10 mg (Fig 6.3.). This involved a filter paper assay and was intended to be used for the quantification of streptomycin in TLC extracts, rather than for use in this experiment and was therefore not done for the other antibiotics. However it may allow a rough idea of the extent to which antibiotic concentrations changed with differences in zone size (although diffusion from the plug onto the assay would be slower than from a filter paper disc and might retain more of the antibiotic). The zone size for 3.5 μg of streptomycin was 18.2 mm on the conventional log dose response curve, whilst 15.7 mm was given as the average zone size corresponding to 3.5 μg/ml in an agar plug (2 μg/ml on the log dose response curve). The zone size from the gradient plate was larger (17.3 mm) and

corresponded to 2.5 μg/ml on the log dose response curve. Some diffusion of antibiotic from the more concentrated to the less concentrated areas of gradient plates has taken place, but the extent of diffusion effects may differ along the gradient (perhaps with more downward diffusion at the lower concentration, but more sideways diffusion at the higher concentrations). The smallest and most polar antibiotics in this study were the aminoglycosides and one might therefore expect that any diffusion observed for other antibiotics would be less than for these; although more experimentation would be required before this could be assumed.

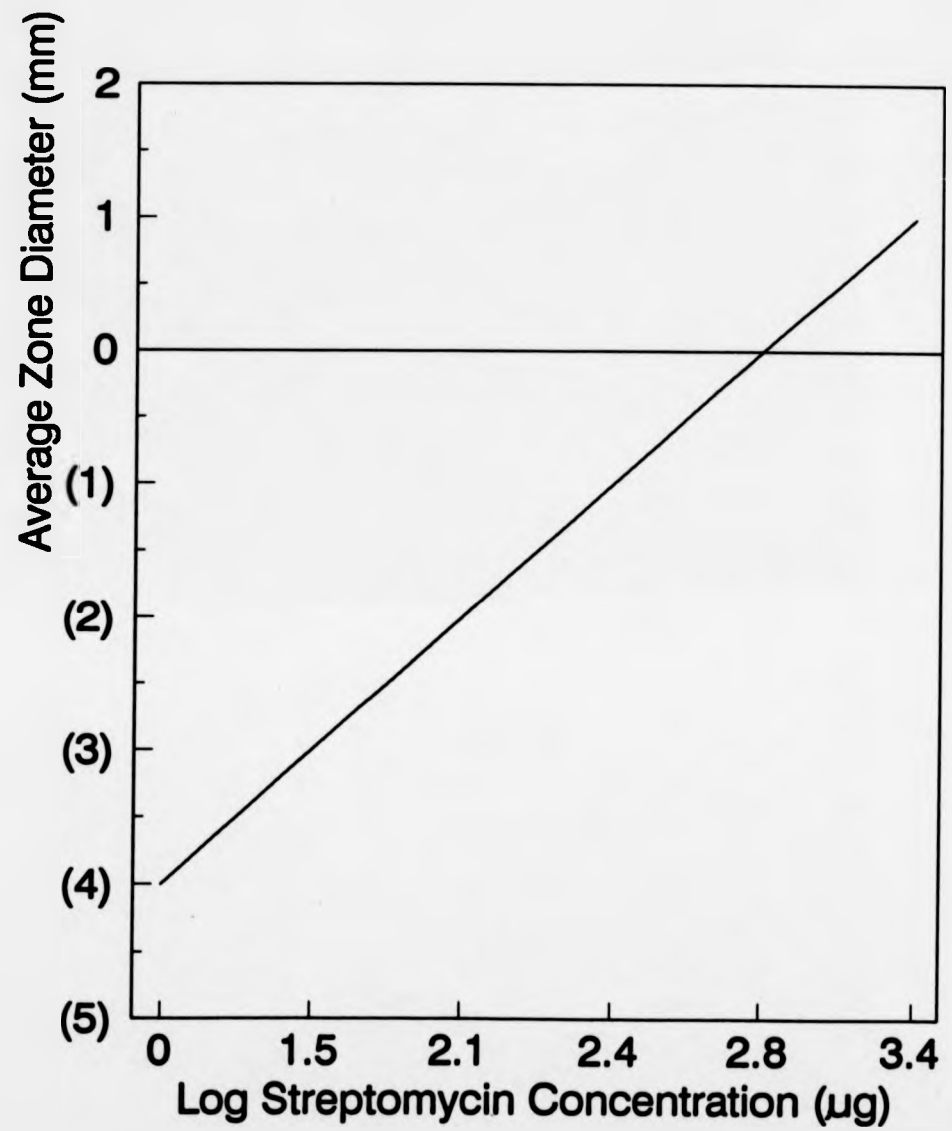
Table 6.6. Comparison of bioassay from antibiotic gradient and pour plates.

Antibiotic	Concent--ration (μg/ml)	Pour Plate mean diam. (mm)	Gradient Plate mean diam. (mm)
Thiostrepton	5	26	25
Neomycin	2	11	12
Kanamycin	2	13	16.7
Streptomycin	3.5	15.7	17.3

Agar plugs were removed from pour plates and gradient plates, containing known and estimated antibiotic concentrations; these were then compared by use of an inhibition assay (section .2.7.2.)

Fig.6.3. Log dose response curve for a streptomycin bioassay.

The graph shows a log dose response curve for streptomycin on filter paper discs. The graph was linear from 1μg to 10mg; the units for the zone diameters were in mm and the antibiotic concentrations in μg.



6.4. Hierarchical Clustering on Qualitative Resistance Profiles.

6.4.1. Comparison of Phenograms Formed Using Different Coefficients and Algorithms.

The nature of the data was carefully considered prior to clustering. It was concluded that a negative match was not always a true measure of similarity between strains, because it was possible that some strains were measured as sensitive when they were observed under incorrect conditions for expression of resistance. Consequentially a similarity coefficient, which did not consider negative matches was thought useful (eg. S_{dice} and S_j). Since duplicates were computed a mismatch might also be due to strain variability and so the Dice coefficient was chosen over Jaccard's because it weights against mismatches relative to matches and would prevent the underestimation of similarity values. The error calculated in section 6.3. relates to clustering using the simple matching coefficient, but could exert a greater effect on the clustering when using a coefficient which ignores negative matches. Clustering was therefore also done using the simple matching coefficient for comparison.

Figs. 6.4. and 6.5. show 4 phenograms made using the resistance data. The first two use S_{sm} and S_{dice} with UPGMA. Close examination of groupings showed that the same strains remained close relatives in each phenogram and although some group distortions were observed, there was good agreement with respect to observations made below (section 6.4.2.). Cophenetic correlation of the phenogram, which used Dice, gave a mantle statistic of 0.78, which showed reasonable agreement with the similarity matrix.

A comparison of different clustering algorithms was also made to minimise the chances of accepting misleading results. The phenograms of Fig. 6.5. show single and complete linkage with the Dice coefficient. Single linkage showed poorer group integrity, whilst complete linkage made groups which were more spread out, although

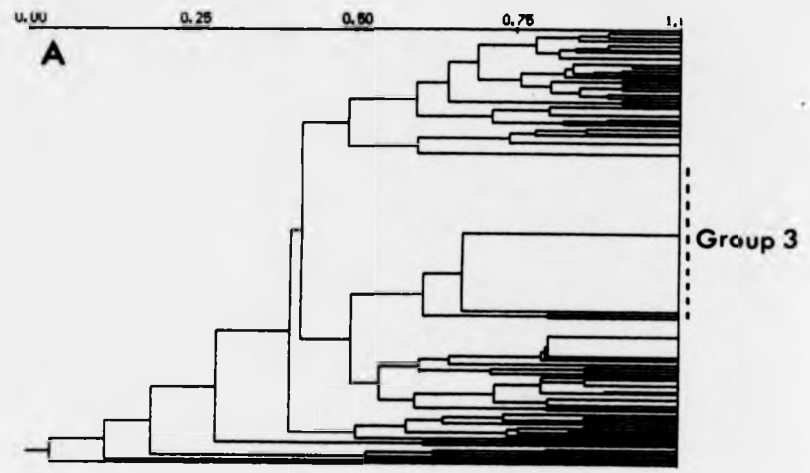
Fig. 6.4. Phenograms, which show relationships between natural isolates based on their antibiotic resistance profiles.

A shows a phenogram created using S_{dice} and UPGMA.

B shows a phenogram created using S_{sm} and UPGMA.

The position of group 3 is highlighted on both phenograms to illustrate that the trends, which are discussed in Section 6.4. were apparent in all of the phenograms studied.

FIG.6.4.



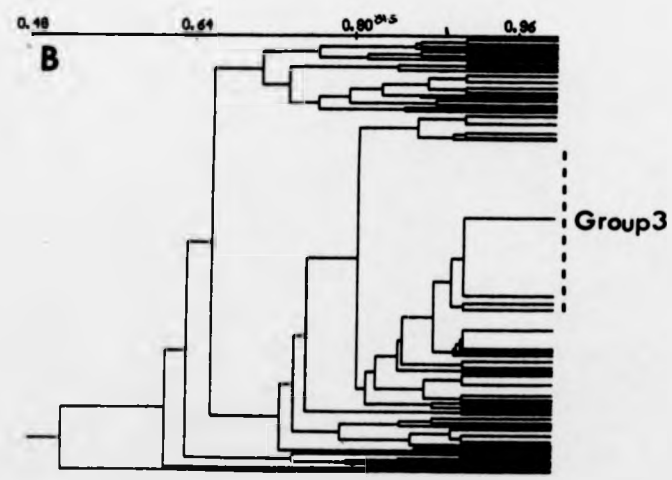
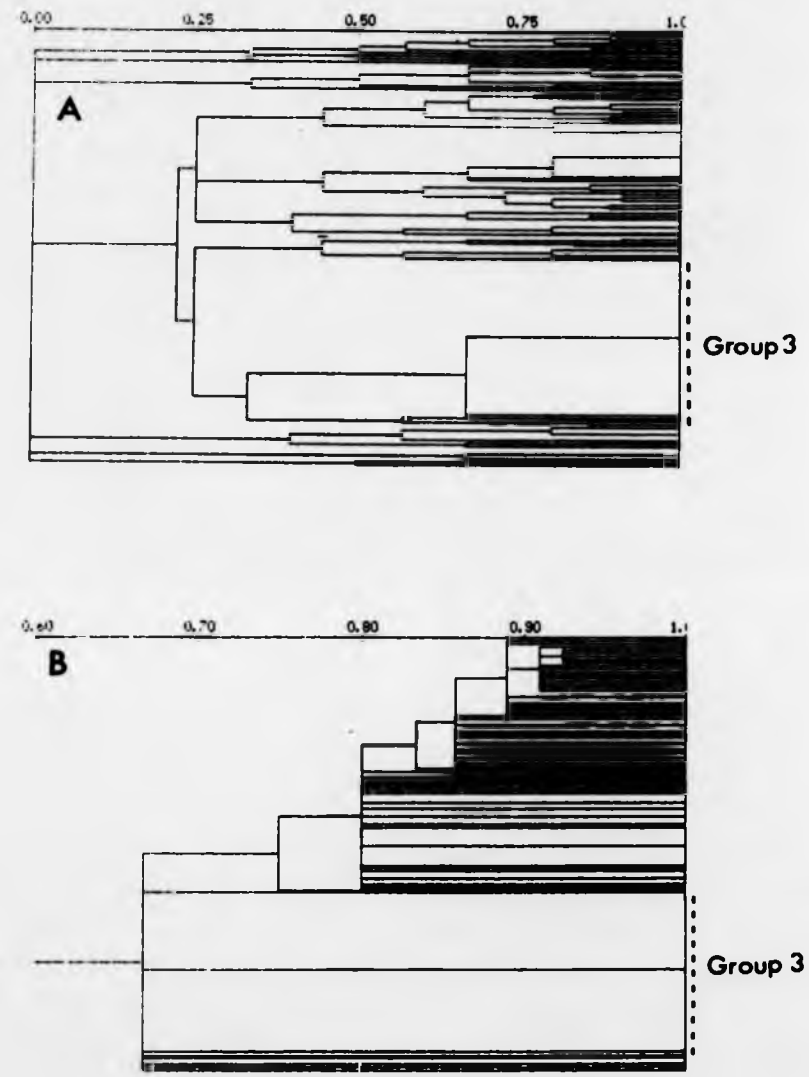


Fig. 6.5. Phenograms, which show relationships between natural isolates based on their antibiotic resistance profiles.

A shows a phenogram created using S_{dice} and Complete Linkage.
B shows a phenogram created using S_{dice} and Single Linkage.

The position of group 3 is highlighted on both phenograms to illustrate that the trends, which are discussed in Section 6.4. were apparent in all of the phenograms studied.

FIG. 6.5.



there was still good agreement with respect to observations made below (section 6.4.2.). The phenogram, which was formed, using average linkage was chosen to present results (Fig. 6.6.), but it is emphasised that all phenograms allowed the delimitation of clusters with differing bioactivity in terms of agrochemical interest (Fig. 6.7.).

6.4.2. The Distribution of Bioactive Strains in the Major and Minor Clusters.

The strains formed 5 major and 5 minor clusters at 51% similarity (Fig. 6.6.). When the distribution was examined with respect to bioactivity (Fig. 6.7.), cluster 3, which contained 37.59% of all strains in the study was comprised mostly of the strains which did not produce biologically active compounds. Those which it did contain were all natural isolates comprising a nonactin producer (C208), which has since been shown to have novobiocin resistance, a producer of either cycloheximide, nonactin or nigericin and two strains which produced antibacterial compounds which were inactive on agrochemical screens. All antibiotic-producing type strains were distributed outside cluster 2. There was no correlation between the class of antibiotic a strain produced and the group to which it clustered.

6.4.3. The Distribution of Resistance Phenotypes in the Major and Minor Clusters.

Table 6.8. illustrates how groups of strains in cluster 3 were phenotypically less resistant than the other groups of strains. The main resistance exhibited by strains in this cluster was penicillin, the most commonly observed resistance for the population (Figs. 6.1. and 6.2.). Penicillin resistance was present in large numbers of strains in all major clusters, but was rare in the minor clusters (6-10). It did not contribute to the separation of bioactive from non-bioactive strains, but its rarity in the less similar clusters (6-10) showed that penicillin sensitivity could be useful in identifying unusual strains.

Fig. 6.6. Phenogram to show the relationships of *Streptomyces* strains based on their antibiotic-resistance phenotypes.

The diagram illustrates the 10 groups of strains, which were defined at 51% similarity. It also shows how the phenogram could be divided into 4 areas.

Area 1 comprised clusters 1 and 2, which contained strains with multiple resistance, many of which produced antibiotics.

Area 2 comprised cluster 3 and antibiotic production was not observed in strains which clustered to this group

Area 3 comprised clusters 4 and 5, which contained strains with multiple resistance, many of which produced antibiotics.

Area 4 comprised the 5 minor clusters (6-10) and contained strains with the more unusual or least similar profiles.

FIG.6.6.

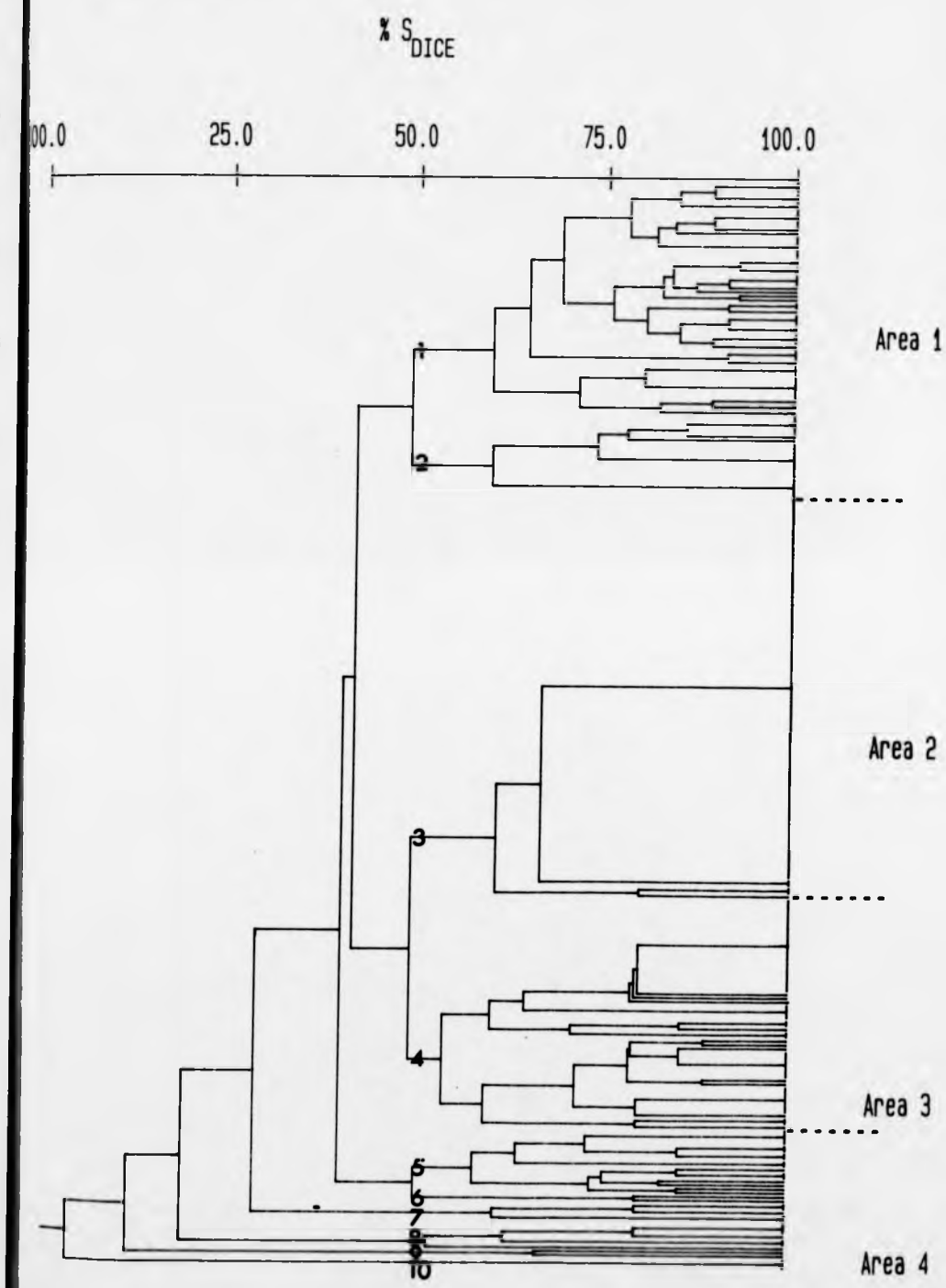


Fig. 6.7. The distribution of multi-resistant and biologically active strains across a phenogram based on antibiotic resistances (Fig. 6.6).

The diagram is a stacked bar chart and illustates the percentage of strains, which produced antibiotics and the percentage of strain, which were resistant to 4 or more antibiotics. Obviously a strain can have both attributes and the height of the bars can exceed 100%.

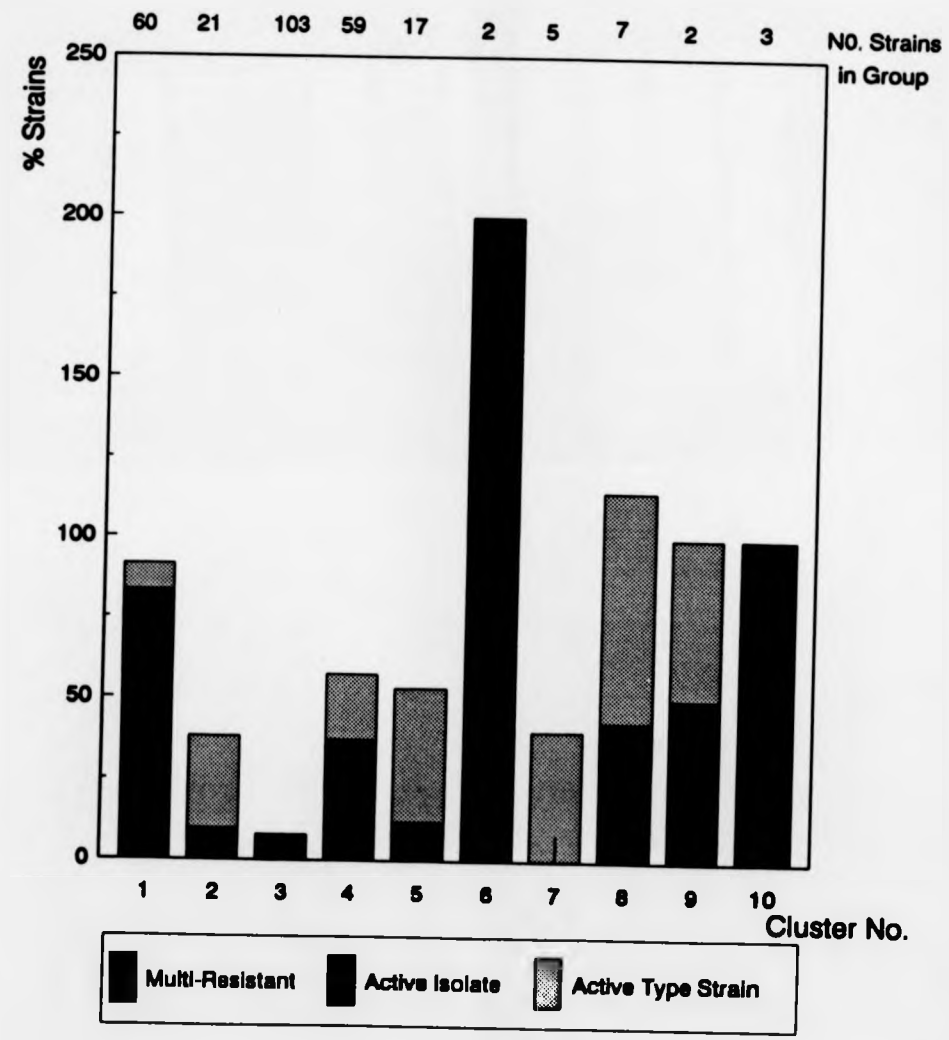


Table 6.7. The distribution of antibiotic resistance in the major and minor clusters.

Cluster (No.)	% Strains with Resistance to: Thio	Strep	Vio	Pen	Blast	Nig
1(60)	35.0	10.0	11.7	76.7	88.3	76.7
2(21)	4.8	71.4	0.0	33.3	0.0	0.0
3(103)	0.0	0.0	2.9	100.0	0.0	1.0
4(59)	35.6	1.7	3.4	83.1	0.0	86.4
5(17)	41.2	5.9	11.8	100.0	35.3	17.7
6(2)	50.0	0.0	0.0	0.0	100.0	0.0
7(5)	20.0	100.0	0.0	60.0	40.0	0.0
8(7)	14.3	71.4	0.0	42.9	28.6	0.0
9(2)	100.0	0.0	0.0	0.0		0.0
10(3)	0.0	0.0	0.0	0.0	0.0	0.0
Mean	19.0	5.0	6.8	87.8	24.0	40.4
SD	18.7	4.2	4.3	23.3	36.6	39.5

Mean values were calculated from the % strains, which were resistant within each cluster group and took into account the number of strains, present in each group.

Key:

Thio = Thiostrepton
Strep = Streptomycin
Vio = Viomycin
Pen = Penicillin G
Blast = Blasticidin
Nig = Nigericin
Novo = Novobiocin
Ery = Erythromycin
Oxyt = Oxytetracycline
Neo = Neomycin
Kan = Kanamycin

Table 6.7. The distribution of antibiotic resistance in the major and minor clusters.

Cluster (No.)	% Strains with Resistance to: Novo	Ery	Oxyt	Neo	Kan
1(60)	23.3	33.3	71.7	0.0	0.0
2(21)	100.0	0.0	0.0	0.0	0.0
3(103)	1.9	0.0	0.0	0.0	0.0
4(59)	13.6	3.4	5.1	5.1	1.7
5(17)	0.0	100.0	11.8	5.9	0.0
6(2)	0.0	100.0	0.0	0.0	0.0
7(5)	0.0	40.0	20.0	0.0	0.0
8(7)	0.0	28.6	14.3	0.0	0.0
9(2)	0.0	0.0	50.0	0.0	0.0
10(3)	33.3	0.0	0.0	0.0	0.0
Mean	0.0	13.4	15.8	1.4	0.4
SD	25.6	26.7	28.6	0.5	0.7

The remaining groups contained a variety of antibiotic resistances and each cluster had a specific profile, which was responsible for its separation from the others. All nine clusters, which contained bioactive strains were distinguished from cluster 3 by containing strains with resistances other than penicillin. The 5 minor clusters (6-10) comprised smaller ranges of resistance than clusters 1, 2, 4 and 5 and contained strains with the most unusual profiles. The milbemycin producer (A1002) was the only producer of a xenotoxic compound in the study. It grouped to cluster 8, showing it had a very different resistance profile.

6.4.6. The Use of Resistance Profiles to Aid the Selection of Strains for Screening.

Four of the strains included in the study had individual activity spectra that indicated the presence of potentially novel compounds and which warranted chemical characterization. This level of interest was typical for the sample size and the screens used. In order to test the hypothesis that antibiotic-resistance profiles could help select strains for screening, a selection of 7 new strains were put through the clustering system. These strains also produced compounds with possible novel agrochemical activity. The results showed that all of the 7 new strains and the 4 original strains had multiple patterns of between 4 and 7 antibiotic resistances (mean = 5.7). Consequentially all of these strains exhibit resistance characteristics which would place them in an area of a phenogram that would reinforce their selection as worthy candidates for screening.

Strains with multiple resistances appeared to be more likely to produce bioactive metabolites and so besides the 21 antibiotic producing type strains included in the phenogram, an additional 21 antibiotic producing type strains were also examined for antibiotic resistance. 81.4% of these had 3 or more resistances and 48.8% had 4 or more. The number of resistances ranged from 1-10 and the mean was 4.2 (Sd = 2.3). Four type strains had only one resistance, but the resistance was not one, which could place them in cluster 3. Consequentially all antibiotic-producing type strains would be selected for screening. A total of 6 type

strains with no reported product were also examined and these also showed multiple antibiotic resistance.

6.4.7. The Distribution of Taxonomically Identified Strains Across the Phenogram.

Fig 6.8. shows the distribution of *Streptomyces* clusters as defined by Williams *et al.* (1983a), across the phenogram (Fig 6.4). It is apparent from this diagram that conventional taxonomy bears no general relationship to antibiotic resistance profiles. Table 6.8. shows the average profile for 4 clusters as defined by Williams *et al.* (1983a). It includes strains shown on the phenogram plus additional strains.

A good distinguishing character would be one which was present in 80% or more of strains in one group and 20% or less in another group. Only clusters 1, 19, 21 and 32 had sufficiently large sample sizes for comments to be made.

Streptomycin resistance distinguished cluster 1 streptomycetes from clusters 19, 21 and 32. Streptomycin resistance has previously been shown to be common in cluster 1 streptomycetes (Wellington and Cross, 1983). Cluster 32 streptomycetes differed to clusters 19 and 21 by their resistance to nigericin. Many more C32s showed resistance to nigericin, but it was below the cut-off point for binary data. Many C32s produce nigericin and some of these producers were scored as sensitive in the binary data.

Fig. 6.8.The distribution of *Streptomyces* clusters as defined by Williams *et al.* (1983a) across the phenogram (Fig 6.4).

The diagram shows the distribution of 8 *Streptomyces* species groups (Williams *et al.*, 1983a) within 10 groups, which were defined at a similarity level of 51% on Fig. 6.4.

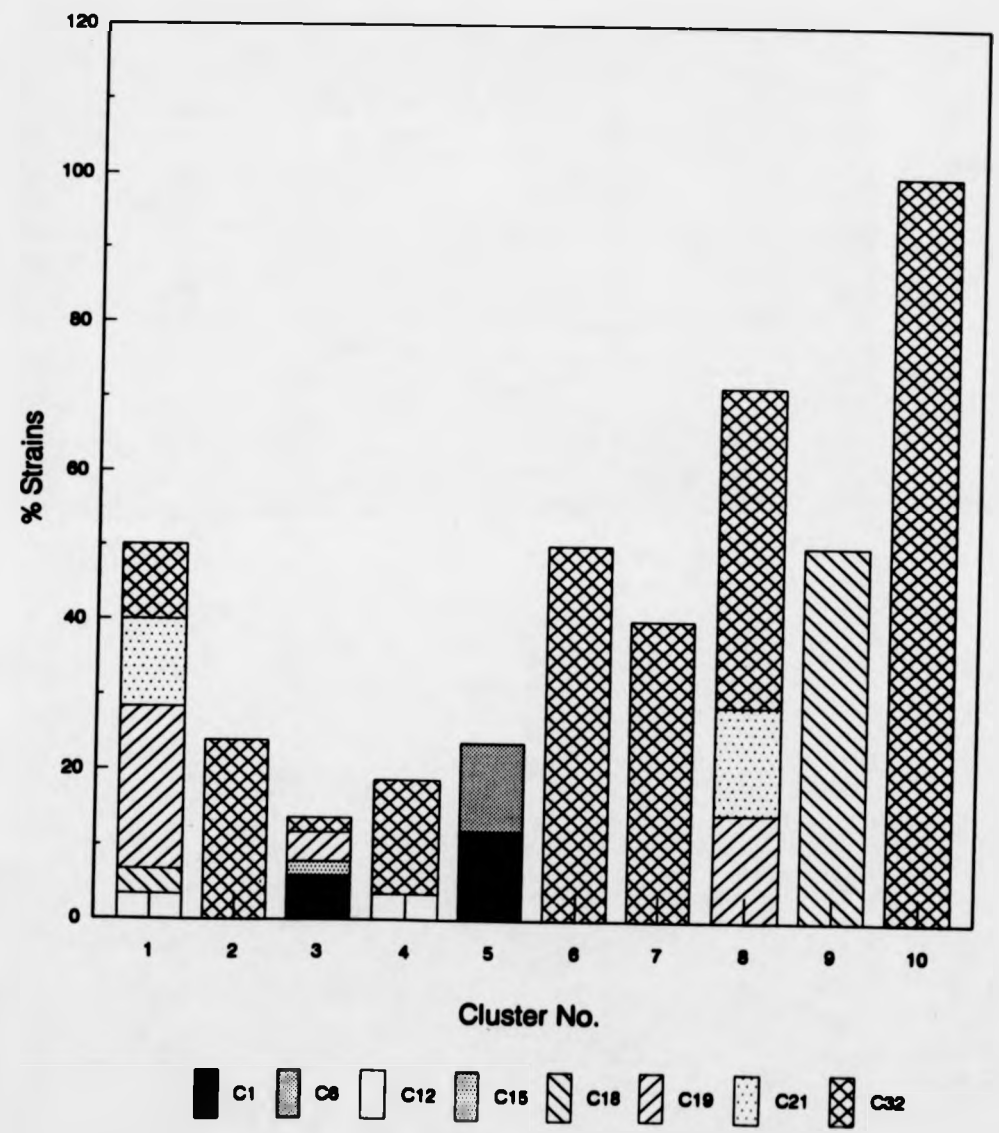


Table 6.8. The distribution of antibiotic resistance in *Streptomyces* species.

Cluster (No)	Thio	Neo	Novo	Vio	Strep	Nig
32 (19)	15.8	5.3	31.6	10.5	10.5	20.1
1 (18)	50.0	27.8	38.9	61.1	83.3	61.1
19 (10)	40.0	10.0	60.0	20.0	20.0	80.0
21 (5)	20.0	0.0	60.0	20.0	20.0	80.0

Cluster (No)	Pen G	Oxyt	Ery	Blast	Kan
32 (19)	57.9	20.1	15.8	26.3	0.0
1 (18)	88.9	44.4	44.4	0.0	5.5
19 (10)	90.0	60.0	30.0	50.0	0.0
21 (5)	100.0	40.0	20.0	80.0	20.0

The table shows the distribution of antibiotic resistance within four cluster groups, which were defined by Williams *et al.* (1983a). The figures in parentheses refer to the number of strains, which were involved in the calculation.

6.5. Relationship Between Phenetic Resistance and Antibiotic Production.

Subsequent to screening for antibiotic resistance a search was made for producers of streptomycin, thiostrepton, novobiocin and blasticidin amongst the resistant isolates. This was done to determine if resistance was confined to producers of the relevant compounds because if this was not the case then the observation of a general relationship between multiple antibiotic resistance and bioactivity, above, was reinforced.

If any specific relationships were found between specific antibiotic producers and bioactivity then this would also be of interest, since one of the problems of drug discovery is that novel compounds can be produced in conjunction with known broad spectrum antibiotics and this can mean that the novel compounds remain unobserved if their spectrum of activity is similar to that of the known antibiotic. For example nigericin is a potent antibiotic produced by many streptomytcetes and many nigericin producers also synthesize other bioactive compounds. A second problem is the rediscovery of known compounds. It would be of value to predict both novel and known broad spectrum compounds prior to screening. The relationship between nigericin producers and their antibiotic resistance profile is discussed below. Table 6.9. summarises the results discussed.

Table 6.9. To show the number of producers of specific antibiotics found in this study.

Antibiotic	No Resistant Strains Tested for Production	No. Producers Found	Total No. Resistant Isolates at any conc.
Blasticidin	39	1	47
Geldanamycin	*	9	*
Nigericin	96	18	96
Novobiocin	11	1?	41
Streptomycin	7	1 (+1)	15
Thiostrepton	7	0	24

The table shows how many of the strains which were phenotypically resistant to one of 6 antibiotics were also found to produce that antibiotic. The full complement of resistant strains was not always tested and so the final column refers to the total number of strains tested. The figure in parenthesis refers to a sensitive strain, which was thought to produce streptomycin.

* A small pilot study to develop gradient plates for the detection of geldanamycin failed to find sensitivity to this compound in 25 randomly selected strains. A larger study was therefore not undertaken. Although not proven to be ubiquitous, resistance to this compound was believed to be highly common. Blanco *et al.* (1986) have shown that certain other producers of RNA polymerase inhibitors, show resistance to geldanamycin at a variety of different levels. For example *Streptomyces lydicus*, which produces streptolydigin, was only resistant to geldanamycin up to 25 μg/ml. This level was within the range of concentrations tested in the pilot study for geldanamycin resistance.

6.5.1. Nigericin Producers.

Nigericin producers did not cluster to a specific region in any of the phenograms studied because each nigericin producer had a unique antibiotic-resistance profile. In order to investigate their distribution across the phenograms and to assess its predictive value, clusters which contained more than 2 SD above the mean number of nigericin producers were selected. 17 strains (64% nigericin producers in the study) were detected by using both single and complete linkage with the Dice coefficient. When using this system an additional 12 strains which did not produce nigericin were diagnosed as being possible nigericin producers.

An alternative means of identifying the producers of specific compounds would be to use resistance profiles based on a single subfamily of compounds as in the work of Hotta *et al.*, (1983), who found that specific aminoglycoside resistance profiles could be used to predict the aminoglycoside products of various strains. Similarly, resistance profiles to a range of polyether antibiotics might prove predictive for strains which produce specific polyether compounds.

Nigericin resistance was not useful in predicting production because although 15% of strains produced it, 40% of strains were resistant to it. Not all of the confirmed nigericin producers showed resistance to this compound above the cut-off level. This may reflect a difference in the type of resistance mechanism favoured by nigericin-producing strains compared to non-producers. Alternatively, the cut-off could have been selected at an inappropriate antibiotic concentration, due to the shape of the graph or range of concentrations used or else the work carried out on an inappropriate medium for the expression of nigericin resistance in some strains.

6.5.2. Producers of Other Compounds.

From 7 natural isolates, which were resistant to streptomycin, one (D147) appeared to produce the compound. Another strain (C11) which was sensitive to streptomycin also appeared to produce this compound. One of the novobiocin

resistant strains was identified as a possible producer of the compound and no thiostrepton producers were found. Similarly, blasticidin resistance did not seem to be an indicator of the production of blasticidin or of any other nucleoside. Of 39 blasticidin-resistant strains analysed, only one strain was a blasticidin producer (JHCC a type strain supplied by ICI).

6.6. Discussion.

Examination of the resistance and bioactivity data of the total collection of strains gave a clear indication that there were two populations of strains; one with multiple resistance and the other with multiple sensitive phenotypes. This may be a reflection of the natural population and so provided an insight into the distribution of antibiotic resistance and biosynthesis genes in the natural environment. If strains which do and do not express antibiotic production under laboratory conditions, showed similar trends for gene expression in the natural environment, then one might expect those which expressed antibiotic production to have a survival advantage. It is then interesting to try to understand why strains which do not express antibiotic production and resistance have managed to survive alongside producing strains. Perhaps antibiotic production does not occur in the natural environment, although there is some evidence to suggest that this is possible (Demain, 1981 and 1984; Rothrock and Gottleib, 1984; Martin and Demain 1980). Otherwise, these strains may have developed other specializations, which are advantageous to survival and might therefore inhabit different microniches than antibiotic producers. These could be separated in either time (the organism might have a specialization, which allows them to proliferate at a different stage in the decomposition cycle) or space (the organism might survive well in microniches, which have extreme environmental conditions).

The clustering data supported the observations of others that a correlation exists between multiple resistance and antibiotic production (Hotta *et al.*, 1983a).

An observation made in this study and that of Fujisawa and Weisblum (1981) is that antibiotic resistance may not be confined to producers of these compounds. Although, it may be that some strains exhibiting resistance possess a silent form of the antibiotic production genes. This is also interesting in ecological terms if reflected in the natural environment. Such strains might represent opportunists, able to share nutrient pools with antibiotic producers, without expending energy on antibiotic production. This distribution of this type of strain may be important for understanding the selection pressures, which are present in the soil, if antibiotics are present. If not, one wonders why such genes are present in non-producers; perhaps they have evolved to carry out other functions or else represent gene transfer events, which then proved useful to the organisms.

Resistance profiles obtained using a range of diverse antibiotics were useful for predicting bioactivity. Reasons for this could be due to resistance being linked with the production of a closely related compound. For for example, Skeggs *et al.* (1987) have shown that single aminoglycoside-resistance determinants can confer resistance to several different 2-deoxystreptamine derivatives, namely kanamycin, apramycin and gentamycin. Resistance may be linked to another class of compound, perhaps with a similar mode of action. An example of this is the *erm* gene encoding for an RNA methylase, which simultaneously confers resistance to a selection of compounds from three antibiotic families; macrolides, lincosamides and streptogramin B's (Fujizawa and Weisblum, 1981; Jenkins et al.,1989; Zalacain and Cundliffe, 1989). This type of mechanism would also explain certain patterns of multiple resistance. Other patterns of multiple resistance may be due to strains having an array of different resistance determinants. It is already known that some antibiotic producers have several self-resistance determinants (Rosteck et al., 1991), and that others have supplementary genes, which confer resistance to foreign products (Skeggs *et al.*, 1986 and 1987; Hotta *et al.*, 1988). Although in these cases the foreign compounds are chemically structurally and biosynthetically related to the product.

Exceptions to the trends seen during this work might be better understood by considering the role of antibiotics in the natural environment. Certain antibiotic resistances in some strains may be present to confer protection against the metabolites of other streptomycetes. For example, organisms which produce nigericin and penicillin are commonly isolated from the soil. If these compounds were produced in the natural environment this could result in a selection pressure for antibiotic resistance in streptomycetes. The large numbers of penicillin- and nigericin-resistant strains observed in this study provide evidence for this hypothesis.

Alternatively a gene conferring resistance could do so fortuitously, having an entirely different function in the host. For example some streptomycetes have two pathways of ammonium assimilation and these organisms are resistant to the herbicide bialaphos, the target of which is glutamine synthetase. Also some resistance genes may not be expressed under laboratory conditions.

These data have lead to many interesting questions about the role of secondary metabolism in the natural environment. It also presented an ideal opportunity to find out more about the genetic and mechanistic nature of antibiotic resistance. By relating antibiotic resistance and sensitive phenotypes to genotype, it was hoped that we might come closer to understanding populations of streptomycetes in natural ecosystems.

Chapter 7.

The Distribution of Sequences Hybridizing to Selected Antibiotic Resistance Gene Probes and their Correlation with Phenotypic Resistance.

7.1. Introduction.

Antibiotic resistance has been used with other phenetic characters to group members of the *Streptomyces* genus (Williams et al., 1983a), but species-groups formed in this way have been shown to be heterogeneous at the DNA level (Labeda and Lyons, 1991) and by 16S rRNA analysis (Stackebrandt *et al.*, 1991). Although selected *Streptomyces* species do have distinctive antibiosis and resistance patterns (Wellington and Cross, 1983), there is generally little correlation between antibiotic production and taxonomic groupings.

Reasons for the poor correlation between conventional taxonomy, which mainly relates to primary metabolism, and antibiotic resistance may include the diversity of resistance mechanisms, which exist in nature (Tables 1.2., 1.3. and 1.4.). For example, producers of the same antibiotic may sometimes rely on different mechanisms for self-defense. Mosher *et al.* (1990) showed that the chloramphenicol resistance gene of *S.venezuelae* did not hybridize to the DNA of *S.phaeochromogenes*, although both strains produced this antibiotic. Other antibiotic producers, such as *S.fradiae*, the tylosin producer, contain several resistance determinants (four in this case) for the inactivation of a single product (Rosteck *et al.*, 1990). Antibiotic resistance has also been found in non-producers (Fujizawa and Weisblum, 1981; Jenkins *et al.*, 1989) and also in antibiotic producers against foreign metabolites (Skeggs *et al.*, 1986 and 1987; Cundliffe, 1987). Some resistance determinants confer resistance to more than one antibiotic in the same chemical family (Skeggs *et al.*, 1987) and others confer resistance to compounds which belong to different chemical classes, but have similar modes of action. For

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example the *erm* gene confers resistance to antibiotics from three chemical classes of antibiotic (Fujisawa and Weisblum, 1981; Jenkins *et al.*, 1989; Zalacain and Cundliffe, 1989). Multiple antibiotic resistance is also an established phenomenon (Fujizawa and Weisblum, 1981; Hotta *et al.*, 1983a and b; Phillips *et al.* in press) and patterns of aminoglycoside or macrolide antibiotics have been shown to correlate with the production of an antibiotic of the same chemical class.

Detailed studies have been carried out on the biosynthesis and genetics of specific antibiotic producers and the regulatory, resistance and production genes of a range of species have been found to exist in clusters on their chromosomes (Chater and Bruton, 1985; Malpartida and Hopwood, 1986; Weber *et al.*, 1990). In addition, the biosynthesis pathways of many antibiotics have been elucidated (Stanzak *et al.*, 1986; Binnie *et al.*, 1989; Pratt, 1989) and certain resistance genes have been shown to play a role in antibiotic production (Murakami *et al.*, 1986). Some workers have related the occurrence of resistance genes to the presence of homologous sequences within producers of the same or related antibiotics; for example, homology to *ermE* has been found in macrolide-producing *Saccharopolyspora* strains, but is not present in macrolide producers from other genera (Stanzak *et al.*, 1990). Similarly, homology to a streptomycin-resistance gene probe was specific to streptomycin-producing strains of *S.griseus*, whilst the kanamycin resistance determinant of *S.griseus* was found to be species specific (Hotta *et al.*, 1988). Virtually nothing is known about how such genes and antibiotic resistance mechanisms are distributed within natural populations in the soil.

Chapter 6 described the grouping of *Streptomyces* strains according to phenotypic antibiotic-resistance patterns. Two distinct populations were observed; one comprised strains with multiple and rare resistance phenotypes and many of these produced antibiotics, whilst the other contained strains with multiple antibiotic sensitivity and did not express antibiotic production. These data provided an opportunity to investigate the genetic basis of the resistance patterns and therefore

was investigated for analysing these profiles further. Determining the distribution of sequences which hybridize to portions of cloned antibiotic resistance genes provides a first step for establishing the frequency with which specific genes occur in the natural environment and may assist in understanding the natural role of secondary metabolism. The probes used are referred to as resistance gene probes, although it is understood that these determinants can also be involved in antibiotic biosynthesis.

7.1.1. Strain Choice.

Seventy-four *Streptomyces* type strains were used for this study and these are indicated in Table 2.2 and summarized in Table 7.1. They represented many species groups (Williams *et al.*, 1983a and b) and produced a variety of antibiotics of different biochemical origin. Certain groups of strains were chosen from culture collections because there was evidence that they might light up a specific probe and these included a variety of cluster 1, 5 and 32 strains (Williams *et al.*, 1983a). The latter were selected because this group has many bioactive members (Arai *et al.*, 1976), cluster 1s were chosen to examine their relationship to the streptomycin resistance gene probe and the cluster 5 streptomycetes were chosen for probing with the bialaphos-resistance determinant because a strain of *Kitasatosporia* was known to produce phosalacine.

One hundred and two natural isolates were selected from strains studied in Chapter 6, including strains with multiple resistance and organisms with phenotypic resistances which might correlate with some of the gene probes used. A small selection of sensitive strains were also chosen. This study was aimed not only at finding more about the distribution of specific resistance determinants in phenotypically resistant strains, but also in antibiotic producers. All strains which produced potentially novel compounds were included as were strains which produced a selection of known antibiotics. Table 2.2. shows the products of the type strains in the study, whilst Table 3.1. shows the bioactivity of the natural isolates.

Table 7.1. Taxonomic identity (Williams *et al.* 1983a and b) of strains used in the Probing Study.

Cluster	Number of Strains Types	Number of Strains Isolates	Cluster	Number of Strains Types	Number of Strains Isolates
1	15	0	32	10	9
3	0	1	37	0	1
5	16	0	42	2	0
6	1	1	44	1	0
7	1	0	55	2	0
12	0	2	61	3	0
15	0	1	64	1	0
18	2	1	68	1	0
19	2	8	S.M.C.	1	0
21	6	0	*S.*spp	9	78
30	1	0			

Key:

S.M.C. means single member cluster group.

Cluster refers to *Streptomyces* groups which were defined by Williams *et al.* (1983a)

7.1.2. Probes and Controls Used in this Work.

DNA probes used in this work are presented in Chapter 2 (Table 2.4). Internal fragments were excised from the appropriate plasmids (Table 2.3) using the restriction enzymes given in Table 2.4. Positive controls were chromosomal DNA from the *Streptomyces* strains from which the original genes were isolated (Table 2.4), but additional positive controls were provided by loading 1 ng of the plasmid

containing the restriction fragment onto the filter. DNA from *Streptomyces lividans* 66 (John Innes 1326), *Erwinia carotovora* (SCRI193) and salmon sperm (Boehringer) was selected as negative controls.

7.2. Interpretation of Data and Evaluation of Test Error.

One aim of the project was to give information on the likelihood that phenotypically resistant strains contained specific antibiotic-resistance genes (so they could be prioritized for future studies). This was assessed using highly stringent conditions and by choosing strains whose DNA samples gave a signal near to that of the positive control. A second aim of this research was to use results from DNA probing experiments as systematic information and for this work all signals which were stronger than the negative controls (usually no signal) were used. These constraints were also used when scoring positives at lower levels of stringency.

Random primed labelling (Feinberg and Vogelstein, 1983) was used to create radioactive DNA for probing and so the size of the hot DNA fragments varied in length. This meant that small highly conserved motifs could bind to labelled DNA at very high stringency, even though they did not indicate the presence of the entire probe sequence. This type of binding may account for some of the weaker signals observed at high stringency and may or may not indicate the presence of sequences which were related to the appropriate gene probe. The high G+C content of *Streptomyces* DNA makes reiterations of small sequences more likely than in organisms which have a more even distribution of nucleotides in their DNA and it is therefore possible that some small homologous motifs which caused positive signals bore no relationship to the gene probes which were used.

The washing conditions were varied by using permissive and stringent salt concentrations so that gene sequences of varying levels of relatedness might be indicated. The specific level of homology varied depending upon the G+C content of the DNA sequence used for probing (Table 7.2).

Table 7.2. The levels of homology, which relate to hybridization to specific gene probes under various conditions of stringency.

Gene	G+C% of Probe	Washing Conditions (0.1% SDS +) 3xSSC	1xSSC	0.2xSSC
aphD	68.55	66.86	74.82	87.43
bar	68.76	66.78	74.74	86.34
vph	76.0	63.81	71.77	83.37
aph	76.18	63.74	71.7	83.3
tsr	59.98	70.6	78.34	89.9
Mean	69.89	66.32	74.28	85.88
Average for *Streptomyces* DNA:	73	65.04	73	84.6

The above data were calculated from the following equation:

$Tm = 81.5 + 16.6 (\log M) + 0.41 (G+C\%)$

Tm was the melting temperature and M was the molarity of the salt solution

A 1° change of temperature was assumed to be equivalent to a 1% change in homology.

The sequence of the novobiocin-resistance determinant is not known, therefore a G+C content of 73%, (Hopwood *et al.*, 1985) has been assumed.

Positive signals declined as expected when stringency was increased from 3xSSC and 0.1% SDS to 0.2xSSC and 0.1% SDS (Table 7.3.). Fig. 7.1. gives an example of various stringent washes, after probing with *aphD*. A grid is provided showing the positions of each strain and the figure legend indicates which strains were scored positive for hybridization with the gene probe (replicate filters, in the same grid format, were used in the other experiments using different probes). The probability of non-specific binding within specific samples, which was due to protein contamination of DNA samples was another possible cause of weak signals, but was thought to be low because only 1.13% of strains hybridized with all six probes at high stringency.

It was possible to evaluate operator error for dot blots, which were probed with *aphD* at the lowest stringency and which were developed, both using the phosphorimager and by autoradiography. Autoradiography was the most common method and spots were read by eye, whilst the phosphorimager allowed the spot intensities to be quantified. An intensity, which was stronger than the count for the negative controls and which resulted in the same percentage positive strains as autoradiography was chosen from the phosphorimage (2000). Comparison of results from the two methods indicated that the P erroneous result (Sneath and Johnston, 1973) was 11.02%. Unfortunately it was not possible to do a similar calculation for other filters or at other levels of stringency, therefore the P erroneous result for the whole of the data is not known.

Fig. 7.1. (A and B); Dot blots, showing results from probing with *aph*D.

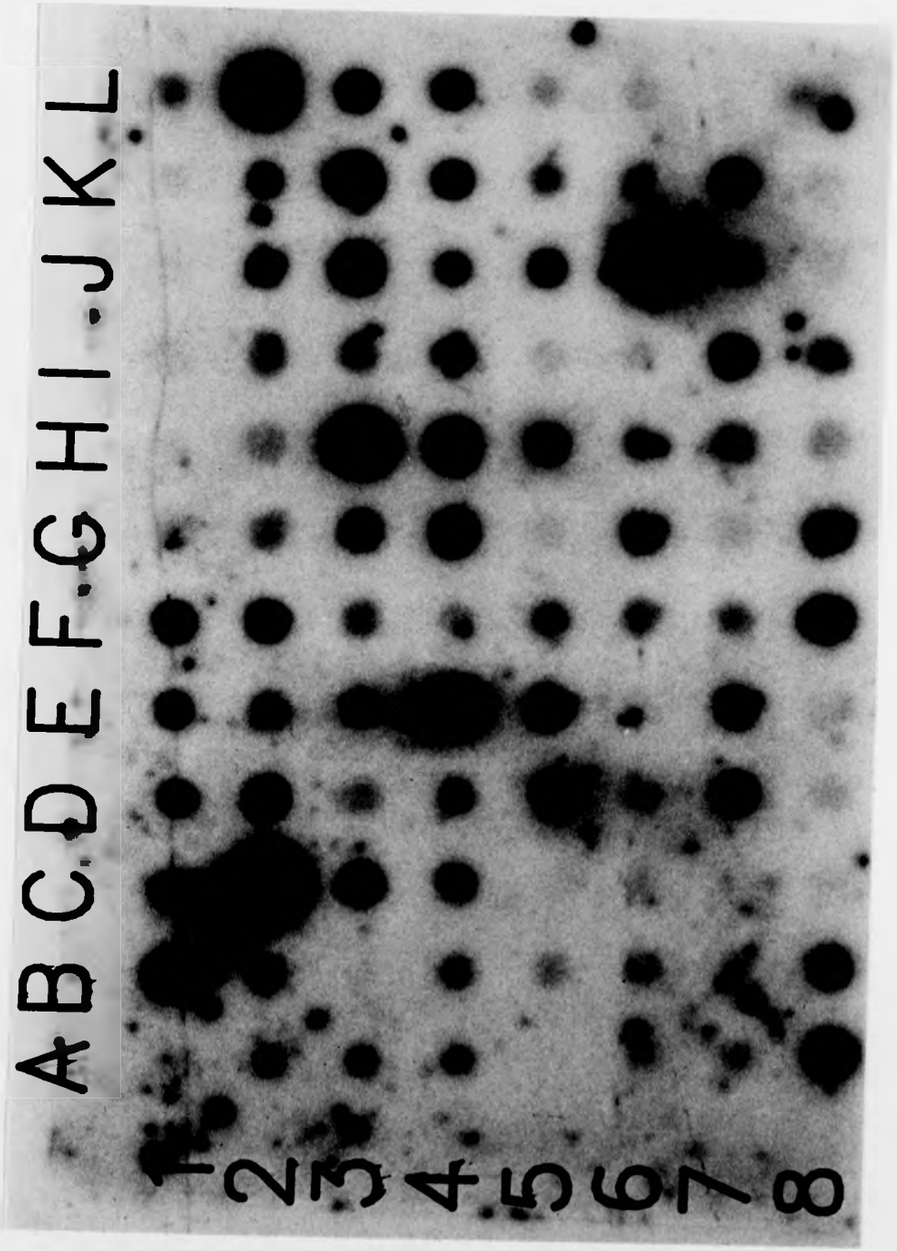
The photographs show one filter, which has been probed using *aph* at various levels of stringency.

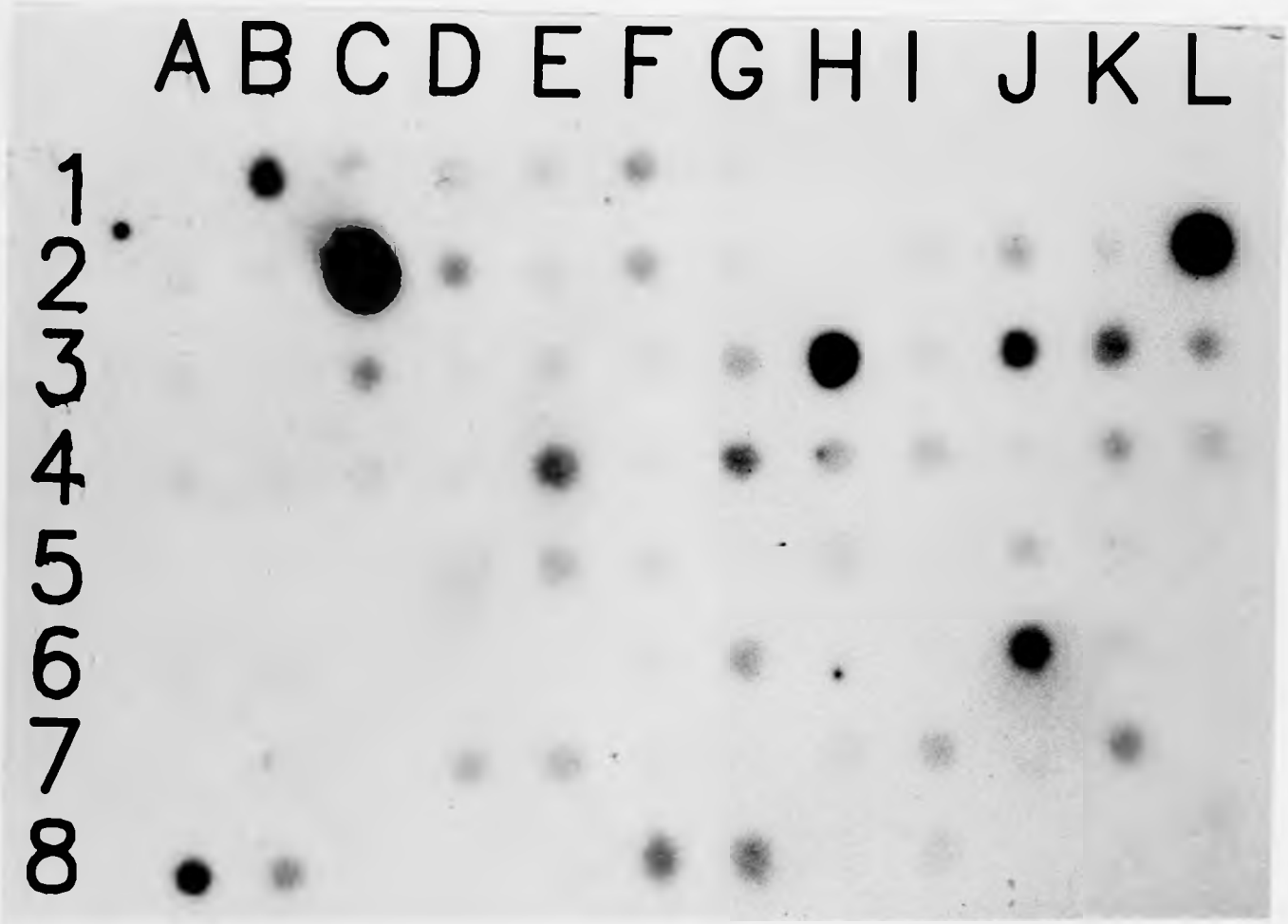
A shows an autoradiograph of the filter after washing with 3 x SSC and 0.1% SDS.

B shows an autoradiograph of the filter after washing with 1 x SSC and 0.1% SDS.

The following strains correspond to the dots and the figures in parenthesis illustrate how each strain was scored for the purposes of cluster analysis:
A1, DSM4112 (0); A2, ISP5060 (11); A3, ISP5174 (11); A4, ISP5016 (11); A5, ISP5064 (0); A6, ISP5022 (11); A7, ISP5278 (111); A8, ISP5122 (111); B1, ISP5196 (11); B2, ISP5556 (11); B3, ISP5329 (0); B4, DSM40023 (11); B5, E35 (0); B6, DSM40077 (0); B7, ISP5069 (11); B8, ISP4213 (111); C1, DSM40232 (11); C2, ATCC12475 (111); C3, ISP5246 (111); C4, KCC S-0313 (11); C5, KCC S-0331 (0) C6, DSM40323 (0); C7, KCC S-0783 (0); C8, DSM40419 (0); D1, 419 (11); D2, DSM40438 (111); D3, DSM40455 (1); D4, KCC S-0446 (11); D5, KCC S-0459 (11); D6, KCC S-0495 (0); D7, DSM40508 (111); D8, KCC S-0519 (0); E1, ISP5550 (11); E2, DSM40598 (11); E3, KCC S-0731 (111); E4, KCC S-0772 (111); E5, KCC S-0785 (111); E6, KCC S-0850 (0); E7, C463 (111); E8, NRRL3664 (0); F1, JHCC1319 (111); F2, JHCC1236 (111); F3, JHCC1390 (11); F4, JHCC1234 (11); F5, 4-739 (11); F6, 734 (1); F7, NRRL B-16130 (0); F8, NRRL B-16185 (111); G1, 734-A (1); G2, AM3672 (11); G3, A10 (111); G4, A19 (111); G5, A26 (1); G6, A39 (111); G7, A73 (0); G8, B4 (111); H1, C3 (0) H2, C5 (0); H3, C11 (111); H4, C14 (111); H5, C15 (11); H6, C20 (11); H7, C23 (11); H8, C24 (1); I1, C32 (0); I2, C38 (11); I3, C91 (111); I4, C92 (111); I5, C129 (0) ; I6, C151 (0) ; I7, C155, (11) ;I8, C167 (11) ;J1, C158 (0) ; J2, C184 (111) ; J3, C186 (111) ; J4, C189 (111) ; J5, C195 (11) ; J6, C212 (111) ; J7, C159 (11) ; J8, C222 (0) ; K1, C227 (0) ; K2, C229 (111); K3, C245 (111); K4, C284 (111); K5, C271 (11); K6, C337 (11); K7, C402 (111); K8, D5 (0); L1, D104 (0); L2, D147 (111); L3, D153 (111); L4, E1 (111); L5, Salmon Sperm DNA (1); L6, JI1326 (0); L7, SCR119 (0); L8, F53 (111).

A

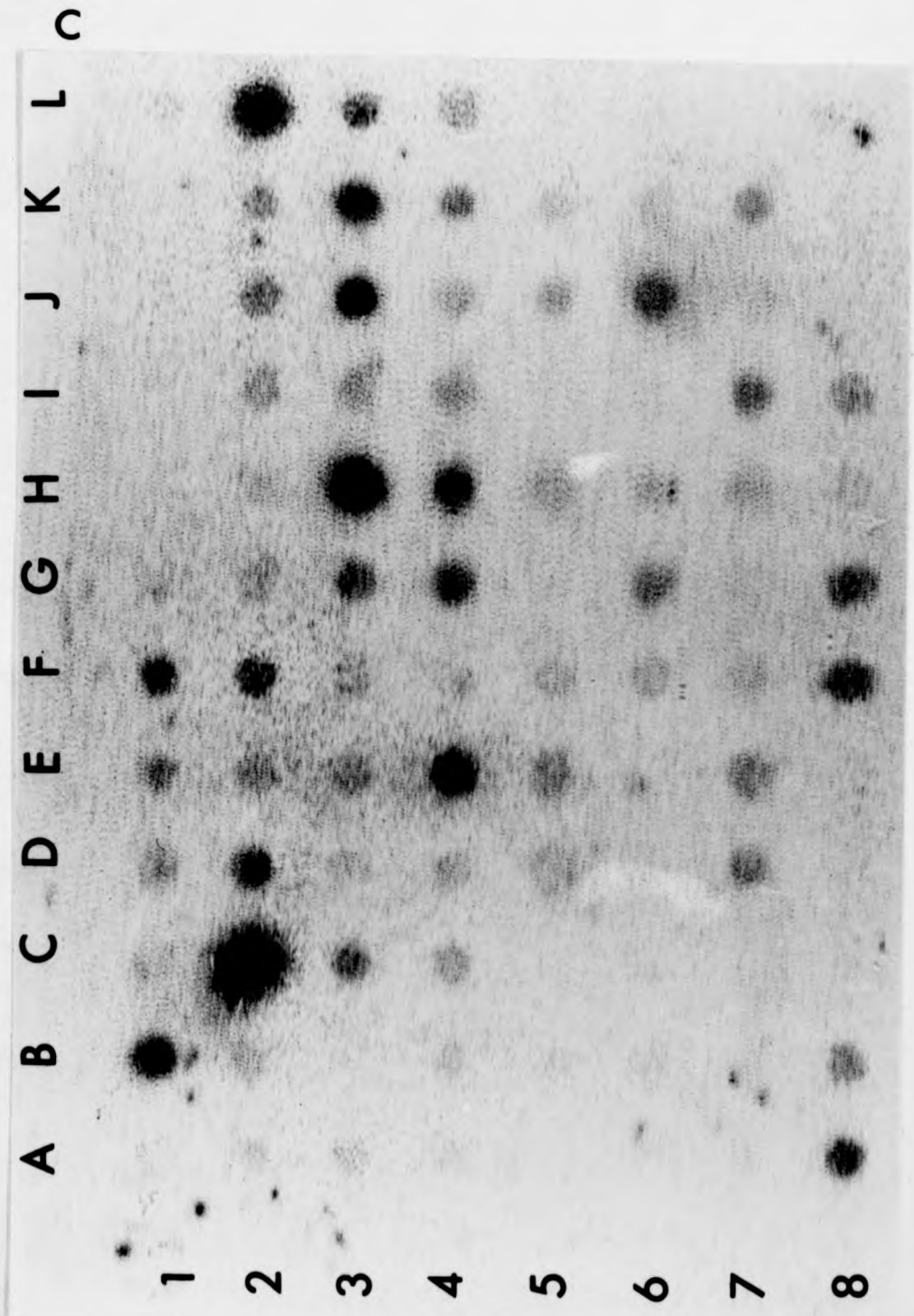




B

Fig. 7.1.C. Dot blot produced using the phosphorimager, where sample DNA was hybridized with a streptomycin resistance gene probe (*aphD*), overnight at 70°C in hybridization solution and washed twice with 3 x SSC and 0.1% SDS for 30 minutes (equivalent to 65% homology, assuming G+C = 73%). DNA from the following strains was probed and the phosphorimager signals (arbitrary units) are given in parenthesis.

A1, DSM4112 (2493); A2, ISP5060 (3095); A3, ISP5174 (2104); A4, ISP5016 (1913); A5, ISP5064 (265); A6, ISP5022 (2434); A7, ISP5278 (1712); A8, ISP5122 (8451); B1, ISP5196 (17338); B2, ISP5556 (4340); B3, ISP5329 (1401); B4, DSM40023 (2439); B5, E35 (1243); B6, DSM40077 (1787); B7, ISP5069 (3182); B8, ISP4213 (3472); C1, DSM40232 (6793); C2, ATCC12475 (96865); C3, ISP5246 (5679); C4, KCC S-0313 (3662); C5, KCC S-0331 (1086) C6, DSM40323 (1701); C7, KCC S-0783 (1508); C8, DSM40419 (140); D1, 419 (3338); D2, DSM40438 (6576); D3, DSM40455 (2271); D4, KCC S-0446 (3276); D5, KCC S-0459 (5581); D6, KCC S-0495 (2967); D7, DSM40508 (5305); D8, KCC S-0519 (256); E1, ISP5550 (3971); E2, DSM40598 (4170); E3, KCC S-0731 (5636); E4, KCC S-0772 (16490); E5, KCC S-0785 95848); E6, KCC S-0850 (2060); E7, C463 (4790); E8, NRRL3664 (545); F1, JHCC1319 (6469); F2, JHCC1236 (5534); F3, JHCC1390 (6469); F4, JHCC1234 (2499); F5, 4-739 (2553); F6, 724 (2134); F7, NRRL B-16130 (1986); F8, NRRL B-16185 (6815); G1, 734-A (2393); G2, AM3672 (2906); G3, A10 (5406); G4, A19 (8362); G5, A26 (1171); G6, A39 (5135); G7, A73 91548); G8, B4 (5604); H1, C3 (1248) H2, C5 (2550); H3, C11 (42567); H4, C14 (15060); H5, C15 (5023); H6, C20 (3058); H7, C23 (3407); H8, C24 (1475); I1, C32 (1131); I2, C38 (2755); I3, C91 (3490); I4, C92 (4070); I5, C129 (959.4) ; I6, C151 (1969) ; I7, C155, (6322) ;I8, C167 (3701) ;J1, C158 (1165) ; J2, C184 (4305) ; J3, C186 (14422) ; J4, C189 (2684) ; J5, C195 (3459) ; J6, C212 (17048) ; J7, C159 (6000) ; J8, C222 (2715) ; K1, C227 (1556) ; K2, C229 (5001); K3, C245 (13885); K4, C284 (4425); K5, C271 (1749); K6, C337 (3987); K7, C402 (6224); K8, D5 (1406); L1, D104 (1638); L2, D147 (81129); L3, D153 (5335); L4, E1 (3424); L5, Salmon Sperm DNA (735.1); L6, JI1326 (1186); L7, SCR119 (251.5); L8, F53 (2145).



The use of replicate filters and replicate DNA samples could have helped with data interpretation and the estimation of test error. This was not done, since it was planned that positive results would be confirmed by carrying out Southern blot analysis (work in progress; Neil Cresswell and Leisa Huddleston, Department of Biological Sciences, University of Warwick). It is emphasised that positive results were used as an indication of homologous sequences to the gene probes, since only sequence analysis can provide information on their precise nature. It could also have been possible to prepare Southern blots from the outset, as in the study of Hotta *et al.* (1988), however this would not have been suitable for the screening of large numbers of strains and the study would have been more limited. The aim was to study a large population of strains, using several gene probes and to observe trends within that population. It was accepted that dot blots would contain more erroneous results than Southern blots. Information from this initial study can now be used to initiate more detailed studies concerning the nature specific positive signals and also of alternative resistance mechanisms.

7.3. The Distribution of Sequences, which Hybridized to Antibiotic Resistance Gene Probes.

7.3.1. The Distribution of Sequences, which Hybridized to Various Antibiotic Resistance Gene Probes.

Table 7.3. shows that the most common hybridizations at high stringency were to the streptomycin and neomycin phosphotransferase gene probes and the least common was the bialaphos resistance fragment. Fig 7.2. gives a graphical representation of this data where differences in the levels of homology, indicated by the stringency washes, have been taken into account.

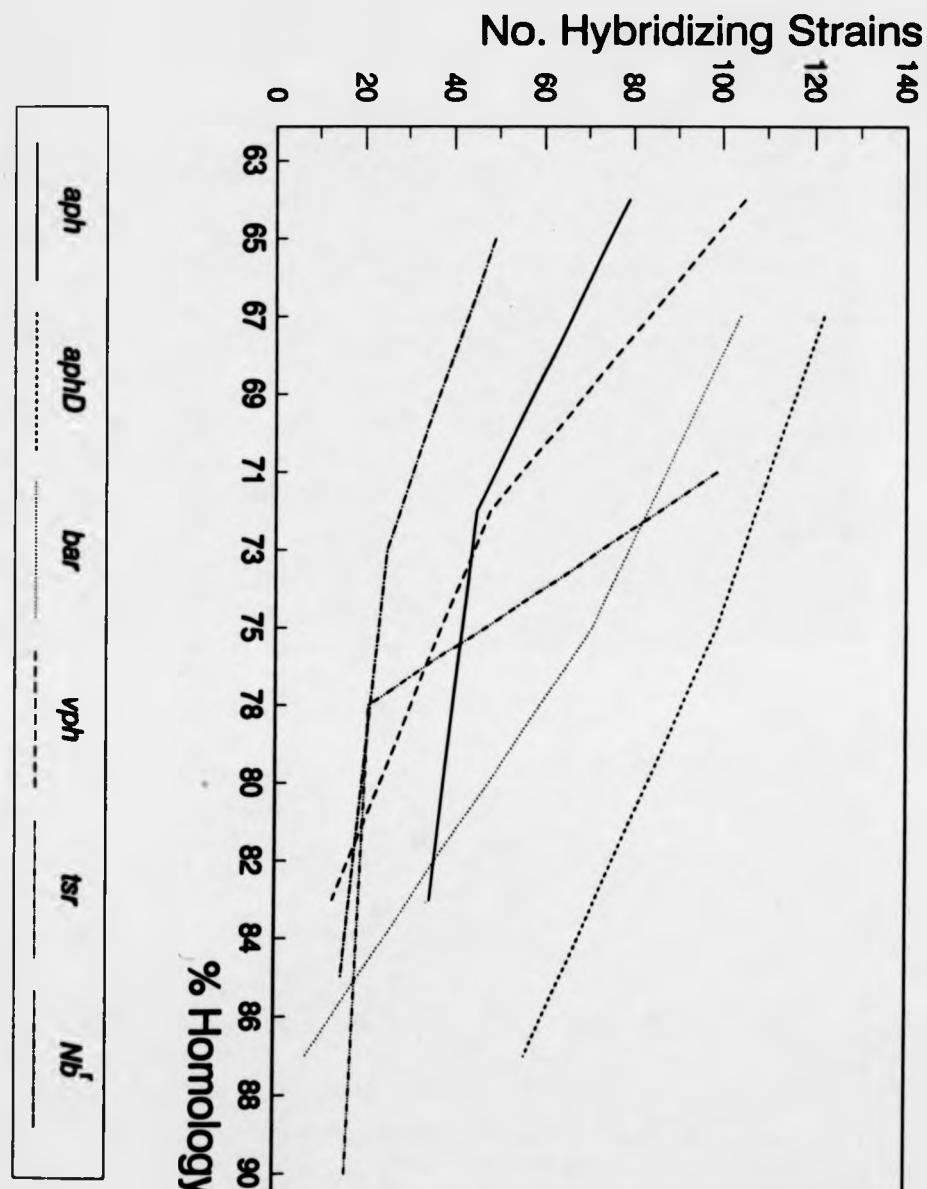


Fig. 7.2. The distribution of DNA from *Streptomyces* strains which hybridized to antibiotic resistance gene probes.

The diagram is a graphical representation of Table 7.3. and shows the distribution of hybridizing sequences with respect to the stringency to which each probe was screened. (NB screening was only carried out at three stringencies for each probe).

Table 7.3. The distribution of positive signals from dot blots within *Streptomces* Strains.

Probe	Stringency (0.1% SDS plus) 3 x SSC	1 x SSC	0.2 x SSC
aphD	69.32 (122)	56.25 (99)	31.82 (56)
aph	44.89 (79)	25.57 (45)	19.89 (35)
tsr	56.25 (99)	11.93 (21)	9.1 (16)
vph	59.66 (105)	27.27 (48)	7.39 (13)
nbr	27.84 (49)	14.21 (25)	8.52 (15)
bar	59.09 (104)	40.34 (71)	3.98 (7)

The numbers given refer to the percentage of strains which hybridized, whilst the figures in parenthesis show the actual number of strains, which hybridized.

Different gene probes gave different homology profiles, possibly showing where there was an abundance or lack of related gene sequences. *tsr*, *aph* and *nbr* showed a sharp decline in the percentage of strains which hybridized to them between 3 x SSC and 1 x SSC and then the numbers of positive signals were more constant from 1 x SSC to 0.2 x SSC. This might suggest that highly related sequences to these genes were rare.

The probes for *vph*, *aphD* and *bar* gave more linear graphs and *vph* also showed a large decline in related sequences from 3 x SSC to 0.2 x SSC homology, but the percentage of hybridizing strains continued to fall, at a reduced slope, to 0.2 x SSC. In contrast the major loss of sequences binding to the *bar* and *aphD* gene probes was from 1 x SSC to 0.2 x SSC..

Table 7.3 describes positive signals which were stronger than the negative controls. However, different intensities were observed amongst the positive signals and Table 7.4 shows the strains for which the most intense signals were observed.

Table 7.4. To show strains giving strong hybridization signals to antibiotic resistance gene probes.

Probe	Strains	Number
aphD	*DSM40236, *ATCC25481 ATCC11062, C11, D147, CAG23.	6 (56)
aph	CAG6, *CAG7, Lu46, *C47, *C284 *KCC S-0772, KCC S-0785, C402.	7 (35)
tsr	KCC S-0772, *KCC S-0785.	2 (16)
vph	*KCC S-0785, CAG7,	2 (13)
nbr	*KCC S-0772, *KCC S-0785.	2 (15)
bar	*1233, *8852.	2 (7)

Positive controls are omitted from the table, but gave strong signals, whilst numbers in parentheses refer to the total number of positive signals.

Strains assigned * gave a signal which appeared as strong as the positive control; ie. the strain from which the relevant gene was isolated, except for Nb^r and *aph* where it was with reference to the spot from the plasmid containing the gene probe (this was because the relevant strains did not light up at 85% stringency).

The other strains on the table gave weaker signals (but these were stronger than the signals which were scored as positive for cluster analysis).

The number of highly related sequences was considerably reduced if only strong signals were scored. These strains were thought to be the most likely to contain similar genes to the actual probes used. The remaining weak signals could comprise strains with small homologous segments of DNA or strains with larger sequences which are not as closely related to the probes as the strong positives, although another consideration was the accuracy of spectrophotometric

quantifications. If 1 μg of DNA was not loaded for each sample then this might also have contributed to differences in spot intensities.

Four strong signals with the *aphD* probe occurred within the DNA of streptomycin producers (DSM40236, ATCC11062) and suspected producers (C11, D147), whilst ATCC25481 *(S.ornatus)* the ornamycin producer also hybridized (none of the CAG or LU series have been screened for bioactivity). Strains, which hybridized to the *aph* probe included C402, which may produce herbimycins and C47, a strain suspected of producing a novel compound (recently found to be borrelidin). Interestingly, *S.hygroscopicus* (KCC S-0772) and *S.lusitanus* (KCC S-0785) hybridized strongly with several probes, including the nb^r probe, which is reported to be specific for the production of novobiocin (Mitchell *et al.*, 1990). The *S.hygroscopicus* strain is known to produce hygromycins and *S.lusitanus* produces tetracyclines. 1233 which hybridized to *bar* is an industrial strain of ATCC21705 the positive control for the *bar* gene, whilst 8852 was *S.vinaceus* the viomycin producer. Most of the strains showing strong hybridization signals did not express resistance to the relevant antibiotic.

7.3.2. The Distribution of Patterns of Hybridization to Various Antibiotic Resistance Gene probes.

The diversity of hybridization profiles, at high stringency is given in Table 7.5. Theoretically there are 720 (6!) combinations of 6 gene probes; this contrasts with the 25 profiles which were observed within 176 different strains. Binding to either or both streptomycin and neomycin phosphotransferase was common, whilst multiple hybridizations were rare and each of these profiles was only ever present in 1 or 2 strains. Examination of similar patterns at lower levels of stringency revealed greater diversity and there were 13 profiles repeated amongst 30 strains and 146 which were unique (at 3 x SSC + 0.1% SDS).

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Table 7.5. The distribution of patterns showing hybridization to antibiotic resistance gene probes.

The diagram shows how various strains hybridized to the gene probes at 85% stringency.

+ refers to a positive signal.

- refers to a negative signal.

Pattern	Streptomycin	Neomycin	Viomycin	Thiostrepton	Novobiocin	Bialaphos	No. strains
[a] 1	-	-	-	-	-	-	76
2	+	-	-	-	-	-	28
[b] 3	-	-	-	-	-	-	24
4	+	+	-	-	-	-	12
5	-	+	-	-	-	-	9
6	-	-	-	+	-	-	3
7	-	-	-	-	-	+	3
8	+	+	+	+	+	+	2
9	+	+	+	+	+	-	2
10	+	-	+	+	+	-	2
11	+	+	+	-	+	-	2
12	+	+	-	+	+	-	1
13	+	+	+	-	-	+	1
14	+	+	+	+	-	-	1
15	+	+	-	-	+	-	1
16	-	+	-	+	+	-	1
17	+	+	+	-	-	-	1
18	+	+	-	+	-	-	1
19	-	-	-	+	+	-	1
21	-	-	+	+	-	-	1
22	+	-	-	+	-	-	1
23	+	-	-	-	+	-	1
24	-	-	-	-	+	-	1
25	-	+	+	-	+	+	1
No. strains	56	35	13	16	15	7	176

[a] These strains hybridized at lower stringencies than 85%.

[b] These strains showed no hybridization at any of the stringencies used.

Delete sample with Table 7.6

7.4. The Expression of Antibiotic Resistance and its Relationship to Homology with Antibiotic Resistance Gene Probes.

Strains were classified into 4 groups based on resistance phenotype and hybridizations at 85% (Table 7.6.). Those strains which were resistant to a specific antibiotic and which hybridized to the relevant gene probe may provide evidence for the presence of that same gene in other streptomycetes and sensitive strains, showing hybridization to the gene probes may possess highly related sequences that are functionally unrelated, but are interesting in evolutionary terms, or else they could harbour silent antibiotic genes. Non-hybridizing resistant strains may exhibit resistance mechanisms other than those conferred by the probes (these are referred to in the text as alternative resistance mechanisms), whilst sensitive strains which did not hybridize did not contain the gene itself, highly related sequences or an alternative resistance mechanism (at least under the conditions tested in this study).

Sensitive non-hybridizing strains were most common for each probe, but the distributions amongst the other three categories varied. *aphD* and *aph* had many homologous sequences in sensitive strains and more strains with alternative resistance mechanisms than strains which were resistant and hybridized. There were high numbers of strains with alternative resistance mechanisms for the antibiotics viomycin, thiostrepton and novobiocin and very few strains with sensitive phenotypes hybridized with these gene probes. There were 16 strains which hybridized to three or more antibiotic-resistance gene probes, representing 48.5% of all positives observed at high stringency and two-fifths of these had resistant phenotypes.

Table 7.6. The distribution of antibiotic resistance and hybridizations (85%) to antibiotic resistance gene probes.

Antibiotic	No. strains (total = 176) Resistant and Hybridized	Resistant and didn't Hybridize	Sensitive and Hybridized	Sensitive and didn't Hybridize
Streptomycin	19	31	27 (12)	74 (13)
Neomycin	6	17	26 (3)	105 (21)
Viomycin	9	33	3 (1)	108 (24)
Thiostrepton	13	53	2 (1)	86 (23)
Novobiocin	8	38	7	100 (25)

Numbers in parentheses refer to the number of strains which have not been tested for antibiotic resistance and could therefore be resistant. In the previous study (Chapter 6) strains were called sensitive below a cut-off value. Low level resistances affected three strains with high homology to novobiocin and these have been included in the above table as resistant.

7.5. The Relationship Between Antibiotic Production and Resistance.

Previous studies provided evidence for a dichotomy in the distribution of antibiotic-resistance phenotypes amongst natural isolates and type strains, where a high proportion of the population expressed multiple antibiotic resistances (3-4 or more) and many produced antibiotics, whilst the remainder showed only a limited resistance phenotype and did not express bioactivity. It was clear that the population of natural isolates and type strains which produced secondary metabolites contained higher numbers of strains, whose DNA hybridized with the gene probes (Table 7.7.). This was compared to a lower number of strains with hybridizing DNA

Table 7.7. The clustering of strains with sequences hybridizing with antibiotic resistance genes.

This table relates to work presented in Chapter 6, where strains, which possessed multiple sensitive phenotypes did not produce antibiotics. Such strains are represented by the columns entitled 'no expression' and are those, which clustered to Area 2 (cluster 3) in the phenogram in Fig. 6.6. Conversely, the column entitled 'expression' refers to strains which clustered to Areas 1, 3 and 4 (clusters 1, 2 and 4 to 10) in the phenogram presented in Fig. 6.6. These strains were shown to express multiple antibiotic resistance and antibiotic production was observed in many of them.

The table indicates that a greater proportion of strains from the Areas 1, 2 and 4 of the phenogram (Fig. 6.6) had DNA, which hybridized with the gene probes, than did DNA from strains from Area 2 (group 3).

No. of	Type strains		Natural isolates	
[a]Hybridizations	No expression	[b]Expression	No expression	Expression
0	2	12	11	17
1	0	5	3	10
2	0	0	1	6
3	0	1	0	1
4	0	3	0	1
5	0	1	0	1
6	0	0	0	0
Cumulative No. positives	0	25	5	34
Total No. strains	2	22	15	36

[a] Hybridization at approximately 85%, assuming a G+C ratio of 73%
[b] Refers to expression of antibiotic production and/or resistance

sequences in the sensitive population, which did not express antibiotic production. Whilst this is not definitive evidence that such strains contain the same gene it does appear to correlate well with the distribution of resistance phenotypes.

7.5.1. Relationship Between Aminoglycoside Production and Antibiotic Resistance.

Eleven type strains, which produce aminoglycosides similar to streptomycin were examined for hybridization to *aphD* (Table 7.8). Six strains hybridized to *aphD* at high stringency (87.43%) and one, a dihydroxystreptomycin producer, hybridized at low stringency (66.86%). Two aminoglycoside-producing streptoverticillia and two streptothricin producers hybridized at 74.82% homology and Table 7.9. shows how this compared to the average homologies of all strains in the study, although the set of aminoglycoside producers includes two natural isolates which were thought to produce streptomycin. The distribution of hybridization to *aphD* for aminoglycoside producers tended towards binding at high stringency, but there was not such a strong correlation between aminoglycoside production and hybridization with the other phosphotransferase genes, *aph* and *vph* (Table 7.9.), although the tendency was towards greater hybridization than the most common mean categories (ie no hybridization at any stringency studied). Aminoglycoside producers followed the average *tsr* trend, but hybridized more often than average to the novobiocin-resistance determinant.

It is interesting to note that Hotta *et al.* (1988) found that the streptomycin phosphotransferase gene from *S.griseus* (SS-1198) was species specific for streptomycin-producing *S.griseus* strains. This gene was reported to be identical, with respect to size and restriction sites, to genes cloned by Distler *et al.* (1985) and Tohyama *et al.* (1984); Lim *et al.* (1989) consider that these latter two genes correspond to *aphD* (Vallins and Baumberg, 1985). The fragment, which was used for probing in the Hotta *et al.* (1988) study was an 0.4 Kb *Sal*1 fragment, compared to a 0.8 Kb (*Pst*1 *Stu*1) fragment, used in this study. The fragment used as probe for this study contained the B, C and D regions and a small part of the E region

Table 7.8. The distribution of signals hybridizing to *aph* and *aphD* in aminoglycoside producers

This table lists strains which were known aminoglycoside producers and shows whether their DNA hybridized to the *aph* and *aphD* gene probes.

Strain	Species	Taxonomic Cluster	Antibiotic Product	Hybridization to aphD (%)	*Hybridization to aph (%)*
DSM40236	*S.griseus*	1	Streptomycin	85	*85*
ATCC12760	*S.humidus*	19	Dihydrostreptomycin	85	*65*
ATCC14607	*S.hygroscopicus*	32	Bluensomycin	85	*<65*
KCC-S 0772	*S.hygroscopicus*	32	Hygromycins	85	*85*
DSM40455	*S.subrutilis*	61	Hydroxystreptomycin	65	*<65*
ISP5550	*S.katrae*	61	Streptothricin	73	*65*
DSM40069	*S.lavendulae*	61	Streptothricin	73	*<65*
ATCC23934	*Stv.mashuensis*	55	Streptomycin	73	*73*
ATCC11062	*S.bikiniensis*	64	Streptomycin	85	*65*
KCC-S 0133	*S.fradiae*	68	Neomycin	[a] *nd*	85
ATCC27441	*Stv.ladakanum*	Single member cluster	5-azacytidine	73	*<65*

[a] *nd = not done.*

Table 7.9. The distribution of positive signals amongst specific antibiotic producers.

Antibiotic Class	Stringency	*aphD*	*aph*	*tsr*	*vph*	Nb^r	*bar*
Aminoglycosides (13)	0-65	0.0	38.5	38.5	15.4	46.2	38.5
	65	7.7	30.8	38.5	46.2	7.7	0.0
	73	30.8	15.4	7.7	30.8	23.1	61.5
	85	61.5	15.4	15.4	7.7	23.1	0.0
Macrolides (5)	0-65	60.0	80.0	40.0	80.0	100.0	60.0
	65	0.0	20.0	40.0	0.0	0.0	20.0
	73	40.0	0.0	20.0	20.0	0.0	20.0
	85	50.0	0.0	0.0	0.0	0.0	0.0
Amino Acid Derivatives(7)	0-65	28.6	42.9	28.6	14.3	42.9	0.0
	65	0.0	28.6	42.9	28.6	28.6	14.3
	73	42.9	0.0	14.3	42.9	28.6	42.9
	85	28.6	28.6	14.3	14.3	0.0	42.9
Cyclo alkane Derivatives (3)	0-65	0.0	33.3	66.7	66.7	66.7	33.3
	65	0.0	66.7	33.3	0.0	33.3	33.3
	73	33.3	0.0	0.0	33.3	0.0	0.0
	85	66.7	0.0	0.0	0.0	0.0	0.0
Benzene Derivatives (1)	0-65	0.0	100.0	100.0	100.0	0.0	100.0
	65	100.0	0.0	0.0	0.0	100.0	0.0
	73	0.0	0.0	0.0	0.0	0.0	0.0
	85	0.0	0.0	0.0	0.0	0.0	0.0

Class of Antibiotic	Stringency	*aphD*	*aph*	*tsr*	*vph*	Nb^r	*bar*
Macro-	0-65	50.0	50.0	25.0	50.0	100.0	50.0
lactams	65	25.0	50.0	25.0	25.0	0.0	0.0
(4)	73	25.0	0.0	50.0	25.0	0.0	50.0
	85	0.0	0.0	0.0	0.0	0.0	0.0
Quinon	0-6	50.0	0.0	25.0	25.0	50.0	0.0
-es and	65	25.0	50.0	50.0	25.0	0.0	50.0
Tetrac-	73	0.0	50.0	0.0	25.0	25.0	50.0
yclines (4)	85	75.0	0.0	25.0	25.0	25.0	0.0
Mean	0-65	30.7	55.1	43.8	40.3	72.3	40.9
(176)	65	13.1	19.3	44.3	32.4	13.6	18.8
	73	24.4	5.7	2.8	19.9	5.7	36.4
	85	32.8	29.9	9.1	7.4	8.5	4.0

This Table uses the stringency values associated with 73% G+C. The homology levels relating to individual genes are shown in Table 7.2. The mean values refer to the proportion of the entire population which hybridized and figures in parentheses refer to the number of strains in each group of antibiotic producers.

(Lim *et al.*, 1989), whilst the fragment in the study of Hotta *et al.* (1988) comprised the B region and portions of the A and C region.

The only other group with a reasonable number of strains was the diverse group of peptide antibiotics. Three probes *tsr*, *vph* and *bar* were isolated from producers of peptide antibiotics. Even when the presence of positive control strains

were taken into account, there was more than the average amount of hybridization shown to the *vph* and *bar* probes within this group.

None of the macrolide or macrolactam producers showed binding at high stringency to any of the probes. This was also rare for cycloalkane and benzene derivatives. All producers of macrolides and macrolactams showed less than 65% homology to the Nb^r probe.

7.6. The Relationship Between Sequences Hybridizing to Antibiotic Resistance Gene Probes and Streptomycete Taxonomy.

There were sufficient strains for four cluster groups (Williams *et al.*, 1983a) to be examined in detail with respect to their average gene homology profiles.

Table 7.10. The distribution of sequences hybridizing to antibiotic resistance genes within *Streptomyces* clusters as defined by Williams *et al.* (1983a).

Cluster Group	% Homol	% Strains with Homology to: *aphD*	*aph*	*tsr*	*vph*	Nb^r	*bar*
1	0-65	14.3	35.7	28.6	28.6	53.5	42.3
(15)	65	7.1	7.1	50.0	14.3	13.3	14.3
	73	42.9	7.1	0.0	42.9	6.7	28.6
	85	35.7	42.9	14.3	14.3	26.7	7.1
5	0-65	18.7	79.0	50.0	50.0	81.3	50.0
(16)	65	18.8	21.0	50.0	37.5	6.3	25.0
	73	43.8	0.0	0.0	12.5	12.5	25.0
	85	18.8	0.0	0.0	0.0	0.0	0.0

Cluster Group	% Homol	% Strains with Homology *aphD*	*aph*	*tsr*	*vph*	Nb^r	*bar*
19	0-65	20.0	50.0	70.0	30.0	100.0	0.0
(10)	65	10.0	40.0	30.0	60.0	0.0	40.0
	73	10.0	0.0	0.0	10.0	0.0	20.0
	85	60.0	10.0	0.0	0.0	0.0	0.0
32	0-65	47.4	57.9	68.4	52.5	73.3	52.6
(19)	65	5.3	21.0	10.5	26.3	21.0	5.3
	73	10.5	0.0	15.8	10.5	0.0	36.8
	85	36.8	21.0	10.5	10.5	5.3	5.3
Mean	0-65	30.7	55.1	43.8	40.3	72.3	40.9
	65	13.1	19.3	44.3	32.4	13.6	18.8
	73	24.4	5.7	2.8	19.9	5.7	36.4
	85	32.8	29.9	9.1	7.4	8.5	4.0

This Table uses the stringency values associated with 73% G+C. For homology levels relating to individual genes please see Table 7.2. The mean values refer to the proportion of strains from the whole population which hybridized to the antibiotic resistance gene probes. Figures in parentheses refer to the number of strains belonging to each taxonomic cluster group (Williams *et al.*, 1983a).

7.6.1. The Distribution of Sequences Hybridizing with Antibiotic Resistance Gene Probes in *Streptomyces albidoflavus* (Cluster 1).

The *aphD* gene probe was isolated from *Streptomyces griseus*. This strain taxonomically identifies to *S.albidoflavus* cluster 1 and specifically to 1b

S.annulatus. Fifteen cluster 1 streptomycetes were probed with *aphD* in order to ascertain any relationship between the gene and taxonomic identity. These strains contained more than the average amount of hybridization to *aphD* (Table 7.10.) and showed more than average resistance to streptomycin (section 6.4.7., Wellington and Cross, 1983; Williams *et al.*, 1983a). In addition cluster 1 streptomycetes showed greater hybridization than average with the other two phosphotransferases. The novobiocin resistance determinant was also isolated from a cluster 1 streptomycete (specifically 1c), *S. niveus* and there was some indication that more C1's than average showed bound to this probe at high stringency.

7.6.2. The Distribution of Sequences Hybridizing to Antibiotic Resistance Gene Probes in *Streptomyces exfoliatus* (Cluster 5).

None of the probes used in the study originated from a cluster 5 strain, although phosalacine, a compound which is related to bialaphos, is produced by *Kitasatosporia*. Cluster 5 strains tended to hybridize less than average with the *bar* gene probe and the *aph* probe, but hybridized more often to *aphD* at 73% than was expected.

7.6.3. The Distribution of Sequences Hybridizing with Antibiotic Resistance Gene Probes in *Streptomyces diastatochromogenes* (Cluster 19).

This group also contained many sequences, which hybridized to the *aphD* probe, but they hybridized less than average to other phosphotransferases; interestingly Distler *et al.* (1987a) has shown that the streptomycin phosphotransferase genes from the taxonomically diverse strains, *S.griseus* and *S.glaucescens*, share 75% homology. The cluster 19 strains also showed less hybridization than average to the other resistance gene probes and this could reflect the fact that this group does not contain a large proportion of antibiotic producers. None of the probes were isolated from a Cluster 19 streptomycete.

7.6.4. The Distribution of Sequences Hybridizing with Antibiotic Resistance Gene Probes in *Streptomyces violaceoniger* (Cluster 32).

The *bar* gene was isolated from a *S.hygroscopicus* strain which is a member of this cluster, however the group profile for hybridization to this probe was very close to the average profile. Lower than the mean hybridization frequencies were shown to *aphD* and *vph* and an average profile to *aph* was observed. The strains also showed less than average binding to the *tsr* probe, although more strains than average hybridized to this probe at 73%.

7.7. Clustering Based on Hybridization Patterns to Antibiotic Gene Probes and its Relationship to Antibiotic Production.

The data were clustered using both a binary form and a semi-quantitative form. For the latter the highest value at which a strain showed hybridization was entered into the matrix. For the former, the data were entered as in the following example:

	probe number 1				
	3xSSC	1xSSC	0.2xSSC		
OTU1	0	0	0	=	0-65% Homology
OTU2	1	0	0	=	65% Homology
OTU3	1	1	0	=	73% Homology
OTU4	1	1	1	=	85% Homology.
OTU5	1	0	0	=	65% Homology

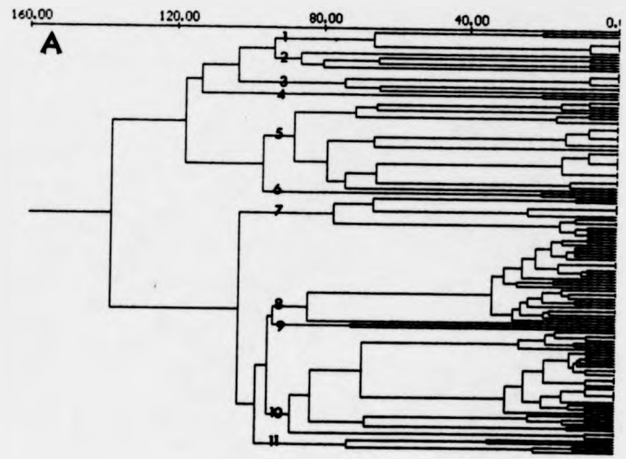
Trees were created using both UPGMA and single linkage and a selection of available coefficients were examined for use with this data. Similarity values were calculated using Euclidean distance and the Manhatten metric for the semi-quantitative data, whilst the Dice and simple matching coefficients were used for the binary data (S_{dice} was chosen to show how weighting against negative matches, affected the clustering, compared to S_{sm} which gave negative and positive matches equal weight). Similarity was scored in the same way as phenotypic data (ie. scored with respect to the test used) which incorporate parallel and divergent evolution and which may therefore contain genetic dissimilarity. A possible alternative form of clustering could have been to weight against characters referring to binding at lower levels of stringency and to give higher regard to results which might be likely to reflect related sequences.

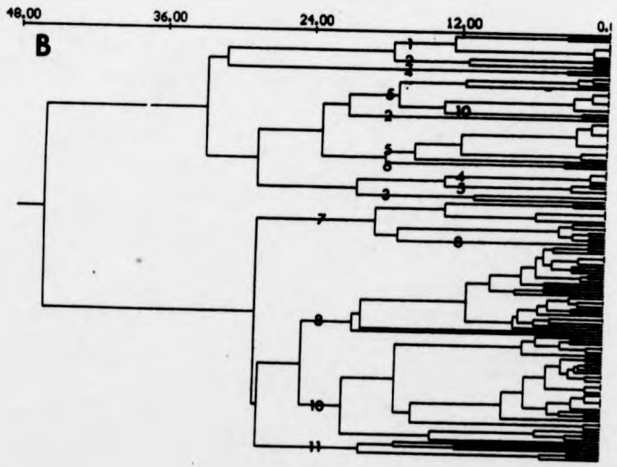
Figs. 7.3. and 7.4. show four relevant phenograms clustered using UPGMA;

Fig. 7.3. Phenograms to show the clustering of strains based on their hybridization patterns to antibiotic resistance genes.

A shows a phenogram created from semi-quantitative data, using Euclidean distance and UPGMA. Eleven groups have been defined on this phenogram at a Euclidean distance of 92.

B shows a phenogram created from semi-quantitative data, using Manhatten distance and UPGMA. The positions of strains belonging to the groups which are indicated for phenogram A.





close examination revealed reasonable agreement between all of the phenograms and very good reproduction of groups for phenograms clustered using the quantitative data. This was better than for those clustered using binary data. The reason could be that when all stringency levels were given an individual character status, the number of negative results increased, causing an overestimation of similarity when S_{sm} was used compared to S_{dice}. Cophenetic correlations showed that phenograms based on quantitative data gave higher Mantle statistics (eg. 0.84 compared to 0.78 for Euclidean with quantitative data versus Dice with binary data and both with UPGMA) and therefore were better representations of the similarity matrix. When new characters were added (erythromycin biosynthesis gene probes; results not discussed) tree topology was hardly altered and this also indicated that the classification was good.

The phenogram derived from Euclidean distances (Fig 7.5) has been chosen to illustrate trends seen within the data and eleven groups were defined at a Euclidean distance of 92. The distribution of 33 antibiotic-producing strains has been superimposed on the phenogram and preliminary observations indicated that the producers of certain classes of antibiotics grouped to specific clusters. All of the macrolide producers clustered to groups 1 or 5, and the macrolactam producers to groups 6 and 10, polyene producers were observed in groups 1, 5, 8 and 11 and those which produced aminoglycosides in 5 and 8. Quinones and benzene and cycloalkane derivatives were found in the latter half of the phenogram (groups 6,8,9 and 10), compared to macrolides and polyenes, which were in the first half. Interestingly producers of peptide antibiotics clustered to either side of the phenogram depending upon whether they were derived from aromatic or non-aromatic amino acids. Two groups in the phenogram were rotated at positions S1 and S2 and a diagram was drawn to illustrate the positions of antibiotic producers across the phenogram (Fig.7.6).

Fig. 7.4. Phenograms to show the clustering of strains based on their hybridization patterns to antibiotic resistance genes.

A shows a phenogram created from binary data, using S_{dice} and UPGMA

B shows a phenogram created from binary data, using S_{sm} and UPGMA

The positions of strains belonging to the group,s which are indicated for phenogram A, Fig. 7.3. are indicated on both diagrams.

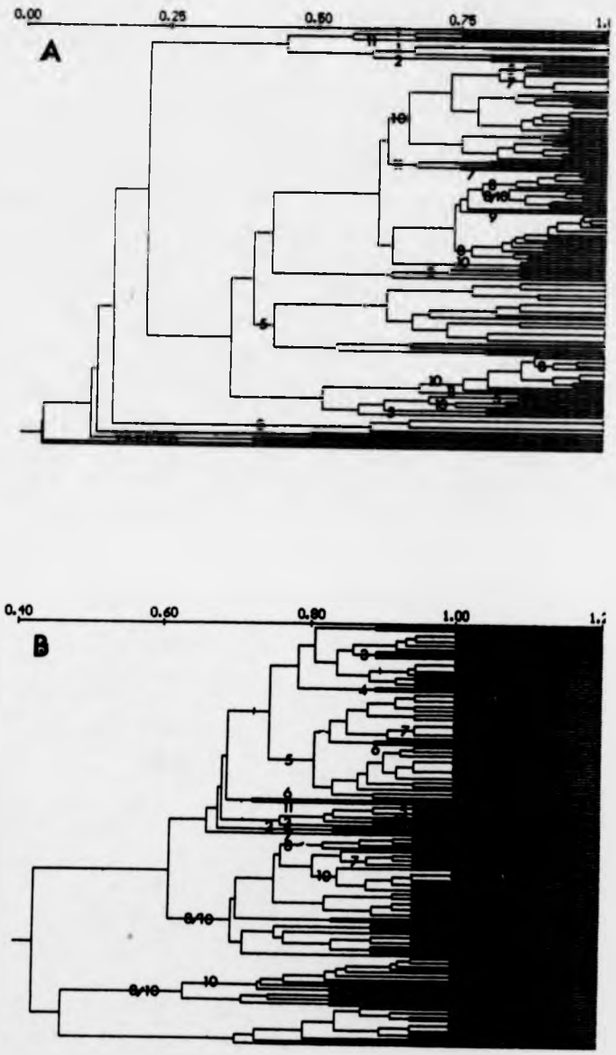


Fig. 7.5. Phenogram to show the clustering of strains based on their hybridization patterns to antibiotic-resistance genes.

The diagram shows the positions of strains belonging to specified classes of antibiotics.

P = Polyenes

M = Macrolides

AA = Amino acid derivatives

C = Cycloalkane derivatives

A = Aminoglycosides

Q = Quinones and tetracyclines

ML = Macrolactams

B = Benzene derivatives.

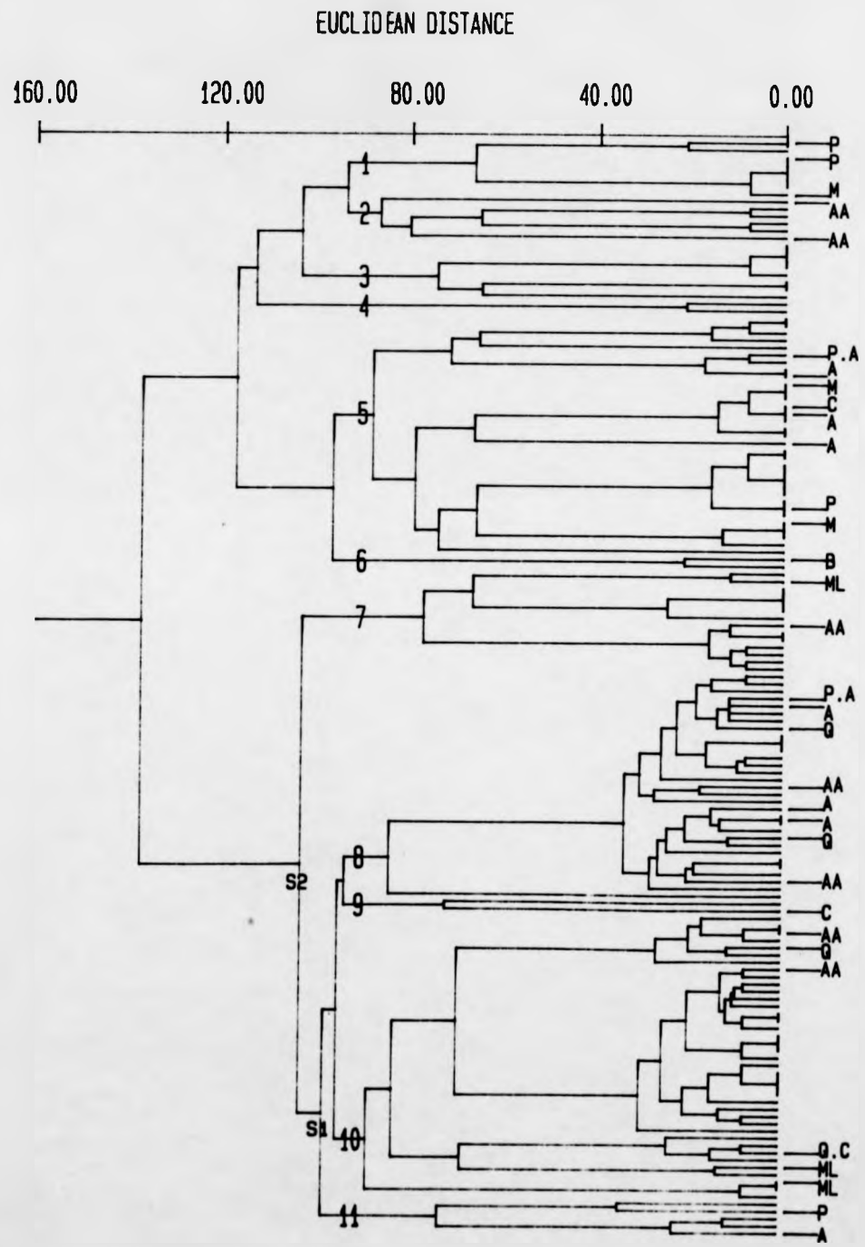
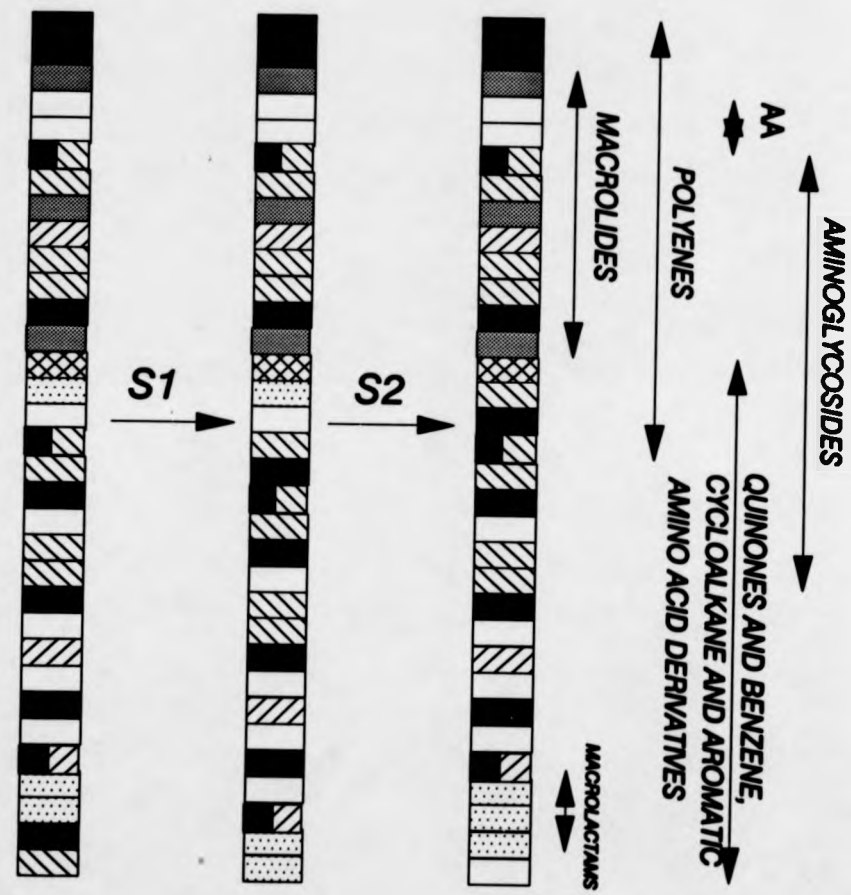


Fig. 7.6. Schematic diagram to shows the clustering of specific classes of antibiotics across a phenogram based on hybridization patterns to antibiotic resistance gene probes.

Two groups on the phenogram shown in Fig. 7.5. were rotated at position S1 and S2 and this schematic diagram shows how the positions of antibiotic producers change when these changes in topology. The diagram also shows the trends which were observed with respect the producers of specific classes of antibiotics clustering to specific groups on the phenogram.



P	POLYENES	*A*	AMINOGLYCOSIDES
M	MACROLIDES	*Q*	QUINONES AND TETRACYCLINES
AA	AMINO ACID DERIVATIVES	*ML*	MACROLACTAMS
C	CYCLOALKANE DERIVATIVES	*B*	BENZENE DERIVATIVES

7.8. Discussion.

At present only a small proportion of the antibiotic biosynthesis pathways and resistance mechanisms in nature are known and virtually nothing is understood about the distribution of production and resistance genes within natural streptomycete populations. The present study helped to prioritize a sizeable proportion (30%) of strains for further studies aimed at determining the nature of positive signals given by dot blot hybridizations. There was also good evidence for the existence of alternative resistance mechanisms and the majority of strains classified as resistant (Chapter 6) failed to hybridize with the gene probes used.

Streptomyces azureus and *Streptomyces laurentii* produce thiostrepton and can methylate rRNA for self-defense (Thompson and Cundliffe, 1980). These determinants also confer resistance to other peptide antibiotics with the same mode of action, namely siomycin, sporangiomycin, nosiheptide and thiopeptin (Thompson and Cundliffe, 1980; Woodman *et al.*, 1991). Producers of these compounds are resistant to thiostrepton and have closely related resistance determinants to *tsr* which could possibly account for some of the positive signals in this study. *S.azureus* actually has two rRNA methylases for thiostrepton resistance and these are not linked to the biosynthetic gene cluster, but are integrated on pIJ101-like plasmids elsewhere in the chromosome Woodman *et al.* (1991). This has lead Woodman *et al.* (1991) to hypothesize that these genes are fortuitous acquisitions obtained later than an original resistance gene, which evolved alongside the biosynthetic apparatus. This supports findings presented in this thesis, which suggest that alternative resistance mechanisms to *tsr* do exist in nature, although further analysis of the resistant strains found in soil is needed to determine if there is more evidence for the mobility of resistance genes within streptomycete populations.

A correlation was found between aminoglycoside production and hybridization with the *aphD* probe. Other workers have found that aminoglycoside phosphotransferases are appreciably homologous within streptomycetes (Distler *et al.*, 1987b; Lim *et al.*, 1989) and may have a common evolutionary origin (Heinzel

et al., 1988; Lim *et al.*, 1990) and some of the positive signals with the *aphD* and *aph* probes may be due to such genes. Neomycin is also known to be inactivated by an acetyltransferase (Thompson *et al.*, 1982b) and so such enzymes may be responsible for some of the alternative resistance mechanisms observed for these aminoglycosides.

Walker (1990) suggested that there has been a selection pressure for the evolution of the streptomycin biosynthesis pathway. Guanidino inositol derivatives could serve as a nutrient reserve, which is readily utilized during starvation and differentiation and this might give organisms a selective advantage for developing this portion of the pathway (and might help the selection of the resistance gene). An alternative proposal for the function of streptomycin exploits its polycationic character, as an accelerator of cell lysis (Szabo *et al.*, 1990). The high proportion of strains whose DNA hybridized to the *aphD* probe may indicate that there are related sequences within streptomycetes. Lim *et al.* (1989) found that *aphD* shared homology with other cloned antibiotic phosphotransferase genes and they also hypothesized that the B and D regions of the sequence encoded ATP binding or phosphate transfer domains, whilst regions A,C and E encoded antibiotic recognition sites. It may be that many of the weak signals were due to the binding of small motifs which encode similar functions to the B and D regions, but on different genes.

Only a small proportion of isolates hybridized to *vph* and Nb^r, although the proportion of phenotypic resistance to sensitivity was similar to that observed for other antibiotics. The only known producer of viomycin is *S.vinaceus*, which has three resistance genes including *vph*, but the capreomycin-resistance determinant also confers resistance to this antibiotic (Skinner and Cundliffe, 1980). Although there is no evidence for an rRNA methylase in *S.vinaceus*, *Mycobacterium smegmatis* does possess such an enzyme, which makes it resistant to this antibiotic (Skinner and Cundliffe, 1980).

The mechanism of the novobiovin-resistance determinant in this study is still unknown as is that of another resistance gene cloned from *S.niveus* (Mitchell *et al.*, 1990). However, Cundliffe and co-workers have cloned a resistance determinant with DNA gyrase activity from another producer *Streptomyces spheroides* (Thiara and Cundliffe, 1989). It is possible that some of the strains with alternative novobiocin-resistance mechanisms in this study also contain such an enzyme.

Studying the diversity of streptomycete populations may help in understanding the evolution and ecological importance of antibiotic production and its relationship to antibiotic resistance. This study reported a novel approach to the analysis of resistance data and exploited taxonomic methodology for pattern recognition. It was shown that groups of strains producing similar antibiotics may be delimited by analysing their patterns of hybridization to antibiotic gene probes. These patterns appeared to be more related to antibiotic production than they were to the taxonomy of the relevant organisms.

Chapter 8

General Discussion.

A variety of theories have described possible functions for secondary metabolite biosynthesis (Bu'lock, 1965; Hütter, 1982; Zähner, 1982; Williams *et al.*, 1989a;), but the natural role of antibiotic production remains unclear. Few attempts have been made to investigate the distribution of bioactivity within natural populations of streptomycetes, although they comprise one of the major antibiotic producing groups. Most of the available data link taxonomic analysis with biased sampling of antibiotic producers (Arai *et al.*, 1976). Whilst the current study did not involve an exhaustive investigation of secondary metabolite production and was still biased towards biologically active products, it attempted to link taxonomic data with phenotypic and genotypic analysis and superimposed biological activity on the clusters produced.

One of the main aims of this project was to take a new approach for analysing natural populations of streptomycetes. Methods from conventional bacterial taxonomy (Dice, 1945; Carmicheal and Sneath, 1969; Sokal and Michener, 1958; Florek *et al.*, 1951; Lance and Williams, 1967) were used to carry out special purpose clustering. The data usually comprised single classes of characters (eg. fatty acid profiles, antibiotic resistance phenotypes and genotypes) which gave monothetic classifications. Groups of strains were delimited from these, allowing trends within the data and correlations with other characters to be discovered. This approach differs from conventional numerical taxonomy which uses large numbers of different tests to obtain polythetic classifications with the usual intention being to generate identification schemes based on general phenetic similarity (Williams *et al.*, 1983 a and b; Langham *et al.*, 1989; Kampfer *et al.*, 1991a and b). Variation and error are an integral part of systematic data (Goodfellow and Dickenson, 1985) and must be rigourously tested as was done in

other numerical studies (Williams *et al.*, 1983a, McCarthy and Cross, 1984 and Kampfer *et al.*, 1991). If the error lies within acceptable limits (Sneath and Johnson, 1972) then cluster analysis can reveal stable relationships for given tests and OTUs.

Fatty acid profiles were observed from two different viewpoints, one which aimed to gain insight into the relationship between fatty acid metabolism and secondary metabolite biosynthesis and another which investigated their use as a taxonomic tool. The success of Saddler *et al.* (1987) in separating *S.cyaneus* from other streptomycetes had indicated that the delimitation of the *S.violaceoniger* group (Williams *et al.*, 1983a and b) from less bioactive clusters was feasible. The attempt failed and reasons for this may lie in differences between the two studies. Like the study in Chapter 4, Saddler and co-workers (1987) used type strains, which represented a tight cluster and some markers from other groupings. However their natural isolates were pre-selected by spore morphology. This compared to an arbitrarily chosen selection, which comprised a variety of species (Williams *et al.*, 1983a and b) and unidentified strains. Work presented in Chapter 2 has shown that many of the streptomycete natural isolates did not conform to described streptomycete taxa. If this was reflected by their fatty acid profiles they may have prevented the delimitation of tight groups for more typical strains, resulting in the relatively homogeneous groupings observed in Chapter 4.

Streptomyces species delimitation may require quantitative data, as opposed to the qualitative data used in Chapter 4. Saddler (1987) based his approach on an assumption that fatty acid profiles remaind constant during logarithmic growth and stationary phase following a study using one strain, grown in Sautons medium, with three day intervals between readings (Saddler, 1986). However, fluctuations in fatty acid levels occurred on a daily basis for D153 in ISP7 medium (section 4.5). Kroppenstedt (personal communication) suggested that changes in fatty acid composition at different growth phases were responsible for his failure to cluster *S.violaceoniger* amongst a tree comprising 12 different *Streptomyces* taxa. Success

was achieved by removing three *S. violaceoniger* strains (Williams *et al.*, 1983a), two of which were present in the study in Chapter 4. The study of Kroppenstedt contained only type strains, which may be biased towards strains which have been patented for antibiotic production and they would therefore differ from many of the antibiotic producers included in Chapter 4. Industrial strains are selected for the production of either one or a group of related antibiotics. Antibiotic-producing isolates often produce several different compounds (Chapter 3), which may or may not relate to fatty acid profiles. Work carried out with D153, a producer of nigericin and geldanamycin, suggested that this was possible. The levels of fatty acids in this strain fluctuated in line with changes in secondary metabolism. However, this study was carried out in a production medium (ISP7), compared to Sautons medium, which aims to optimize biomass for taxonomic studies. D153 did not produce antibiotics in Sautons medium.

Information about the bioactivity of isolates (Chapter 3) was of central importance to the project because it was used to find correlations with all of the other data sets. Data used for these comparisons were generated using a wide range of culture conditions, extraction procedures and screening methods to increase the chances that antibiotic production would be expressed. This also yielded information about the distribution of antibiotic production in natural *Streptomyces* isolates (within the experimental limits of the study). Twenty percent of the strains studied produced a bioactive compound. The most common antibiotics were nigericin (15.5%) and geldanamycin (10%). It was hypothesized that the presence of these metabolites in such large numbers of streptomycetes could indicate that they play a useful role in their producers. For example, a suggested function for nigericin was as an ion scavenging agent. The preferential binding of nigericin to K^+ as opposed to Na^+ could suggest a function in maintaining intracellular K^+ concentrations. A similar role was put forward and a survival advantage indicated for the siderophores, which act as iron scavengers (Hutter, 1982). A role in the inhibition of germination has been suggested for other polyethers, which have been shown to

bind to spore surfaces in a similar manner to the germination inhibitor of *S.viridochromogenes* (Ensign, 1976; Grafe *et al.*, 1986). It is not known whether nigericin binds to spores in this way. The proposed function of nigericin is further supported by the work of Williams *et al.* (1989a) and Grably *et al.* (1990), who have shown that a highly sophisticated receptor-antibiotic complementation exists between this antibiotic and chemically-related compounds and the ions to which they bind.

An additional discovery made was that geldanamycin production was always associated with nigericin biosynthesis. A portion of the geldanamycin molecule is derived from polyketide biosynthesis (Ghisalba, 1985) and it was suggested, therefore, that the two compounds might share early biosynthesis steps or else have evolved separately in the same progenitor strain (and possibly from the same progenitor pathway). In the latter case, nigericin producers which did not express geldanamycin production might contain silent biosynthesis genes or represent a line of descent in which the ability to make this compound has been lost. The producers of many polyketide antibiotics show hybridization to the early genes in actinorhodin biosynthesis (Malpartida *et al.*, 1987) and the consensus of scientific opinion is that the majority of polyketide biosynthesis pathways share a common evolutionary origin (O'Hagan, 1988; Professor D.A. Hopwood, personal communication).

Most strains which produced compounds, thought to be good candidates for novel agrochemicals (comprising various different activities) also produced nigericin. Because both nigericin and geldanamycin are broad spectrum antibiotics it was hypothesized that novel compounds, which were produced concomitantly with them, might not be detected (ie their activity spectrum could be hidden within that of the broad spectrum antibiotic). Antibiotic expression in the producers of these compounds was examined more closely.

Expression studies revealed a greater diversity of responses to different nutrient concentrations and oxygen tensions. Reducing oxygen tension, in cultures of strain D153, lead to differential expression, between nigericin production, which was repressed and geldanamycin biosynthesis, which remained unchanged. This effect may have been responsible for the expression of nigericin production on solid media, rather than in liquid media in certain strains where shear factors may have given rise to small mycelial fragments, which were inefficient at oxygen transfer. Polyethers are biosynthesized from a polyene precursor via a triepoxide intermediate. In monensin biosynthesis, the formation of this intermediate is the only point in the pathway which requires molecular oxygen (Cane *et al.*, 1983). A similar pathway is likely for related polyethers, such as nigericin, and it is possible that denying sufficient molecular oxygen to D153 prior to nigericin biosynthesis would block the pathway at the point prior to the formation of this tri-epoxide intermediate.

Growth on nutrient gradients showed that a group of streptomycetes (similar in the sense that they all produced nigericin and that some produced geldanamycin) did not show the same response to possible stimuli for differentiation (Chapter 3). It was thought that this might reflect differences in rates of substrate utilization, thresholds for responses to nutrient limitation and pH or the presence of special mechanisms for the uptake of limited nutrients, which helped to delay the onset of starvation responses. Grafe (1989) has suggested that the characteristic cytodifferentiation and polymorphic behaviour of streptomycetes is a form of adaptation to a constantly changing natural environment. Grafe (1989) states that this could be done either by choosing between an array of pre-set developmental programs throughout the growth cycle or via rearrangements of chromosomal or extrachromosomal genetic elements. These were also thought to be possible explanations for the behaviour of some strains, which frequently exhibited variable expression in this work (Chapters 3 and 6).

The expression of antibacterial and antifungal activity was also strain specific with respect to the nutrient environment and altering nutrient conditions led to a changed product spectrum for most strains. This is in line with observations made for the different responses of rifamycin-producing strains to nitrate concentrations (Ghisalba, 1984). There was evidence that some of the strains could produce compounds which had not been previously observed. Exposure to certain nutrient conditions caused some strains to repress antibiotic production, whilst under others they behaved as would be expected from the preceding work (Chapter 3). Differential expression at varying nutrient concentrations has also been reported by Hall and Hassal (1970) who found that *S.jamaciensis* produced a different antibiotic at 0.1mM Pi than it did at 0.4mM Pi. In addition, Grabley *et al.* (1990) have shown that the antibiotic spectrum of DSM 3816 (a nigericin producer, which also produces several other antibiotics) was also dependent on the culture medium and conditions. The switch on of antibiotic production within the group of strains presented in Chapter 3 required a diversity of nutrient conditions and nutrient gradient plates offered a novel approach to investigate this in some detail. Gradients were also used to investigate antibiotic resistance (Chapter 6).

Several facts emerged when antibiotic-resistance profiles were examined for correlations with bioactivity. Resistance was not always present in producers of the relevant compound and was found in strains which showed no evidence of antibiotic production; an observation also made by Fujisawa and Weisblum (1982). There was, however, a general correlation between resistance and secondary metabolite biosynthesis. A strain was more likely to be bioactive the more resistances it possessed, and also if these resistances were rare within the population examined. Strains which did not express antibiotic production during the current study showed mainly sensitive phenotypes apart from common resistances such as penicillin resistance. Interestingly, Hotta *et al.* (1983a, 1983b, 1985), Yamashita *et al.* (1985) and Bibikova *et al.* (1990) have shown that resistance profiles using specific classes

of antibiotics correlate with the production of antibiotics belonging to the same chemical group.

Where sensitivity correlated with no antibiotic production, strains may not have been exposed to the conditions required to express the relevant genes. However, if there are streptomycetes which neither produce antibiotics nor possess antbiotic resistance genes then this has ecological implications. Perhaps these strains have other advantages for survival in the environment and might be suited to growth in more extreme conditions, such as high salt microenvironments. Alternatively they could have specializations for utilizing recalcitrant substances.

Resistances which were not associated with antibiotic production have been described by Fujisawa and Weisblum, 1981, Jenkins *et al.*, 1989 and Mosher *et al.*, 1990.). Antibiotic resistance (Chapter 6) was defined in the context of a population study and may not always reflect the levels of resistance required by an antibiotic producer. However, some strains which possessed antibiotic resistance but did not produce an antibiotic could have a survival advantage against external attack from the bioactive products of foreign microorganisms and might also benefit from the reduction of other competetive species without having to expend energy on antibiotic production. This assumes that antibiotic production occurs in the soil and some evidence for this was given by Rothrock and Gottleib (1984), who described the production of geldanamycin in soil by *Streptomyces hygroscopicus* var *geldanus*. The concept also is supported by Martin and Demain, (1980), Demain (1981) and (1984), Williams and Vickers (1986) and Weller and Thomashow (1990).

All the above types of strains (with respect to the expression of antibiotic resistance and production) were isolated from the same soil samples, but could inhabit different microniches. For instance, antibiotic producers may inhabit more competitive micro-environments, such as the rhizosphere, It would be interesting to know whether populations from different types of locations have higher or lower diversity with respect to antibiotic production and resistance. Most strains studied in Chapter 6 came from grassland or agricultural soil samples.

The majority of phenotypic resistances did not appear to be correlate with the production of the relevant antibiotic by the resistant strain. Resistance directed against the compounds studied (Chapter 6) might be due to a variety of factors. Some determinants may have evolved to confer resistance against a different compound, which may be structurally related to the test antibiotic. For example, aminoglycoside self-resistance determinants can confer resistance to other antibiotics of the same class (Skeggs *et al.*, 1987). Alternatively a different compound might have a similar mode of action as seen in the MLS resistance phenotype (Fujizawa and Weisblum, 1981; Jenkins *et al.*, 1989; Zalacain and Cundliffe, 1989). Other resistanccs may be present to confer protection against the products of competitors. This type of resistance would not necessarily correlate with bioactivity, but by measuring how often a resistance occurs in a natural population could give a measure of the selection pressures, due to antibiotic production, present within that environment. Neither penicillin resistance nor a proportion of the nigericin resistances correlated with bioactivity and beta-lactams and polyethers are common products of soil isolates (J. Benner, personal communication). Assuming antibiotic production in the natural environment (Weller and Thomashow, 1990), the high occurrence of these resistances could be related to defence against external attack. Multiple resistances might indicate an array of different mechanisms or a general mechanism. C.J.Thompson (personal communication) has isolated a promoter, which can be switched on by many different antibiotics, irrespective of mode of action or structure. It was associated with a pristinamycin-resistance gene, which was thought to be involved in drug export. Apart from rRNA methylases (Cundliffe, 1989), drug export is implicated in multiple resistance (MLS) in many macrolide producers (C. Hershberger, personal communication).

The fact that specific phenetic resistance could be due to many underlying mechanisms {eg. resistance to kanamycin can be conferred by acetyltransferase (Cundliffe, 1986) and rRNA methylase (Skeggs *et al.*, 1987)} means that resistance profiles are very useful for preselecting strains for screening. A resistance expressed

to a certain antibiotic could be due to a resistance determinant for a novel antibiotic. This hypothesis was strengthened by probing data (Chapter 7), which suggested that resistances exhibited by most strains in the study were due to mechanisms other than those which were related to the gene probes used. The data is also supported by the results from an antibiotic resistance pre-screen set up at ICI Agrochemicals Plc., which has shown that strains chosen because they have multiple antibiotic resistance patterns give many more positive activities on *in vivo* screens than do sensitive strains and the number of strains being further investigated is also greater.

In an attempt to understand the nature of antibiotic resistance at the genetic level, strains were probed with a relevant selection of antibiotic-resistance genes. Out of 9 strains with only penicillin resistance and 8 strains with only penicillin and nigericin resistance 2 showed positive hybridizations to one gene probe. Of the strains, which had three or more resistances, 45% hybridized to between 1 and 6 probes. The probing data was of a preliminary nature, but if the homologies were to represent the presence of resistance genes then the phenetic trend would be supported by a similar genetic trend.

Tentative observations suggested that there could be a relationship between the production of specific antibiotic classes and the presence of sequences that hybridized to antibiotic resistance gene probes. Special purpose clustering uncovered two populations. Strains which showed a close relationship to the gene probes (ie hybridized to them) included aminoglycoside and macrolide producers. Producers of other antibiotics, derived from fatty acid metabolism, showed a lower relationship to the gene sequences used.

Potentially highly related sequences which gave strong signals were present in streptomycetes other than those from which the genes were isolated. Weaker signals were present at high stringency for many other streptomycetes and may have indicated the presence of less related sequences or highly related sequences of a shorter length. In order to assess how successful using dot blots as a primary screen for finding genes in large populations of streptomycetes confirmation using southern

hybridization and sequencing studies will be required. The findings of these future studies may have a bearing on the evolution of secondary metabolism, antibiotic resistance and gene transfer. The approach appears to have been useful in determining strains, which could have novel or different resistance mechanisms.

A recurring theme throughout this work was that the raw data were not definitive and referred to phenotype rather than genotype. For instance, the growth conditions were important for the expression of both antibiotic production (Chapter 3) and antibiotic resistance (Chapter 6). Different nutrient concentrations and oxygen tensions affected whether or not antibiotics were produced and could change the product spectrum and which particular strains expressed antibiotic resistance was influenced by the growth medium. A consequence of this was that although a strain was negative under test conditions, it was not known whether that strain was capable of expressing a relevant set of genes if the environmental conditions were made favourable. Similarly, conditions could be found to repress gene expression by strains, which gave a positive score under the test conditions (Chapter 3). Strains, which express the same character under identical conditions, could be assumed to be more like one another than strains which express this character under different conditions. However, the latter is more like the former type of strain than a strain which cannot express the character at all.

Detection limits may also have influenced the scoring of negative results and low level expression might not always have been detected by the instrumentation or assay procedures used. Examples of where this may have occurred included, Gas Chromatography Mass Spectrometry (Chapter 4), thin layer chromatography (Chapters 3 and 5), and bioautography (Chapter 6). Likewise positive results could be due to different things. For example, phenetic antibiotic resistance was scored in Chapter 6 and a resistant phenotype could be due to one or several of a range of underlying mechanisms (a fact which was exploited). Even when a strain has the same mechanism the enzymes and genes responsible may be different. Similarly, in Chapter 5 different compounds may have identical Rf values. These problems are

common to other taxonomic studies, which use the systematic approach for phenetic data. For example, carbon source utilization tests could show positive as a result of different enzymes, pigmentation can be due to different compounds and negative results might show positive under slightly different conditions. All these factors can lead to strains grouping against general trends during cluster analysis. In order to minimise the influence of 'mis-scored' negative results coefficients were chosen which weighted against negative matches.

Positive results using the dot blot approach gave a range of intensities and therefore a certain type of interpretation was necessary. Weak hybridizations were scored positive, although they could be due to non-specific binding resulting from protein contamination in DNA samples or, since random primed labelling was used, they might be due to short homologous motifs. Weak positive results may also have been due to related sequences, which might or might not comprise portions of resistance genes. In view of this equal weight was given to positive and negative signals, for cluster analysis.

8.2. Future Work.

This project has provided a variety of opportunities for future research and the knowledge gained, whilst carrying out the work has led to the development of several interesting hypotheses. The theory that common occurrence of certain antibiotics might indicate that they are useful to their producers could be developed further by carrying out experiments aimed at discovering what their natural functions might be. For example, if nigericin was involved in maintaining ionic balance or if it functioned as an ion scavenging agent then its producers might survive better in excessively high or low ionic (Na^+, K^+) environments than would non-producers.

Another fact which emerged was that geldanamycin appeared to only be produced by strains, which also produced nigericin. This is interesting from an evolutionary point of view and more insight might be gained into this relationship

by carefully examining their chemical structures and pathways of biosynthesis. Professor Don Ritchie (personnal communication) has cloned the geldanamycin production genes and if relevant probes and sequence information became available then these could be used to examine nigericin and geldanamycin producing strains in detail. Experiments using strain D153 indicated that changes in the levels of fatty acids might be related to production of geldanamycin and nigericin and this relationship could be investigated in more detail. For example radioactively-labelled precursors might establish whether the decline in fatty acid levels relates directly to antibiotic production.

It is still not clear as to whether antibiotic production is a feature of all streptomycetes or if there are some strains, which do not express this aspect of secondary metabolism. Strains which did not express antibiotic production and resistance were identified during this research and a more detailed look at the effect of environmental factors on these strains might help to assess how the strains might behave in the natural environment. Questions to be addressed include whether they are incapable of expressing bioactivity and resistance, and if not whether they might inhabit a different type of micro-niche to the strains which expressed antibiotic production and resistance. If evidence suggested that the latter case was correct then it would be of ecological interest to determine if relevant strains were specialized for survival in the natural environment in other ways (eg. extreme concentrations of ions, salt, heavy metals or the ability to utillize recalcitrant compounds).

Little is known about secondary resistances (those not connected with antibiotic production), although this work has identified a series of strains which expressed antibiotic resistance, but did not produce an antibiotic under the experimental conditions used. It would be useful to use other methods for determining bioactivity in these strains. If this further supported the existence of secondary resistance in these strains then it would be challenging to pursue the ecological and evolutionary relationship between determinants, which confer secondary resistance and those which confer primary resistance.

The study also indicated strains, which might contain specific antibiotic resistance genes or related genes and has allowed a smaller number of strains to enter a secondary screen, involving southern analysis, which will permit further selections to take place. The chosen strains will then undergo detailed analyses aimed at determining evolutionary relationships within their secondary metabolisms. For example, sequence specific and consensus primers from known genes (eg. *aphD*) might be used and sequence analysis of interesting genes might then be carried out.

Although a variety of antibiotic resistance mechanisms are known it is likely that more exist in nature. It might therefore be enlightening to examine the nature of resistance mechanisms in resistant nonhomologous strains. This could be achieved by using assays for specific enzymes that could be involved in antibiotic resistance or by using additional probes if they became available

The study also allowed the development of several new methodologies. For example nutrient and antibiotic gradient plates were useful tools for population and expression studies; both of these methods could undergo further development. Streptomycetes could be grown on two dimensional nutrient gradients and then overlayed with test organisms to provide useful information on conditions required for bioactivity in individual strains. Antibiotic gradients could also provide a means of stimulating antibiotic production. Diffusion effects on gradient plates could also be studied using dyes of varying solubilities, or by direct assay in the form of plug assays for the antibiotic plates; perhaps colorimetric and flourimetric enzyme assays could be adapted for use with nutrient gradients.

Another application of the work was that a pre-screen based on antibiotic resistance profiles was incorporated into the screening system at ICI Agrochemicals P.l.c. and proved useful in prioritizing strains for screening. This system could be developed to include a much wider range of antibiotic resistances, which might allow prediction of the producers of known compounds as in the work of Kunimoto Hotta.

Although TLC profiles were not a useful taxonomic tool, it might be interesting to analyse the rare compounds which were observed in Chapter 5, both chemically and for biological activity. In addition chemical analysis of the variable spots might help to understand why the results were not consistent and timecourse experiments might show how important standardized conditions were to scoring profiles. Image analysis could be useful for reducing operator error.

Chapter 9.

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