THE DISTRIBUTION AND DIVERSITY OF ACTINOMYCETES IN SOIL FRACTIONS

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This thesis is submitted for the Degree of Docter of Philosophy

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November, 1997.

ACKNOWLEDGEMENTS

I wish to thank my supervisor Dr Elizabeth Welllington for her guidance and help during my PhD studies. I would also like to thank Holger Heuer for his useful discussions, and his co-operation on experiments to solve the problems which we encountered with actinomycete PCR products on denaturing gradient gels. It was also appreciated that he was able to order many different PCR products at the expense of the BBA in Germany. Thanks to Hanne Gürtler for obtaining financial support from Novo-Nordisk which covered international conference expenses and my industrial placement at Novo-Nordisk. During my placement, I particularly wish to thank Rita and Annette for their technical guidance and help; and Uwe for helping me interpret the data obtained from the HPLC. At Warwick University I wish to thank Jane Green for technical assistance; Pamela Weiner for proof reading; Joy Watts and Martin Krsek for useful discussions concerning some of the studies. And finally to Novo-Nordisk and the BBSRC for jointly funding my PhD.

Declaration

The work presneted in this thesis was carried out by myself, except for the 16S rDNA amplified products of 27-701, 226-701, 27-797 and 226-797 which were used in experiments jointly performed by Holger Heuer and myself.

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Abstract

The results presented were concerned with the survival of *Streptomyces coelicolor* A3(2) (pJI673) inoculated into soil microcosms, which were destructively fractionated so that the total propagules and spore counts could be determined in each of the soil fractions. It was found that this microorganism became associated with the smallest soil aggregates at the time of inoculation but with incubation of the soil microcosms the mycelia and spores became attached to the larger soil aggregates. In the sterile soil, the streptomycete growth was much greater than in nonsterile soil, perhaps due to the increased supply of nutrients created by autoclaving the soil, and the lack of competition. Many of the newly formed spores in sterile soil were not attached to the soil aggregates, which may have enabled them to be distributed to new microsites.

When the distribution of indigenous actinomycetes in soil was investigated, it ressembled the distribution of *Streptomyces coelicolor* in nonsterile soil after the inoculant had been through one life cycle. Actinomycetes were then isolated from each of the soil fractions, as well as the unfractionated soil, and each of these strains were identified to genera, if possible. It was found that many of the micromonosporas and streptosporangia were isolated from the 63-251 μm soil aggregates, probably because this fraction contained low eubacterial and streptomycetes populations caused by the low organic content within this soil fraction. There was a high eubacterial count in the 2-20 μm soil aggregates and although the actinomycetes were outcompeted within this soil fraction, their diversity was greatest within this fraction. This diversity was also reflected by their production of different secondary metabolites.

DNA was extracted from each of the isolates and amplified using specifically designed primers for high GC microorganisms. Each of the products were individually run on denaturing gradient gels. It was found that the amplified products from actinomycetes formed bands on the denaturing gels which migrated to 3 positions. Each of these positions corresponded to major groups of actinomycetes of which streptomycetes formed one group. The patterns corresponding to the isolates of each soil fraction would be compared with the amplified products derived from in situ soil DNA extracts. It was found that the results were not comparable but this work is still being investigated.

Abbreviations

16S rDNA 16S rRNA gene on the chromosome

16S rRNA 16S gene transcribed into RNA

AV low nutrient and salts medium

BSA bovine serum albumin

DAP diaminopimelic acid

DAPI diamidinophenylindole

DGGE denaturing gradient gel electrophoresis

DMSO dimethyl sulphoxide

DNA deoxyribonucleic acid

FTIR Fourier Transform Infrared Spectroscopy

GC guanine and cytosine

HGC primer specific for high G+C microorganisms

HPLC high pressure liquid chromatography

HV humic acid medium

MPN-PCR polymerase chain reaction carried out using the principles of most probable number viable dilution methods to assess bacterial populations

MSD minimum significant difference

p.s.i. pounds per square inch

PCR polymerase chain reaction

RASS reduced arginine, starch and salts medium

RNA ribonucleic acid

SC starch casein medium

SDS sodium dodecyl sulphate

SEM scanning electron microscopy

TLC thin layer chromatography

1. Introduction

1.1 Soil Composition

The soil is composed of particles which are the product of weathering of rocks. Soil particle analysis can be performed to determine the texture of the soil based on the fixed diameters of the different soil particle classes. These are the <2 μm clay particles, 2-20 μm silt particles, 20-2000 μm fine sand and >2 mm coarse sand. As the clay particles are very small, they possess a much higher surface area in comparison to an equivalent weight of the larger silt and sand particles (Parkinson *et al.*, 1971). The major types of clay particles include illite, kaolinite and montimorillinite (Griffiths, 1965). Illite consists of single clay lattices and, in comparison to other clay particles, they have a lower surface area which means that less water can be absorbed. Kaolinite consists of 2 intercalating clay lattices whereas montimorillinite has 3 and when either types of clay particles are immersed in water, they expand by ratios of 1:1 and 2:1, respectively. Consequently, 80% of the total surface area of montimorillinite clay particles is within the lattices which means that organic molecules which become attached to these internal layers are unavailable for microbial decomposition (Knaebel *et al.*, 1994). Metal ions which are alkaline and alkaline earth metals in soil, form an ionic layer around the negatively charged clay particles. The ions maintain the soil at near to neutrality but in soils where there is heavy rainfall, these ions may be leached out as they are replaced with sodium ions so that the soil becomes acidic (Dixit and Sharma, 1993). The biological material, mainly plant matter in grassland and agricultural soils, often constitutes a small percentage of the total weight of the soil although some soils such as peat may have a much higher organic content (Kabir *et al.*, 1994). The separate classes of soil particles and the biological matter do not exist independently but combine to form aggregates. The smallest class of aggregates which happen to be the most stable are water stable aggregates. They are held together by organic polymers produced from animal, plant and microbial activity as well as the adhesive properties of clay particles. These are collected by a method known as soil fractionation which relies on soil aggregates remaining intact when immersed in water during sieving and sedimentation.

Using this method it has been possible to collect soil aggregates ranging from <2-500 μm in diameter (Jocteur-Monrozier *et al.*, 1991).

The crumbs which are larger soil aggregates, measuring several millimetres in diameter, are formed from water stable soil aggregates. They are stabilised by the network of roots as well as by the wetting and drying cycles of the soil which act to pull the soil particles together. The soil crumbs may also form larger aggregates of soil described as peds, which are several centimetres in diameter. The weathering of the soil may occur by repeated cycles of drying and wetting or freezing and thawing. This causes the aggregates to expand and then contract to form stable clumps. It is possible to form artificial large aggregates of soil, termed clods, by wetting and mixing the soil (Oades, 1984). In sandy soils, water stable aggregates do not form due to the extremely low clay content and the low biological matter, but instead larger, unstable aggregates are formed.

1.2 Soil Horizons

The soil can be divided into layers with decreasing depth which appear physically different from one another (Coleman and Crossley, Jr, 1996). The top layer of the soil is the phyllosphere which consists of the growing plants. Bacteria and fungi may grow on the leaves of the plants. The next layer is the leaf litter layer, which in forest soils is quite dense whereas in agricultural soils it is almost non-existent (Gray and Williams, 1971). The leaf litter layer in forest soils may consist of dead fallen leaves or pine needles, depending on the type of forest. This layer is often described as the A0 horizon but some soils have several more distinct layers called, A1 and A2, which consist of litter at various stages of decomposition. Previous studies which investigated the horizons where different actinomycete genera were to be found, showed that the streptosporangia were predominantly isolated from the A horizon perhaps as actively growing mycelia on litter (Zenova *et al.*, 1995). This would not be surprising because streptosporangia grow in weakly acidic conditions which would occur within the A horizon. Acidophilic streptomycetes have been found to grow within the litter layer but they are restricted to discrete sites despite the high organic content of this layer (Khan and Williams, 1975).

The next layer consists of decomposing plant material, soil and plant roots. It is within this fraction that neutrophilic streptomycetes are predominant, usually as spores but

sometimes as mycelia, growing on dead root fragments when they were observed by scanning electron microscopy (Williams *et al*, 1972). The streptomycetes which grow within this soil horizon are of major interest in ecological studies, in understanding their modes of survival and dispersal, in an oligotrophic environment. They are also useful in the pharmaceutical industry for their production of secondary metabolites. The B horizon may be several meters deep and below this there will be the parent rock, the C horizon.

1.3 The Irregular Distribution of Bacteria in Soil

Much of the precipitation on the soil runs through cracks through the soil layer formed by roots. When the water is in close contact with the roots, some of the water enters the xylem vessels of the plant by capillary pressure. This results in the water running along the root surface, either laterally along root mats or vertically to the bedrock (Williams, 1985). The younger roots exude nutrients and other products, and together with the availability of water, the rhizoplane provides ideal conditions for bacterial colonisation. The bacterial density in soil nearby the young roots, the rhizosphere, may also be quite high. Investigations into the bacterial distribution within the rhizosphere of the soil have shown that bacterial densities are 50 fold greater near the roots than much further away (De Leij *et al.*, 1993).

Periodic precipitation should cause the soil to dry out after the water has passed through the soil by gravitational forces to the base rock layer. However, some of the water is captured within soil aggregates by capillary forces and by clay particles which absorb water (See Figure 1). Consequently, the water is held within the soil aggregates for some time. The dissolved nutrients and oxygen within water may enable bacteria inside the soil pores to grow as the water flows into the soil aggregates. Soil fractionation studies revealed that water stable aggregates ranging from 2-20 μm in diameter have higher bacterial densities due to their high clay content and high microporosity (Jocteur-Monrozier *et al.*, 1991). Conversely, the larger aggregates had a much lower microbial population, perhaps due to their lower microporosity. The majority of bacterial cells were found to reside within the pore spaces of the soil aggregates rather than on the surface of the aggregates when they were viewed by the scanning electron microscope. Probably the bacteria grow within the aggregates on nutrients which diffuse through the

pores and are prevented from desiccation by the water retained within the aggregates. Studies by Hattori (1988) revealed that many of the Gram negative bacteria were present within the soil aggregates whereas the Gram positive bacteria were generally found in the outer part of the soil aggregates. It was postulated that Gram negative bacteria were perhaps binding the aggregates together by their excreting polysaccharides. The Gram positive bacteria form spores that are resistant to dry conditions and so the spores could exist outside soil aggregates. An explanation for the high bacterial densities in the 2-20 μm soil fraction may be attributed to the finer pores which enable nutrients and oxygen to flow quickly into the aggregates, whereas wider pores in the larger soil aggregates would have a lower capillary flow (Hattori, 1988; Jocteur-Monrozier *et al.*, 1991). Consequently, the larger soil aggregates have anaerobic centres caused by the low oxygen flow rates through their wide pores (Hattori, 1973; Jocteur-Monrozier *et al.*, 1991). It is probable that anaerobically respiring bacteria may grow within these aggregates on nutrients trapped within the aggregates. In previous studies it was shown that the anaerobic bacteria, in proportion with the aerobic bacterial densities, formed a significant component of the microbial population particularly in the top layer of the soil (Hattori, 1973).

Nutrients within the soil are heterogeneously distributed which may be indicative of the bacterial distribution in soil, as microorganisms will grow where nutrients are present (Williams, 1985). Recent studies suggested that the bacterial growth rates in soil were equivalent to the growth rates determined on the laboratory medium (Murray *et al.*, 1995). It was postulated that the problem with most ecological sampling studies is that all the bacteria are assumed to grow at the same rate on laboratory medium whereas many of the microorganisms may take longer to germinate since they were in a dormant state in soil. This implies that the soil is an oligotrophic environment, where some bacteria grow continuously but slowly, whilst others have phases of rapid growth followed by long periods of dormancy (Williams, 1985). The dormant state is particularly easy to observe with streptomycetes as only a small percentage form actively growing mycelia, whilst most of the population consists of dormant spores. The streptomycete spore population can be differentially detected using a streptomycete spore specific extraction method (Herron and Wellington, 1990). The morphological difference between the active and dormant states is not so obvious for other bacteria

inhabiting the soil. Agrobacteria cells become dwarf and coccoid because of the onset of starvation and can remain in this state for long periods of time but their morphologically altered states are much more difficult to detect in soil populations.

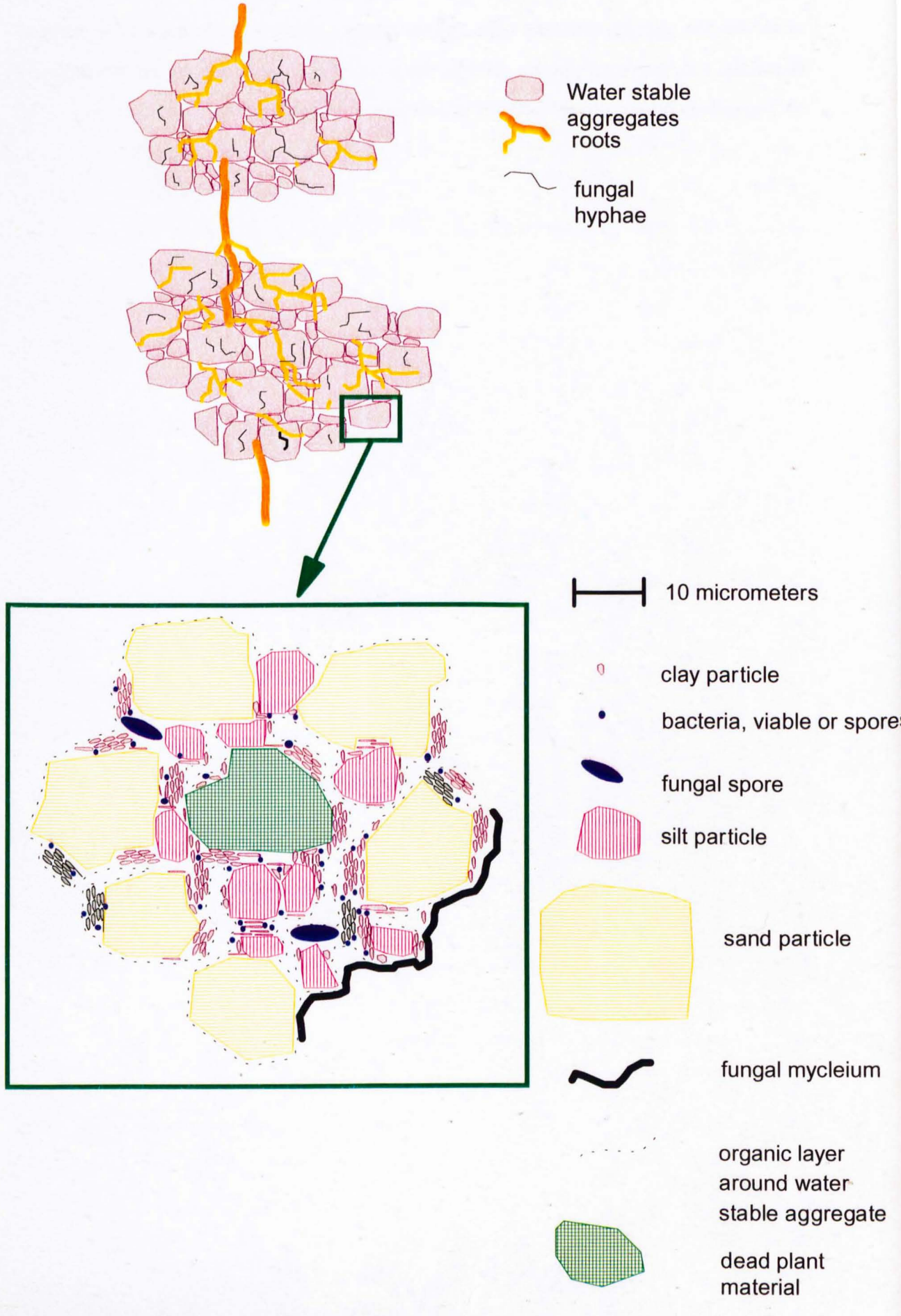

Water stable aggregates
roots
fungal hyphae
10 micrometers
clay particle
bacteria, viable or spores
fungal spore
silt particle
sand particle
fungal mycleium
organic layer around water stable aggregate
dead plant material

Figure 1. Structure of soil aggregates.

Unstable soil aggregates formed by entanglement of plant roots and fungal hyphae. These are composed of water stable aggregates held together by microbial by-products and clay binding.

1.4 Soil Fractionation

There are several different methods to fractionate soil into either original soil particles, water stable aggregates or soil crumbs. Whichever method is chosen, the principle remains the same, which is to determine whether the micro-environments which the bacteria inhabit influences their distribution as well as nutrient availability. One method of soil fractionation involves completely disrupting the structure of the soil using homogenisation, then sieving to collect the sand fraction and collecting the silt and clay fractions by sedimentation (Christensen, 1987). It was found that the clay fraction had the highest proportion of bacteria. This was explained by the organic molecules adhering to the surface of the clay particles which likewise encourage the attachment of bacteria. Clay particles are also negatively charged due to the substitution of trivalent aluminium for tetravalent silicon in the lattices of these particles. This negative charge attracts cations, particularly alkaline and alkaline earth metals, such as potassium and calcium, which in turn results in the formation of an increasing gradient of anions away from the cations. This gradient is known as the double electric layer in which negatively charged bacterial cells are attracted by Van Der Waals forces near to but not onto the surface of clay particles (Gray and Williams, 1971). The very low negative charge on sand particles prevents the attachment of microorganisms unless the cells are particularly hydrophobic (Stenström, 1989).

An alternative fractionation method investigated the water stable soil aggregate distribution from which it was possible to ascertain where the different bacteria resided within the separate fractions (Jocteur-Monrozier *et al.*, 1991). These soil aggregates are disrupted to break up the crumbs by gently shaking whilst they are immersed in water in a sieve. The larger soil aggregates are collected in this way but the smaller aggregates must be collected either by sedimentation or low speed centrifugation, otherwise the finer graded sieves would become blocked. These smaller aggregates settle at a rate depending on the diameter of the soil aggregates which assumes that they all have the same density (Darbyshire *et al.*, 1993). The disadvantage of sedimentation is that it is time consuming. A quicker alternative is centrifugation but this method requires standardisation to collect the desired size range of the soil aggregates.

It has been found that the undegraded organic matter appears with the largest soil aggregates. There is also a high carbon and nitrogen content, comprised of stable molecules, in the 2-20 μm soil aggregates. Consequently, it has been found that the bacterial counts are highest in the largest and 2-20 μm soil aggregates as well as with the <2 μm soil aggregates (Jocteur-Monrozier *et al.*, 1991). It has been hypothesised that the high density of bacteria in the 2-20 μm soil aggregates could also be attributed to the high microporosity detected within this fraction compared to the other soil fractions. The high bacterial counts within the smallest soil aggregates may be detached cells from the surface of larger soil aggregates. High bacterial populations were associated with these particular soil fractions in fractionated grassland soil, whilst in continuously cultivated soil a more even distribution throughout the different soil fractions was observed (Kandeler and Murer, 1993). Lower bacterial counts within the expected soil fractions may have been caused by the ploughing of agricultural soil which reduces the stability of water stable aggregates.

A unique method was developed by Hattori (1988) to measure the bacteria in the outer and inner part of water stable soil aggregates by immersing them in water and gently sonicating for several minutes. The bacteria which were easily removed, appeared in the supernatant perhaps because the cells were near the outer surface of the aggregates. Other bacteria remain with the soil aggregates as these cells are firmly attached or deep within the aggregates. It was found that the bacterial population within the soil aggregates were mainly Gram negative bacteria whereas the Gram positive bacterial spores were identified in the outer part of the soil aggregates.

In another method the soil crumbs can be collected by using tweezers to hand pick the larger aggregates and dry sieving to collect the smaller aggregates. With the largest soil crumbs higher bacterial counts were found on minimal medium compared to nutrient agar but this difference in counts did not occur with the smaller soil crumbs (Dabek-Szreniawska, 1993). It was concluded that many of the bacteria which grew were oligotrophic bacteria forming colonies on the minimal medium. These bacteria may have been growing within the soil crumbs, in narrow pores which were inaccessible to grazing protozoa. This observation may be true based on other studies using rhizobia inoculants (Ozawa and Yamaguchi, 1986).

Using this method of separating soil crumbs, it was also found that protozoa were often restricted to soil aggregates (Hattori and Hattori, 1993). It is also hypothetically possible that bacterial populations may be restricted to soil aggregates. Therefore, some microorganisms would require dispersal mechanisms so they could be distributed throughout the soil.

1.5 Actinomycetes

The actinomycetes are Gram positive microorganisms which grow in aerobic conditions as branching substrate mycelium, and later form spore chains at the end of aerial hyphae. Many of the different types of actinomycete genera can be identified by their sporulating structures, such as streptosporangia, which forms a cluster of spores contained within a sheath. They are mesophilic and some are either acidophilic or neutrophilic. They produce a wide range of enzymes which can degrade complex molecules such as lignin, hemicellulose, starch and chitin.

The largest actinomycete genus is the streptomycetes which form either straight, curly or spiralled spore chains. Aerial hyphae are formed during the latter part of their life cycle, as the growth rate declines which is accompanied by antibiotic production (Lechavalier and Lechavalier, 1981). The aerial hyphae which form spore chains within a sheath extend up from the surface of the solid medium on which the substrate mycelium is growing. In *Streptomyces coelicolor*, the formation of spores is initiated by the *WhiG* gene which results in a cascade of regulatory genes producing the proteins which effect each stage of spore development (Mendez and Chater, 1987). Similar regulatory controls may exist within other streptomycetes. Spores may preserve the microorganism in nature when nutrients become low but Williams *et al.* (1972) found that streptomycete spores lasted for a limited time of about 24 weeks. This may be caused by the quantity of ribosomal RNA in spores, which is only two fold lower than in mycelia of equivalent dry weight, suggesting that the metabolic activity in spores is in a reduced state rather than in a completely dormant state (Quiros *et al.*, 1989). The main function of the spores may be to disperse the streptomycete to new sites of nutrient availability. Spore formation is linked in a complicated way to antibiotic production by a process which has never been fully understood. Like sporulation, antibiotic production in *Streptomyces coelicolor* is regulated by a global antibiotic regulatory gene, *Asb* (Adamidis *et al.*,

1990). This gene may in turn affect a cascade of genes up to the final regulatory control of each antibiotic cluster. The role of antibiotics has never been elucidated despite their antagonistic effects on bacteria and fungi. The reason for this is that the indigenous streptomycetes do not appear to produce antibiotics in soil or perhaps the methods to detect antibiotic production in soil are too insensitive (Williams, 1982). Others have postulated that antibiotics are produced as a result of poor primary metabolism by actinomycetes or they are produced to protect lysing mycelium during spore formation from bacteria competing for nutrients (Chater *et al.*, 1988).

1.6 Identification of Actinomycetes

The actinomycetes can be subdivided into 6 different groups on the basis of the diaminopimelic acid (DAP) isomer and the types of amino acids present within the cell wall. The analysis is performed by running the extracted cell products on thin layer chromatography (TLC). Four of these groups contain the DAP although the streptomycetes are the only major group which possesses the LL-DAP isomer whereas 3 other groups have the meso-DAP (Staneck and Roberts, 1974). Whichever group the unidentified bacteria falls into may give an indication to which genus it may belong. A clearer indication may be obtained if the sporulating structures are viewed under the microscope, as some actinomycete genera have distinctive sporulation structures. Further characterisation may be performed by analysing the sugar extracts run on TLC since there are 4 specific groups which possess distinctive patterns. Consequently, by a process of elimination it is usually possible to identify the genera (Lechavalier and Lechavalier, 1970). Sometimes it is necessary for a more detailed analysis, by studying the menaquinones which are the only type of quinones present in Gram positive bacteria unlike the Gram negative bacteria which also have ubiquinones. The menaquinones are used for identification, as there are 4 major groups to which they could belong but the carbon chain length and the degree of saturation are also important factors in identification to the generic level. The menaquinones may be either run on TLC plates or analysed by high pressure liquid chromatography (HPLC) and then the spots from the plates, or the eluted fractions from the column, can be further characterised by running them on silver impregnated TLC plates or silver-loaded HPLC system to determine the number of double bonds. The menaquinones which have many double carbon bonds

either migrate slowly up the plate or are eluted late from the column (Kroppenstadt, 1982).

One particular group, the streptomycetes, has proven important commercially because of the extracted secondary metabolites which have antimicrobial activities. Consequently, this actinomycete genus has received more attention which necessitated a method being developed to identify which species an isolate was most closely related by performing a series of biochemical tests (Williams *et al.*, 1983). The results are scored either positive or negative for each characteristic test and the data is entered into a program called Matiden. After the program has been run the output shows 3 species to which the microorganism is most closely associated along with the Wilcox probability. A high Wilcox probability indicates that the microorganism may be closely related to the streptomycete to which it was identified, although low values would indicate poor identification.

An alternative method compares the fatty acids which are formed within the cytoplasmic membrane of a microbe. There are usually unbranched fatty acids in Gram negative bacteria whereas in Gram positive bacteria, they are branched. The microorganisms which are to be evaluated must be grown under identical conditions and incubated for the same length of time. The fatty acids are extracted from the biomass and analysed by gas-liquid chromatography. The chromatogram obtained will be unique for each particular microorganism. It is therefore possible to identify to which species it may belong providing known type strains have been treated under the same conditions. However, it is not always necessary to know the identity of the type strains because in ecological studies a large collection of isolates may be compared to each other and plotted onto a 2-dimensionally.

Identification can be made more accurate by using molecular tools than with the classical methods using cell wall, sugar and menaquinone analyses because the results from the latter may cluster actinomycetes which are different but have similar biochemical traits. Consequently, an identification made to the genus level based on biochemical characteristics may differ from the identification predicted by molecular information using sequencing. It is also possible that some genera are much more closely related than

were previously supposed. Sequence information on the 16S rRNA has been obtained which can be used to develop genus specific DNA probes for hybridisation identification using the DNA extracted from the microorganisms. Further characterisation may be performed using primers in PCR to amplify the hypervariable region of the 16S rRNA gene which is then sequenced (Stackebrandt *et al.*, 1991). By matching the sequence found with those already existing on the database, it is possible to identify to which species the isolate may belong. However, there are many natural isolates that have sequences which do not match anything stored on the databases and so remain unidentified. In the classical methods the microorganism would be identified to a subgeneric level even if species identification was not possible.

1.7 The Microbial Population in Soil

Altogether the quantity of microbial propagules may number 10^9 cells per g soil but the fungi may comprise up to 50% of the microbial biomass even though their population is quite low at around 10^5 propagules per g soil. The fungi can be subdivided into 4 different groups which are the phycomycetes, ascomycetes, basidiomycetes and fungi imperfecti (Hattori, 1973). The phycomycetes grow to form a highly developed mycelium which has no cross-walls and then produce stolons leading away from the mycelium. At intervals along the stolon, sporangia are formed which contain non-motile spores. The ascomycetes comprise the largest group of fungi and these produce 8 spores contained within an ascus as part of the sexual cycle. However, branching spore chains are often produced which are described as conidia, as a consequence of the asexual cycle. The typical members of this group of fungi are penicillia and aspergillia. The next largest group of fungi are the basidiomycetes which form the distinctively large sporangium called the basidium. These fungi are usually found in fields and forests, perhaps because these soils are rarely disturbed which would enable the basidiomycete hyphae to grow over large distances without becoming fragmented. The final group of fungi, fungi imperfecti, appear not to form the sexual stage but do form conidia. All these fungi are commonly found in soils and generally their mycelium may cover large areas of soil. However, in some soils, particularly of forests, the fungi may be restricted to growing within certain soil horizons such as the leaf litter layer and B soil horizon. Obviously, the deposition of the leaf litter layer is seasonal, so not surprisingly a succession of functionally different fungi were detected in a beech forest soil (Kjøller and

Struwe, 1994). The first fungi detected were pectin degrading fungi, then cellulolytic fungi and finally chitinolytic fungi although lignin degrading fungi survived through all the different fungal successions.

Many of these morphological and biochemical properties are shared by a group of bacteria described as streptomycetes which exist at 10^7 propagules per g soil and make up to 20% of the microbial biomass. The streptomycetes, like the fungi are involved in the degradation of complex molecules which may result in some overlap of their niches. Consequently, it has not been surprising to find that fungal populations decreased whilst streptomycete populations increased as soil samples were obtained from more southerly regions from the Arctic (Hattori, 1973).

The majority of streptomycete propagules in soil exist as spores while the remainder are mycelia growing on organic debris, usually on dead roots (Mayfield *et al.*, 1972). They form long mycelia with few branching points and they grow within the crevices of soil aggregates (Cresswell *et al.*, 1992). Thin sectioning of soil crumbs has revealed that highly branched mycelia and globose bodies are formed within the pore spaces although these mycelia may be attributed to other actinomycetes growing in the soil (Mayfield *et al.*, 1972). Streptomycetes may grow at a much slower rate in soil than on laboratory medium as determined by calculations of the specific growth of germinating spores. From this it was concluded that the soil is an oligotrophic environment, making it necessary for streptomycetes to survive as spores through periods when there are few nutrients. They may also grow on the complex substances remaining after copiotrophic bacteria have used the freely available metabolites. Inoculated *Streptomyces lividans* spores in soil microcosms germinated forming extensive mycelia, which survived for several days before the onset of sporulation (Cresswell *et al.*, 1992). This does not necessarily mean that all the mycelia die once sporulation has begun. Within these particular experiments, more mycelia may have been produced, had the larger dead root fragments not been removed by sieving in preparation of the soil for microcosm experiments.

Besides the streptomycetes, there are pseudomonad, corynebacteria and bacilli which form almost the remaining 30% of the microbial biomass and are detectable at 10^8

propagules per g soil. The corynebacteria may thrive on the exudates released by young roots, while bacilli are involved in the breakdown of plant material. The pseudomonad population may play an important role in the nitrogen cycle by denitrification, thereby returning the nitrogen back to the atmosphere. The minor part of the microbial population in soil is formed by algae and protozoa which number 10^4 cells per g soil. The protozoa may play an important role in grazing on the faster growing bacteria in the soil unlike the slow growing bacteria which may be physically protected within narrow soil pores (Ozawa and Yamaguchi, 1986). There are other microorganisms in the soil which are at very low concentrations, e.g. *Bradyrhizobium sp.*, which plays an important function in the nitrogen cycle by fixing nitrogen into higher plants (Hattori, 1973).

1.8 Viable Counting as a Means to Follow the Fate of Streptomycetes in Soil

The standard procedure used for determining the number of viable actinomycetes in soil involves shaking the soil in saline solution for a standard time and then counting colonies which have grown on spread plates. After 20 minutes, the maximum quantity of mycelial fragments may be obtained as they fragment although these results were based on an experiment conducted using microbial inoculants (Mayfield *et al.*, 1972). If the mycelia were shaken for much longer then the fragments became so small that they no longer formed viable units. However, these fragments may often vary in length, which means that the quantity recovered determined by the plate counts may not accurately reflect the active biomass. Alternative methods to fragment the mycelia are sonication and homogenisation. Sonication is a harsh method which often produces erroneous results (Ramsay, 1984) whereas homogenisation is equally effective as shaking but cannot be performed quickly on a large number of replicates (Baecker and Ryan, 1987).

Streptomycetes have 2 stages during their life cycle in soil, as mycelia which are presumably actively growing, and spores which are often described as dormant. Consequently, to gain a better understanding of the growth of streptomycetes in soil, it was necessary to devise methods to monitor one of the streptomycete stages independently from the other. Methods to extract the mycelia from soil and concentrate them are ineffective because the mycelia become entangled around the soil particles but methods have been developed to investigate the spore population alone. One such technique entails shaking the soil for 1 hour in saline solution so that the mycelia are

completely disrupted, leaving only the spores (Skinner *et al.*, 1951). However, in this original study, mycelia and spores were used which had been grown on laboratory medium for several days although it was possible that as mycelia aged the cell wall became more rigid and therefore less liable to disruption. Another method was developed which separated the spores from the mycelia and the bulk of the soil by a process of differential centrifugation (Herron and Wellington, 1990). The advantage of this method was that the spores were detected at very low quantities although the yield of spores were often lower than the original spore population within the soil, as many were retained within the bulk of the soil.

1.9 Antibiotic Production by Actinomycetes in Soil

Antibiotics have never been directly detected in natural soil but studies have been carried out to investigate secondary metabolite production in unamended and amended sterile soil microcosms inoculated with the producing microorganisms. One of the best examples showed *Streptomyces venezuelae* produced chloramphenicol in sterilised soil when the microorganism grew (Gottlieb, 1976). It has been found that there was a direct correlation between the extent of antibiotic production by a bacterial inoculant, and the amount of nutrients which were mixed into sterile soil microcosms. (Soulides, 1965). In the natural soil, the organic content is very low, which results in only a small percentage of streptomycete spores germinating to form mycelia (Cresswell *et al.*, 1992). This may lead to a much lower yield of antibiotics in the natural soil, which would test the limits of the detection for extracted antibiotics. It must also be assumed that the antibiotics do not diffuse far away from the producing mycelia growing in the soil. Hence sampling the whole soil rather than trying to recover where the mycelia are localised, such as on dead roots, would result in the antibiotic concentration being diluted below detectable levels. It is worth considering that some antibiotics, particularly those that are basic, readily adhere to clay particles, so that these antibiotics are rendered inactive in the soil and may be difficult to extract. Those not attaching to soil particles may be soon degraded by microbial activity in the soil (Williams, 1982).

Many studies have been carried out to observe the affect of controlling a fungal pathogen in soil using bacteria expressing an antifungal secondary metabolite. In one such study,

the separate effects of each of 10 different streptomycetes were examined on the fungus, *Phytophthora megasperma,* when the streptomycete and fungus were inoculated into soil together. It was found that only one streptomycete restricted the growth of the fungal pathogen preventing root rot on the soyabeans even though this microorganism happened not to express any antibiotics. A more promising result was found when the soils were inoculated with each of these streptomycetes and pre-incubated for several days, prior to inoculation of the fungal pathogen into soil. It was then found that most of the streptomycetes prevented the plants from becoming diseased (Rothrock and Gottlieb, 1981). An explanation for the lack or low antimicrobial activity of the streptomycetes may be that the niches of these 2 microorganisms had little overlap, perhaps as they grew on different substrates or occupied distinct microbial habitats. In another experiment, *Pseudomonas aureofaciens* strains were isolated from a soil which did not contain the root rotting fungus, *Gaeumannomyces graminis.* It was found that these strains exhibited antifungal activity which effectively suppressed the growth of *G. graminis.* It could be argued that the *P. aureofaciens* and *G. graminis* strains competed for growth space in the same microbial habitat, on the wheat roots, but when a mutant of *P. aureofaciens* was inoculated into soil it only slightly suppressed the growth of the *G. graminis* (Harrison *et al.*, 1993).

The results of many of these experiments have highlighted the different interactions that may occur among microorganisms, especially between streptomycetes and fungi. These interactions are not necessarily attributed to antibiosis. Streptomycete competition with fungi may encompass parasitism, where the chitin in the fungal cell wall is degraded causing lysis; competition for nutrients; and the production of volatile inhibitors. These may affect some or all of the stages of the fungal life cycle (Williams, 1985). Inhibitory substances to fungal spores have been detected in soil extracts. It was found that the toxins were destroyed by sterilisation which confirmed their presence, so that when the fungal spores were added to soil extract, they germinated and grew (Gottlieb, 1976).

Perhaps the most convincing evidence for antibiotic production was found by observing the effects of natural actinomycete strains producing antibiotics in soil on the fungal hyphae of *Helminthosporium sativum.* It was found that the antibiotic caused similar

distortions to the mycelium whether they were grown in soil or in culture (Stevensen, 1954). Other results indicate that the role of antibiotics by the producing microorganism does not appear to show a selective advantage (Gottlieb, 1976).

It was found that when *Streptomyces lividans* strains were grown separately or together in soil microcosms containing thiostrepton, that the strain which carried the lysogen KC301 did not grow any better than the strain without the lysogen (Marsh and Wellington, 1994). This was surprising because the KC301 conferred resistance to thiostrepton which should have enabled the microorganism containing this lysogen to outcompete the other strain. The results indicated that the antibiotic had little affect on the *S. lividans* without KC301 even though it was proven that the antibiotic was not lost by adherence to clay particles or by microbial degradation.

Despite the lack of conclusive proof that antibiotics are produced in soil, it cannot yet be ruled out as a possibility, but others have suggested alternative mechanisms, such as a role for secondary metabolites in morphological differentiation during spore formation (Williams and Vickers, 1986). It is also possible that each secondary metabolite confers a particular characteristic advantage for the bacteria expressing it, perhaps not necessarily as an antimicrobial compound. For instance, it was found that bacteria expressing bleomycin were also resistant to alkylating agents which may be readily found in the environment and be damaging to the microorganism.

1.10 Soil Microcosms

Prior to inoculation the soil microcosms may be sterilised in order to observe the growth of the inoculant without bacterial competition. The microcosms have no input of nutrients from plants, either through decaying plant material in the leaf litter horizon or exudation by young roots. Animal activity is also excluded from the soil microcosms because products formed by the animals and dead remains may act as sources of nutrients but their main effect would be on the disturbance of soil which would contribute to mixing of nutrients and to a lesser extent the dispersal of microorganisms (Thorpe *et al.*, 1996). Obviously to create microcosms to include these parameters would be practically impossible as there would be too many parameters to contain and

also it would be difficult to identify which factor could be attributed to the observed effect (Leser, 1995).

Previous studies have shown that air drying the soil may increase the stability of the aggregates, particularly the larger aggregates, as soaking in water with gentle agitation may result in water failing to penetrate within the soil clods, thereby maintaining their stability (Beare and Bruce, 1993). However, this observed aggregate stability may be negated by crushing the soil with pestle and mortar before wet sieving. This makes the soil within these microcosms homogeneous unlike the natural soil. In the soil microcosms there would be plenty of freely available nutrients composed of the dead organic remnants of fine roots, fungi and bacterial cells, especially within the sterilised soil. The air dried conditions of the nonsterile soil may induce the indigenous soil bacteria into the dormant state, either as spores or minute bacterial cells which have low metabolic activities. The metabolic activities of the indigenous soil microorganisms may require several days to increase compared with the higher metabolic state of inoculated bacteria (Teuben and Verhoef, 1992). The streptomycete spores from the surface of laboratory medium after several days growth have reasonably high ribosomal RNA contents (Quiros *et al.*, 1989). An investigation into the nucleic acid contents of starved cells which had become much smaller indicated that they were lower than larger viable cells (Bakken and Olsen, 1989). Consequently, indigenous soil streptomycete spores which have had to endure oligotrophic conditions microcosms may germinate much slower than bacteria inoculated into the soil.

1.11 Soil Sterilisation for Microcosms

There are 3 methods which are used to sterilise soil and these are gamma irradiation, exposure to vapours of methyl bromide and autoclaving (Eno and Popenoe, 1964). Gamma irradiation of the soil effectively kills all the bacterial cells but it is necessary to use small quantities of soil, usually one gram, otherwise the radiation is unable to penetrate the soil. The disadvantage of this method is the expense compared to the other techniques but it is sometimes used as it does not mineralise organic carbon and nitrogen, and leaves the destroyed microorganisms as intact cells. The next least destructive method involves using methyl bromide vapour which reacts with the microorganisms and destroys them as the vapour penetrates the soil but it leaves organic molecules intact.

Not all the bacteria are destroyed as sometimes the methyl bromide vapour fails to penetrate through the pores into the aggregates where the bacteria reside and also it may be ineffective against spores. When Hattori (1988) investigated the effect of ethylene dibromide on soil bacteria, it was found that the Gram positive bacteria often survived while the Gram negative bacteria were killed. This was expected because the greater part of the spore population was represented by the Gram positive bacteria. Much larger quantities of soil can be sterilised with methyl bromide unlike the method using gamma irradiation. The limitations of the method may also be caused by the clay content of the soil which could affect aggregation therefore preventing adequate sterilisation within the pore spaces. For large quantities of soil that need to be sterilised, the method used is autoclaving. With this method all the microorganisms are destroyed, causing disruption of the cell walls and the cell contents are partially mineralised along with freely available organic detritus. The bacilli spores are heat resistant so it is necessary to autoclave the soil twice leaving an incubation period allowing the bacilli spores to germinate. All the bacterial cells are destroyed and the method is cheap which explains why it is so commonly used.

1.12 Analysing Microbial Communities using Molecular Methods

The conventional methods to study the fate of microorganisms in soil involve bacterial isolation on either selective or non-selective media. Although these methods are very sensitive enabling bacterial detection at very low levels, not all bacteria are culturable (Akkermanns *et al.*, 1994). Moreover, nutrient rich media may select for the copiotrophic bacteria rather than the dominant microorganisms in a sample environment (Moyer *et al.*, 1994). As an alternative to these methods there are molecular techniques which can be used to evaluate the bacterial diversity of total DNA extracts from soil samples, as well as to compare isolated strains with one another.

A technique was developed to extract DNA directly from a sample, such as a soil (Ogram *et al.*, 1987). In the previous studies, the bacterial fraction was isolated from the sample and then the DNA was extracted from this fraction, but the disadvantages of this method were that some bacteria were so firmly attached to soil particles that they could not be removed. This meant that the bacterial fraction was unrepresentative of the actual

microbial population in the soil sample, because filamentous microorganisms such as fungi would be entrapped within the soil fraction (Holger and Myrold, 1991). The methodology for in situ isolation of DNA from soil samples proceeded by SDS lysis at 70°C and then followed by bead beating in phosphate buffer, which helps to remove DNA adsorbed to soil particles. The method indicated that 95% of cells were disrupted. One of the problems with this method is that extracellular DNA may also be recovered although this may be overcome by washing the soil in phosphate buffer before pursuing the lysis steps. The analysis of the extracted DNA may provide an indication of the population density of the microorganisms, but it would also be interesting to know the active bacterial population in an environmental sample. This can be accomplished after the RNA has been extracted from soil, by quantitative densitometric measurements of the RNA hybridisations (Odenyo *et al.*, 1994a).

DNA hybridisation studies are commonly used to study either a specific microorganism within a microbial community or the fate of the release of a genetically engineered microorganism (GEM) in an environment. In one particular example, a probe was especially developed for *Pseudomonas stutzeri* that has an important ecological role in denitrification in oceans (Kerkhof, 1994). The fate of *P. Stutzeri* has been specifically monitored by hybridisation to a fragment of eukaryotic DNA cloned into the microorganism (Chaudhry *et al.*, 1989). In another experiment, the RNAs from different *Ruminococcus* species were extracted and hybridised with specifically designed probes whereas previously it had only been possible to measure the rates of substrate degradation or end product formation (Odenyo *et al.*, 1994b). It has been found in hybridisation studies that the combination of small probes and many genes on an unfragmented bacterial chromosome, yielded the optimum results (Steffan *et al.*, 1989; Griffiths *et al.*, 1996). Using these conditions positive hybridisation signals were obtained when a bacterial population of 10^4 cells or higher were probed although contaminants which were co-purified with the DNA extract from soil reduced the signal intensity by 10 fold (Tas *et al.*, 1995; Tebbe and Vahjen, 1993). When attempting to completely remove the probe from the filter membrane, sometimes residual signal remained due to irreversible binding to humic acids (Moran *et al.*, 1993). It must be realised that successful hybridisation results may be obtained even if the probe of several hundred

bases does not have complete homology with the target gene. Hence it has been proven that hybridisation studies are less sensitive to sequence differences in comparison to other methods, such as PCR (Ward *et al.*, 1990).

An unusual hybridisation method called reverse sample genome probing (RSGP) comprising of a dotblot of bacterial DNA derived from natural isolates, known strains and soils were repeatedly probed with DNA extracts from the natural isolates (Telang *et al.*, 1994). The known bacterial strains were those which were expected to be found within the soils and which may have resembled the microorganisms that were isolated. The results closely matched isolates with the known strains and also showed which isolates are similar to one another.

Another unique hybridisation study was performed using a dotblot of soil DNA extracts which were repeatedly hybridised with different soil DNA extracts. From the results, it was possible to determine the diversity of the bacteria in the soil and whether the microbial population in the soil was composed of dominant and rarer species (Griffiths *et al.*, 1996).

A commonly used technique is amplification of the DNA using polymerase chain reaction (PCR) which enables specific genes to be detected. Using this technique it was found that one bacterial cell per g soil could be detected although if the dilutions were accounted for, then realistically 10^3 cells were detected (Tsai and Olsen, 1992). When PCR was used to selectively amplify microorganisms in sterile and nonsterile soil, it was found that released DNA from the other bacteria did not reduce the detection level (Tebbe and Vahjen, 1993). It was postulated that the PCR may have been inhibited by humic acids which were extracted with the DNA.

In one study, repetitive extragenic palindromic -PCR (REP-PCR) was used to amplify extracted DNA from isolated microorganisms (Ka *et al.*, 1994). This PCR was carried out using a short primer which hybridised with any part of the bacterial chromosome to create several products which were then separated on a gel. Consequently, the products

from identical microorganisms formed the same patterns while those from similar bacteria had a proportion of DNA fragments that matched.

Another method called restriction length polymorphisms (RFLP) may be used in which the PCR product is digested with a mixture of restriction enzymes. This technique may be performed on the amplified products derived from the DNA of isolated microorganisms or the cloned products from soil DNA extract to reveal similarities between different products (Moyer *et al.*, 1994; Ward, 1995). Furthermore, it may also be used on the amplified products obtained from soil DNA extracts as it is possible to determine patterns which may be attributed to particular microorganisms (Herrick *et al.*, 1993). Either the amplified products from isolated bacteria or cloned PCR products may be sequenced so that their sequences can be compared to the sequences available on the databases which reveal the identity of the microorganisms (Wong *et al.*, 1994; Liesack and Stackebrandt, 1992). However, there are many bacteria for which there has been little or no sequence information obtained.

Once the non-culturable bacteria in the environmental sample have been identified, it is possible to develop fluorescently labelled probes which would hybridise with the bacteria in situ (Amann *et al.*, 1995; Giovannoni *et al.*, 1988; Kane *et al.*, 1993). Therefore, it would be possible to visually monitor the bacteria, whilst undertaking enrichment procedures in order to create the optimum conditions for growth of the microorganism, so that they may be selectively cultured. There were many experiments carried out which either proved bacteria inoculated into soil entered into a non-culturable state, or that the total viable counts were much lower than the total bacterial direct counts indicating that a large proportion of the microbial population was non-culturable. One particular experiment, using a spectrophotometric technique called CoT analysis measured the hybridisation kinetics of DNA extracted from 206 isolated bacteria (Torsvik *et al.*, 1990a). The experiment was repeated using purified soil DNA extract from where the bacteria had been isolated, and it was found that microbial population was 200 times more diverse in soil, than the bacteria which grew on media (Torsvik *et al.*, 1990b).

1.13 The Analysis of Strains on the Basis of 16S rDNA Sequence Variation

The 16S rRNA gene is a useful gene to study because it is present within all bacteria, because it is absolutely necessary for their survival. For this reason, the 16S rRNA gene remains quite well conserved since a single mutation could distort the secondary structure of the interacting proteins and rRNA molecules which form the ribosome, therefore preventing its function. As a consequence, the 16S rDNA gene has been used for taxonomic identification and in combination with 16S rRNA sequence of closely related bacteria, it is possible to ascertain the evolutionary descent of the organism. Most of the sequence variation between closely related species occurs within the hypervariable regions, whereas between different genera there are minor base pair differences throughout the 16S rRNA gene. It is possible for the sequence of the hypervariable region to show greater variability between different species than between different genera. The 16S rRNA gene has 3 hypervariable regions for actinomycetes which occur at about positions 200, 800 and 1000 (*Escherichia. coli* numbering) (Brosius *et al.*, 1981). These hypervariable regions are not necessarily the same for bacteria which are outside the actinomycete lineage. However, these hypervariable regions could not be used in previous studies to form a streptomycete specific probe for hybridisation studies because they show too much variability between species. Hence the 16S rDNA had to be formed using the sequence of the remaining gene to form a probe, which differed by only one base from other eubacteria (Stackebrandt *et al.*, 1991). Consequently, to ensure that the probe only hybridised to streptomycete DNA, very high stringent conditions were required to prevent non-specific hybridisation of the probe to the DNA of other bacteria.

1.14 Microbial Populations Analysed on Denaturing Gradient Gel Electrophoresis (DGGE)

Traditionally DGGE has been used to discover the mutant genes of clonal cell populations, either bacterial or eukaryotic. These genes have either been amplified by PCR or restricted digestion and subsequently run on a denaturing gradient gel. If restricted DNA fragments are run on the gel, then it has been found necessary to use a specific probe to hybridise with the specific DNA fragments which have migrated on the gel (Myers *et al.*, 1988). However, PCR products are more convenient for analysis on DGGE because all that is required is a simple staining method such as ethidium bromide.

Using high stringency PCR conditions to amplify the DNA extract from the microbial community, avoided non-specific PCR products from appearing (Muyzer *et al.*, 1993). If other products appeared on the normal gel then it would be impossible to determine where they would appear on the denaturing gradient gel particularly if their sequence was unknown. Without the sequence information, it would therefore not be possible to calculate the melting conditions of these products. There may be sequence information relating to the desired PCR products for a few strains but there are many different types of strains in the soil and it is not known by how much their sequences would differ from the type strains. Consequently, it would not be possible to accurately predict where they would run on the denaturing gel. To overcome the problem of spurious products being formed, Muyzer *et al.* (1993) developed a method called touchdown PCR which encouraged the formation of the required amplified products at good yields. The products were then run on a parallel denaturing gradient gel, with a gradient previously determined using the amplified products from a few selected strains that were expected to be found within the microbial community. Ideally, the amplification products of the different bacterial species should all separate well on the denaturing gradient gel. Each product should correspond to a sequence which is slightly different from the other bacteria. However, it is possible that closely related sequences with only a base pair difference may migrate together as a single band. Moreover, the sequence of one band may represent more than one type of bacteria. Nevertheless, the pattern obtained does provide some representation of the microbial community, which can be supported by running the amplified products from extracted DNAs of bacterial isolates, recovered from the environmental sample that was analysed, on a similar denaturing gradient gel. Using denaturing gradient gels to analyse bacterial populations is a quick method compared to either identifying microorganisms which have been isolated or cloned, followed by sequencing of the 16S rRNA products. The latter method requires many clones to be formed in order to gain a representative sample of the population, whereas running the amplified product from the environmental sample DNA extract directly on the denaturing gradient gel, means that only the bands formed need to be sequenced. This entails much less work than what is required for cloning and sequencing. It is also possible to easily make comparisons between different environmental samples although it is necessary to know the sequences of the bands since the position of the bands does not

conform to any pattern. Alternatively, the bands corresponding to isolated microorganisms can be aligned with those from the soil DNA extract. This DGGE method also provides a visual representation of the outcome of altering PCR conditions which enables further development by implementing slightly different steps. It also offers the opportunity to investigate quantitative PCR using a competitive product that differs by only a few bases (Henco and Heibey, 1990).

1.15 Amplification of DNA for DGGE Analysis

To the 5' end of one of the PCR primers, a sequence of 40 GCs are added which are generally guanosine residues rather than cytosine residues, as it has been found that problems are incurred on the DNA synthesiser in forming sequences with long chains of cytosine residues (Sheffield *et al.*, 1989). The other primer in the PCR remains unchanged. Using the optimum annealing temperatures for the primers, calculated by excluding the GC region, the PCR amplification is performed and then the amplified product is run on the denaturing gradient gel. In the gel the GC region at one end of the product prevents the DNA becoming completely single stranded which would result in diffuse bands being formed at the high denaturant concentration (Sheffield *et al.*, 1989). Instead the DNA partially melts as it migrates through the increasing denaturant, and then when completely melted except for the GC region, the DNA band becomes sharply focused. Consequently, the GC rich region acts as a clamp on the melting amplified DNA product. Ideally, this GC clamp enables 95% of single base pair changes to be detected by a shift in the band position (Myers *et al.*, 1985b). The transitions result in a larger shift of the band position while many of the transversions shift only slightly from the original PCR product. The rest of the transversions are undetectable by DGGE.

When PCR products are amplified without the GC clamp, single base pair mutations cannot be detected by a shift in the band position, but when they are run on the DGGE using a variation of the method called heteroduplex analysis, 25% of single base mutations are detectable (Myers *et al.*, 1985c). Heteroduplex PCR products are formed from the annealing DNA strands of mutant and wild type DNA so that these products become more unstable in the denaturant gradient in comparison with the homoduplex product formed from the identical annealed DNA strands. The heteroduplex bands are retarded much sooner compared to the homoduplex bands and migrate as a double band.

It has also been shown that single base mutations can only be detected by heteroduplex analysis but not by homoduplex analysis, even if the amplified PCR products contain a GC clamp (Baetow *et al.*, 1992).

An alternative approach to the GC clamp is the Chemiclamp which is a modified adenosine on the second base from the 5' end of one of the primers (Costes *et al.*, 1993). The Chemiclamped primer is used in the standard PCR protocol to amplify a region of DNA but afterwards the amplified product is exposed to long wave UV irradiation which results in a covalent bond being formed between the modified base and the thymidine on the opposite DNA strand. Subsequently, this also acts as a clamp and preliminary investigations indicated that it acted similarly to the GC clamp although the bands in the denaturing gel were slightly more diffuse.

1.16 Aims

1) To fractionate Warwickshire soil and determine whether the streptomycetes and eubacteria occur within different soil fractions. The eubacterial distribution would be determined for each of the different size classes of water stable soil aggregates and would be compared with the results obtained from fractionation studies of similar soil types.

2) To determine which of the soil fractions showed the highest streptomycete diversity and find whether the diversity was proportionally linked to a high secondary metabolite diversity. Streptomycetes would be isolated from the different soil fractions and screened for secondary metabolites by Novo-Nordisk.

3) To develop a molecular technique to detect actinomycete populations in soil using denaturing gradient gel electrophoresis. Actinomycete isolates would be recovered from soil fractions and in situ soil fraction DNA extracts would be amplified using group specific primers and run on DGGE.

CHAPTER TWO

2. Methods

2.1 Media

All media were made up to 1 litre with distilled water, the pH was checked and autoclaved at 121°C for 15 minutes at 15 p.s.i.

Medium	Ingredients	Quantity
Oatmeal agar	fine oatmeal (boiled 20 minutes)	20 g
	yeast extract (Oxoid)	5 g
	Lab M agar (Amersham)	15 g
Reduced arginine, starch and salts agar (RASS) (Herron & Wellington, 1990)	L-arginine	0.1 g
	soluble starch (BDH)	12.5 g
	Di-potassium phosphate	1 g
	Sodium chloride	1 g
	Magnesium sulphate	0.5 g
	Iron III sulphate (1%(w/v))	1 ml
	Zinc sulphate (1%(w/v))	1 ml
	Maganese sulphate (1%(w/v))	1 ml
	Copper sulphate (0.1%(w/v))	1 ml
	Lab M agar (Amersham)	15 g
TSB plus sucrose	Tryptone soya broth (Oxoid)	20 g
	Sucrose	100 g
Starch casein agar (Küster and Williams 1964)	Soluble starch	10 g
	Vitamin free casein (Difco)	0.3 g
	Potassium nitrate	2 g
	Sodium chloride	2 g

	Di-potassium hydrogen phosphate	2
	Magnesium sulphate	0.05 g
	Calcium carbonate	0.02 g
	Iron II sulphate	0.01 g
	Lab M agar (Amersham)	20 g
Soil extract agar	Extract from filter 1 kg soil autoclaved with 1 litre distilled water 15 minutes	<1 litre
	Lab M agar	15 g
	pH 7-7.5	
HV agar	Humic acid	1 g
	Di-sodium hydrogen phosphate	0.5 g
	Magnesium sulphate	0.05 g
	Potassium chloride	1.7 g
	Iron II sulphate	0.01 g
	Calcium carbonate	0.02 g
	Lab M agar (Amersham)	18 g
	pH 7.2	
after autoclaving add:	B vitamin solution	10 ml
AV agar	L-arginine	0.3 g
	Glucose	1 g
	Glycerol	1 g
	Dipotassium phosphate	0.3 g

	Magnesium sulphate	0.2 g
	Sodium chloride	0.3 g
	Lab M agar (Amersham)	15 g
	pH 7.2	
after autoclaving add:	B vitamin solution	10 ml
0% denaturant solution (100 ml)	40% Acrylamide/Bis (37:5:1)	18.8 ml
	50x TAE buffer	2 ml
100% denaturant solution (100 ml)	40% Acrylamide/Bis (37:5:1)	18.8 ml
	50x TAE buffer	2 ml
	Formamide (deionised with 1 h stirring AG501-X8(D) resin (Biorad)	40 ml
	Urea	42 g

2.2 Bacterial Strains

The organism used was *Streptomyces coelicolor* A3(2) which carried the plasmid pIJ673. The plasmid encoded resistance to viomycin, neomycin and thiostrepton (Cresswell *et al.*, 1992). The antibiotics, neomycin and viomycin were used in the media to select for this organism.

2.3 Preparation of Spore Suspension

Streptomyces coelicolor A3(2) was grown on oatmeal agar spread plates for 7 days at 28°C until grey aerial mycelium could be clearly seen. To three plates 3x10 ml ¼ strength Ringers solution was added and the surface of the plate was scraped with a sterile loop. The suspensions were poured into a universal which was then centrifuged at 3,000g for 5 minutes. The supernatant was discarded whilst the pellet was resuspended in 1 ml ¼ strength Ringers solution by whirly mixing for 1 minute. The suspension was

then filtered, using a syringe with cotton wool plugged at one end which removed the mycelium. The filtrate contained only spores which would be used to inoculate soil microcosms. The quantity of spores in the suspension was determined by directly counting them under a haemocytometer.

2.4 Storage of Actinomycete Isolates

The actinomycetes were grown on oatmeal agar plates for 7 days at 28°C. Some mycelia was scraped from the surface using sterile loops and placed into bijous containing sterile 20% (v/v) glycerol. These were kept until required in the -20°C freezer.

2.5 Soil Microcosms and Fractionation

Nonsterile Warwickshire soil (120 g) was put into 6 plastic beakers and sealed with tin foil and autoclave tape. The pots were sterilised at 121°C, 15 p.s.i. for 15 minutes with 1 day incubation at room temperature between each autoclaving session. Each soil was inoculated with 10^6 *Streptomyces coelicolor* A3(2) spores per g soil and the water content was adjusted to 15% (v/w). The soil microcosms were incubated at room temperature and destructively sampled in duplicate on either days 0, 2 or 15. On these respective days 10 g (for spore extraction), 1 g (for total count determination) and 2 g (for DNA isolation and humidity determination) were removed from each soil microcosm. The remaining soil was fractionated using wet sieving and samples were removed as above for the unfractionated soil. To determine the total viable count and spore counts respectively, the extracts were plated onto RASS + 50 μg/ml thiostrepton + 10 μg/ml neomycin + 50 μg/ml nystatin and incubated at 28°C for 3 days. All colonies which grew were counted. Nonsterile soil microcosms were prepared as for sterile microcosms except they were not sterilised.

The soil was fractionated by placing soil (100 g) into a 251 μm sieve and covering with 1500 ml sterile distilled water and shaking gently for 10 minutes on a IKA-Vibrax-VXR shaker. The sieve was removed from the soil suspension and left to drain for 2 minutes. The soil suspension was poured onto a 63 μm sieve and gently shaken for 10 minutes. This sieve was also removed from the soil suspension and left to drain for 2 minutes. The soil suspension was poured into a large glass vessel and left to sediment for the required number of minutes obtained from the sedimentation tables. This was repeated

twice to collect the soil fraction ranging from 20-63 μm soil aggregates. The glass vessel containing the soil suspension was placed into the cold room at 4°C for a required number of hours to sediment the soil particles from 2-20 μm in diameter. The remaining soil suspension was decanted and 5M CaCl2 was added to a final concentration of 0.025M and left for 1 hour at 4°C. This was centrifuged at 6,000g for 20 minutes at 4°C in the Beckman centrifuge. All the soil fractions were collected, weighed and samples were taken for total viable count determination (1 g), spore extraction (10 g) and humidity determination / DNA extraction (2 g).

To determine the water content in the soil, the soil was weighed in an Eppendorf tube and placed into an oven at 80°C for 4 hours, uncapped. The tubes were removed, allowed to cool and reweighed. It was then possible to calculate the amount of water loss on heating.

2.6 Soil Fractionation of Fresh Warwickshire soil

The soil (100 g), from Cryfield, University of Warwick, was placed into an ethanol sterilised box to which 250 ml sterile distilled water was added. The box was covered and placed into the refrigerator at 4°C for 16 hours. The soil mixture was poured through a 2 mm sieve and the roots were collected using sterilised tweezers. The soil mixture which had passed through the 2 mm sieve was kept for further soil fractionation. The soil on the sieve was covered with 1250 ml sterile distilled water and shaken on the IKA Vibrax shaker for 10 minutes. The soil fraction remaining on the sieve was collected whilst the soil suspension which had passed through the sieve was fractionated as described above.

2.7 Electron Microscopy of Soil Aggregates

Soil was stuck onto 1 cm aluminium pin stubs (Agar Aids) using Electrodag 915 (Acheson) and left in the desiccator for several days. They were sputter coated with gold for 120 seconds at 20 mA using the E5200 auto sputter coater (Biorad) and then viewed in the scanning electron microscope (Joel T330A).

2.8 Total Direct Count Determination

Soil (1 g) and 5 ml sterile distilled water were ground with a pestle and mortar for 5 minutes. The soil suspension was poured into a tube and allowed to settle for 10

seconds. An aliquot (1 ml) was removed from 1 cm below the surface of the suspension and dispensed into an Eppendorf tube. DAPI solution (10 µl) was added to the soil suspension and left for 5 minutes. The soil suspension was centrifuged in the microcentrifuge for 2 minutes. The supernatant was discarded and replaced with 1 ml isopropanol. This was mixed for 20 seconds on the whirly mixer and spun at 13,000 g for 2 minutes. The isopropanol supernatant was discarded and replaced with sterile distilled water to a final volume of 1 ml and resuspended using a whirly mixer. An aliquot (2 µl) was placed into one well of a 15 well multitest slide (ICN Biomedicals), partially air dried and viewed using a HV-A filter block (Nikon) under the epifluorescence microscope (Nikon Optiphor) at 200x magnification. The cells within ten fields of view were counted and averaged.

2.9 Monitoring the Inoculant

To determine the total number of propagules per g soil 9 ml ¼ strength Ringers solution were added to 1 g soil and shaken hard for 20 minutes on the flask shaker. A dilution series was made using 9 ml ¼ strength Ringers solution and 100 µl aliquots were dispensed onto the appropriate media spread plates in triplicate. The plates were incubated for 7 days at 28°C.

To determine the spore count, 10 g soil, 2 g Chelex and 10 ml 0.1% sodium deoxycholate (w/v) plus 2.5% PEG6000 (w/v) was added. This was shaken gently on the flask shaker for 2 hours at 4°C. The soil was spun down in a bench centrifuge at 2,250g for 30 seconds and the supernatant filtered. The pellet was re-extracted as before and the filtrates were pooled. The latter was centrifuged at 4,000g for 15 minutes in a bench centrifuge and the supernatant discarded. The pellet was resuspended in 9 ml ¼ strength Ringers solution and shaken hard on the flask shaker for 20 minutes. A dilution series was prepared from the extracted spore suspension using 9 ml ¼ strength Ringers solution. Aliquots (100 µl) were dispensed in triplicate onto RASS + 10 µg/ml rifampicin + 50 µg/ml nystatin + 50 µg/ml thiostrepton + 10 µg/ml neomycin and incubated for 7 days at 28°C.

2.10 Determination of Indigenous Actinomycete Counts

The soil fractions were serially diluted and plated onto media which selected for actinomycetes and streptomycetes. Many bacteria grew and the actinomycete-like colonies were counted by the appearance of aerial mycelium and sporulation. However, many actinomycetes which sporulate slowly were not counted.

2.11 Statistical Analysis

The bacterial counts determined from each of the soil fractions were adjusted to the values according to one gram of dried soil fraction. Each soil sample was plated in triplicate onto medium and the colonies which formed were counted and averaged. Duplicate soil samples were taken from separate soil microcosms at each particular time point. Only these values were used in the statistical analysis which was determined by minimum significant difference (Fry, 1989).

Minumum significant difference (MSD) = $Qa[mse(1/n_i+1/n_j)/2]$

- Qa Using the number of sample points (n) which were days, and the degrees of freedom calculated by one-way analysis on Mimitab, the value from studentised range table (Q) at 95% confidence limits was determined.

- mse Mean sequare error calculated by one-way analysis on Minitab.

- n_i Lowest number of replicates.

- n_j Highest number of replicates.

2.12 Isolation of Actinomycetes from Soil Fractions

Soil (1 g) was shaken vigorously on the shaker (Stuart Scientific) for 20 minutes in ¼ strength Ringers solution and then serially diluted. These dilutions were plated in triplicate onto 3 streptomycete selective media which were Starch casein medium, Reduced arginine, starch and salts medium and AV medium. They were also plated onto HV medium which is selective for actinomycetes. The plates were incubated for 7 days at 28°C. A plastic grid containing 64 squares was stuck onto the back of each spread plate at the dilution one higher than would normally be used for counting

colonies. The actinomycetes were isolated from each square using a sterile toothpick and plated onto oatmeal agar. These plates were incubated at 28°C for 3 days and examined for growth. The purity of each culture was checked under the light microscope and by plating onto nutrient agar.

2.13 Cell Wall Analysis of Actinomycete Isolates

The actinomycetes were grown on oatmeal agar for 7 days at 28°C. Some mycelia was scraped off into a glass bijou to which 1 ml 6 M hydrochloric acid was added. The bijou was capped and autoclaved for 20 minutes at 121°C and 15 p.s.i. The caps were removed and the bijous were placed in the oven at 80°C in the fume hood. The acid was evaporated off and the pellet was washed once using sterile distilled water and evaporated to dryness. The pellet was resuspended in 100 µl sterile distilled water and run on a cellulose TLC plate in methanol (160 ml), distilled water (54 ml), pyridine (20 ml) and 6 M hydrochloric acid (8 ml) for 3 hours. A standard control containing both forms of the diaminopimelic acid found was spotted onto the TLC at either end. The TLC was removed, allowed to dry and developed with 0.2%(w/v) nihydrin (BDH) in acetone in the oven at 80°C for 3 minutes. The LL-DAP ran further than the meso-DAP.

2.14 Phase Contrast Light Microscopy of Actinomycete Mycelium

Actinomycetes were grown on oatmeal agar plates up against coverslips inserted at 45°. The plates were incubated at 28°C for 7 days or until sufficient sporulation could be observed. Some actinomycetes sporulated poorly and subsequently were grown on soil extract agar with coverslips inserted at 28°C for 2-4 weeks. The coverslips were removed air dried and viewed under the microscope at 100x magnification. Fragmentation of substrate mycelia, number of spores in spore chains, shape of chains, e.g. spiralled, degree of sporulation and alternative sporulating structures were the main characteristics observed and recorded.

2.15 Metabolic analysis of actinomycetes on solid oatmeal agar

The actinomycetes were grown on oatmeal agar by first preparing a spore suspension in 10 ml sterile distilled water containing (10% (v/v)) Tween. The flasks containing the oatmeal agar were inoculated with 5 ml spore suspension and incubated for 13 days at 30°C. The metabolites were extracted from the flasks with 80 ml ethyl acetate for 12

hours at 250 rpm on a rotary shaking table (IKA Labortechnik, Denmark). The solvent mixture was centrifuged at 3000 g for 10 minutes in a bench centrifuge and 10 ml of the upper solvent layer was removed. This was evaporated to dryness in a SpeedVac Concentrator (Savant A290) and the pellets were resuspended in 100 µl DMSO. These were run on the liquid chromatograph (Hewlett Packard Series 1090) using a reverse phase column. The peaks which occurred on the chromatograms were compared to the compounds stored in the HPLC library and some were identified.

2.16 Characterisation of actinomycetes using Biolog

The actinomycetes were grown on oatmeal agar plates for 6 days at 30°C. Using a wetted sterilised cotton wool bud the spores were removed from the plate and suspended in sterile distilled water. The spore suspension was measured spectrophotometrically at 600 nm and adjusted until it had an optical density of 0.2. An aliquot (1.5 ml) was removed and dispensed into 13.5 ml sterile carrageenan solution (0.2%(w/v)). It was mixed for 20 seconds and 100 µl were pipetted into each Biolog plate well using a multichannel pipetter. The plates were incubated for 5 and 8 days at 26°C and then measured using the microtitre plate reader with the Biolog computer package installed.

2.17 Characterisation of actinomycetes using Fourier Transform infrared spectrometer (FTIR)

The actinomycetes were grown for 4 days in TSB liquid media within glass boiling tubes. They were incubated at 30°C on the rotary shaking table at 250 rpm. The cultures were centrifuged for 10 minutes at 3,000 g and the liquid medium was discarded. The pellets were washed three times and finally resuspended (5 ml) using sterile distilled water. They were then sonicated for 30 minutes in a sonicating water bath (Branson 3210) and 15 µl was pipetted onto the FTIR disc and dried under vacuum for 10 minutes. The samples were measured in the FTIR machine.

2.18 Macromorphological Characterisation of actinomycetes

The actinomycetes were grown for 6-10 days on oatmeal agar spread plates at 30°C. Each of them was compared to the others by the colour of their substrate and aerial mycelium. Other features such as the extent of sporulation and the formation of exudates also helped to group actinomycetes.

2.19 HPLC analysis of streptomycete strains isolated from Warwickshire soil aggregate fractions

The *Streptomyces* species isolated from Warwickshire soil aggregates (See Appendix) were grown for 6 days at 30°C on oatmeal agar slants. A spore suspension was made using 10 ml sterile distilled water containing Tween (10%(v/v) and 5 ml was inoculated into a 100 ml pre-germination liquid medium. These flasks were incubated at 30°C for 2 days on the rotary shaker at 250 rpm. Then aliquots (5 ml) were removed and dispensed into 2 types of liquid medium (100 ml) which were soya-meal based medium and oatmeal based medium. Streptomycetes inoculated into the media were incubated for 6 days and were shaken at 250 rpm in a 30°C room. The metabolites were extracted using 100 ml ethyl acetate which was evaporated to dryness using liquid nitrogen and resuspended in 6 ml DMSO. This extraction was performed by a robot designed specifically for Novo-Nordisk. The samples were then analysed on the Hewlett Packard 1090M series II high performance liquid chromatograph mainframe with diode array detector series II and HPLC 3D Chemstation. These samples were run through a pre-column 4.0 mm I.D. x 5 mm and then a stainless steel column 4.0 mm I.D. x 60 mm, which were both filled with C-18 material measuring 3 μm in diameter. The solvents used in this HPLC system were 0.1% aqueous ortho-phosphoric acid and acetonitrile.

2.20 Extraction of DNA from Actinomycete Cultures

Each isolated actinomycete was grown in tryptone soya broth liquid medium plus (10%(w/v)) sucrose at 28°C for 3 days. They were centrifuged at 3,000 g for 10 minutes in the bench centrifuge (MSE Mistral 2000) and washed in sterile distilled water. The pellet was resuspended in 10 ml TE, pH 8 and sonicated 5 times for 1 minute at 6 μHz. Betwe en each sonication cycle, the samples were maintained on ice. The samples were spun in the bench centrifuge (MSE Mistral 2000) at 3,000 g for 10 minutes and 700 μl of the supernatant was put into an Eppendorf tube. Phenol chloroform (500 μl) was added, mixed and spun in the microcentrifuge (Sanyo) at 13,000 g for 15 minutes at 4°C. The upper phase was removed and if a white precipitate was still present the phenol chloroform extraction was repeated. Any remaining phenol was removed using 500 μl chloroform, centrifuging and removing the top phase. The DNA was further purified by adding 0.05 volumes spermine-HCl, mixing and leaving to stand at room temperature for 5 minutes. It was then centrifuged for 5 minutes at 13,000 g in the microcentrifuge

(Sanyo) and the supernatant was poured off. It was centrifuged again for 2 minutes and the remaining supernatant was pipetted up and discarded. The pellet was resuspended in 1 ml 0.3 M sodium acetate, 10 mM magnesium chloride and 70%(v/v) ethanol, mixed and left at room temperature for 5 minutes. The method for DNA precipitation was carried out as before using spermine-HCl. The final DNA pellet was resuspended in 50 µl TE, pH 8 and stored at -20°C.

2.21 DNA Extraction from Soil

Soil (1 g) was placed into a bead beating bottle (Braun) along with 2 g glass beads (0.1-0.11 mm diameter) (Braun) and 7 ml phosphate buffer, pH 8. This was placed into the bead beater (Braun) and shaken for 5 minutes with carbon dioxide cooling. The bottles were spun at 2,000 g for 5 minutes in the bench centrifuge and the supernatant was poured into a Beckmann centrifuge tube together with 0.5 volumes PEG6000 and 0.2 volumes sodium chloride. This was placed onto the vertical shaker (Griffin) and shaken very gently at 4°C, overnight. It was then spun in the Beckmann centrifuge at 6°C for 30 minutes at 10,000 g and the supernatant was discarded. The tubes were inverted over paper towels to allow excess liquid to drain out. The pellet was resuspended in 700 µl TE, pH 8 and phenol chloroform extraction was performed. After the chloroform extraction 500 µl of the upper phase was removed and further purified using spermine-HCl followed by 0.3 M sodium acetate, 10 mM magnesium chloride and 70%(v/v) ethanol as described for the extraction of DNA from actinomycete cultures.

2.22 Polymerase Chain Reaction

Each reaction contained 1 µl purified DNA, 1 µl dNTPs master mixture, 1 µl (soil DNA and dag gene amplification) or 0.1 µl (actinomycete DNA amplification) forward primer, 1 µl (soil DNA and dag gene amplification) or 0.1 µl (actinomycete DNA amplification) reverse primer, 5 µl 25 mM magnesium chloride and 5 µl PCR buffer. Some reactions were carried out using 2.5 µl DMSO and 5 µl 10 mM bovine serum albumin, particularly for PCR of soil DNA which was slightly contaminated with humic acids. The mixtures were put into siliconised Eppendorfs (Biorad) and overlaid with 50 µl DNase free mineral oil (Sigma). They were placed into the PCR machine (Hybaid) and the first cycle for 10 minutes at 94°C was initiated. The temperature then reduced to 82°C when 2 µl Taq polymerase (GibcoBRL) (diluted 0.2 times in sterile distilled water) was added.

After this the temperature increased to 94°C for 1 minute, a specifically chosen annealing temperature for 1 minute and then 2 minutes at 72°C for the polymerase extension. These three steps were repeated for 30 cycles and ended with 10 minutes at 72°C. The PCR products were run on a 1% (w/v) agarose gel containing 0.25 μg/ml ethidium bromide.

2.23 High GC Clamp PCR Primer

A 60 base oligomer was synthesised in which the 5' end possessed a string of 40 guanosine and cytosine residues whilst the 20 bases at the 3' end were specific for a region on the DNA which would be amplified. The other primer to be used in the amplification would remained unchanged. After several cycles of PCR, the PCR products all possessed the sequence between the primers and including the primers as well as a 40 base pair high GC rich sequence at one end. The reason for this high GC region was to ensure complete DNA denaturation could not occur as the DNA strands would remain together at this particular region.

2.24 Chemiclamp PCR Primer

An oligomer was synthesised which was specific for the region of DNA to be amplified (Appligene, UK). Two adenosines were synthesised at the 5' end of the oligomer, although the second one was a modified form enabling covalent bonding to the opposite strand on exposure to 354 nm UV light. Immediately after these adenosine residues there was the restriction site followed by the primer specific region. The restriction site was necessary should any of the PCR products need to be sequenced since the covalent bond at the second adenosine residue would prevent dissociation of the DNA strands.

2.25 PCR with the High GC Clamp Primer

This was performed using exactly the same protocol as for normal PCR. The PCR products were run on a 1% (w/v) agarose gel containing 0.25 μg/ml ethidium bromide. If non-specific PCR products were observed then the PCR was repeated using more stringent conditions.

2.26 PCR with the Chemiclamp Primer

This was performed as with the normal PCR except after the amplification 20 μl of the product was removed and pipetted onto a Parafilm M (American National Can) and

exposed to 354 nm UV light at a distance of less than 1 cm for 20 minutes. To avoid evaporation of the sample this was carried out in the 4°C room.

2.27 Perpendicular Denaturing Gradient Gel Electrophoresis

An initial 0-100% denaturant gradient was established using the Model 475 Gradient Delivery System (Biorad). The gradient was formed at right angles to where the comb was inserted and a perpendicular gel was created in the D Gene denaturing gradient gel system (Biorad). The gel was loaded into 0.5x TAE buffer, pH 8 at 60°C. The PCR product (formed using either the high GC clamp primer or Chemiclamp primer) from one reaction (50 µl) was diluted to 200 µl using TE followed by the addition of 200 µl loading buffer. The PCR product was loaded in the single well at the top of the gel and it was run for 90 minutes at 150 V from the top to the bottom of the gel in recirculating buffer. Once the melting position had been ascertained a narrower gradient, i.e. 40-70% denaturant, was made which would enable to determine the optimum separation of two different PCR products generated using the same PCR primers.

2.28 Travel Denaturing Gradient Gel Electrophoresis

Once the optimum gradient had been established using perpendicular DGGE, the same concentration of denaturants were used from the top to the bottom of the gel. Two different PCR products formed using the same primers were mixed and an equal volume of loading dye was added. The maximum volume which could be added to each well was 40 µl. Aliquots of these PCR product mixtures were added at specific time intervals (generally 5-30 minutes) until all 16 wells had been loaded. During this time the gel was run at 150 V from top to bottom in 60°C, recirculating 0.5x TAE buffer.

2.29 Parallel Denaturing Gradient Gel Electrophoresis

Once the optimum time for separation of different PCR products was determined from the travel DGGE, the PCR products derived from actinomycete DNA extracts and soil DNA extracts could be run for the length of time where maximum separation of the PCR products occurred as determined from the perpendicular denaturing gradient gel. The PCR products, either separate or as a mixture were loaded into each of the wells and the gel was run.

2.30 Denaturing Gradient Gel Staining

The gel was initially stained using 1 μg/ml ethidium bromide in 0.5x TAE buffer (100 ml) for 5 minutes on a horizontal shaker (Luckham, UK). This solution was then removed and replaced with 100 ml distilled water and shaken for 15 minutes. The gel was visualised under the UV transilluminator (UVP Inc., California, USA) and photographed (Polaroid P665 film) for 20 seconds. The gel was then silver stained using the procedure of Rosenbaum and Riener (1987).

CHAPTER THREE

3. Streptomyces coelicolor A3(2) Growth Associated with Different Sized Soil Aggregates

3.1 Introduction

Soil fractionation is a technique based on wet sieving and sedimentation to separate different sized water stable aggregates into fractions based on the size of the aggregates (Jocteur-Monrozier *et al.*, 1991). Previous studies have shown that culturable bacteria grown on spread plates are associated with the >251 μm and 2-20 μm soil aggregates, and <2 μm soil particles (Kabir *et al.*, 1994). A proportion of the largest soil aggregates that are greater than 251 μm in diameter, are solely undegraded organic matter, such as root and leaf fragments. The larger aggregates have wider pores as they are composed of coarser particles which cannot be arranged compactly, so the capillary flow through these wide pore spaces is relatively slow, resulting in the centre of the larger soil aggregates becoming anaerobic (Jocteur-Monrozier *et al.*, 1991). A gram of the 2-20 μm aggregate fraction has a high surface area and a high water holding capacity because there is a large proportion of clay particles that form intercalating layers. The surface area and water holding capacity of each of the soil fractions are linearly proportional to each other (Jocteur-Monrozier *et al.*, 1991). The 2-20 μm soil fraction also has the highest microporosity compared to the other soil aggregates where microporosity is described as the total volume of pores ranging from 0.2 to 3 μm in diameter, per gram of soil fraction measured between two different matric potentials. Also in this soil fraction most of the bacteria were observed, using scanning electron microscopy, within the pore spaces rather than on the surface of the aggregates and were usually separate from other bacterial cells or in small microcolonies of 2-3 cells (Jocteur-Monrozier *et al.*, 1991). A method was developed to separate the microbial biomass and it was found that fungal mycelia became entangled around soil particles and subsequently sedimented with the large soil particles when centrifuged at low speeds (Faegri *et al.*, 1977). Therefore, it was anticipated that actinomycete mycelia may grow around some aggregates and perhaps within the pore spaces. However, previous studies have shown that most

Streptomyces mycelia are associated with the dead root fragments (Mayfield *et al.*, 1972). Therefore it was expected that the mycelia of the inoculant should be growing in the >251 μm soil fraction where root fragments are expected to be found. The streptomycete spores may appear within the <2 μm soil fraction since Hattori (1988) found that most of the Gram positive bacteria were in the outer soil fraction, and he concluded that many of them were probably spores as they were resistant to ethylene dibromide. It may however be possible that many of them were bacilli spores. Competition from other bacteria in the soil may also influence where the streptomycetes grow, for example, the 2-20 μm soil aggregates have a large bacterial population (Jocteur-Monrozier *et al.*, 1991) which may restrict streptomycete growth. In a soil microcosm run for 60 days, which had 20% (W/W) of its soil replaced every fifteenth day, it was shown that the *Streptomyces lividans* inoculant decreased at a rate greater than the dilution effect of replacing the soil (Cresswell *et al.*, 1992). This was because either there were insufficient available microsites to colonise as they were already occupied by microorganisms, or perhaps the decrease occurred due to limited predation by grazing protozoa. Using soil fractionation, it might be possible to determine whether a *Streptomyces coelicolor* inoculant would be able to compete with soil bacteria which are most numerous in the >251 μm, 2-20 μm and <2 μm soil fractions.

Streptomyces lividans spores inoculated into sterile soil microcosms germinated to produce long mycelia with infrequent branching which contrasted with its growth on laboratory medium (Clewlow *et al.*, 1990). In their studies, it was found that the highest number of mycelial propagules determined by plate counts was observed after 2 days incubation of the soil microcosms. Using scanning electron microscopy, the mycelia were observed at high levels of growth at particular microsites whilst most of the soil contained no mycelium. By the fifth day, the mycelia had declined as nutrients had decreased and sporulation had occurred which was detected by viable counts and visually under the scanning electron microscope. A similar pattern of *Streptomyces* growth was observed in nonsterile soil although spore germination was much lower (Wellington *et al.*, 1990). The aim of this chapter was to determine where the *Streptomyces coelicolor* inoculant grew with respect to the different sized soil aggregates and so they were sampled on the days which were believed to reveal the greatest differences between mycelia and spores. It was anticipated that it would grow around the larger soil

aggregates and the spores would appear in the <2 μm soil fraction. The soil bacteria were also enumerated using acridine orange as a fluorescent stain to determine the total numbers of active respiring bacteria, and grown on nutrient agar to determine the total viable counts. These counts from air dried fractionated soil would be compared with the counts obtained from previous studies on untreated soil samples (Kabir *et al.*, 1994).

3.2 **Procedure**

Sterile and nonsterile air dried soil microcosms (120 g) were set up and inoculated with 10^6 *Streptomyces coelicolor* A3(2) pIJ673 spores per g soil. They were destructively sampled in duplicate on days 0, 2 and 15. A sample (20 g) was removed asceptically before wet sieving and sedimentation (Jocteur-Monrozier *et al.*, 1991) to compare the streptomycete population in the unfractionated sample with the combined fractionated samples. A sample (1g) was taken for the total streptomycete viable count. The spores were extracted from another sample (10 g) using a streptomycete spore specific method (Herron and Wellington, 1990). The soil and spore suspensions were plated onto RASS agar plates containing 50 μg/ml nystatin, 50 μg/ml neomycin and 10 μg/ml thiostrepton, and then incubated for 3 days at 28°C.

3.3 **Results**

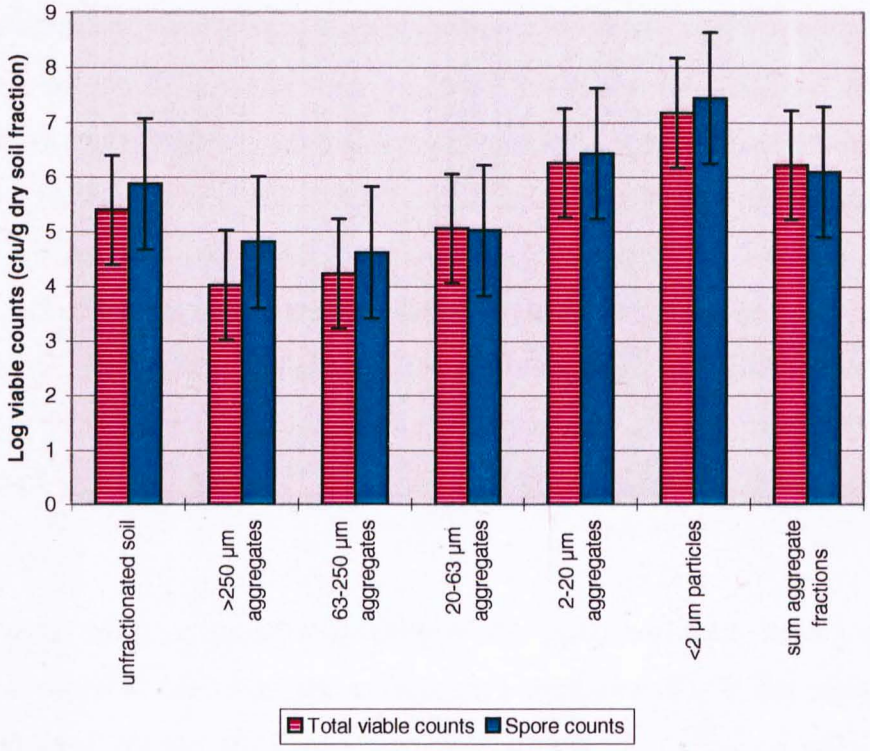
3.3.1 Experiments Using Sterile Soil Microcosms

On the day of inoculation the soil was fractionated and most of the spores were found unassociated with the soil aggregates (See Figure 2). The spores were predominately in the <2 μm soil particle fraction although the majority of the water stable soil aggregates were in the size range of 63-251 μm whilst the smaller fraction contained the <2 μm soil particles (See Figure 3). The soil was fractionated on the second day of incubation which had thereby enabled the inoculated streptomycete to germinate and grow in the soil microcosms. There were significantly more mycelia than spores in the >251 μm, 20-63 μm and 2-20 μm soil aggregate fractions, but the highest streptomycete viable counts were observed in the <2 μm soil particle fraction, 2-20 μm and 20-63 μm soil aggregate fractions (See Figure 2). From day 0 to day 2 there were increased total counts in the soil aggregates ranging from <2 to 63 μm although there was only a significant difference between total propagules and spore counts in the 20-63 μm aggregates. It was not possible to determine the mycelial counts from the difference of total counts from

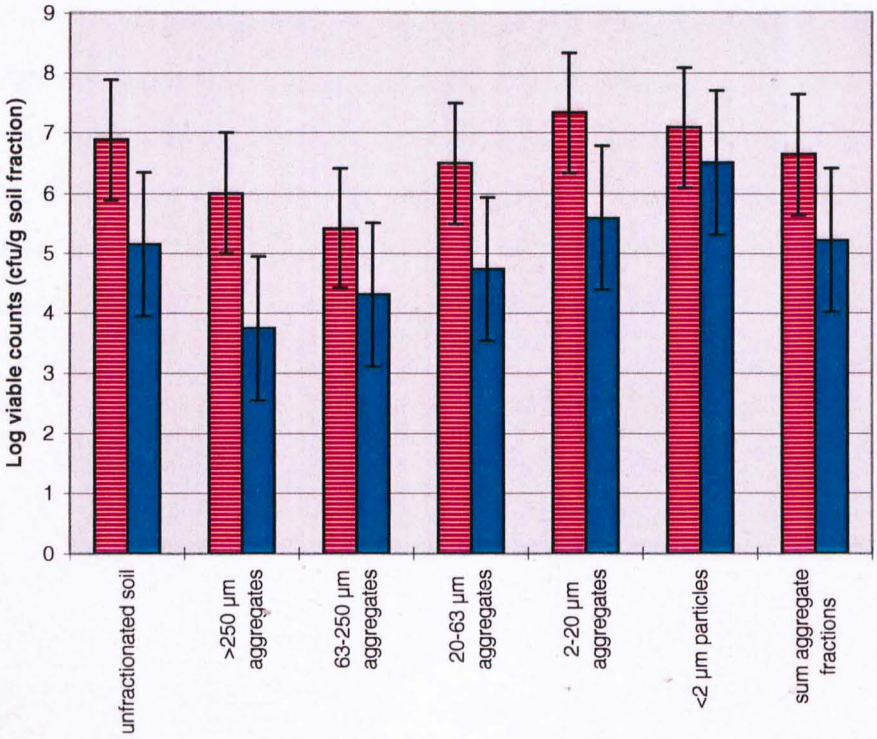
spore counts but this does not necessarily preclude that mycelia were present at high levels within the two soil fractions containing the smallest aggregates. The distribution of the aggregate fractions did not differ from the soil fractionated on the day of inoculation. After the streptomycetes had resporulated and the population had stabilised, another fractionation was completed on day 15 to determine any differences in this population (See Figure 2). There were no significant differences between the total number of soil propagules, i.e. spores plus mycelial fragments, and spore counts. The streptomycetes were distributed throughout the soil aggregate fractions although many more spores were observed in the <2 μm soil particle fraction. Only the >251 μm soil aggregate fraction increased in proportion from day 0 to 15 whilst the other fractions remained unaffected.

The soil fractions were examined using scanning electron microscopy but very little mycelium was observed which may have been due to the low field of view at high magnification (1000x to 3000x). In the fraction containing 20-63 μm soil aggregates a germinating spore was found within the crevice of a soil aggregate. Also some mycelia may have been growing through the pores of an aggregate although the diameter of these mycelia would have been much smaller than laboratory grown streptomycetes cultures. In the 2-20 μm aggregate fraction some mycelia were observed growing away from the aggregate. No other mycelia were observed despite the presence of many fluorescent green streptomycete mycelia which were observed in soil aggregate fractions when the mycelia were stained with acridine orange and observed under epifluorescence microscopy at low magnification.

A


Log viable counts (cfu/g dry soil fraction)
0
1
2
3
4
5
6
7
8
9
unfractionated soil
>250 µm aggregates
63-250 µm aggregates
20-63 µm aggregates
2-20 µm aggregates
<2 µm particles
sum aggregate fractions
Total viable counts
Spore counts

B


Log viable counts (cfu/g soil fraction)
0
1
2
3
4
5
6
7
8
9
unfractionated soil
>250 µm aggregates
63-250 µm aggregates
20-63 µm aggregates
2-20 µm aggregates
<2 µm particles
sum aggregate fractions

C

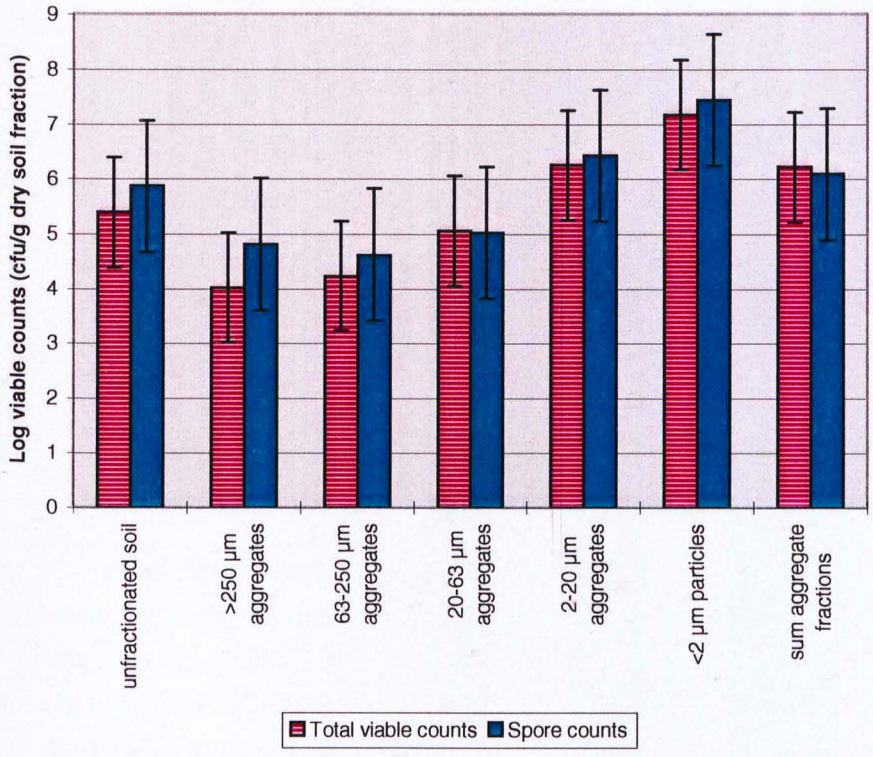


Figure 2 *Streptomyces coelicolor* A3(2) pIJ673 distribution in sterile soil at day 0, 2 and 15.

Soil fractionation was carried out and the number of total propagules and spores were independently determined using ¼ strength Ringers extraction and spore specific extraction methods, respectively. The viable counts were made on RASS plus 10 µg/ml neomycin, 50 µg/ml thiostrepton and 50 µg/ml nystatin. The viable counts determined by the sum of the aggregate fractions are the cumulative counts made for each fraction multiplied by the percentage of each particular fraction. Microcosms were sampled destructively on days 0 (A), 2 (B) and 15 (C). Error bars indicate minimum significant difference (MSD).

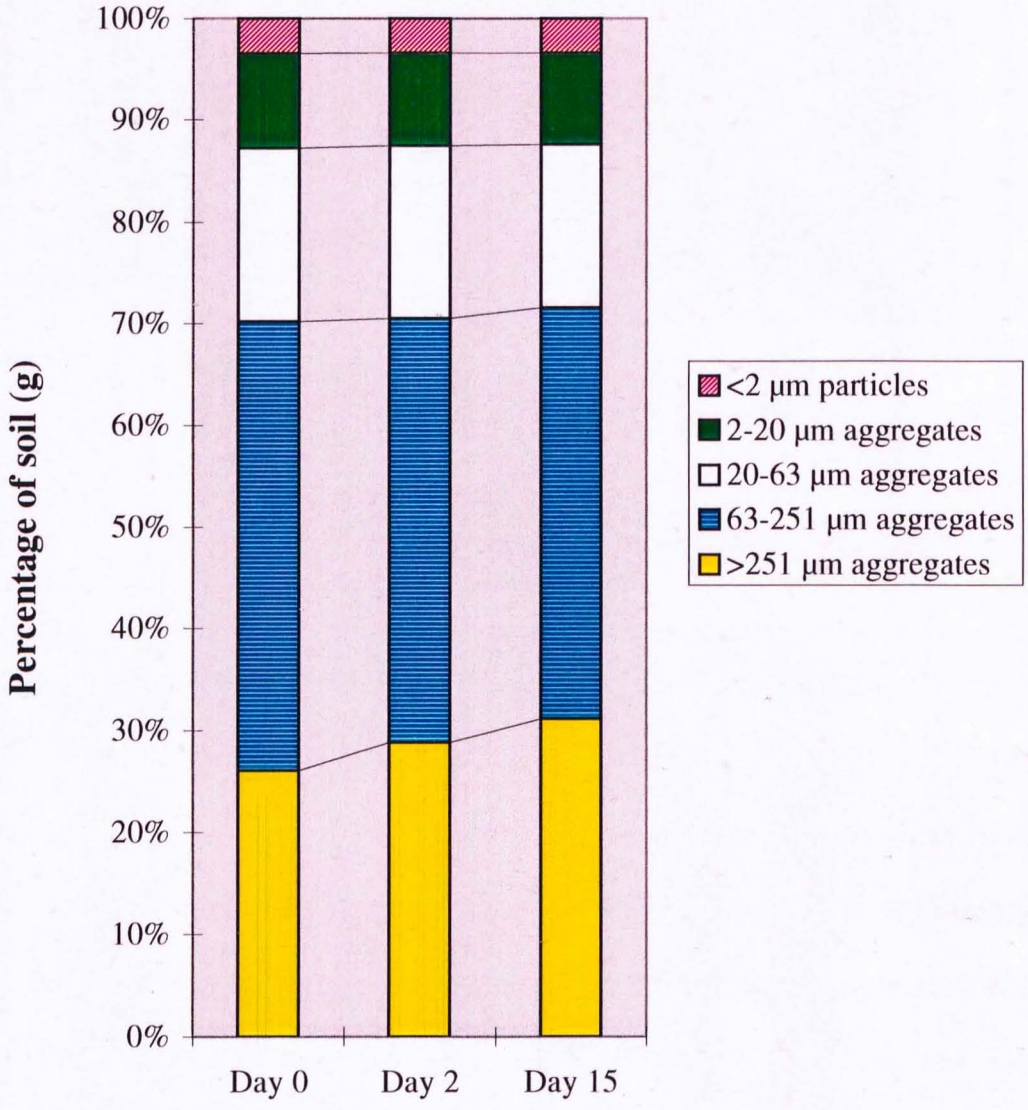

Percentage of soil (g)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Day 0
Day 2
Day 15
<2 µm particles
2-20 µm aggregates
20-63 µm aggregates
63-251 µm aggregates
>251 µm aggregates

Figure 3. The percentage distribution of the soil aggregate fractions.

Soil fractionation carried out on days 0, 2 and 15 using sterile Cryfield soil from Warwickshire inoculated with *Streptomyces coelicolor* A3(2) pIJ673 spores and incubated at 22°C.

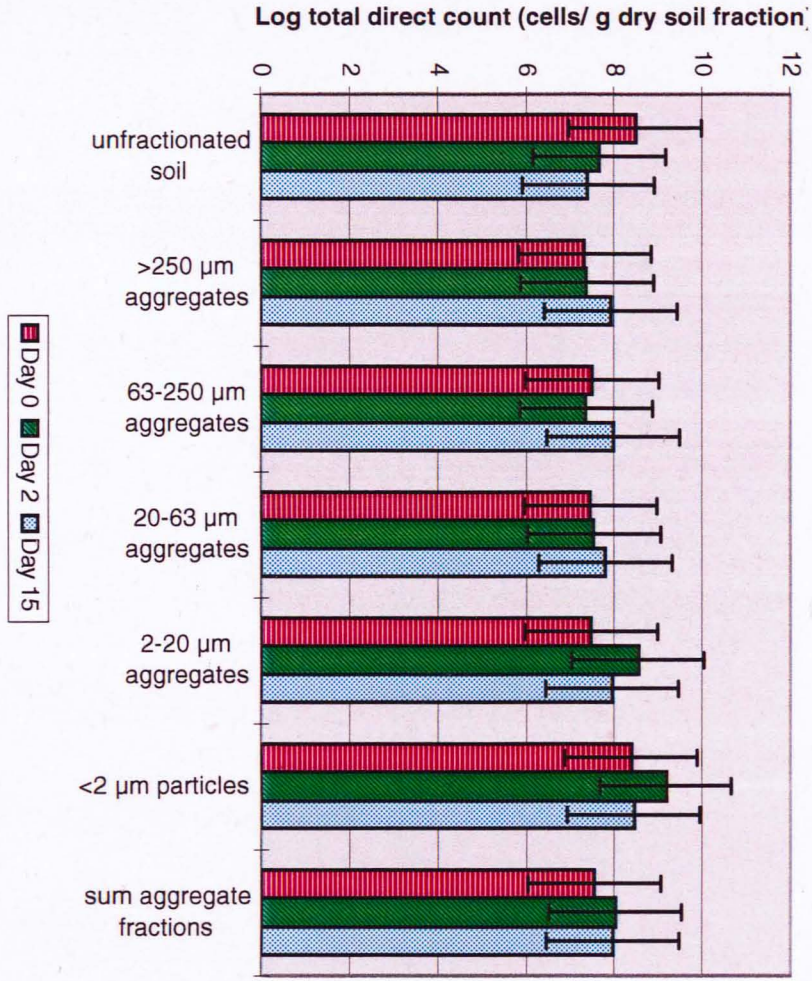

Log total direct count (cells/ g dry soil fraction)
0
2
4
6
8
10
12
unfractionated soil
>250 µm aggregates
63-250 µm aggregates
20-63 µm aggregates
2-20 µm aggregates
<2 µm particles
sum aggregate fractions
Day 0
Day 2
Day 15

Figure 4. Total direct counts of *Streptomyces coelicolor* A3(2) pIJ673 in sterile soil.

Spores inoculated into sterile soil microcosms were fractionated on days 0, 2 and 15. Spores counted under epifluoresence microscope using staining of cells with acridine orange. Error bars indicate minimum significant difference (MSD).

3.3.2 Experiments Using Nonsterile Soil Microcosms

At day 0, the spores were mainly found within the <2 μm soil particle fraction and also in the 2-20 μm aggregate fraction (See Figure 6A). The other larger soil aggregate fractions contained equally low numbers of spores. At day 2, propagules were at highest levels in the soil aggregate fractions ranging from <2-63 μm in diameter (See Figure 6B). However, only in the 20-63 μm aggregate fraction was the total propagules greater than the spore counts. Furthermore, a significant increase was observed for the total viable *Streptomyces coelicolor* counts from day 0 to day 2 only in the 20-63 μm aggregates. This does not mean that many of the propagules detected in the 2-20 μm aggregates and <2 μm particles were not mycelia, even though there was no difference between the total propagules and spore counts. From day 2 to day 15, the spores significantly increased in the >251 aggregate fraction perhaps indicating mycelial growth between these two days within this fraction (See Figure 6C). The growth of streptomycete inoculant in soil resulted in an increase in the proportion of >251 μm aggregates over the 15 day incubation of the microcosms. The quantity of 20-63 μm aggregates increased from day 2 to day 15 (See Figure 5).

In the <2 μm soil fraction, the highest total direct counts were determined using acridine orange (See Figure 7). The counts in the <2 μm soil fraction were only significantly different, on day 0 and 15 from the 20-63 μm aggregate fraction on day 2. No significant differences were observed between any of the other soil fractions because the statistical errors were so large.

The total bacterial viable counts which were determined on nutrient agar were significantly higher in the 2-20 μm aggregates compared to the >251 μm and 63-251 μm aggregate on day 0 (See Figure 8). When the microcosms were incubated there was a significant increase for the unfractionated soil from day 0 to day 2. None of the aggregate fractions showed a corresponding increase except for the <2 μm particle fraction which was not determined on day 0. Consequently, this fraction may have exhibited the greatest increase over the 2 days. From day 2 to day 15, there were no significant increases in any of the soil fractions. Overall the highest numbers of propagules were in the 2-20 μm aggregates and <2 μm particles on days 2 and 15.

Significant differences were observed between the total direct counts and the viable counts within the unfractionated soil and the aggregate fractions ranging from >251-20 μm in diameter at day 0. On days 2 and 15, the only significant difference was in the 20-63 μm aggregate fraction.

The comparison between the fractionated sterile and nonsterile soil microcosms inoculated with *Streptomyces coelicolor* showed that at day 0, the spore distributions were the same throughout all the soil fractions. At day 2, the total counts in sterile soil were more than ten fold higher and statistically different from the total counts in the nonsterile soil for the >251 μm, 63-251 μm, 2-20 μm soil fractions and the unfractionated soil. As previously described within this chapter, there were significant differences between the total counts and the spore counts for the >251 μm, 20-63 μm, 2-20 μm soil fractions and the unfractionated soil on day 2. In the nonsterile soil there was only one significant difference between total counts and spore counts in the 20-63 μm soil fraction on day 2. It would appear that mycelia grew throughout most of the sterile soil fractions but growth was restricted to one soil fraction in the nonsterile soil. At day 15, the spores were significantly greater in the <2 μm soil fraction for the inoculated sterile soil microcosms compared to all the other soil fractions. In the inoculated nonsterile soil microcosms the spores were significantly greater in the <2 μm soil fraction compared to the >251 μm and 63-251 μm soil fractions.

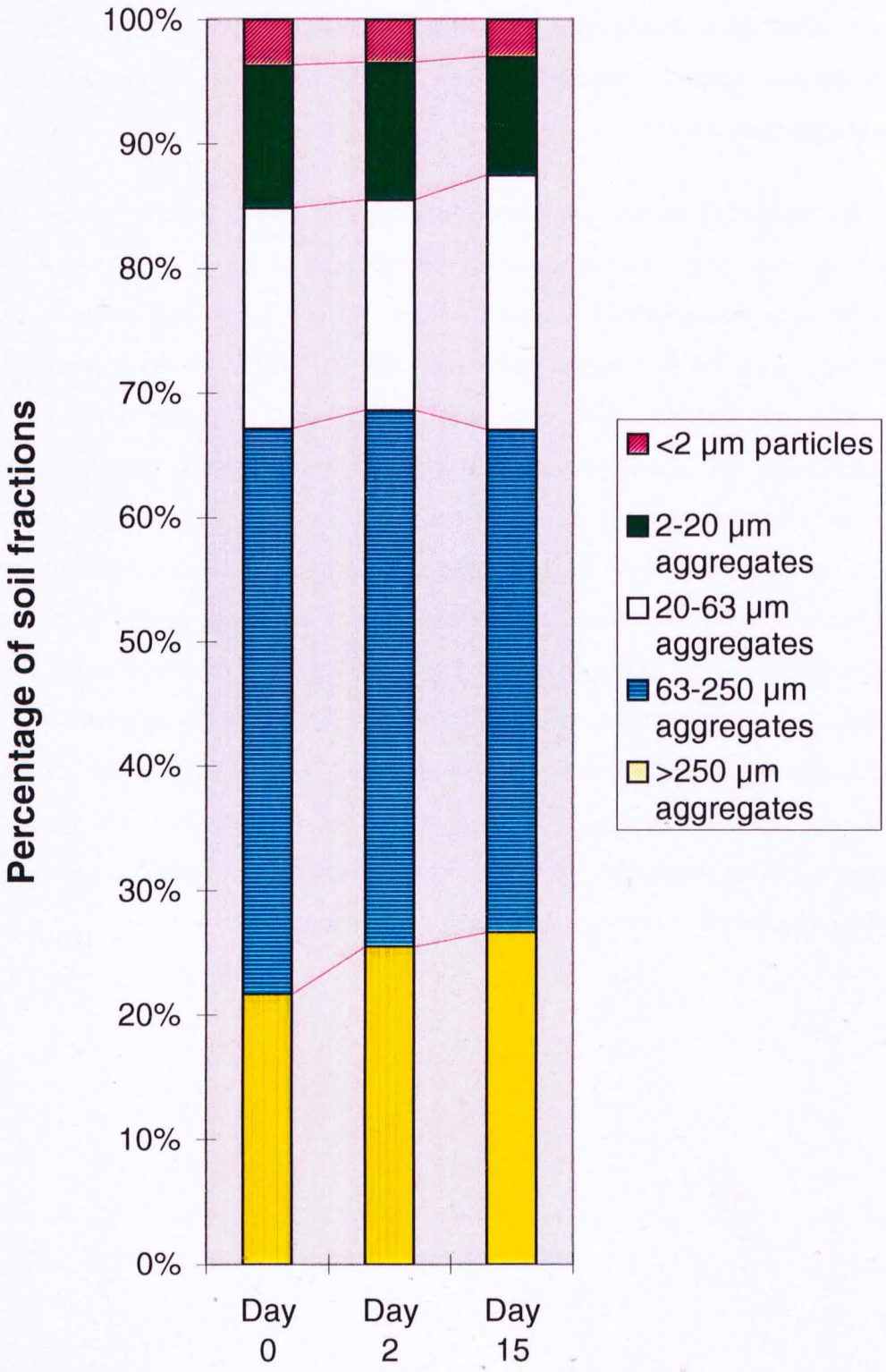
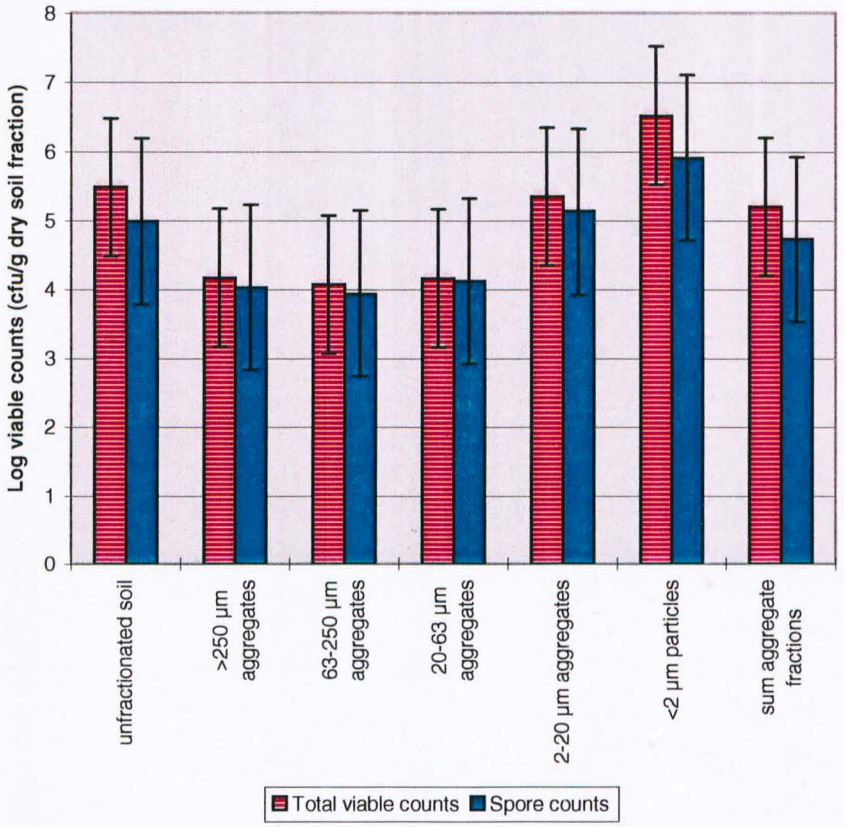
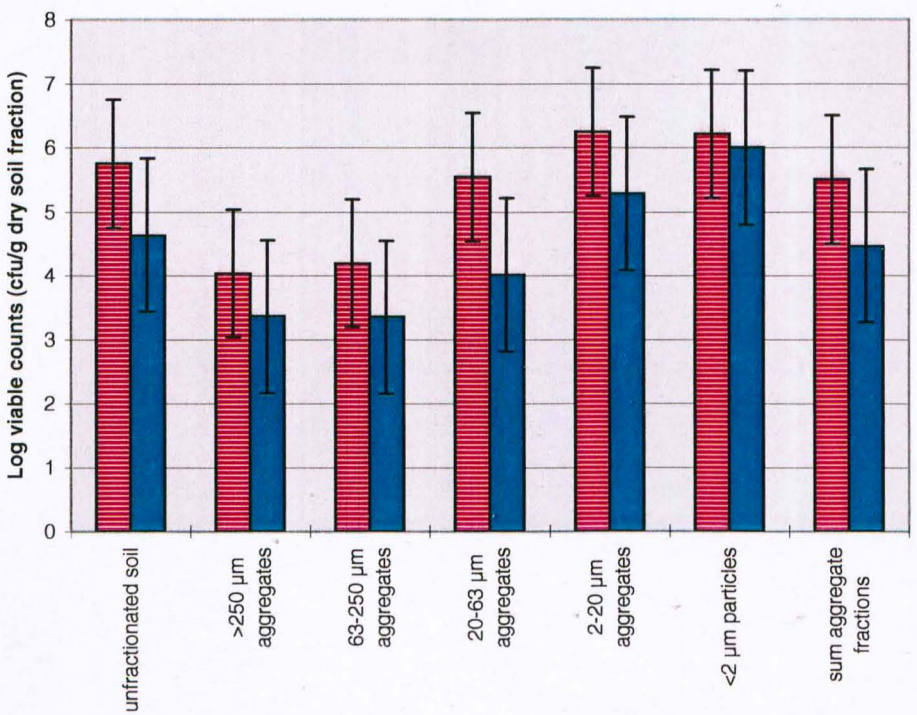

Percentage of soil fractions
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Day 0
Day 2
Day 15
<2 µm particles
2-20 µm aggregates
20-63 µm aggregates
63-250 µm aggregates
>250 µm aggregates

Figure 5 Distribution of nonsterile soil aggregate fractions inoculated with *Streptomyces coelicolor* A3(2) pIJ673.

A


Log viable counts (cfu/g dry soil fraction)
0
1
2
3
4
5
6
7
8
unfractionated soil
>250 µm aggregates
63-250 µm aggregates
20-63 µm aggregates
2-20 µm aggregates
<2 µm particles
sum aggregate fractions
Total viable counts
Spore counts

B


Log viable counts (cfu/g dry soil fraction)
0
1
2
3
4
5
6
7
8
unfractionated soil
>250 µm aggregates
63-250 µm aggregates
20-63 µm aggregates
2-20 µm aggregates
<2 µm particles
sum aggregate fractions

C

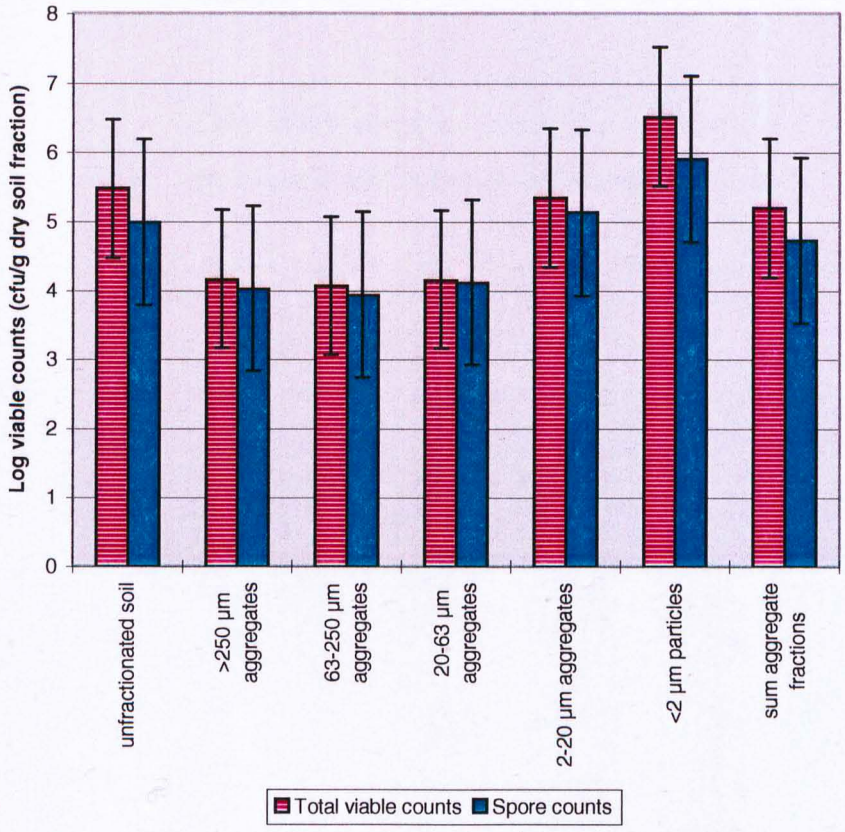


Figure 6 Distribution of *Streptomyces coelicolor* A3(2) pIJ673 in fractionated air dried soil.

The organism was inoculated as spores into Cryfield Hall soil, Warwickshire and incubated at 22°C. Total *Streptomyces* propagules were determined on by ¼ strength Ringers extraction and plated onto RASS plus 10 µg/ml neomycin, 10 µg/ml thiostrepton and 50 µg/ml and 50 µg/ml nystatin. Spore counts were determined using the spore extraction specific method and plating on the same medium. Viable counts can be directly compared to results of fractionated sterile soil (See Figure 2). Soil microcosms destructively sampled on days 0 (A), 2 (B) and 15 (C). Error bars indicate minimum significant difference (MSD).

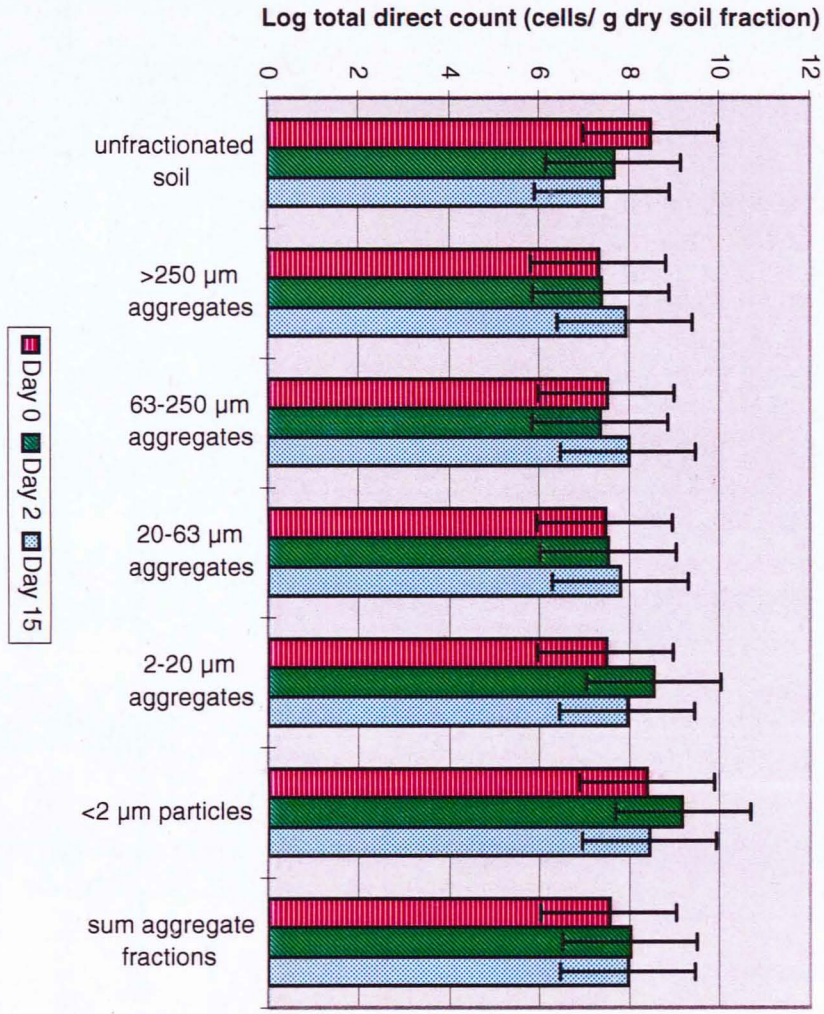

Log total direct count (cells/ g dry soil fraction)
0
2
4
6
8
10
12
unfractionated soil
>250 µm aggregates
63-250 µm aggregates
20-63 µm aggregates
2-20 µm aggregates
<2 µm particles
sum aggregate fractions
Day 0
Day 2
Day 15

Figure 7 Total direct counts of bacteria in soil fractions.

Cells were counted under the epifluoresence microscope after staining with acridine orange.

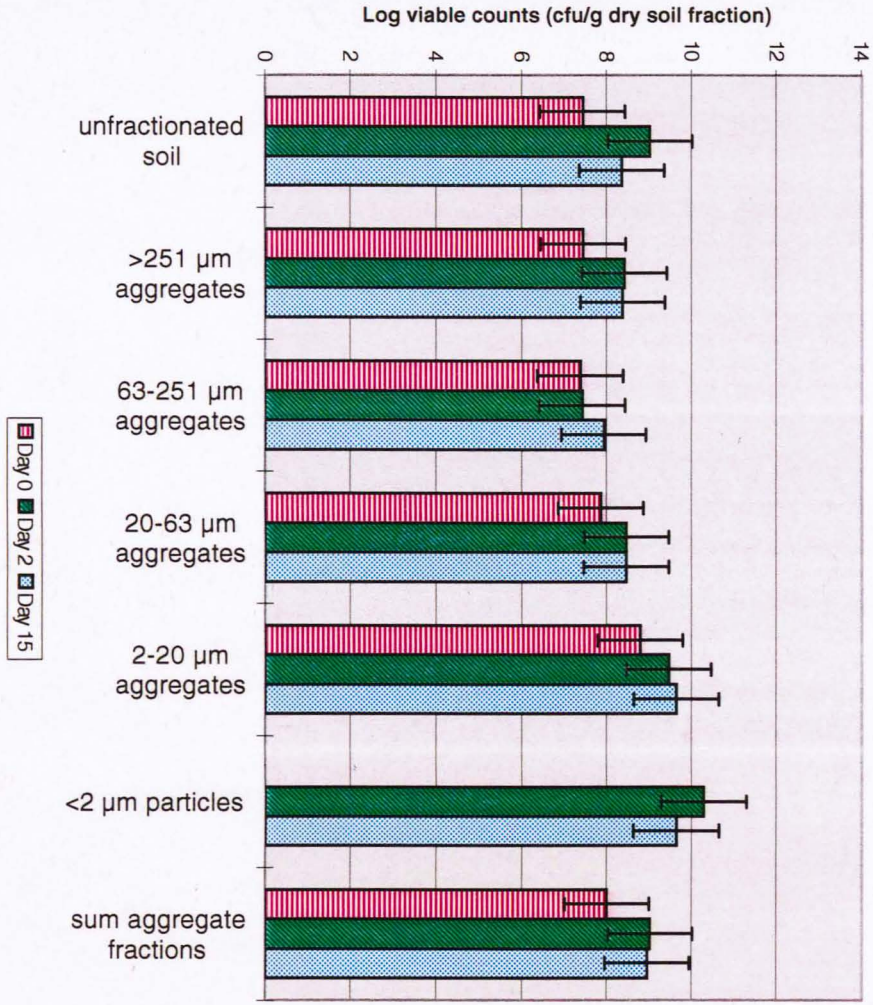

Log viable counts (cfu/g dry soil fraction)
0
2
4
6
8
10
12
14
unfractionated soil
>251 µm aggregates
63-251 µm aggregates
20-63 µm aggregates
2-20 µm aggregates
<2 µm particles
sum aggregate fractions
Day 0
Day 2
Day 15

Figure 8. Total viable bacterial counts in soil fractions.

These were determined by plating onto nutrient agar plates and incubating for one week at 30°C.

3.4 Conclusions

3.4.1 *Streptomyces coelicolor* *A3(2) in the Soil Aggregates*

Streptomyces coelicolor A3(2) (pIJ673) spores inoculated into soil were associated with the clay particles in the <2 μm soil particle fraction. The spore specific extraction method which uses an ion exchange resin to disperse clay aggregates showed that the spores and small mycelial fragments could be selectively recovered from soil (Herron and Wellington, 1990). Other fractionation studies using *Bradyrhizobium* cells, have shown that half the inoculated cells were associated with larger soil aggregates (Ozawa and Yamaguchi, 1986). This could depend upon how the cells were inoculated into soil. For example, mixing the inoculated bacteria and soil and then adjusting the water content, will encourage the cells to be washed into soil aggregate pores. If the water is added to the desired matrix potential before the microorganisms are inoculated then the bacteria will remain outside the pores and it is unlikely that these cells will enter the pore spaces in a static batch microcosm (Beare and Bruce, 1993). However, the pore structure of air dried soil may be severely disrupted when the water content is adjusted, unless less inevasive methods of rewetting the soil are used to ensure the stability of the pore structure (Beare and Bruce, 1993). In the natural environment, rainwater and movement of soil by animal activity will increase the chance of bacterial adsorption and adhesion to soil aggregates (Rijnaarts *et al.*, 1993). Hydrophobic cells, especially streptomycete spores may become attached to sand particles (Stenström, 1989), although they are generally thought to attach to clay particles via elements attached to the cations surrounding the clay particles (Douglas *et al.*, 1971).

In the experiment many spores germinated and there were high levels of growing mycelia within the sterile soil microcosms when they were sampled on the second day which correlated well with microcosm experiments carried out using *Streptomyces lividans* (Cresswell *et al.*, 1992). The enhanced growth may be due to nutrients released from dead bacteria destroyed by autoclaving (Parkinson *et al.*, 1971). Many of these bacteria would be in the 2-20 μm and <2 μm soil fractions (Jocteur-Monrozier *et al.*, 1991) and therefore it was not surprising to find the mycelia were mainly detected within the smaller soil aggregate fractions that were <63 μm. Sedimentation according to Stoke's Law assumes that the particles are of equal density and spherical which is not true for soil aggregates (Darbyshire *et al.*, 1993) therefore the size ranges of soil aggregates

collected in each fraction will not be exactly as expected. The propagules observed within the smallest soil particle fraction may be the result of dislodged mycelial fragments and spores particularly in nonsterile soil where the microorganism will be restricted in growth due to fewer nutrients (Ozawa and Yamaguchi, 1986). Streptomycete growth may occur as long mycelia extending over the surface of soil aggregates (Wellington *et al.*, 1990) but these mycelia may become detached and fragmented during wet sieving. The streptomycetes may not necessarily be restricted to growing around soil aggregates but may also grow within the aggregates like so many other bacteria. Mayfield *et al.* (1972) observed streptomycetes growing within pore spaces of soil crumbs and these streptomycetes formed highly branched mycelia producing globose bodies. Other studies have also shown that many *Streptomyces* and *Streptoverticllium* species can survive semi-anaerobic conditions which would enable them to grow beneath the surface of the aggregates (Locci, 1988). They may also survive within the thin film of water around soil particles and in drier conditions within the pores of aggregates (Fenchel, 1994). This may have been indicated by previous studies which showed that they grew at low moisture tensions (Karagouni *et al.*, 1993) because water would be restricted to these pore spaces (Van Gestel *et al.*, 1991).

Mycelial propagules were observed using fluorescence microscopy to determine the total direct counts on day 15, in the largest sterile soil aggregate fraction. Within this fraction previous studies have shown much of the organic material, such as, dead roots may be found (Jocteur-Monrozier *et al.*, 1991), although there were many more spores associated with the smallest soil particles. Other studies have also shown that streptomycetes are associated with the dead organic remains of plants, insects and possibly fungi (Williams *et al.*, 1972), and perhaps higher counts would have been obtained in this experiment, had many of the roots not been sieved out using the 2 mm sieve. Within the finest soil particles, the spores may be derived from aerial hyphae produced above the substrate, e.g. root fragments, (Williams, 1985) or the larger aggregate surfaces to aid dispersal of streptomycete spores to new microenvironments (Locci, 1988).

The comparison of the distribution of *Streptomyces coelicolor* between the sterile and nonsterile soil fractions showed that the microorganism grew wherever organic matter

was available in the sterile soil fractions, although some of these nutrients would have become available through lysis of cells after autoclaving. After nutrients had become depleted, sporulation occurred and many spores were produced which were associated with the smallest soil particles enabling them to be dispersed to new microsites. In nonsterile soil, the streptomycete grew only in the 20-63 μm soil fraction which has been shown by other studies to contain the least, organic carbon, organic nitrogen and microbial biomass compared to the other soil fractions (Jocteur-Monrozier *et al.*, 1991). It would appear that the streptomycete did not grow as well in the >251 μm and 2-20 μm nonsterile soil fractions compared to the equivalent sterile soil fractions. The high organic carbon in these soil fractions would encourage growth of the streptomycete in sterile soil but the high microbial populations within these nonsterile fractions would provide effective competition for the streptomycete inoculant. *Streptomyces coelicolor* grew in the nonsterile soil fraction with the lowest organic matter which was probably irregularly distributed throughout the aggregates and could only be used by a microorganism which was mycelial as it grew from one site of substrate to another. The spore counts in the <2 μm soil fraction, were much lower in the nonsterile soil compared to sterile soil and may affect the overall survival of this microorganism in soil, as was observed by Cresswell *et. al.* (1992) for the survival of *Streptomyces lividans* in nonsterile soil microcosms.

3.4.2 Mycelia Observed in Soil Aggregates Using SEM and Epifluoresence Microscopy

There was little mycelium observed by scanning electron microscopy within any of the soil fractions which was consistent with previous studies (Wellington *et al.*, 1990). Locci (1988) observed that streptomycetes are usually thinner and less branched in soil than on laboratory medium which may enable them to grow through the pore spaces within the soil aggregates. This would have made it difficult to detect streptomycete mycelia if they were residing within the soil aggregates. Using acridine orange staining, mycelia were clearly observed as this method for determining total direct counts relies on breaking the soil aggregates thereby releasing the bacteria from the soil particles, so they can be counted. Mycelia were in all the soil fractions, except the smallest soil particles and they were observed on the second and final day of the experiment after sporulation had occurred but there were insufficient mycelial fragments to obtain a quantitative yield. Mycelia have been detected indirectly during the later stages, within soil microcosm

experiments (Karagouni *et al.*, 1993; Cresswell *et al.*, 1992) although their presence may not necessarily indicate growth (McCarthy and Williams, 1992). Using CTC may help determine whether these mycelia are still actively respiring (Rodiguez *et al.*, 1992).

3.4.3 Soil Aggregate Stability

The aggregate stability of an agricultural soil, such as the one used in this experiment, would be lower than a permanently cultivated soil (Beare and Bruce, 1993), as ploughing disrupts the soil aggregates (Kabir *et al.*, 1994). However, by air drying the soil the proportion of the largest aggregates would slightly increase because of adhesion by clay, mineral particles and organic matter to each other (Beare and Bruce, 1993). Consequently, the effect of streptomycetes on soil aggregate formation, if any, would be noticed in poorly aggregated cultivated soil without other microorganisms as in sterile Cryfield Hall Soil. Only the largest soil aggregates in the sterile and nonsterile soil microcosms showed an increase in the percentage recovered over the duration of the experiment perhaps due to streptomycete growth. However this presents an anomaly because the total streptomycete counts were no lower in this fraction compared to the other soil fractions. As the counts appeared to be similar throughout all the soil fractions, this means that viable counts may not accurately reflect the actual microbial biomass because of the mycelial nature of these microorganisms (Wellington *et. al.*, 1992). Consequently, longer mycelial fragments may originate from the larger soil aggregates to become entangled around these soil aggregates, thereby contributing to their stability. Increased aggregation was also observed with the 20-63 μm nonsterile soil aggregates from day 2 to day 15 perhaps due to microbial activity (Parkinson *et al.*, 1971).

3.4.4 Total Bacterial Counts in Soil Aggregates

The method for determining total direct counts using acridine orange was subject to interference because non-specific autofluoresence of soil debris occurred making it difficult to count the bacteria. Soil debris has also hindered other ecological studies using fluorescence (Porter and Feig, 1980). The inactive or low respiring soil bacteria fluoresced green due to binding of acridine orange to DNA whilst the bacteria which fluoresced red may have been active because the dye reacted with RNA, or dead due to nonspecific binding of the dye to cells (Hobbie *et al.*, 1977). Large errors occurred in

trying to distinguish between fluorescing images which were bacteria as well as problems encountered with the depth of focus of the soil bacterial suspensions (Anderson and Slinger, 1975). An alternative approach was to use DAPI or Hoechst 33342 (Monger and Langer, 1993) although total direct counts may become lower compared to using acridine orange, as some cells do not stain with DAPI (Suzuki *et al.*, 1993). Total direct count determination using DAPI resulted in only non-specific yellow staining of inoculated streptomycete spores (Colemann, 1980) and no fluorescence of mycelia or other soil microoganisms. Soil bacteria in Cryfield Hall Soil stained with DAPI failed to fluoresce perhaps due to a high inhibitory salt concentration (Zweifel and Hagström, 1995).

An unexpected result was obtained when total direct counts were determined throughout all the sterile soil aggregate fractions. However, previous studies have also noted that a small proportion of viable bacteria still remained, even after a number of successive soil sterilisations which was attributed to steam not fully penetrating the pores (Meikle *et al.*, 1995). This problem was overcome by adjusting the air dried soil to the desired water potential before autoclaving.

The total direct counts in nonsterile soil fractions remained unchanged over the 15 days of the experiment, despite rewetting the air dried soil which would have encouraged bacteria to grow and recover to their original population in natural soil. Previous studies have shown that air drying soil only results in a 10% decrease of bacterial cells (Scheu and Parkinson, 1994). When Kabir *et al.* (1994) examined a continuously cultivated soil, they found there were equivalent proportions of bacteria within all the soil fractions. These results were confirmed in the soil microcosms containing the streptomycete inoculants, probably because similar soil types had been used in both experiments. In contrast, high bacterial counts were associated with the roots, 2-20 μm aggregates and <2 μm soil particles in the grassland or pasture soils (Richaume *et al.*, 1993; Jocteur-Monrozier *et al.*, 1991). This could be directly correlated with the high proportion of vegetation (Kandeler and Murer, 1993).

3.4.5 Viable Bacterial Counts in Soil Aggregates

The total viable counts significantly increased in the unfractionated soil from day 0 to day 2 and then remained stable but none of the aggregate fractions exhibited significant increases due to the increase in microbial activity, except for the <2 μm particle fraction which was not sampled. Other studies showed that viable counts of air dried soil greatly increased within this fraction after several days (Scheu and Parkinson, 1994). The biovolume of many soil bacteria will increase, especially if the cells have become smaller after a starvation period or as a consequence of spore germination and then will continue to multiply (Scheu and Parkinson, 1994). There was a significant difference between the total direct counts and the viable counts in the 63-251 μm aggregate fraction. These bacteria may have been slow growing oligotrophs which would fail to form visible colonies on spread plates (Dabek-Szreniawska, 1993). They may have resided within aggregates to prevent being predated faster than their rate of growth (Ozawa and Yamaguchi, 1986).

3.4.6 Overall conclusion

It was originally believed that the streptomycetes would occur within the >251 μm and <2 μm soil fractions rather than in the other soil fractions. When the spores were inoculated into the soil, they were unattached to the soil particles but gradually became associated with the larger aggregates during incubation. This occurred at a greater rate in the sterile soil compared to the nonsterile soil, and significantly more mycelial propagules were formed in the sterile soil due to the increased nutrients. Even though spores were found at significant levels throughout all the soil fractions, there was a higher quantity in the <2 μm soil fraction in sterile soil. It would be anticipated that air dried and fresh soils would contain a high number of spores within this fraction due to the available supply of dead roots which would form a greater proportion of nutrients than that observed in sieved, air dried nonsterile soil. In fresh soil, it would be expected that the streptomycete population would be significantly high in the largest soil aggregate fractions which would contain decomposing plant material.

It was surprising to discover that there were many spores which remained attached to the soil aggregates. Consequently, it was not possible to show that there were only mycelial propagules, excluding spores, within the intermediate soil fractions. This technique would not enable ecological studies to be performed on the streptomycete mycelia in

soil. However, it would be expected that the streptomycete spores would form a significant proportion of the total bacterial population within these particular soil fractions. This may be useful in isolation studies to help evaluate the full streptomycete diversity within soil and recover may unusual isolates.

CHAPTER FOUR

4. The Distribution of Eubacteria and Streptomycetes in Soil Aggregates

4.1 Introduction

When *Streptomyces coelicolor* spores were inoculated into nonsterile soil microcosms, they germinated and mycelia grew. The mycelia were mainly detected in the 20-63 μm soil fraction. There were high numbers of streptomycete propagules throughout the soil fractions ranging from <2-63 μm in diameter and it was probable that mycelia were a significant proportion in the smallest soil aggregates but they could not be distinguished from the spore counts. At the end of the experiment there was little variation in the spore distribution throughout all the soil fractions. It was expected that the spores would be unattached to the soil aggregates enabling them to be dispersed freely to new microsites. It is possible that they have become attached firmly to the soil particles or may be residing deep within pore spaces of soil aggregates. This would prevent them being disassociated from the soil aggregates. The indigenous streptomycetes are already part of the soil matrix and therefore it is interesting to determine with which aggregates the spores are associated and whether the mycelia grow on the same soil aggregates.

One hypothesis is that if indigenous streptomycetes produced antibiotics within their microenvironments in soil, then this would enable them to effectively compete with other soil bacteria. On this basis it would seem reasonable that streptomycetes should follow the same distribution pattern as bacterial populations in soil which are mainly associated with the roots and within the 2-20 μm and <2 μm soil aggregates (Kabir *et al.*, 1994). The soil aggregates larger than 251 μm contained undegraded organic matter while the smaller soil aggregates were composed of complex, stable organic molecules (Jocteur-Monrozier *et al.*, 1991). The undecomposed, dead plant root fragments, in previous studies were shown to have streptomycetes growing as dense colonies of mycelia, which were observed by scanning electron microscopy (Mayfield *et al.*, 1972). However, it is also likely that streptomycete mycelia grow on organic debris within the smaller soil aggregates but observations by scanning electron microscopy would reveal few mycelial

fragments because most of the mycelia would be growing through the pores inside the soil aggregates. However one problem in studying streptomycete inoculants in soil microcosms is that Cresswell *et al.* (1992) found few spores germinated perhaps due to the limited availability of nutrients. When *S. coelicolor* spores were inoculated into soil microcosms, in the experiments described in the previous chapter, it was found that the spores were associated with the smallest soil fractions immediately after they had been inoculated into the soil, and after they had been growing for several days. It would appear that many of the spores at the end of the experiment may have been the same spores which were initially inoculated into the soil, especially if only some of the original spores germinated. By studying the indigenous streptomycetes, it can be assumed that their populations have reached a stable distribution over many successive generations of streptomycete growth cycles, and it would be expected that they would be found associated with the soil aggregates as spores, where they had previously grown and perhaps where the spores were distributed. For this reason, it is possible that the distributions of inoculated streptomycetes would differ to that of indigenous streptomycetes in soil fractions. In fresh soil, it may be possible to show that mycelial counts are significantly greater than spore numbers in particular soil fractions indicating growth within these soil aggregates.

An alternative hypothesis is that streptomycetes may occur within some, if not all of the soil fractions containing complex microbial products because streptomycetes release enzymes to break down compounds which include hemicelluloses and chitin. They have been found to be responsible for humic acid degradation (Dari *et al.*, 1995). It was found in previous studies that many of the stable organic microbial products were within the soil aggregates ranging from <2-50 μm in diameter, and that the 20-50 μm soil aggregates possessed the least organic carbon content of all the soil fractions (Jocteur-Monrozier *et al.*, 1991). The predominant microbial products are the humic acids, which are precipitated under alkaline conditions and are almost recalcitrant to further degradation by microbial activity. These observations correlated well with other studies on straw decomposition in separate sand, silt and clay fractions which indicated that complete decomposition occurred only in the sand fraction, whereas a low residual organic content remained in the other fractions (Christensen, 1987). This could be explained by the weakly acidic conditions which exist around the sand particles enabling

microorganisms to completely degrade the straw whereas in the silt and clay fractions they create a slightly alkaline environment. The silt particles may have organic molecules stuck to their surface whereas these molecules may become intercalated between the clay particle lattices to become inaccessible to microbial degradation. It was likely that clay and silt were the main soil particles forming the smaller soil aggregates which would explain why stable organic compounds were mainly associated with these aggregates. However, the slightly larger soil aggregates, primarily composed of silt particles surrounded by humic acids, provide ideal conditions for streptomycetes which are capable of using this complex molecule.

A further prediction is that the streptomycete spores would not be associated with the soil particles, as this would allow their dispersal to new microsites. If this is true, they should appear in the <2 μm soil fraction after soil fractionation. This observation was borne out as *S. coelicolor* spores grwoing in sterile soil were mainly found in this soil fraction in the experiment from the previous chapter (See Figure 3). However, in nonsterile soil, many of the *S. coelicolor* spores were also found in the other soil fractions indicating that they were firmly attached to the microaggregates, although the highest spore counts were in the <2 μm soil aggregates (See Figure 6C). Growth of streptomycetes in soil, by scanning electron microscopy observations, showed that the aerial hyphae grew above the substrate (Williams, 1985), so once the spores were detached they were distributed throughout the air spaces between soil aggregates to new microsites. This would suggest that the spores should collect in the <2 μm soil fraction during soil fractionation but this disregards that many streptomycete spores were found in the other soil fractions indicating that they were firmly attached to the microaggregates. However, streptomycete spores have been shown, in previous studies to readily attach to clay particles (Goodfellow and Williams, 1983) that hold the microaggregates together. It is possible the streptomycete spores are not dispersed far from where sporulation took place and that they soon become attached to nearby clay particles. During soil fractionation some of the less firmly held clay particles with the associated streptomycete spores, perhaps on the outside of the soil microaggregates, will be removed from the microaggregates and appear in the <2 μm soil fraction. Obviously, it is an advantage if the spores are removed from the original nutrient sources so that

they may be dispersed to uncolonised areas and to prevent them being degraded by protozoa or other bacteria (Ritz, 1995).

The aim was to determine the distribution and growth of indigenous streptomycetes in soil microcosms by viable counting of propagules in different sized, rewetted, air dried soil aggregates. These microcosms would be incubated and sampled several times to detect the initial spore inoculum, growth of mycelia and sporulation. The results from this experiment can be compared directly to the previous soil fractionation studies of *S. coelicolor* inoculated into sterile and nonsterile soil microcosms. These microcosms were specially prepared by air drying and sieving through a 2 mm sieve to remove roots and large particles which created a homogeneous soil environment. Freshly sampled soil was also studied to examine the distribution of indigenous soil bacteria, actinomycetes and streptomycetes within soil fractions and the results were compared with those found in previous studies on agricultural soils by Kabir *et. al.* (1994). The unusual actinomycetes were isolated on selective media which contain complex carbon substrates, such as starch casein (Kuster and Williams, 1964) and HV agar (Hayakawa and Nonomura, 1987). Also they were isolated on media with low carbon contents, such as AV (El-Nakeeb and Lechavalier, 1963) and HV agar. The HV and RASS media were used in this part of the experiment to determine the actinomycete and streptomycete populations which would grow on the respective media.

4.2 Procedure

Soil microcosms were created using air dried soil, rewetted to 40% water holding capacity and were sampled on days 0, 2 and 15 for total streptomycete counts and streptomycete spore counts using the method described in chapter 3.2. The samples were plated onto RASS medium containing 10 µg/ml rifampicin and 50 µg/ml nystatin and after the plates were incubated for 7 days at 28°C, the sporulating streptomycete colonies were counted. The streptomycetes selected for on RASS medium plus 10 µg/ml rifampicin and 50 µg/ml nystatin, would be species which are resistant to rifampicin while there would be others which could not grow on this medium (Wellington *et al.*, 1987).

Soil microcosms may not represent the natural environment so fresh soil was used. The fresh soil (120 g) was placed into a sterilised plastic box to which 250 ml sterile distilled

water was added and left for 16 hours at 4°C. This was carried out in duplicate for subsequent statistical analysis of the results. The boxes containing the soil suspensions were then gently shaken for 2 minutes and the roots were collected together as a fraction by using tweezers. The soil was fractionated by wet sieving but 3 more soil fractions of the larger soil aggregates were collected, compared to the standard method. The soil fractions collected using wet sieving were: >2000 μm, 251-2000 μm and 63-251 μm soil aggregates. The last three soil fractions: 20-63 μm, 2-20 μm and <2 μm soil aggregates, were collected using sedimentation. Each of these fractions was serially diluted and plated in triplicate onto nutrient agar for viable bacterial counts. They were also plated onto RASS medium plus 50 μg/ml nystatin for predominantly streptomycete counts and onto HV medium plus 50 μg/ml nystatin for actinomycete counts. The plates were incubated at 28°C for 7 days and all the colonies forming on the nutrient agar plates were counted whilst only the sporulating colonies were counted on the RASS and HV media.

4.3 Results

4.3.1 Streptomycetes In Rewetted Air-Dried Soil Aggregates

On the day the microcosms were established, the streptomycete propagules were significantly greater in the <2 μm particle fraction compared to the >251 μm and 63-251 μn aggregate fractions (Figure 9). In the <2 μm particle fraction compared to the other aggregate fractions, the spore counts were significantly higher. The total streptomycete counts on day 2 were significantly lower in the 20-63 μm and 63-251 μm aggregates than those in the 2-20 μm aggregates and <2 μm particles. Between the different soil fractions, there was very little variation in the spore counts. There were significantly higher streptomycete propagules on day 15, in the <2 μm particles, 2-20 μm and >251 μm aggregates in comparison to the counts in the 63-251 μm aggregates. Total counts were greater than spore counts in the 20-63 μm aggregate fraction although this difference was notstatistically different. To determine whether there was a significant population of mycelia present in this fraction at day 15, it would have been necessary to observe the mycelia stained with in situ fluorescent dyes under an epifluorescent microscope.

There was an increase in total number of propagules within the 2-20 μm aggregates from day 0 to day 2 (Figure 9). The spores counts increased throughout all the soil fractions

although the counts detected in the 20-63 μm and 63-251 μm were unusually higher than the total streptomycetes perhaps caused by experimental error. A comparison of the total streptomycete propagules showed that there were no significant differences between days 2 and 15 whereas from day 0 to day 15, the mycelia plus spores significantly increased within the <2 μm particles, 2-20 μm and >251 μm aggregates. Moreover, the soil fractions, >251 μm and 63-251 μm aggregates had significant spore population increases from day 0 to day 15.

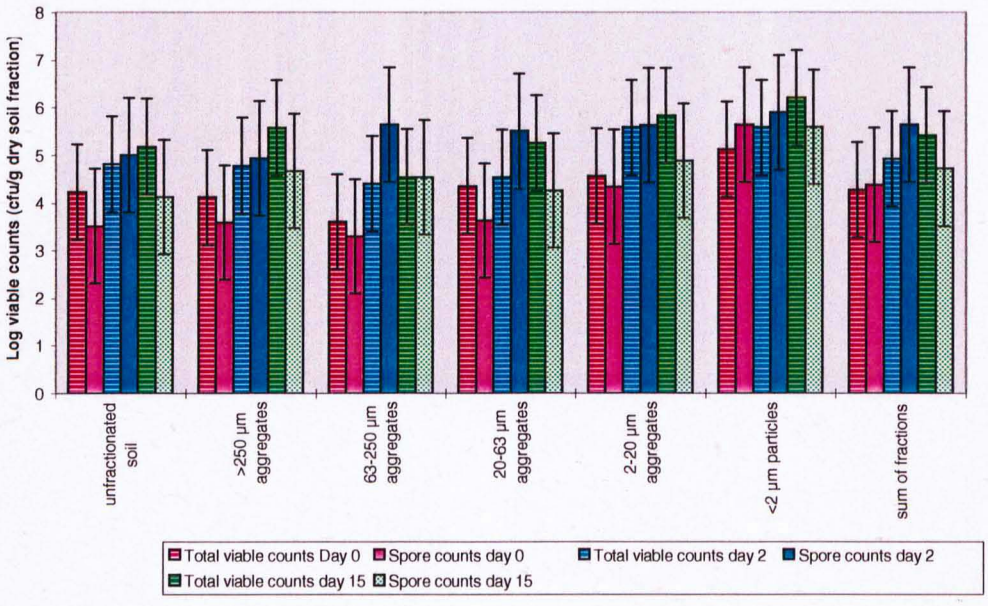

Log viable counts (cfu/g dry soil fraction)
0
1
2
3
4
5
6
7
8
unfractionated soil
>250 µm aggregates
63-250 µm aggregates
20-63 µm aggregates
2-20 µm aggregates
<2 µm particles
sum of fractions
Total viable counts Day 0
Spore counts day 0
Total viable counts day 2
Spore counts day 2
Total viable counts day 15
Spore counts day 15

Figure 9. Indigenous streptomycete populations in air dried soil aggregates.

Uninoculated nonsterile soil from Cryfield Hall, Warwickshire, incubated at 22°C. Sporulating streptomycetes were counted on a RASS medium plus 10 μg/ml rifampicin and 50 μg/ml nystatin for one week. This medium selects for the growth of *Streptomyces violaceoniger* but inhibits the growth of other *Streptomyces* species. Sporulating streptomycetes were counted on the selective medium. Error bars are minimum significant differences (MSD).

4.3.2 Bacterial, Streptomycete and Actinomycete Populations in Fresh Soil Aggregates

The bacteria were significantly higher in the root fraction compared to the unfractionated soil and the aggregate fractions ranging from 20-2000 μm in diameter (Figure 10). Each of these fractions was air dried and the bacterial counts were determined for one gram of air dried material. However, the roots are mainly composed of organic material unlike much of the soil fractions so the loss of water was greater. Consequently, this may have led to the roots having a much higher bacterial population than within the soil fractions, even if their densities within each of these fractions were originally comparable. Among the aggregates, the <2 μm particles and the 2-20 μm aggregates had significantly higher bacterial counts than the counts in the 63-251 μm aggregates. The counts of streptomycetes were likewise significantly higher in the root fraction compared to the other soil fractions, even though the roots were a minor fraction of the soil (See Figure 11). They were also significantly higher in the <2 μm particle, 251-2000 μm and >2000 μm aggregate fractions compared to the 63-251 μm aggregate fraction. There were significant differences between the viable bacterial and lower streptomycete counts for nearly all the soil fractions, particularly in the 2-20 μm soil fraction. These differences were not observed in the 251-2000 μm aggregate fraction indicating that most of its bacterial population were streptomycetes.

The actinomycete counts were statistically higher in the root fraction compared to the unfractionated soil and aggregates ranging from 2-251 μm in diameter. Throughout these soil fractions, the actinomycete population was fairly evenly distributed. The 63-251 μm soil aggregates possessed a much lower actinomycete count the <2 μm particles. The actinomycete counts on HV were significantly lower than the viable bacterial counts in all the soil fractions like the predominantly streptomycete population grown on RASS plates. Particularly large differences were detected in the root fraction and 2-20 μm aggregates. No significant differences were observed between the streptomycete and actinomycete population within the different soil fractions because many of the actinomycetes were probably streptomycetes.

A comparison of the viable bacterial counts in the fresh soil fractions to those in the air dried soil fractions showed that there were no significant differences between counts on days 0 and 15 (See Figure 8). In the fresh soil, the bacterial population was significantly

lower than the population in the <2 μm air dried soil fraction at day 2. The bacterial counts in the >251 μm soil fractions could not be compared with the 3 fractions collected in the fresh soil because one of the fresh soil fractions included roots which were removed from soil prepared for the microcosms.

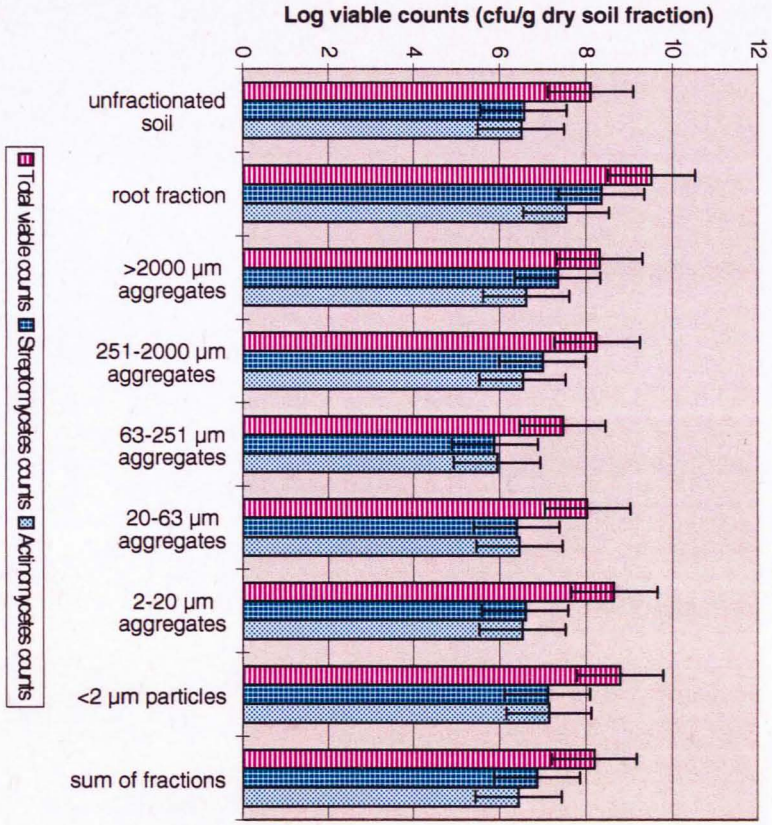

Log viable counts (cfu/g dry soil fraction)
0
2
4
6
8
10
12
unfractionated soil
root fraction
>2000 µm aggregates
251-2000 µm aggregates
63-251 µm aggregates
20-63 µm aggregates
2-20 µm aggregates
<2 µm particles
sum of fractions
Total viable counts
Streptomycetes counts
Actinomycetes counts

Figure 10. Bacterial, streptomycete and actinomycete distributions in fractionated fresh natural soil.

Bacteria were grown on nutrient agar, streptomycetes on RASS medium and actinomycetes on HV medium. Sporulating colonies were counted on the RASS and HV media. Plates were incubated at 28°C for 7 days. Error bars are minimum significant differences (MSD).

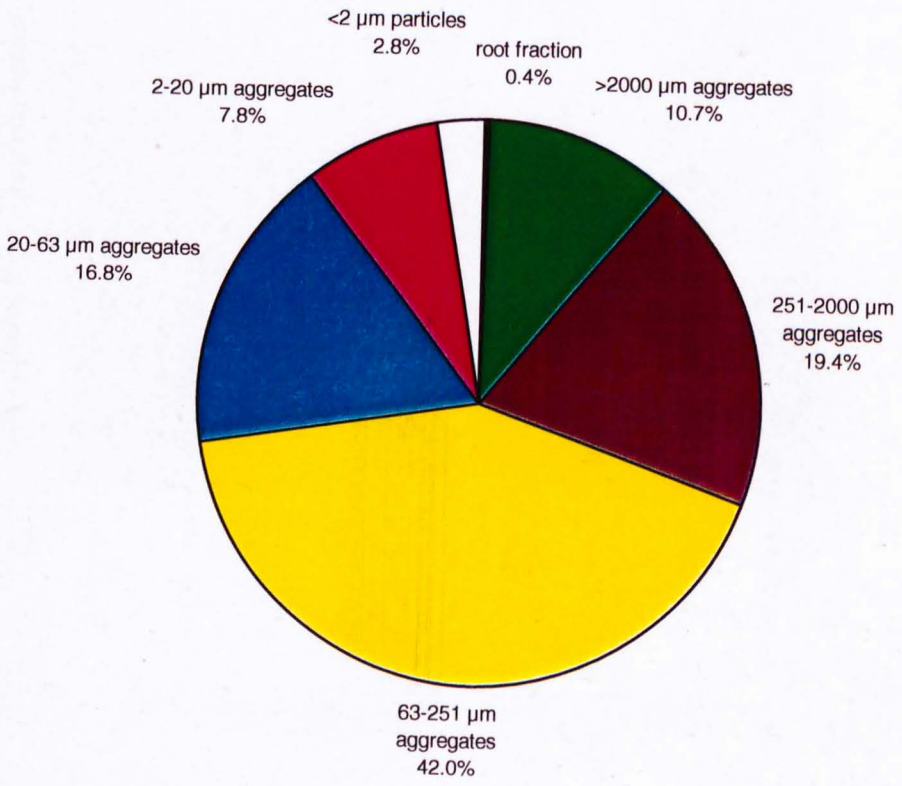

<2 µm particles
2.8%
root fraction
0.4%
>2000 µm aggregates
10.7%
2-20 µm aggregates
7.8%
20-63 µm aggregates
16.8%
251-2000 µm
aggregates
19.4%
63-251 µm
aggregates
42.0%

Figure 11. Distribution of soil fractions after fractionation of fresh Warwickshire soil.

4.4 Conclusions

4.4.1 Indigenous Streptomycete Distribution in Air Dried Soil

The results from these experiments showed that the highest streptomycete counts were associated with the <2 μm soil particle fraction at day 0. Within this dispersible fraction Hattori (1988) found many Gram positive spores, which presumably would have included streptomycetes. If it is assumed that the larger aggregates contained most of the organic material, as proven in other soil fractionation studies (Jocteur-Monrozier *et al.*, 1991), indigenous bacteria within them may have become dormant or non-viable as a consequence of air drying. It is also possible that many of the bacterial cells will become detached from soil aggregates and during fractionation, they will appear with the smallest soil aggregates. From this, it can be concluded that they will be distributed to new microsites possessing optimal conditions for growth. Nearly all the streptomycete population in the rewetted air dried soil, at the beginning of the experiment, probably would be spores because most of the mycelia would have died during air drying. However, streptomycete mycelia have been revealed by scanning electron microscopy studies which showed that that they are only a minor part of the natural streptomycete population in the soil. This was because many of the spores did not germinate due to insufficient nutrients available within their immediate microenvironment in the soil (Mayfield *et al.*, 1972). Consequently, it must be assumed that the streptomycete population would have remained largely unaffected due to the effect of air drying.

On the second day of the experiment, the streptomycetes germinated and the highest counts, indicating growth, were in the 2-20 μm soil aggregates. This concurred well with previous soil fractionation experiments which determined that the highest bacterial density occurred in the 2-20 μm soil fraction (Jocteur-Monrozier *et al.*, 1991; Kabir *et al.*, 1994). On day 15, streptomycetes were detected in the >251 μm soil aggregates associated with the larger fragments of organic matter, and also in the 2-20 μm and <2 μm soil aggregates. Within these particular soil fractions, the streptomycete distribution did not differ from the total bacterial distribution found in previous soil fractionation experiments (Kabir *et al.*, 1994). However, the indigenous streptomycete distribution on day 15 was different from the *S. coelicolor* pattern in soil also on day 15 (See Figure 6C), but did appear to be similar to the *S. coelicolor* distribution at day 2, perhaps due to indigenous streptomycetes continuing to grow within the soil aggregates at day 15. The

soil microcosms showed that there were no significant differences between the indigenous streptomycete propagules and spores, yet the total number of streptomycete propagules significantly increased with time. This could be accounted for by the results discovered by Babich *et al.* (1994), which indicated that different streptomycete species in unamended rewetted soil grew at different times during soil incubation, thereby preventing direct competition. This also led to a high diversity of streptomycetes being maintained. The streptomycetes on the medium, RASS plus rifampicin, represented only a proportion of the total streptomycetes in soil because many were not resistant to the antibiotic. However, these selected streptomycetes grew as detected within the soil fractions and this pattern may be representative of the total streptomycete population. This assumes that most of the streptomycetes occur with particular soil aggregates in soil and that their association with soil fractions is not an artefact of the fractionation method. An increase in the number of streptomycetes seems possible since Lloyd (1969) found that when soil was left for several days, the streptomycete population increased and mycelia were frequently detected, perhaps growing on the remnants of dead bacteria, as well as dead plant material, from the affects of air drying.

The lowest streptomycete populations were detected in the 63-251 μm aggregates during the 15 days of the experiment, despite similar total direct counts being found throughout all the soil fractions (Figure 6). This discrepancy may be accounted for in a previous study which showed there were many more colonies on the minimal medium compared to those on the nutrient agar, only for the larger soil aggregates. On the minimal medium, the other bacteria which grew, may also represent the oligotrophic bacterial population (Dabek-Szreniawska, 1993). It was suggested that oligotrophs may live within the aggregates to protect them from predation by protozoa.

4.4.2 The Distribution Of Bacteria, Streptomycetes And Actinomycetes In Fresh Soil

The populations of both the total viable bacteria and actinomycetes were greatest in the root fraction. This observation may be due to copiotrophic bacteria which thrived on the exudates released by live roots while the actinomycetes would be decomposing the dead roots (Mayfield *et al.*, 1972). But the highest bacterial counts were associated with the roots, 2-20 μm aggregates and <2 μm particles fractions and this distribution correlated

well with the viable bacterial determinations made on agricultural soil (Kabir *et al.*, 1994). Streptomycete counts were greatest within the >251 μm soil aggregates which may have contained high organic contents as found in previous soil fractionation studies (Jocteur-Monrozier *et al.*, 1991). They were also detected at high levels with <2 μm soil particles and their population may represent freely dispersed spores. The actinomycete populations were not significantly different throughout the soil fractions, except the <2 μm soil fractions, as this fraction contained a higher number of propagules which were presumably spores. The general distribution of actinomycetes was similar to streptomycetes within the soil fractions, probably because many of the colonies on HV medium were streptomycetes, since actinomycete isolation work carried out before has shown that 80% were streptomycetes (Xu *et al.*, 1996). The actinomycete and streptomycete populations were outcompeted on and within the 2-20 μm soil aggregates, as there were huge differences between viable bacterial counts and, actinomycete or streptomycete counts. It would appear that the soil bacteria in this soil fraction were not inhibited by any antibiotics that the indigenous actinomycetes may have produced to enable them to effectively compete. Consequently, they are restricted to growing at sites where the copiotrophic bacteria do not grow. These conditions may occur after root death because no more exudates can be derived from them, which would lead to the nutrients in the surrounding microaggregates becoming limited, thereby enabling streptomycetes to colonise.

A direct comparison of the total viable bacteria and indigenous streptomycetes in rewetted, air dried soil microcosms and the freshly sampled soil cannot be made, because only selected streptomycetes resistant to rifampicin were determined in the air dried soil, whereas the total streptomycete population was determined without selective antibiotics. Another argument preventing directing comparison was that a greater range of soil fractions was collected in the freshly sampled soil compared to the rewetted, air dried soil. The air dried soil was prepared to remove roots and stones which were collected as separate fractions in the fresh soil. But it was noticed that there were significant differences observed between the higher total streptomycete counts and the selected streptomycetes within all the soil fractions. However, these soils can be compared on the basis of their patterns of bacterial populations within the different soils. It would appear that by air drying the soil, the bacteria were reduced through death or dormancy,

especially within the smaller soil aggregates, 2-20 μm. This was concluded from the streptomycetes which predominately occurred in the 2-20 μm soil fraction of fresh soil, if the roots are excluded in the analysis. The effect of air drying on the bacterial population determined in previous studies has shown that only 10% of the population is reduced (Scheu and Parkinson, 1994). A comparison between rewetted, air dried soil microcosms in Chapter 3 (Figure 8) with the fresh soil of these experiments showed, that the bacterial densities in the soil fractions were not significantly different. Nevertheless, fewer competing bacteria, perhaps as a result of dormancy, enabled the streptomycetes to grow more effectively within the 2-20 μm soil fraction in the rewetted, air dried soil microcosms after several days incubation.

The streptomycete spores in the air dried soil were mainly disassociated from the soil aggregates when the soil microcosms were initially established. This result was expected from the previous studies using *Streptomyces coelicolor* inoculants. The distribution patterns of indigenous streptomycetes and *Streptomyces coelicolor* in soil were similar to each other. It was however apparent that there were no significant differences between the total propagules and spores within any of the soil fractions for the indigenous streptomycetes. This may be accounted for by the many different types of streptomycete species which have different lag times and growth rates.

In the fresh soil, the indigenous actinomycete population were associated with the large soil aggregates and in the soil fraction containing the smallest soil particles, although there was a smaller proportion of streptomycete propagules associated with the other soil fractions. This result was expected on the basis of the results obtained using *Streptomyces coelicolor*. A closer examination revealed that the 2-20 μm soil aggregates showed a high proportion of bacteria but a much significantly lower streptomycete population. This indicated that the streptomycetes competed poorly with the other bacteria for easily metabolisable nutrients.

Once a pattern of growth and distribution of streptomycetes had been determined from the soil fractionation studies, it was then interesting to discover the species diversity between the different soil fractions. It must be realised that some of the colonies which grew on the streptomycete medium may have been other actinomycete genera. Some of these genera may be more readily found within certain soil fractions due to the unique physical properties exhibited within the soil aggregates. A high species diversity or the

occurrence of particular actinomycete genera within certain soil fractions would be useful in isolation work and studies into the streptomycete diversity within soils.

CHAPTER FIVE

5. Actinomycetes Isolated from Air-Dried Soil Fractions

5.1 Introduction

Previous studies have shown that many of the bacteria were associated with the 2-20 μm and <2 μm soil fractions. It is conceivable that bacteria grew well in the 2-20 μm water stable aggregates because there was sufficient oxygen necessary for aerobic respiration and plenty of available nutrients for growth. Within this particular soil fraction, soluble nutrients and gases could rapidly flow through the extensive network of narrow pores whereas the flow rate through the wider diameter pores was found to be much slower. As a consequence of these narrow pores, there may be a high internal surface area which was covered by stable microbial products, enabling bacteria to attach while fresh nutrients and gases flowed past. This environment within the pores may have enabled bacteria to grow continuously, as the microorganisms were not subjected to rapid dry and wet cycles because the pores remained hydrated even under quite dry soil conditions (Jocteur-Monrozier *et al.*, 1991). The bacteria within these microaggregates may have derived exudates from young roots which provided soluble nutrients to enable bacteria to multiply. The surface of many bacteria were found to be covered with polysaccharides and as the quantity of microcolonies within soil aggregates increased, more soil particles were possibly integrated into the water stable soil aggregate structure, thereby increasing the size of the microaggregates (Degens and Sparling, 1996). Eventually, the roots would have died either resulting in the bacteria dying or entering a dormant state. It is possible that at this stage the streptomycete spores would have then germinated and grown, degrading the dead roots, dead bacteria and microbial products holding the microaggregates together. It would appear that the streptomycete spores were dispersed throughout the soil but they soon became attached to clay particles that were already part of the microaggregate structure. Sometimes there may have been organic debris nearby which might have been colonised by mycelia, originating from spores which have successfully germinated. Mayfield *et. al.* (1972) found that only 4% of all streptomycete spores germinated in soil whilst the remainder are thought to disappear eventually due to

grazing by protozoa and other bacteria. This has been shown to be the case in previous studies which indicated that streptomycete spores significantly decreased in numbers from when they were washed into lakes during the rainy season to the beginning of the following season (Jiang and Xu, 1996). The fall in streptomycete counts could also have been attributed to their poor growth within this environment, whereas the micromonospora population remained steady.

There are about 45 actinomycete genera besides streptomycetes, that are not so readily isolated on selective media which include *Streptosporangium*, *Micromonospora*, *Actinomadura* and *Nocardia*. *Streptosporangium* have been found associated with leaf litter, root mats and in sandy soils. On this basis, it would have been expected that streptosporangia would not have been present in any of the agricultural soil fractions due to the almost negligible leaf litter layer and the high clay content of this soil type. Conversely, sandy soils may have had a lower streptomycete population so that streptosporangia would have been more easily detected, whereas in loamy soils, streptosporangia probably grew but the much higher streptomycete population masked their population on viable count plates. By fractionating the soil, it may be possible to recover a particular soil fraction which predominantly contains streptosporangia. Another group of commonly isolated actinomycetes is *Micromonospora*. *Micromonospora* spores are hydrophilic which means that they are dispersed by water unlike the hydrophobic streptomycetes which are dispersed by air currents (Goodfellow and Williams, 1983). Their hydrophobic spore walls would enable them to enter pores within the soil aggregates by capillary pressure of the water in the pore spaces, during periods when the soil is drying. Within the centre of these aggregates, organic matter has been found on which micromonosporas could grow, using their extensive enzymatic degrading activity, especially the cellulases. *Micromonospora* has also been found to grow quite well in submerged conditions where there were low oxygen potentials. Evidence for the existence of bacteria selectively occupying either the inner or outer part of soil aggregates may be concluded, indirectly from results of previous experiments. In one such experiment, a soil amended with a hydrocarbon to facilitate the isolation of nocardia, it was found to be unsuccessful but the *Rhodococcus* population increased. It was not known where the nocardia resided with respect to the soil particles but the rhodococcus present possessed amphoteric polymers which enabled them to collect at

the air to water interface. Hence *Rhodococcus* could attach to a surface, as on the outside of the soil aggregates but not to a surface under submerged conditions which would be expected in soil aggregates (Rijnaarts *et al.*, 1993). This seemed to suggest that *Nocardia* and *Rhodococcus* grew in different microenvironments enabling the *Rhodococcus* access to the hydrocarbon, unlike the *Nocardia*.

The aim of this study was to determine whether the particular actinomycete genera could be isolated from a specific soil fraction, or whether the actinomycetes are randomly distributed throughout the soil aggregates irrespective of the different properties exhibited between the soil fractions. One would expect a higher streptomycete diversity in the 20-63 μm and <2 μm soil fractions compared to the 63-251 μm and 2-20 μm soil fractions. This is because as mentioned in the previous chapters, the streptomycetes grew in the 20-63 μm soil fraction whilst their spores were found in the soil fraction with the finest soil particles. Many of the streptomycete propagules in a sample of air dried soil would only exist as spores in any of the soil fractions. Slow growing bacteria, possibly the actinomycetes, may have survived and grown in the larger soil aggregates where incidentally the oxygen tensions are lower but the chances of predation by grazing protozoa are also much reduced (Dabek-Szreniawska, 1993). It is not known what type of actinomycetes would be able to compete with the other copiotrophic bacteria in the 2-20 μm soil fraction but perhaps many of them were just dormant streptomycete spores, attached to clay particles. Alternatively, they may have been associated with the larger soil aggregates in this soil fraction because the conditions are more suitable for streptomycete growth than faster growing bacteria.

5.2 Procedure

The actinomycetes were isolated on different types of selective media for streptomycetes (RASS, AV and starch casein) and actinomycetes (HV) which would ensure a diverse range of actinomycetes. The actinomycetes were isolated from three squares of a grid of 66 squares, on a transparency attached to the underside of the spread plate. The isolates were plated onto nutrient agar to check that they were not contaminated with other bacteria and from the plate, a scraped portion was viewed under the phase contrast microscope to check that only mycelia were present. Once the purity had been confirmed each strain was stored in a glycerol suspension. To partially identify each of the

actinomycete isolates, their cell wall chemotype was determined to discover whether they were a streptomycete, and further identification was performed by analysing their sporulation structures under phase contrast microscopy. For the microscopic analysis, the strains were plated onto oatmeal agar, with a coverslip inserted at 45°C and after 7 days growth at 28°C the sporulating structures were viewed. If none could be seen, then they were replated onto soil extract agar and incubated until aerial mycelium was visible on the colonies growing on the plates.

5.3 Results

Altogether 283 actinomycetes were isolated from the air dried Cryfield Hall soil and its soil aggregate fractions. Many of these bacteria were streptomycetes which was determined by observing spore chains of each strain under phase contrast microscopy. Additionally, cell wall analysis was performed to confirm whether they were streptomycetes by the presence of the LL-diaminopimelic acid (DAP) isomer in the cell wall. Other genera within the actinomycetes have the meso-DAP and can subsequently be differentiated from streptomycetes. They may also possess uniquely identifiable sporulating structures to specific genera.

The unfractionated soil yielded 62 isolates of which 40 were streptomycetes while 14 were not streptomycetes (See Table 1). Not all the isolates grew without contamination so those that were, were discarded, whilst some failed to grow after several generations (8 strains). A few isolates were lost throughout all the soil samples used for actinomycete isolations. The streptomycetes produced spore chains, one of which was *Streptoverticillium* that was identified by the characteristic verticillate spore structure. Three of the other actinomycetes could have been *Streptosporangium*, as sporangia were observed under the microscope. Of the five actinomycetes with the meso-DAP cell wall composition, it was found that 3 produced spore chains, one produced single spores and the other strain had no spores.

From the >251 μm aggregate fraction, 49 actinomycetes were extracted (See Table 2). Of these, there were 35 streptomycetes, 30 which produced spore chains, 2 produced single spores and 3 had no spore chains. Four strains containing the meso-DAP had spore chains whilst no sporulation was visible for 3 strains. One of the non-sporulating

strains was morphologically similar to *Micromonospora* when viewed as colonies on plates.

Thirty-nine actinomycetes were extracted from the 63-251 μm aggregates and 17 were found to be streptomycetes (See Table 3). All the streptomycetes sporulated except one and it was noticed that one of them was *Streptoverticillium*. A preliminary examination of the other actinomycetes revealed that there was 5 *Micromonospora*, 4 *Streptosporangium*, 1 *Nocardia*, 1 *Microtetraspora* and 5 unidentified strains with no sporulation.

The initial actinomycete isolation work from 20-63 μm aggregates recovered 66 strains (See Table 4). Forty-six were streptomycetes although 4 produced no spore chains. Two of the sporulating strains may have been *Pseudonocardia*, due to their fragmenting substrate mycelium. The actinomycetes with the meso-DAP isomer comprised 4 *Streptosporangium*, 2 *Micromonospora*, 7 unidentified strains with spore chains and 2 unidentified strains without sporulation.

Fewer actinomycetes were extracted from the 2-20 μm aggregates because other bacteria outnumbered them (See Table 5). However, 27 were recovered, 12 with the LL-DAP isomer indicating they were streptomycetes and 9 with the meso-DAP isomer. Three streptomycetes had no spore chains. Another streptomycete produced fragmenting aerial mycelium and also had a particularly distinctive substrate mycelium. Its colony had a crumbly texture unlike streptomycetes in general with hard colonies. The other actinomycetes which were tentatively identified included 1 *Streptosporangium*, 1 *Micromonospora*, 4 unidentified strains with spore chains and 2 unidentified strains without sporulation.

Forty actinomycetes were isolated from the <2 μm soil particles (See table 6). There were 29 streptomycetes although 4 strains did not produce any spore chains. The actinomycetes with the meso-DAP cell wall composition numbered 10. Three of these have no clearly discernible spores but 7 isolates produced spore chains.

For each of the aggregate fractions, the percentages of streptomycetes were calculated to streptomycete percentages per g dry unfractionated soil. These were added together

which showed that 69.6% of all isolated actinomycetes were streptomycetes. This corresponds well with the actual recovery of streptomycetes from unfractionated soil at 76.9%. The highest streptomycete recovery was from the >251 μm aggregates at 82.2% whilst the lowest recoveries were from the 63-251 μm and 2-20 μm aggregates at 51.4% and 61.9%, respectively (See Table 7). Other actinomycete genera were present in these fractions that were not streptomycetes.

strain number	sporulating structures	number of spores	colour substrate mycelium	colour aerial mycelium	isolation medium	cell wall DAP
1	straight spore chains	20	buff	grey	RASS	LL
2	straight spore chains	30	buff	grey	RASS	LL
4	straight spore chains	>30	buff	white	RASS	LL
5	none		buff	none	RASS	LL
6	straight spore chains	20	buff	none	RASS	meso
7	straight spore chains	20	buff	white	RASS	meso
8	straight spore chains	20	buff	grey	RASS	LL
9	straight spore chains	20	buff	grey	RASS	LL
10	straight spore chains	30	buff	white	RASS	LL
11	straight spore chains	30	buff	grey	RASS	LL
12	straight spore chains	20	buff	grey	RASS	LL
15	straight spore chains	10	buff	white	RASS	LL
16	fragmenting aerial hyphae	10	buff	grey	RASS	LL
17	straight spore chains	20	buff	white	RASS	LL
18	sterile aerial hyphae		buff	none	RASS	meso
20	sporangia		buff	pink	RASS	meso
21	straight spore chains	20	buff	light grey	RASS	LL
22	straight spore chains	20	buff	grey	AV	LL
23	spiralled spore chains	20	buff	white	AV	LL
24	straight spore chains	>30	buff	white	AV	LL
25	curly spore chains	20	buff	grey	AV	LL
26	straight spore chains	>30	buff	white	AV	LL
28	straight spore chains	10	buff	grey	AV	LL
29	straight spore chains	20	buff	grey	AV	LL
30	verticillate	20	buff	cream white	AV	LL
31	straight spore chains	30	buff	grey	AV	LL
32	straight spore chains	20	buff	grey	AV	LL
33	curly spore chains	30	buff	white	AV	LL
36	none		yellow	none	AV	none
37	straight spore chains	<10	buff	grey	AV	LL
38	straight spore chains	>30	buff	grey	AV	LL
39	straight spore chains	>30	buff	grey	AV	LL
40	straight spore chains	>30	buff	grey	HV	LL
41	single spores		pink	white	HV	meso
42	straight spore chains	<10	buff	white	HV	meso
44	straight spore chains	>30	buff	white	HV	LL
45	spiralled spore chains	>30	buff	white	HV	
46	straight spore chains	>30	buff	grey	HV	
47	straight spore chains	>30	buff	grey	HV	LL
49	sporangia		buff	white	HV	meso
51	straight spore chains	30	buff	grey	HV	meso
52	straight spore chains	30	buff	grey	HV	LL
53	fragmenting aerial hyphae	20	buff	white	HV	LL
54	sporangia		buff	pink	HV	meso
55	straight spore chains	<10	buff	white	HV	LL
56	spiralled spore chains	30	buff	white	HV	LL
57	straight spore chains	10	buff	white	HV	meso
58	straight spore chains	20	buff	grey	SC	none
59	straight spore chains	>30	buff	light grey	SC	LL
60	straight spore chains	20	buff	grey	SC	LL
61	straight spore chains	20	buff	grey	SC	LL
62	straight spore chains	20	buff	grey	SC	

Table 1. Actinomycetes isolated from unfractionated soil.

strain number	sporulating structures	number of spores	colour substrate mycelium	colour aerial mycelium	isolation medium	cell wall DAP
1	single spores		buff	white	HV	LL
2	straight spore chains	20	yellow	grey	HV	LL
3	spiralled spore chains	30	buff	brown	HV	LL
4	straight spore chains	20	buff	grey	HV	LL
5	clusters spores	2 or 4	orange	none	HV	meso
6	spiralled spore chains	20	buff	light grey	HV	LL
7	straight spore chains	<10	buff	grey	HV	LL
9	curly spore chains	20	buff	white	HV	LL
10	fragmented aerial hyphae	20	yellow	light grey	HV	LL
11	spiralled spore chains	20	yellow	grey	HV	LL
12	spiralled spore chains	20	buff	white	HV	LL
13	straight spore chains	20	buff	none	HV	meso
14	spiralled spore chains	<10	brown	white	HV	meso
15	straight spore chains	20	buff	grey	HV	LL
16	straight spore chains	20	buff	grey	HV	LL
17	single spores		yellow	white	HV	LL
18	straight spore chains	<10	buff	grey	HV	LL
19	straight spore chains	<10	buff	grey	AV	LL
20	straight spore chains	20	buff	grey	AV	LL
21	spiralled spore chains	20	buff	white	AV	LL
22	spiralled spore chains	20	yellow	grey	AV	LL
23	spiralled spore chains	>30	buff	white	AV	LL
24	straight spore chains		yellow	grey	AV	LL
25	spiralled spore chains	20	buff	light grey	AV	LL
26	straight spore chains	10	buff	none	AV	meso
27	none		yellow	none	RASS	LL
28	fragmented aerial hyphae	20	yellow	grey	RASS	meso
29	spiralled spore chains	20	buff	grey	RASS	LL
30	spiralled spore chains	20	buff	grey	RASS	LL
31	spiralled spore chains	20	buff	white	RASS	LL
32	straight spore chains	20	buff	grey	RASS	LL
33	straight spore chains	20	buff	grey	RASS	LL
34	straight spore chains	20	buff	grey	RASS	LL
35	straight spore chains	30	buff	grey	RASS	LL
36	straight spore chains	10	yellow	white	RASS	LL
37	straight spore chains	10	yellow	grey	RASS	LL
38	fragmented aerial hyphae	20	yellow	grey	RASS	meso
39	none		buff	light grey	RASS	meso
40	straight spore chains	10	buff	grey	RASS	LL
41	spiralled spore chains	20	buff	grey	RASS	LL
42	straight spore chains	<10	buff	none	RASS	meso
43	verticillate	20	yellow	light grey	RASS	LL
44	straight spore chains	20	yellow	light grey	RASS	LL
45	straight spore chains	10	buff	grey	AV	LL
46	straight spore chains	10	yellow	grey	AV	LL
49	straight spore chains	20	yellow	grey	AV	LL

Table 2. Actinomycetes isolated from the >251 μm soil aggregates.

strain number	sporulating structures	number of spores	colour substrate mycelium	colour aerial mycelium	isolation medium	cell wall DAP
1	sporangia		buff	pink	AV	meso
2	none		buff	white	AV	LL
3	straight spore chains	20	buff	none	AV	meso
4	sporangia		buff	pink	AV	meso
5	single spores		orange	none	AV	meso
6	spiralled spore chains	20	buff	light grey	AV	LL
7	single spores		orange	none	AV	meso
8	none		buff	none	AV	meso
9	straight spore chains	20	yellow	grey	AV	LL
10	straight spore chains	>30	buff	white	AV	LL
11	straight spore chains	20	yellow	grey	AV	LL
12	single spores		orange	none	AV	meso
13	spiralled spore chains	20	buff	light grey	AV	LL
14	straight spore chains	20	buff	white	AV	LL
15	straight spore chains	20	yellow	grey	AV	LL
17	single spores		orange	none	HV	meso
18	single spores		orange	none	HV	meso
19	straight spore chains	10	buff	none	HV	meso
20	aerial hyphae		buff	none	HV	meso
21	sporangia		buff	white	HV	meso
22	straight spore chains	20	buff	grey	HV	LL
23	straight spore chains	20	buff	grey	HV	LL
25	aerial hyphae	none	buff	white	HV	meso
26	straight spore chains	20	buff	grey	HV	LL
27	straight spore chains	20	buff	grey	RASS	LL
28	reticulate	20	buff	white	RASS	LL
29	sporangia		buff	white	RASS	meso
30	single spores		orange	none	RASS	meso
31	straight spore chains	4	pink	white	RASS	meso
32	straight spore chains	<10	green	white	RASS	LL
33	fragmenting aerial hyphae		buff	white	RASS	meso
34	spiralled spore chains	20	buff	light grey	RASS	LL
35	spiralled spore chains	20	buff	light grey	RASS	LL
36	straight spore chains	20	buff	grey	RASS	LL
37	straight spore chains	10	buff	white	RASS	LL
38	straight spore chains	10	buff	none	RASS	LL
39	none		buff	none	RASS	meso

Table 3. Actinomycetes isolated from 63-251 μm soil aggregates.

strain number	sporulating structures	number of spores	colour substrate mycelium	colour aerial mycelium	isolation medium	cell wall DAP
1	straight spore chains	20	buff	light grey	RASS	LL
2	straight spore chains	20	buff	light grey	RASS	LL
3	single spores		buff	none	RASS	LL
4	straight spore chains	<10	buff	none	RASS	meso
5	straight spore chains	10	buff	light grey	RASS	LL
7	straight spore chains	30	buff	none	RASS	meso
8	straight spore chains	20	buff	light grey	RASS	LL
9	fragmenting aerial hyphae		orange	purple	HV	meso
10	straight spore chains	30	brown	green	HV	LL
11	single spores		orange	none	HV	meso
13	single spores		green	none	HV	meso
14	straight spore chains	<10	yellow	light grey	HV	LL
15	spiralled spore chains	20	buff	light grey	HV	LL
16	straight spore chains	<10	buff	grey	HV	LL
17	straight spore chains	30	yellow	light grey	HV	LL
18	straight spore chains	20	buff	white	HV	meso
19	straight spore chains	20	buff	grey	HV	LL
20	straight spore chains	20	buff	grey	HV	LL
21	straight spore chains	>30	buff	none	HV	meso
23	straight spore chains	20	buff	grey	HV	LL
24	fragmenting aerial hyphae	<10	red	white	SC	LL
25	straight spore chains	20	buff	grey	SC	LL
26	straight spore chains	20	buff	grey	SC	LL
27	straight spore chains	20	buff	grey	SC	LL
28	spiralled spore chains	30	buff	grey	SC	LL
29	straight spore chains	20	yellow	grey	SC	LL
30	fragmenting aerial hyphae	>30	orange	white	AV	meso
31	straight spore chains	20	buff	grey	AV	LL
32	straight spore chains	20	buff	grey	AV	LL
33	straight spore chains	20	buff	grey	AV	LL
34	straight spore chains	20	buff	grey	AV	LL
35	sporangia		buff	pink	AV	meso
36	sporangia		buff	pink	AV	meso
37	straight spore chains	<10	buff	grey	AV	LL
38	sporangia		buff	pink	AV	meso
39	straight spore chains	20	buff	grey	AV	LL
40	straight spore chains	5	light gey	dark grey	AV	LL
42	straight spore chains	>30	yellow	white	AV	LL
43	straight spore chains	20	buff	white	AV	LL
44	curly spore chains	30	pink	white	AV	LL
46	curly spore chains	30	pink	white	AV	LL
47	straight spore chains	20	buff	white	AV	meso
48	straight spore chains	20	yellow	light grey	AV	LL
49	straight spore chains	20	yellow	light grey	AV	LL
51	straight spore chains	<10	brown	light grey	AV	LL
52	straight spore chains	20	yellow	grey	AV	LL
53	straight spore chains	30	buff	grey	AV	LL
54	straight spore chains	30	brown	light grey	AV	LL
55	straight spore chains	<10	buff	white	AV	LL
56	fragmenting aerial hyphae		pink	none	AV	LL
57	straight spore chains	10	buff	none	AV	meso
58	sporangia		pink	white	AV	meso
59	curly spore chains	20	buff	light grey	AV	LL
60	fragmenting aerial hyphae	30	buff	none	AV	LL
61	curly spore chains	20	buff	white	AV	LL
62	spiralled spore chains	20	green	light grey	AV	LL
63	aerial hyphae	none	cream	none	AV	LL
64	straight spore chains	>30	pink	white	AV	LL
65	curly spore chains	<10	buff	light grey	AV	LL
66	spiralled spore chains	20	buff	light grey	AV	LL

Table 4. Actinomycetes isolated from 20-63 μm soil aggregates.

strain number	sporulating structures	number of spores	colour substrate mycelium	colour aerial mycelium	isolation medium	cell wall DAP
1	straight spore chains	20	buff	grey	RASS	LL
2	straight spore chains	20	yellow	grey	RASS	LL
3	sporangia		red	white	RASS	meso
5	straight spore chains	20	buff	grey	RASS	
6	spiralled spore chains	20	buff	white	RASS	meso
7	straight spore chains	20	yellow	grey	RASS	LL
8	straight spore chains	<10	buff	dark grey	RASS	meso
11	aerial hyphae	none	buff	none	RASS	meso
13	straight spore chains	10	buff	grey	HV	LL
14	straight spore chains	<10	buff	none	HV	meso
16	none		orange	none	HV	LL
17	straight spore chains	20	orange	black	HV	LL
18	straight spore chains	20	orange	grey	HV	meso
19	fragmenting substrate mycelium		green	none	HV	LL
20	fragmenting aerial hyphae	none	white	white	HV	LL
21	straight spore chains	30	buff	white	HV	LL
22	none		orange	none	HV	meso
23	curly spore chains	20	green	white	AV	LL
24	straight spore chains	<10	buff	none	AV	LL
25	spiralled spore chains		green	light grey	AV	LL
27	single spores		buff	none	AV	meso

Table 5. Actinomycetes isolated from 2-20 μm soil aggregates.

strain number	sporulating structures	number of spores	colour substrate mycelium	colour aerial mycelium	isolation medium	cell wall DAP
1	none		orange	none	RASS	meso
2	curly spore chains	30	buff	none	RASS	meso
3	curly spore chains	30	buff	none	RASS	meso
5	straight spore chains	20	buff	grey	RASS	LL
6	straight spore chains	<10	red	pink	RASS	meso
7	straight spore chains	20	buff	grey	RASS	LL
8	straight spore chains	20	buff	grey	RASS	meso
9	fragmenting aerial hyphae		brown	white	RASS	meso
10	straight spore chains	20	buff	grey	RASS	LL
11	straight spore chains	20	buff	white	RASS	LL
12	straight spore chains	20	buff	grey	AV	LL
13	aerial hyphae		buff	white	AV	LL
14	straight spore chains	20	buff	grey	AV	LL
15	straight spore chains	20	buff	grey	AV	LL
16	straight spore chains	20	yellow	grey	AV	LL
17	straight spore chains	20	buff	grey	AV	LL
18	straight spore chains	20	buff	white	AV	LL
19	straight spore chains	20	buff	grey	AV	meso
20	none		brown	white	AV	LL
21	straight spore chains	20	brown	white	AV	LL
22	spiralled spore chains	20	buff	grey	AV	LL
23	spiralled spore chains	20	buff	grey	AV	LL
24	spiralled spore chains	30	buff	light grey	AV	LL
25	aerial hyphae	none	pink	white	HV	meso
26	straight spore chains	20	buff	grey	HV	LL
27	straight spore chains	<10	buff	grey	HV	LL
28	straight spore chains	20	buff	grey	HV	LL
29	straight spore chains	20	buff	grey	HV	LL
30	straight spore chains	10	buff	grey	HV	LL
31	curly spore chains	20	buff	grey	HV	LL
32	spiralled spore chains	20	buff	grey	HV	LL
33	straight spore chains	20	buff	white	HV	meso
34	fragmenting aerial hyphae	>30	buff	white	HV	LL
35	straight spore chains	<10	buff	white	HV	meso
36	straight spore chains	<10	yellow	grey	HV	LL
37	straight spore chains	20	yellow	grey	HV	LL
38	straight spore chains	<10	yellow	light grey	HV	LL
39	spiralled spore chains	20	yellow	grey	HV	LL
40	fragmenting aerial hyphae	20	yellow	white	HV	LL

Table 6. Actinomycetes isolated from <2 μm soil particles.

	Percentage	
	LL-DAP	meso-DAP
Unfractionated soil	76.9	19.2
>251 μm soil aggregates	82.2	17.8
63-251 μm soil aggregates	51.4	48.6
20-63 μm soil aggregates	76.7	23.3
2-20 μm soil aggregates	61.9	38.1
2 μm soil particles	74.4	25.6

Table 7 Percentage of actinomycetes isolated from each soil fraction.

The isolates possessing LL-cell wall corresponded to streptomycetes whilst meso-DAP related to other actinomycete genera.

5.4 Conclusions

Nearly all the actinomycetes isolated from the soil fractions were streptomycetes followed by *Micromonospora*, *Streptosporangium*, *Actinomadura* and *Nocardia*. Similar results have been shown by other isolation studies (Williams and Wellington, 1982; Xu *et al.*, 1996). Most of the streptomycetes would have been *Streptomyces sp.* although representatives of other genera such as *Kineosporia sp.*, *Kitasatoa sp.*, *Nocardiodes sp.* and *Streptoverticillium sp.* could not be excluded (Goodfellow and Williams, 1983). The largest proportion of streptomycetes in comparison to other actinomycete genera were found within the >251 μm aggregate fraction, which was previously described as the soil fraction containing the highest organic content (Jocteur-Monrozier *et al.*, 1991). Streptomycete mycelia have been observed growing on dead plant material, particularly roots fragments by scanning electron microscopy studies. However, it is probable that actinomycetes detected within the soil fractions were in the spore form or as inactive mycelial fragments (McCarthy and Williams, 1992) and that they would remain buried within the nutrient sources on which they had been growing. Several isolates putatively identified as streptosporangia and micromonosporas were recovered from the 63-251 μm aggregate fraction. This was surprising as streptosporangia are usually found within the phyllosphere of plants and leaf litter. They are also found in sandy soils as they prefer weakly acidic conditions (Zenova *et al.*, 1995). The composition of the larger aggregates would have been sand particles, favouring *Streptosporangium* growth provided suitable nutrients were available. Previous studies have shown that streptomycetes grew at pH 7-8.5 and presumably they will grow in microsites around these pH ranges (Nonomura and Ohara, 1969). Micromonosporas were also isolated which have usually been found growing in wet soils and in microaerobic conditions (Goodfellow and Williams, 1983). They may either be degrading cellulose (McCarthy and Williams, 1992) which streptomycetes cannot readily breakdown or growing within the deeper pore spaces of these larger aggregates. The ability to use different nutrients enables the microorganism to occupy a different niche. Previous studies have shown that the same plants in different geographical locations have similar bacterial associations (Wong *et al.*, 1994). Furthermore, a high vegetation diversity in soil showed an equally high actinomycete diversity perhaps indicating that particular streptomycetes performed a specific role in degrading nutrients (Xu *et al.*,

1996). The actinomycetes isolated other than streptomycetes, formed 45% of those recovered from 63-251 μm soil aggregates and it was generally noticed that they were slow growing. These results correlated well with previous studies which indicated that many of the bacteria within the larger aggregates may have been autochthonous (Dabek-Szreniawska, 1993). Furthermore, these bacteria may have replaced the faster growing bacteria (Hattori and Hattori, 1993). The bacterial population within aggregates ranging from 20-500 μm was usually lower (Kabir *et al.*, 1994), perhaps favouring the isolation of rarely recovered bacteria. These may include rarely isolated streptomycetes because it was discovered on the basis of colony comparisons that there were many different streptomycetes extracted from the 20-63 μm aggregate fraction. Some of these may have been growing on the organic matter not easily available to all bacteria. A possible example are humic acids which are resistant to most microbial degradation, particularly as they bind well to silt particles (Christensen, 1987). Conversely, easily metabolisable nutrients flowing through the pores of the smaller 2-20 μm microaggregates were proven to be ideal conditions for faster growing bacteria (Jocteur-Monrozier *et al.*, 1991). Slightly larger aggregates would have equally larger pore diameters and therefore there would be a reduced capillary pressure through the pores resulting in less soluble nutrients for bacterial growth. Consequently, in such low nutrient conditions, the slower growing streptomycetes would become more successful. Many streptomycetes were also found within the <2 μm particle fraction perhaps due to their prolific sporulation compared to other actinomycete genera. The isolates from this fraction were morphologically compared to one another. Assuming that morphologically similar strains were similar biotypes then the actinomycete distributions were log normal. Identification of the isolates to a species level would reveal little further information on the microbial community because a proper comparison of actinomycete communities from each of the soil fractions would require much larger sample number (Kindel *et al.*, 1992). This problem could be overcome by focusing on a smaller group of bacteria (Ka *et al.*, 1994).

In the original aim, it was expected that the streptomycete diversity would be high within the 20-63 μm due to the apparent growth observed within this soil fraction. This idea was true on the basis of colony morphologies although this is a poor indicator of *Streptomyces* species diversity. Further tests would be necessary to identify the species to which each of the isolates belong although this is a time consuming process.

However, the actinomycete diversity based on generic differences was highest in the 63-251 μm and 2-20 μm soil fractions. The former soil fraction contained mainly *Streptosporangium* species and *Micromonospora* species whilst the latter soil fraction exhibited a mixed actinomycete diversity. It would appear that the larger soil aggregates possess characteristics which favour the growth of *Streptosporangium* and *Micromonospora* species, although it is not known whether this would occur within all soils. This may also prevent direct competition occurring between these genera and the faster growing streptomycetes. Alternatively the spores belonging to these actinomycete genera are hydrophilic which would enable them to enter pores through capillary suction of water, particular when the soil becomes dehydrated. In contrast the streptomycete spores are hydrophilic and are more likely to remain outside the soil aggregates.

The actinomycetes recovered from the 2-20 μm soil aggregates exhibited high diversity even though the population was outcompeted by other soil bacteria. It was possible that they had represented propagules which once grown at specific microsites which had possessed the ideal conditions for their growth. In contrast the actinomycete populations in the soil fractions containing the largest soil aggregates and smallest soil particles produced only a limited diversity consisting of grey sporulating streptomycetes. These may have been the faster growing streptomycetes which also produce many spores. Hence a fuller understanding of actinomycete diversity may be obtained by investigating the bacteria recovered from the 2-20 μm soil fraction.

CHAPTER SIX

6. Analysis of Secondary Metabolites Produced by Actinomycetes Isolated from Soil Fractions

6.1 Introduction

It can be concluded from the previous experiments that the streptomycetes were primarily associated with the 20-63 μm soil aggregates of the rewetted air-dried soil (See chapter 4). Isolation of actinomycetes from each of the remoistened air-dried soil fractions showed that the 20-63 and 2-20 μm soil aggregates possessed the greatest number of isolates which were phenotypically different from one another. However, many of the isolates from the 2-20 μm soil aggregates were not streptomycetes but other actinomycete genera. Actinomycetes other than streptomycetes have produced novel antibiotics, such as gentamicin produced by *Micromonospora purpurea,* although the majority of patented antibiotics have been derived from streptomycetes (Goodfellow and Williams, 1983). The search for new secondary metabolites becomes increasingly difficult when so many have already been discovered. To overcome this problem streptomycetes are being isolated from unusual and extreme environments, such as desert sands, in anticipation that these isolates may reveal a different metabolic diversity from the diversity usually detected in forest, agricultural and grassland soils (Langley *et al.*, 1991). An ingenious method was devised using antibody capture methods to selectively recover *Streptosporangium* isolates, in preference to streptomycetes which are usually dominant microorganisms on selective medium (Mullins *et al.*, 1995). It is possible that the microbial diversity may be affected by invading bacteria, spread by human encroachments into environments that had previously remained intact, often as a result of using the land for agricultural purposes.

One of the aims was to determine the diversity of the secondary metabolites produced by the streptomycetes isolated from the different soil fractions. It was expected that the smaller soil aggregate fractions would possess a wider range of different streptomycetes which may have led to more diverse metabolites being detected. During this experiment,

a selection of actinomycetes were investigated to gauge the diversity of the streptomycete isolates by using Biolog and Fourier Transform Infrared Spectroscopy (FTIR). The results from Biolog can be used to cluster similar microorganisms together based on their utilisation of 95 different substrates (Dammann-Kalinowski *et al.*, 1996) while the results from FTIR can be used to group the bacteria together by the infra red spectrum produced from air dried cells (Borel and Lynch, 1992). In FTIR, it was concluded that each peak on the spectrum corresponded to a particular chemical bond (Breton *et al.*, 1994). From the Biolog and FTIR data, the cluster patterns formed, would help determine if the isolates were diverse and whether certain antibiotics may have been attributed to each cluster.

6.2 Procedure

Nineteen actinomycetes were randomly chosen of which 10 were streptomycetes possessing buff substrate mycelium and grey aerial mycelium. The other actinomycetes comprised *Streptosporangium*, *Micromonospora* and *Actinomadura* isolates (See Table 7). On all these strains, the Biolog and FTIR data were collected which were clustered together using the alogorithms available on the Biolog and OPUS microstations, respectively. The 19 actinomycetes were compared to each other to discover similar strains on the basis of macromorphological characteristics. This is possible because actinomycetes produce a variety of different coloured substrate and aerial mycelia unlike many other bacteria which may all appear similar when grown on agar. The actinomycetes were also grown on an oatmeal based agar for 14 days and any organic soluble secondary metabolites which they produced were extracted using ethyl acetate and concentrated by liquid nitrogen for analysis by HPLC. Using Biolog, FTIR, morphological comparisons and metabolite HPLC analysis, data were obtained which were pooled to gain an insight into the production of antibiotics by different groups of actinomycetes isolated from the same soil. It was hoped that this would provide a general overview of the complete actinomycete population isolated from Warwickshire soil fractions.

The 161 streptomycetes isolated from the unfractionated soil and the soil fractions (See appendix for new labelling) were grown for 2 days in yeast extract pregermination medium at 30°C. Then after this period of growth, 5 ml aliquots were transferred to

oatmeal and soya-meal based fermentation media which were incubated for 6 days at 30°C. From these media, the secondary metabolites were extracted using ethyl acetate as the extraction solvent which is selective for organic soluble metabolites. This extraction method was carried out using a robot laboratory specifically designed for Novo-Nordisk. The extracts were analysed on a reverse phase HPLC chromatograph and chromatograms were printed, showing the secondary metabolites produced by the isolates. These metabolites were analysed by determining their diversity and concentrations.

STRAIN NUMBER	GENUS	SOIL FRACTION
11 (HCA00372)	streptomycete	>251 μm aggregates
18 (HCA00379)	streptomycete	>251 μm aggregates
19 (HCA00380)	streptomycete	>251 μm aggregates
20 (HCA00381)	streptomycete	>251 μm aggregates
21 (HCA00382)	streptomycete	>251 μm aggregates
22 (HCA00383)	streptomycete	>251 μm aggregates
23 (HCA00384)	streptomycete	>251 μm aggregates
24 (HCA00385)	streptomycete	>251 μm aggregates
25 (HCA00386)	streptomycete	>251 μm aggregates
3 (HCA00411)	streptosporangium	63-251 μm aggregates
4 (HCA00412)	streptosporangium	63-251 μm aggregates
5 (HCA00413)	micromonospora	63-251 μm aggregates
12 (HCA00420)	micromonospora	63-251 μm aggregates
21 (HCA00429)	streptosporangium	63-251 μm aggregates
29 (HCA00437)	streptosporangium	63-251 μm aggregates
33 (HCA00441)	actinomadura	63-251 μm aggregates
29 (HCA00474)	streptomycete	20-63 μm aggregates
30 (HCA00475)	actinomadura	20-63 μm aggregates
35 (HCA00480)	streptosporangium	20-63 μm aggregates

Table 8. Actinomycetes strains used for Biolog and FTIR analysis.

Actinomycetes isolated from Warwickshire soil aggregate fractions. They were identified by cell wall type and their sporulating structures seen under the microscope. They were grown on oatmeal based agar for metabolic production

6.3 Results

6.3.1 Morphological Comparison of Actinomycetes

The actinomycetes strains were compared by texture of colony type, colours of substrate, spore and aerial mycelia and their general appearance of growth on oatmeal agar. These comparisons showed that there were three pairs of similar actinomycetes isolates. Two of these pairs were streptomycetes which were HCA00372 and HCA00379 and the second pair were HCA00380 and HCA00474. The third pair of morphologically similar strains were a *Streptosporangium* isolate HCA00412 and an *Actinomadura* isolate HCA00475. This was surprising as both microorganisms were putatively identified to different genera but it was possible that HCA00475 was incorrectly identified by the failure to observe sporangia even though aerial hyphae were seen. The isolates which possessed a meso-DAP cell wall often produced aerial hyphae but it was not always clear whether minute spore chains were observed.

6.3.2 Biolog Characterisation of Actinomycetes

The Biolog plates were incubated at 26°C for 5 days and then analysed (See Figure 12). After this incubation period, all the streptomycete strains produced confluent growth in the majority of the wells, while *Streptosporangium* and *Actinomadura* isolates grew forming minute colonies in almost half of the wells. The strains identified as *Micromonospora*, like *Streptosporangium* grew poorly in the wells and they were found to grow on fewer substrates. The same plates were analysed again on day 8 to determine whether these poorly growing actinomycetes grew on more substrates, if they were left to incubate for longer. Their growth at day 8 appeared to be unchanged. There were some similarities in the clustering patterns of day 5 (See Figure 12) compared to day 8 (See Figure 13) although 5 streptomycete strains of the 10 that were analysed, regrouped into different clusters. Analysis of the plates on days 5 and 8, indicated that there were 7 and 8 clusters, respectively. The clustering patterns showed that the 10 streptomycetes isolates formed 3 separate clusters despite the strains being morphologically similar. The streptomycetes isolates HCA00372, HCA00379 and HCA00383 from the >251 μm aggregates remained together within one cluster from day 5 until day 8. Likewise streptomycetes, HCA00380 and HCA00385 from the >251 μm aggregates, clustered into the same group on days 5 and 8. The *Streptosporangium* isolates formed 1 and 2

clusters on days 5 and 8, respectively, while one strain, HCA00429, fell into a cluster with streptomycetes on both sampling days. On day 5, all the *Streptosporangium* strains clustered together except HC00429. This strain was the only *Streptosporangium* isolate analysed which produced confluent growth in the Biolog wells. On day 8, the *Streptosporangium* isolate HCA00412 became an outgroup of the large streptosporangia cluster. A strain HCA00475 which was identified as *Actinomadura* clustered together with the large streptosporangia group but apparently this strain was macro-morphologically similar to HCA00412, a strain which was thought to be *Streptosporangium*. It is possible that HCA00475 was incorrectly identified on the basis of its sporulating structures. Alternatively, it may be that these strains belonged to different genera and that they just happened to look very similar on plates despite the unusual red colour of their substrate mycelia.

Other strains which were similar to one another were HCA00380 and HCA00474 forming one pair and HCA00372 and HCA00379 forming another similar pair. Neither of these paired strains were neighbours in the cluster patterns although they did cluster within the same group on day 5. On day 8, strains HCA00372 and HCA00379 from 20-63 μm aggregates remained within the same cluster whereas HCA00380 and HCA00474 became members of different clusters.

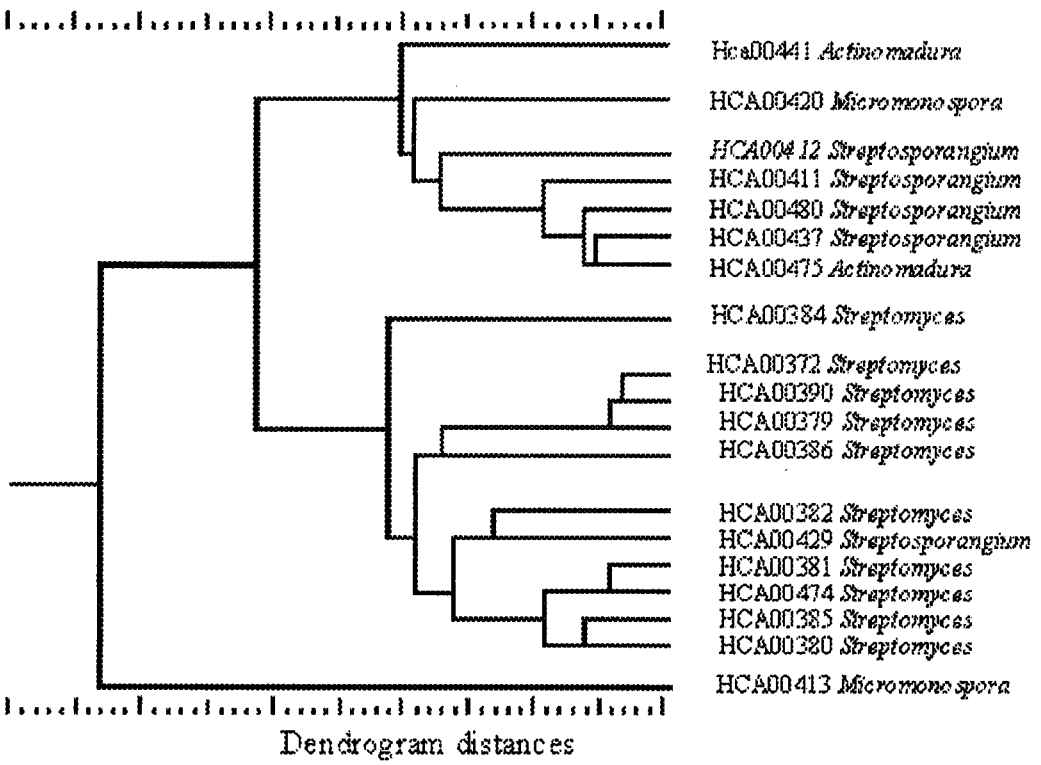

Hca00441 Actinomadura
HCA00420 Micromonospora
HCA00412 Streptosporangium
HCA00411 Streptosporangium
HCA00480 Streptosporangium
HCA00437 Streptosporangium
HCA00475 Actinomadura
HCA00384 Streptomyces
HCA00372 Streptomyces
HCA00390 Streptomyces
HCA00379 Streptomyces
HCA00386 Streptomyces
HCA00382 Streptomyces
HCA00429 Streptosporangium
HCA00381 Streptomyces
HCA00474 Streptomyces
HCA00385 Streptomyces
HCA00380 Streptomyces
HCA00413 Micromonospora
Dendrogram distances

Figure 12. Biolog clustering pattern of actinomycetes at day 5.

The 19 selected actinomycetes (See Table 7) belonging to streptomycete, *Streptosporangium*, *Actinomadura* and *Micromonospora* were grown on Biolog plates for 5 days at 26°C.

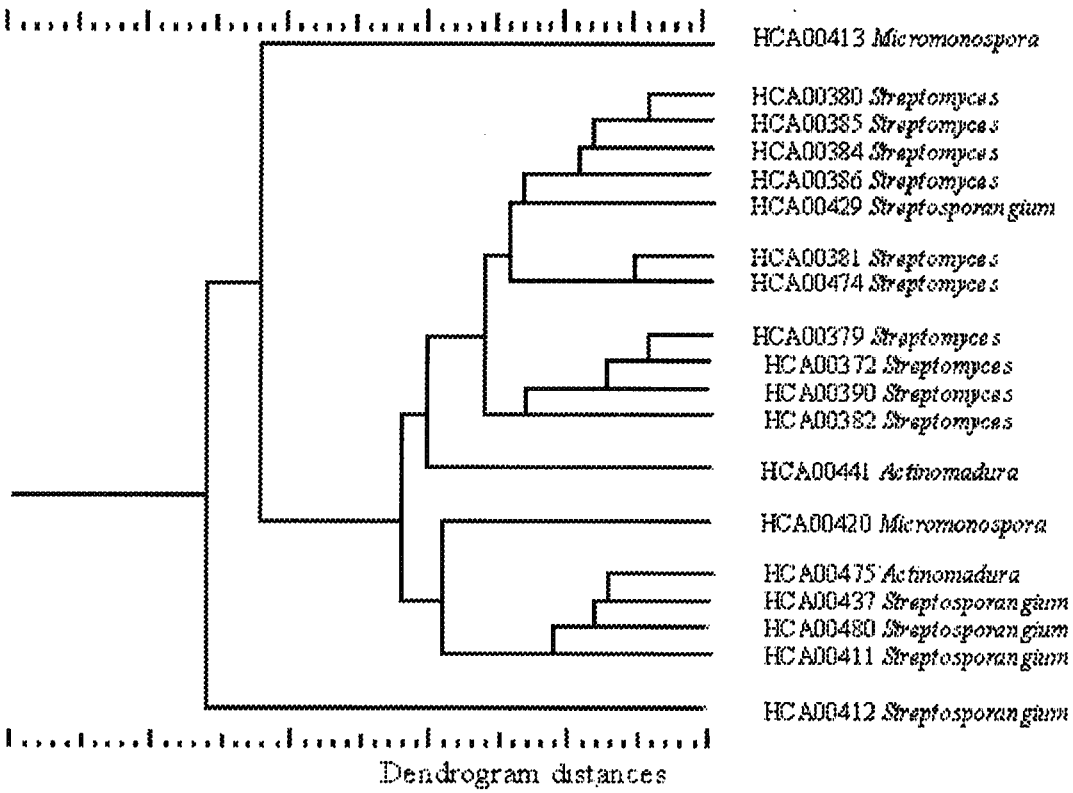

HCA00413 Micromonospora
HCA00380 Streptomyces
HCA00385 Streptomyces
HCA00384 Streptomyces
HCA00386 Streptomyces
HCA00429 Streptosporangium
HCA00381 Streptomyces
HCA00474 Streptomyces
HCA00379 Streptomyces
HCA00372 Streptomyces
HCA00390 Streptomyces
HCA00382 Streptomyces
HCA00441 Actinomadura
HCA00420 Micromonospora
HCA00475 Actinomadura
HCA00437 Streptosporangium
HCA00480 Streptosporangium
HCA00411 Streptosporangium
HCA00412 Streptosporangium
Dendrogram distances

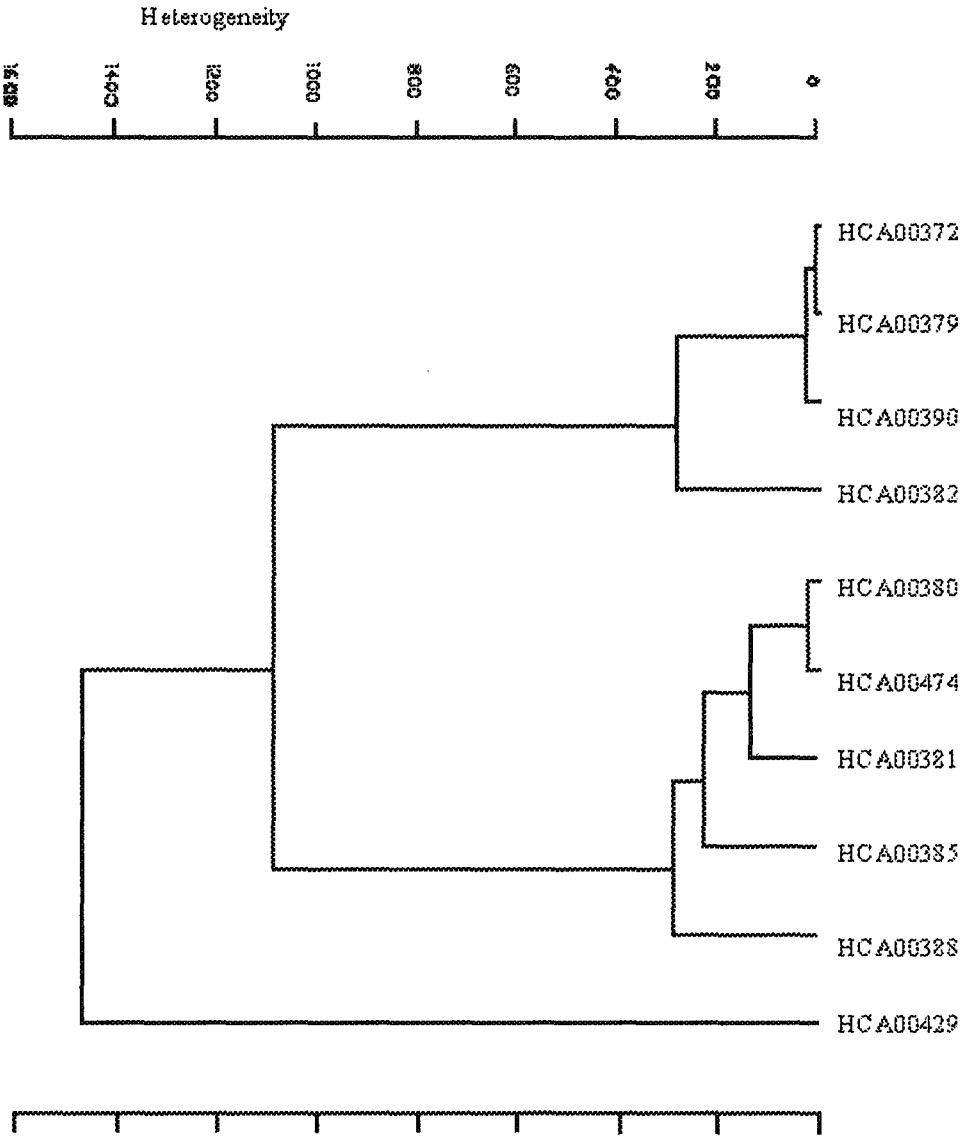
Figure 13. Biolog clustering pattern of actinomycetes at day 8.

The 19 selected actinomycetes (See Table 7) were grown on Biolog plates for 8 days at 26°C.

6.3.3 FTIR Characterisation of Actinomycetes

The same actinomycetes were used for FTIR characterisation as those used for Biolog analysis. Many of those which were not streptomycetes which were HCA00384, HCA00411, HCA00412, HCA00413, HCA00420, HCA00437, HCA00475 and HCA00480, did not grow in the yeast extract based pre-germination medium. This medium was used because its components were soluble, and they could be removed by washing the cell material. The only streptomycete strain which did not grow was HCA00384. There were 2 strains which grew but could not be used in the FTIR analysis. These were a *Streptosporangium* strain, HCA00411 and an *Actinomadura* strain, HCA00441, which both formed tough mycelial pellets that resisted sonication. When their cell material was measured in the FTIR machine they both consistently failed the quality test even after many attempts. Hence both of these strains were omitted from the analysis.

Three clusters were formed from the 10 actinomycetes analysed (See Figure 14). Two of these clusters contained the streptomycete isolates while the *Streptosporangium* strain, HCA00429, clustered by itself. Both streptomycete clusters contained strains which were morphologically distinct from one another. The two pairs of morphologically identical isolates which were HCA00372 and HCA00379; and HCA00380 and HCA00474, were nearest neighbours within their separate streptomycete clusters.



Ward's Alogrithm correlation with staging to first stage

Frequency ranges (weights) =
901 - 699 /cm (1.0)
1200 - 900 /cm (3.0)
3001 - 2799 /cm (1.0)

Figure 14. FTIR clustering pattern of actinomycetes.

The 10 actinomycetes which included 9 streptomycetes and 1 *Streptosporangium* were isolated from Warwickshire soil fractions. Originally 19 strains (See Table 7) were selected but many of the genera other than streptomycetes grew as either clumped pellets that could not be disrupted or failed to grow. The strains were grown in yeast extract based pre-germination liquid medium for 4 days at 30°C, washed, air dried and measured by FTIR.

6.3.4 Metabolic Profiles of Selected Actinomycetes Grown on Solid Medium

All 19 actinomycete isolates showed that a macrolide was produced between retention times of 4.18 and 4.21 minutes which was either unidentified or was putatively identified as a josamycin or a midecamycin derivative. This macrolide was produced at low quantities which was just detectable by the HPLC chromatograph. It was considered that this compound had not been identified as a cell component from the data of cell extracts stored on the HPLC library. To determine whether this was possible, chromatograms of previous secondary metabolic extracts from streptomycetes were viewed but no peaks were found between retention times of 4.18 and 4.21 minutes. It was possible that the unknown macrolides, josamycin or midecamycin derivatives were the same compounds but because of drift in the retention times they were identified differently. If each of these compounds were taken separately, it was found that none of them were restricted to particular clusters obtained from Biolog data on days 5 and 8 and the FTIR data.

There were 2 streptomycete isolates, HCA00384 and HCA00383 which produced the quinone identified as a miamycin derivative, referred to as analogue 2. These 2 strains were morphologically dissimilar and were also present in 2 separate clusters based on the Biolog results for days 5 and 8. Their difference had been previously noticed when the strains were prepared for FTIR because strain HCA00383 grew in a yeast extract based medium but strain HCA00384 did not grow. Another miamycin derivative producer with a retention time of 4.18 minutes, an *Actinomadura* strain, clustered separately from the Biolog data obtained on days 5 and 8. Another compound, a quinone which was identified as a mithramycin A derivative was produced by 2 morphologically similar isolates, HCA00380 and HCA00474. Both of these isolates were in the same cluster groups formed from the Biolog data on day 5 and the FTIR data. However, these strains on the basis of Biolog data obtained on day 8, they fell into 2 separate clusters. The only other compound that was produced was an aminoglycoside identified as a sporaviticin derivative with a retention time of 3.91 minutes. This secondary metabolite was produced by strain HCA00441, identified as *Actinomadura*. This isolate also clustered by itself based on the Biolog data obtained on days 5 and 8 and was morphologically dissimilar to the other 19 actinomycetes.

One of the clusters formed from the Biolog data on day 5 comprised streptosporangia and one *Actinomadura* which produced a compound, most probably a quinone with a retention time of 4.13 minutes. These streptosporangia were all morphologically dissimilar except for the HCA00412 and HCA00475 which were morphologically similar. This quinone was not produced by one *Streptosporangium* isolate, HCA00429 which clustered with streptomycetes on the basis of Biolog data from days 5 and 8 and clustered alone on the basis of FTIR data.

6.3.5 Metabolic Profiles of All Streptomycetes Isolated from Soil Aggregate Fractions

The 161 streptomycete extracts yielded peaks on the HPLC chromatograms which were identified as medium components but no secondary metabolites were detected. One possibility for the lack of secondary metabolites may be attributed to peaks that were not identified using the data stored in the HPLC library on standard compounds which were previously run through the HPLC chromatograph. It would be impossible to form an HPLC library from all the secondary metabolites produced by every actinomycete. Even if the compound exists in the library, for a good identification to be made, the spectra of the unknown and standard compounds measured from 200-600 nm on the HPLC chromatograph must be 99.9% comparable. However, the unknown compounds in the microbial extracts may contain medium components unlike the standard compounds which were used to create the library. These medium components may cause the secondary metabolites to drift from their expected retention times and may also influence their spectra. If either or both factors were affected, identification would be difficult.

There were many compounds which were putatively identified between 95% to 99.9% similarity to known compounds stored in the library but the best identifications would have to be greater than 99.9% similarity. These low levels of identifications may have been caused by either slight drifts in retention times or small inaccuracies in the measurement of spectra preventing identification. It was also possible that the compounds only resembled those which existed within the HPLC library. These poorly identified compounds were mithramycin A and miamycin, both of which were commonly produced by streptomycetes isolated from the soil fractions. Both of these antibiotics were shown to be formed at high quantities. They were coloured compounds, quinones which yielded a characteristic spectrum when measured from 200-600 nm.

Spectrophotometric measurement of the compounds eluted from the HPLC column was carried out by diode arrays which preferentially detect fluorescent compounds. However, a weakly fluorescent compound may form an endpoint spectrum which shows little or no absorbance in the spectrum range from 200-500 nm but a large absorbance between 500-600 nm. The compounds which were identified on the basis of their endpoint spectrum, meant that these compounds had to be excluded from the final analysis of secondary metabolites produced by isolates recovered from Warwickshire soil fractions. These eliminations were necessary because identifications based on spectra limited to such a small range of the ultra violet wavelength could not be accurately differentiated from the endpoint spectrum of other metabolites.

Many streptomycetes growing in the oatmeal based liquid medium produced 2 secondary metabolites which were predicted as mithramycin derivatives with retention times of 3.67 and 3.99 minutes (See Table 8). They were both produced in high quantities, determined by the volume of the peaks on the chromatogram, calculated from the full height and the width, halfway up the peak. These calculations were performed automatically by the HPLC chromatograph. The width of the peaks barely differed between compounds meaning that the height of the peaks could be used as an indicator for quantity. An absorbance measurement of greater than 0.2 absorbance units was arbitrarily taken as a value for good production of a secondary metabolite. This absorbance value will be discussed later with reference to good production. Consequently, good production levels were associated with many isolates which were thought to produce mithramycin A derivatives. However, there were other mithramycin A derivatives with quite different retention times which were produced at much lower quantities and therefore they were not immediately recognised from the other peaks on the chromatograms. There were 2 streptomycete producers of the mithramycin A derivatives, HCA00380 and HCA00385, which clustered together when the data from FTIR and Biolog was analysed. These strains appeared to have identical chromatograms, although many of their peaks corresponded to medium and cell wall components rather than secondary metabolites. This observation was deduced because many of the peaks regularly occurred throughout the chromatograms of all the isolates. The streptomycete isolate HCA00380 was morphologically similar to the isolate, HCA00474, although HCA00474 did not produce the predicted mithramycin A derivative. It was possible that these quinones were present

but their peaks were not identified due to drift from their expected retention times of 3.66 and 3.99 minutes. The chromatograms of the microbial extracts from the actinomycetes grown on solid oatmeal based medium indicated that the isolates HCA00474 and HCA00380 produced mithramycin A derivatives unlike the isolate HCA00385. These mithramycin derivatives were at the lower limits of detection for the HPLC chromatograph perhaps due to either poor production or inefficient extraction of secondary metabolites formed by the isolates grown on the oatmeal based agar. It was therefore conceivable that mithramycin A derivatives were produced by HCA00385 but were not at detectable levels.

Another commonly identified quinone was a miamycin derivative, specifically referred to as analogue 2, produced by the streptomycete isolates growing in the soya-meal based liquid medium. This quinone had a retention time of 4.12 minutes and differed from the other identified miamycin derivative which possessed a retention time of 4.18 minutes. There were no streptomycete producers of the miamycin analogue 2 derivative which were found to produce the mithramycin A derivative.

Ten percent of isolated streptomycetes from soil fractions produced either a diverse range of metabolites, especially between retention times of 3 and 5 minutes, or had good production of more than 5 metabolites with peak heights greater than 0.2 absorbance units (See Table 9). The metabolites with retention times between 3 and 5 minutes were particularly focused on, because usually few compounds were eluted from the column during these times. There were many peaks with earlier and later retention times perhaps corresponding to medium and bacterial cell wall components. The remaining 90% of the streptomycetes isolated, sometimes produced a few metabolites at extremely low quantities but the majority of isolates produced nothing. A good quantity and a diverse range of secondary metabolites were produced by 20% of previous Novo-Nordisk streptomycete culture collection, when their extracts had been previously run through the HPLC chromatograph. Of this collection of strains, 60% of their extracts based on HPLC chromatographs, possessed either a high yield or a good diversity of secondary metabolites but not both characteristics, while the remaining 20% of the strains produced almost nothing apart from the bacterial and medium constituents. It was possible that Novo-Nordisk streptomycete culture collection was highly diverse in terms of different

streptomycete variants. This would therefore explain why good secondary metabolite production for these streptomycetes was proportionally higher compared to streptomycetes isolated from Warwickshire soil. Novo-Nordisk achieved this high streptomycete diversity by recovering strains which were morphologically dissimilar whereas the isolates from the Warwickshire soil fractions may have been very similar since they originated from one soil. This apparent similarity certainly appeared to be true since morphological characterisation of isolates (See chapter 5) showed that many of them had buff coloured substrate mycelium and grey aerial mycelium. It was also probable that such similar isolates were recovered because only the most abundant streptomycetes were isolated. These bacteria may also have metabolic profiles which do not differ much among them. However, the isolates from two soil fractions, 20-63 μm and 2-20 μm soil aggregates, showed slightly higher percentages of good producing secondary metabolite strains, in comparison with the percentages obtained from the >251 μm soil aggregate fraction and the unfractionated soil. The result was statistically significant at a probability of 95% for the 20-63 μm soil fraction and at 90% for the 2-20 μm soil fraction (See Table 10). This indicated that the streptomycete diversity within these 2 soil fractions was greater than the >251 μm soil aggregate fraction and the unfractionated soil, although a more significant result may have been obtained had a much larger sample of streptomycetes been analysed in the 2-20 μm soil fraction. This analysis was performed independently at Novo-Nordisk, where they had no prior knowledge of the actinomycete diversity in the different soil fractions, thereby ensuring an unbiased result. The unfractionated soil yielded streptomycetes which had the least number of interesting chromatograms (3%), perhaps reflecting the metabolic diversity of the <2 μm soil fraction isolates which would have contributed the largest proportion of streptomycetes to the unfractionated soil. However, this explanation cannot be confirmed because the chromatograms showing the secondary metabolites of the <2 μm soil fraction isolates were not examined.

Soil fraction	Strain number	Mithramycin A	Miamycin, analogue 2	others
unfractionated	1 (HCA00309)	+		
>251 µm aggregates	7 (HCA00369)		+	Midecamycin
>251 µm aggregates	9 (HCA00370)		+	Josamycin
>251 µm aggregates	10 (HCA00371)		+	
>251 µm aggregates	16 (HCA00377)	+		Lincomycin
>251 µm aggregates	19 (HCA00380)	+		
>251 µm aggregates	24 (HCA00385)	+		
>251 µm aggregates	45 (HCA00406)	+		
63-251 µm aggregates	10 (HCA00418)		+	
63-251 µm aggregates	13 (HCA00421)		+	
63-251 µm aggregates	27 (HCA00435)	+		
20-63 µm aggregates	8 (HCA00454)			Midecamycin
20-63 µm aggregates	20 (HCA00465)	+		
20-63 µm aggregates	27 (HCA00472)	+		
20-63 µm aggregates	40 (HCA00485)	+		
20-63 µm aggregates	49 (HCA00492)	+		
20-63 µm aggregates	52 (HCA00494)	+		
20-63 µm aggregates	53 (HCA00495)			Midecamycin

Table 9. Streptomycetes producing identified metabolites.

Identified metabolites produced by strains isolated from Warwickshire soil aggregate fractions. ❶ Produced 6 types of Mithramycin A compounds.

Unfractionated soil	
strain	media
36 (HCA00338)	oatmeal
>251 μm soil aggregates	
strain	media
21 (HCA00382)	soyameal
33 (HCA00394)	soyameal
63-251 μm soil aggregates	
strain	media
13 (HCA00421)	soyameal
27 (HCA00435)	soyameal
20-63 μm soil aggregates	
strain	media
2 (HCA00449)	oatmeal
3 (HCA00450)	oatmeal
10 (HCA00456)	soyameal
23 (HCA00468)	soyameal
40 (HCA00485)	oatmeal
46 (HCA00489)	soyameal
53 (HCA00495)	oatmeal
55 (HCA00497)	soyameal
60 (HCA00502)	soyameal
2-20 μm soil aggregates	
strain	media
HCA00523	soyameal
HCA00527	oatmeal
HCA00529	oatmeal

Table 10. Streptomycetes exhibiting interesting metabolic patterns.

Strains isolated from Warwickshire soil aggregate fractions grown in two different media.

	Unfractionated soil	>251 μ soil aggregates	63-251 μm soil aggregates	20-63 μm soil aggregates
>251 μm soil aggregates	**0.69**	-	-	-
63-251 μm soil aggregates	**1.13**	**0.69**	-	-
20-63 μm soil aggregates	**2.69**	**1.98**	**0.80**	-
2-20 μm soil aggregates	**1.76**	**1.72**	**0.90**	**0.39**

Table 11. Statistical determination of actinomycete metabolic diversity from different soil fractions.

Actinomycetes were isolated from different sized soil aggregates and each of the strains was grown in oatmeal and soyameal based media. Secondary metabolites were extracted and examined on the HPLC. If values were greater than 2.58; 1.96 and 1.64 then significant at 99%; 95% and 90% probability, respectively assuming actinomycetes have a Poisson distribution

Determined using the equation: $sd^2 = nP(1-P)$, where P= probability of success =n (successes)/ n (total)

(Weatherburn, 1962).

6.4 Conclusions

When the actinomycetes were analysed by Biolog, the clustering of the data indicated that they were different clusters from day 5 to day 8, especially for the streptomycetes. This result could be accounted for by the different growth rates of actinomycetes on the different carbon substrates in the Biolog plate wells. Previous studies on readily metabolised substrates showed that specific growth rates on 8 separate carbon sources on selected substrates was fast while on other substrates the growth was much slower (Garland and Mills, 1991). While growth on the easily degradable carbon sources may occur rapidly even though the microorganism may grow poorly on the substrate, the growth on the substrate will be scored positive but later may be recorded as negative. Conversely, growth on slowly metabolisable carbon substrates will be recorded as being negative but the result may switch to become positive as the Biolog plates are incubated for a longer time. The positive results are obtained when the absorbance is equal to or greater than 60% of the highest absorbance measurement while negative results are lower than this value. These measurements are determined using the Biolog program installed on the Biolog microstation. Not surprisingly it is possible to obtain borderline results which lie close to the distinction between being positive and negative. These are treated accordingly by whichever Biolog clustering program is used.

From the Biolog and FTIR data, the clustering patterns which were obtained appeared to show some similarities although a *Streptosporangium* HCA00429, was grouped within a streptomycete cluster for the Biolog data but not the FTIR data. It was unlikely that this strain was misidentified since 2 characteristics, cell wall composition and sporulating structures, distinguished it from streptomycetes. However, unlike the other *Streptosporangium* isolates, this strain produced confluent growth in the wells of the Biolog plates, similar to streptomycete growth. It has been suggested before that the growth of bacteria on different substrates in the Biolog plate may represent bacterial functional diversity rather than the bacterial taxonomic diversity when the results are clustered (Haack *et al.*, 1995). In contrast a relationship between the taxonomic diversity and the clustering patterns from the FTIR data was shown to exist by the apparently good correlation between the results from DNA-DNA hybridisation to FTIR (Curk *et al.*, 1994). It could be presumed on the basis of these previous FTIR data, that the streptomycetes isolated from the Warwickshire soil fractions represented 2 different

species as determined from the 2 clusters that were formed despite many of these streptomycetes having morphologically similar colonies. However, very morphologically dissimilar strains have been proven to represent more than one streptomycete species (Kurylowicz and Gyllenberg, 1989). Obviously it is most likely that morphologically similar streptomycetes would cluster together whichever method is used, and this was shown to be true when similar strains grouped together within the same cluster, based on either Biolog or FTIR data. It is possible that morphologically similar strains may express different enzyme levels which would affect their growth on the different carbon substrates so that they may cluster apart when the Biolog data is analysed. This certainly occurred with at least one pair of morphologically identical strains. Even if the functional diversity was represented by the clustering patterns obtained from the Biolog data, it was noticed that the data for actinomycetes were clustered into different genera. Previous studies showed that poor results were obtained when Biolog was used for the identification of streptomycetes (Wünsche and Babel, 1996). These different clusters probably reflected the ability to grow on the full range of different substrates which was best for streptomycetes, then streptosporangia while *Micromonospora* grew poorly on many of them.

The Biolog data from day 5 and the FTIR data each showed a single streptomycete cluster containing 3 representatives which produced a quinone identified as a mithramycin derivative that was determined from the chromatograms of the 19 actinomycetes grown on oatmeal based agar and liquid medium. These same Biolog data also showed that all the strains in another cluster, probably streptosporangia, produced an unidentified quinone that was retained in the column for 4.13 minutes. Another separate cluster represented an actinomadura isolate that produced an aminoglycoside identified as sporaviticin B, which was not produced by the other actinomycetes. Hence, it would seem that particular antibiotics were specifically produced by similar strains perhaps within the same species. These antibiotics encoded by genes may be restricted to similar bacteria due to the increasing difficulty in genetic transfer among increasingly different bacteria. This was proven in previous experiments, where plasmid transfer of pIJ702 was shown to occur more frequently between microorganisms of the same species compared to those of different species (Bleakley and Crawford, 1989). The identified antibiotics mentioned before may therefore have been encoded on plasmids

which can transfer among different varieties of bacteria which belong to the same species. However, there was one antibiotic, a macrolide, lincomycin which was only produced by only one streptomycete from the 161 streptomycetes examined from Warwickshire soil fractions. From this single example, it may be concluded that some antibiotics were only produced by one particular variety of streptomycete. Conversely another macrolide with a retention time between 4.18 and 4.21 minutes, appeared to be produced by all 19 actinomycetes growing on oatmeal based agar. This macrolide was sometimes loosely identified as either midecamycin or josamycin which are both very closely related leucomycins with minor differences between 2 alkyl side groups (Glasby, 1976). It was therefore conceivable that this macrolide was produced by all the actinomycetes. If this was true then horizontal transfer may have occurred between all the actinomycetes within Warwickshire soil by either a broad host specific phage or a broad range plasmid. However, there was no transfer of genes encoding mithramycin A or miamycin from the streptomycetes to other actinomycete genera since these antibiotics were not identified from the chromatograms of each of the actinomycete metabolic extracts. This was to be expected since genetic transfer to other genera is an event less likely to occur than between different species belonging to the same genera.

In this study, streptomycetes were isolated from one soil and the antibiotics produced were at low quantities and exhibited little diversity. The low secondary metabolite diversity may have reflected the low microbial distribution. Hence, it could be possible that the streptomycetes isolated consisted of a few species capable of producing some antibiotics that were not detectable by the methods employed. For instance, streptomycin is a water soluble secondary metabolite which therefore cannot be detected in the organic phase by the HPLC chromatogram. The preliminary investigation showed that there were several streptomycete species formed from only 10 streptomycetes examined by the FTIR. Some of these strains were so similar that it was possible that they were identical. Although, the FTIR analysis on the streptomycetes only represented a small proportion, the fact that several clusters were formed indicated that a large number of isolates from the Warwickshire soil fractions must have been similar, perhaps belonging to the same species. The majority of the streptomycetes possessed grey aerial mycelium perhaps related to a few species of streptomycetes which grow well on selective medium. This correlated well with previous studies which showed that most

isolated streptomycetes formed grey aerial mycelium (Williams *et al.*, 1983). The selective isolation may have occurred for a few streptomycete species on RASS medium whilst very few *Streptosporangium* species were selected on nutrient low HV medium even though 4 different selective media were used to obtain the best overall actinomycete diversity. Evidence for selective isolation was provided in previous studies which showed that spores of *Streptomyces albidoflavus* were commonly isolated (Huck *et al.*, 1991).

It was observed that the streptomycete cultures growing in the soyameal based medium and oatmeal based medium generally produced the mithramycin A and miamycin derivatives, respectively. It could be assumed that many of the streptomycetes from Warwickshire soil produced similar compounds but if a medium was developed which would facilitate the antibiotics produced by the actinomycetes extracted from this soil, then the full secondary metabolic diversity would be revealed. The production of antibiotics in certain media is not unusual and studies have focused on antibiotic production in media. It has been shown that slight modifications to the constituents may dramatically effect the production of the antibiotic (Vilches *et al.*, 1990). Consequently, the metabolic profiles of streptomycetes isolated from some soils would yield good results if only the soyabean and oatmeal based media were used. If this occurred then it would be worthwhile recovering a large number of streptomycetes isolated from this type of soil. As a consequence of the low secondary metabolite yields obtained from all the streptomycetes recovered from these soil fractions, it was difficult to identify whether any particular soil fraction would yield streptomycetes possessing a diverse range of secondary metabolites. It appeared that the best chromatograms produced by a large proportion of streptomycetes were recovered from the 20-63 μm, significant at 95% probability, and from 2-20 μm soil fractions at 90% probability. From the 2-20 μm soil aggregates, the streptomycetes produced the highest proportion of interesting chromatograms, at 25% of their population, while the unfractionated soil had the lowest at 3% of their population. It has already been suggested that different species may produce a range of different secondary metabolites and it was found that the actinomycetes recovered from the 2-20 μm soil fractions were very diverse between and within genera on the basis of morphology and cell wall chemotype. This would seem to indicate that the production of diverse secondary metabolites by isolates specifically from

the 2-20 μm was the correct observation. Consequently, collecting this soil fraction would be advantageous in increasing either the actinomycete or just the streptomycete diversity from a chosen soil, particularly if interesting metabolic profiles had already been obtained from strains obtained from one soil.

The results of these studies indicated that the actinomycete and metabolic diversities were directly related to each other. This was based on the differences in actinomycete diversity throughout the different soil fractions which was evaluated by determining the genus of each isolate and then comparing their morphologies. Although there may have been many identical isolates based on the clustering results obtained from Biolog and FTIR, it evident that there at least several different streptomycete species. The secondary metabolites produced by all the isolates recovered from this soil were apparently similar. Therefore it is possible that isolates recovered from other soils may exhibit such similarities in metabolic diversity. If good metabolic profiles were obtained with a few isolates from a specific soil, then it would be worthwhile to recover a greater actinomycete diversity from the 2-20 μm soil fraction.

CHAPTER SEVEN

7. Analysis of Soil Actinomycetes on Denaturing Gradient Gel Electrophoresis

7.1 Introduction

Denaturing gradient gel electrophoresis is a technique used to separate amplified DNA of several different sequences, which may differ from each other by as little as one base (Myers *et al.*, 1985a). The standard condition for DGGE is that a linear denaturant gradient, ranging from 0% to 100%, is established from one end of the gel to the other. These denaturants consist of formamide and urea. The concentrations of the denaturants may be changed to create a narrower gradient, if required. The gel is run at a temperature close to the melting point of the DNA, usually at 60°C, although some low melting DNA requires a lower running temperature (Poddar and Maniloff, 1989).

Initially a perpendicular gel is run, with the sample loaded along the top of the gel and the denaturant gradient at right angles to the direction the DNA samples run in the gel. The DNA is allowed to migrate for several hours, then the gel is stained with ethidium bromide, and afterwards or instead, silver stained. The DNA should be visible on the gel as a sigmoid band, and the DNA running slowest corresponds to completely melted DNA while the DNA migrating furthest is the double stranded DNA (Myers *et al.*, 1988). Between these phases, the concentration of the denaturant is determined so that another gel with a narrow gradient can be run.

Once the concentration of denaturants for the optimum DNA melting has been determined, a parallel gel is prepared, which has the denaturant gradient in the same direction as the DNA samples are run. A DNA mixture of 2 different PCR products are loaded into each well at periodic time intervals until all the wells have been filled. The purpose of this experiment is to determine the optimum time required for separation of the 2 PCR products on the gel, because the relative migration of the separate products may constantly change during the running time (Theophilus *et al.*, 1989). It has been observed that maximum separation of the amplified products often occurred when they

ceased to migrate further up the denaturing gradient (Muyzer *et al.*, 1993). Further samples may be compared with each other once the best time has been sought.

The DNA samples run on the denaturing gradient gel may be amplified products created by PCR. The PCR is carried out using two primers of about 20 bases although for the analysis of amplified products on denaturing gradient gels, one primer must have a high GC sequence at the 5' end, which is termed a GC clamp. Previous studies carried out using a GC clamp of different lengths, between 40-200 base pairs, showed that the longer GC clamps possessed a higher melting temperature, although a 40 base pair GC clamp was usually sufficient for DGGE studies (Myers *et al.*, 1985c). The presence of the GC clamp showed that 95% single base transitions and transversions were detected, otherwise without the GC clamp fewer single base mutations were detected (Myers *et al.*, 1985b). The products without the GC clamps were dependent not only on whether there were at least 2 melting domains, but also if the melting temperatures between these domains were great enough to enable one of them to act as a GC clamp, which would enable single base mutations to be detected within the other melting domain (Myers *et al.*, 1987). Whether the products contain a GC clamp or not, the mutated amplified DNA fragment migrates at a different rate in comparison to the amplified parental DNA, on the denaturing gradient gel.

It was decided that a primer should be designed which was specific for actinomycetes because many different actinomycetes had been isolated from the different soil fractions. Both the actinomycetes isolates and the soil fractions were going to be analysed on the DGGE. It was not possible to isolate just streptomycetes because many actinomycetes belonging to other genera exhibit similar morphologies. Two different strategies would be used to design the primers. One method was to chose sequences around 20 bases in length which possessed the greatest homology between different actinomycetes but exhibited less homology with other bacteria. Alternatively, a very short sequence would be found which was unique to actinomycetes and a primer would be designed around this region even if there were a few mismatching bases within the primer sequence.

When PCR is carried out on soil DNA, it is important that there is sufficient target DNA for the amplification to be successful. PCR of the actinomycete 16S rDNA from a soil

DNA extract should form a product because actinomycetes usually number 10^7 propagules per gram of soil. However, some of the actinomycete genera may be at much lower levels. If the variable region was amplified, then many PCR products would be formed with different sequences corresponding to the many different actinomycete species. Thus it would be likely that some of the amplified products may be at such low quantities that they do not form a visible band on the denaturing gradient gel. This result would occur because some bacterial species would be below the level of MPN-PCR detection. Obviously this also assumes that there is no PCR bias. Another factor to consider when amplifying the variable region of the 16S rDNA gene is that some genera show little sequence variation between their species, as for *Micromonospora*. Accordingly, *Micromonospora* may form far fewer bands on the denaturing gradient gel compared with streptomycetes even if one considers that there may be more streptomycete species in soil compared to *Micromonospora*.

Despite the loss of species resolution when amplifying a highly conserved region of the actinomycete 16S rDNA as opposed to its variable region, the pattern of bands from amplified products of soil DNA extract on the denaturing gradient gel should reveal actinomycete 16S rDNA extracted from soil. This pattern could be compared to the amplified products derived from DNA extracts of the actinomycetes isolated from soil. The region of DNA amplified from 226 to 513 (*E. coli* positioning) was almost completely conserved for all actinomycetes with occasional base pair differences between genera and it was unlikely that there would be much sequence variation between the species.

The aim of the experiment was to amplify a conserved region of the 16S rDNA from DNA extracted from each actinomycete isolated from the soil fractions, and run the amplified products separately on denaturing gradient gels. The position of each amplified 16S rDNA product would be determined relative to other PCR products. The extracted DNA from all the actinomycetes isolated from a particular fraction would be put together in a mixture for PCR amplification of the 16S rDNA and the resulting products would be run on the denaturing gradient gel. Some products amplified from the DNA mixture may disappear due to PCR bias. Finally it was intended to compare this pattern obtained with the pattern of the PCR products amplified from DNA

extracted out of each soil fraction. The pattern on the denaturing gradient gel may contain bands corresponding to actinomycetes which could not be isolated. There may also be bands from the cultured actinomycetes which are not visible in the soil fraction pattern, because these microorganisms could be at a low population in the soil fraction compared to other soil bacteria or as a result of PCR bias against that microorganism.

7.2 Procedure

A number of different primers were designed which would selectively amplify actinomycete 16S rDNA. The DNA extracts from type strains were amplified and then the products were run on a perpendicular denaturing gradient gel to determine the melting point of the DNA. Once it had been elucidated, a narrow gradient gel was made so that the DNA would melt in the middle of the gel. The optimum time for separation of the PCR products was determined on a travel parallel denaturing gradient gel. Then a parallel denaturing gradient gel was prepared, and the products from DNA extracts of actinomycetes were run on the gel and compared with the products from soil DNA extracts.

Actinomycete strains		
1	*Actinomadura malachitica*	DSM 43462
2	*Planobispora lonigispora*	ATCC 23887
3	*Arthrobacter oxydans*	JCM 2521
4	*Dactylosporangium aurantiacum*	ATCC 23491
5	*Promicromonospora citrea*	JCM 3051
6	*Oerskovia turbata*	JCM 3160
7	*Amorphosporangium auranticolor*	JCM 3038
8	*Geodermatophilus obstcutus*	ATCC 25078
9	*Planomonospora parontospora*	ATCC 23864
10	*Sporichthya polymorpha*	JCM 3089
11	*Spirillospora albida*	JCM 3041
12	*Nocardiopsis anta*	ATCC 31511
13	*Streptoverticillium abikoense*	NRRL B-1516
14	*Streptoverticillium fievens*	NRRL B-1676
15	*Brevibacterium flavum*	JCM 1308
16	*Kineosporia curantica*	JCM 3230
17	*Streptosporangium roseum*	CBS 31356
18	*Actinosynnema mirum*	JCM 3225
19	*Actinomyces* species	QM B-814
20	*Actinoplanes philippinesis*	JCM 3001
21	*Ampurallariella digitata*	ATCC 15349
22	*Streptoalliteichus hindustamis*	JCM 3268
23	*Microbispora rosia*	ATCC 21946
24	*Arthrobacter crystallopoietes*	DSM 20744
25	*Micrococcus luteus*	DSM 20030
26	*Clavibacter michiganense*	DSM 20744
27	*Streptomyces aureofaciens*	DSM 40127
28	*Nocardia* natural isolate species	HCA
29	Rhizosphere potato DNA	
30	*Nocardia* natural isolate species No. 1	
31	*Nocardia* natural isolate species No. 2	
32	*Rhodococcus rhodochrous*	E 120
Non-actinomycetes strains		
33	*Agrobacterium tumefaciens*	DSM 30205
34	*Agrobacterium rhizogenes*	DSM 30148
35	*Rhizobium meliloti*	DSM 30135
36	*Rhizobium leguminosarum*	DSM 30132
37	*Burkholderia gladioli*	DSM 4285
38	*Burkholderia solanacearum*	DSM 1993
39	*Pseudomonas fluorescens*	R2F
40	*Clostridium pasteurianum*	DSM 525
41	*Cytophaga heparina*	

Table 12. Bacterial strains to test specificity of HGC primers.

DNA recovered from actinomycetes which were obtained from the culture collection at Warwickshire University, Coventry. DNA recovered from the non-actinomycetes were obtained from the Federal Biological Research Centre for Agriculture and Forestry, Braunsweig, Germany. The DNA was amplified using primers to amplify a specific region of the 16S rDNA.

7.3 Results

7.3.1 Specificity of the Actinomycete Specific Primers

DNA was extracted from 31 different actinomycetes, 3 were isolates and the remainder of them were type strains (See Table 11). The negative controls to test the specificity of the actinomycete primers were from the alpha, beta and gamma subclasses of Proteobacteria, a representative of the Clostridium-Bacillus branch, which has low GC content, and *Cytophaga heparina*. Using the actinomycete primer which anneals to position 226 to 243 (*E. coli* numbering) and a universal primer containing the GC clamp at position 701 to 721 (*E. coli* numbering), the DNA from actinomycetes, except for *Planobispora longispora* and *Streptoverticillium fievens*, produced an amplified product 495 base pairs long. The negative controls did not produce any amplified products except for the DNA from *Cytophaga heparina*, which formed a weak nonspecific and longer product. When the bacterial DNAs were amplified using universal primers, products were obtained with all strains, except *Streptoverticillium fievens*.

7.3.2 Amplified PCR Products from Actinomycete Isolates Run on DGGE

Nucleic acids were extracted from each of the isolates that was recovered from the soil fractions. A region of 16S rDNA was PCR amplified from 226 to 513 using one primer specific for actinomycetes whilst the other was a general eubacterial primer. This eubacterial primer had a 40 base pair GC rich chain at one end which became incorporated into the PCR products. The products were run on a perpendicular denaturing gradient gel to establish the melting point which was found to occur at 53% denaturant. Once the denaturant concentration at the melting point was determined it was possible to create a parallel denaturant gradient gel from 40-70% denaturants. On this gel, the separation of two PCR products with slightly different base composition was determined after periodic time intervals. This was found to occur after 205 minutes (See Figure 15) although the bands of the PCR products on the gel became slightly unfocused. The denaturing gradient gels were run at different temperatures but it had no effect. It seemed as though the GC rich clamp may be causing problems in combination with the reasonably high GC content of the amplified actinomycete 16S rDNA, at 65%. It was postulated that the demarcation between the GC clamp and the amplified 16S rDNA region was ill defined. To overcome this problem, a modified psoralen amidite was incorporated into the 5′ end of the primer (Chemiclamp; Costes *et al.*, 1993) to replace

the 40 GC nucleotides. After PCR amplification of the 16S rDNA, the double stranded PCR product was permanently cross-linked to form a covalent bond at the psoralen amidite under UV irradiation. It was possible for the whole PCR product to become single stranded under denaturing conditions except at the psoralen cross-linked amidite. This amplified product was run on the 40-70% parallel denaturing gradient gel and the products were retarded after 210 minutes, but the band was less focused compared to the GC clamped product run under the same denaturing conditions (See Figure 16). However, it was discovered that if the amplified products were not cross-linked, the different products could be separated and yet remain focused bands. It was apparent that the positions of the uncross-linked products on the denaturing gradient gel differed from those of the products containing either a high GC clamp or Chemiclamp, which were run on a similar gel. Using the high GC clamp, the problem with unfocused bands was finally resolved after many denaturing gradients had been run by incorporating 40% acrylamide: bisacrylamide solution (37.5:1) supplied by Fisons instead of Biorad into the denaturing gradient gels, resulting in sharp bands.

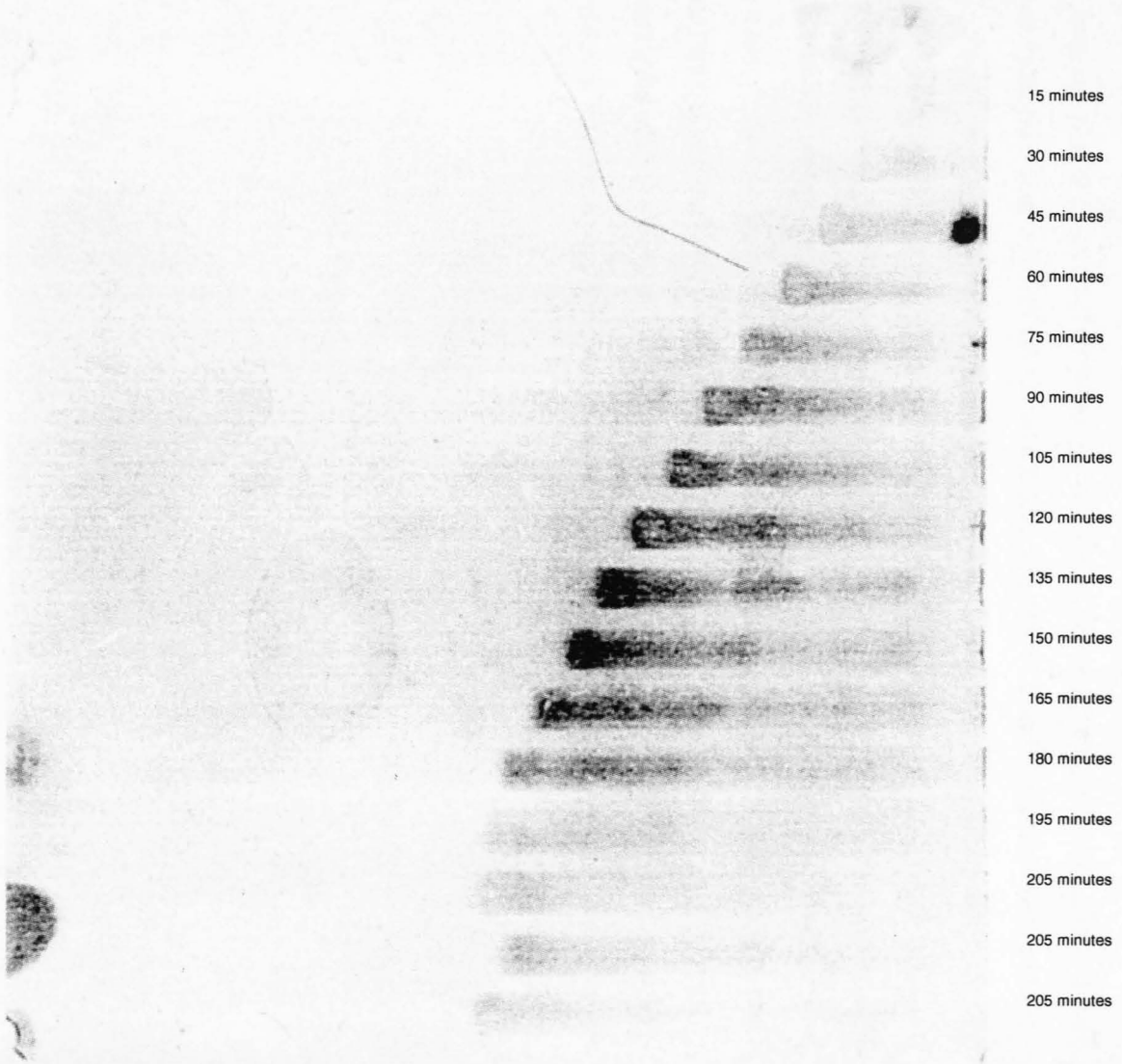
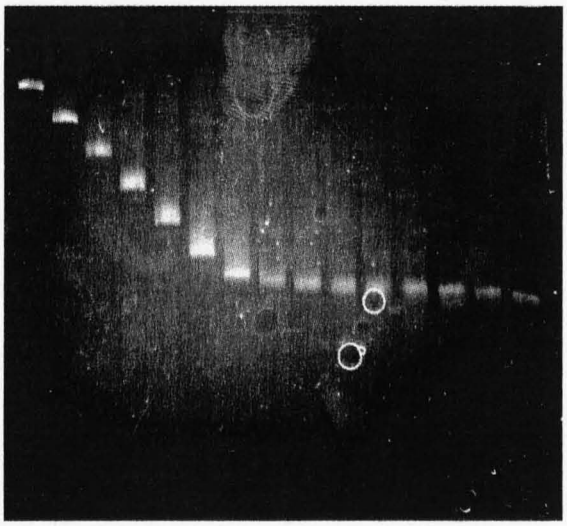

15 minutes
30 minutes
45 minutes
60 minutes
75 minutes
90 minutes
105 minutes
120 minutes
135 minutes
150 minutes
165 minutes
180 minutes
195 minutes
205 minutes
205 minutes
205 minutes

Figure 15. Time travel DGGE to determine optimum separation of PCR products.

PCR products amplified from natural actinomycete isolates from 226-513. Run on a 40-70% denaturing gradient gel.



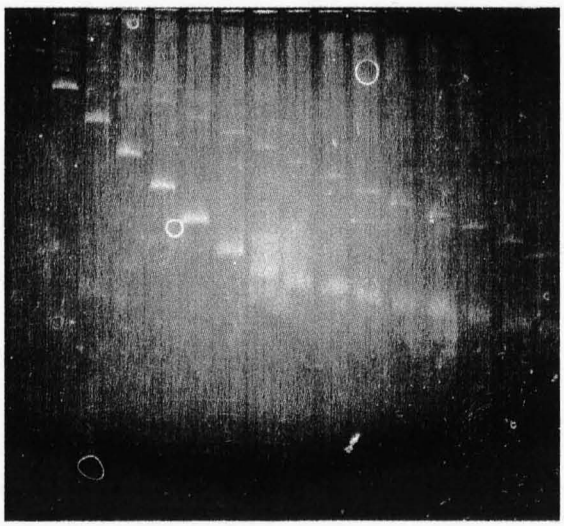


Figure 16. Comparison of GC clamped and Chemiclamped PCR products on DGGE.

DNA from one actinomycete isolate amplified using the same PCR conditions and primers amplifying from 243-513 (*E. coli* positioning) on the 16S rDNA which were run on denaturing gradient gel at 60°C with gradient of 45-75%. The products were loaded onto the gel at every 15 minutes beginning from the left. (Top) GC clamped product. (Bottom) Chemiclamped product.

16 streptomycete spore chains (LL)

46 streptomycete spore chains (LL)
45 streptomycete spore chains (LL)
44 streptomycete spore chains (LL)
43 *Streptoverticillium* spore chains (LL)
41 streptomycete spore chains (LL)
40 streptomycete spore chains (LL)
39 actinomycete non-sporing (meso)
38 actinomycete non-sporing (meso)
37 streptomycete spore chains (LL)
36 streptomycete spore chains (LL)
35 streptomycete spore chains (LL)
34 streptomycete spore chains (LL)
15 streptomycete spore chains (LL)

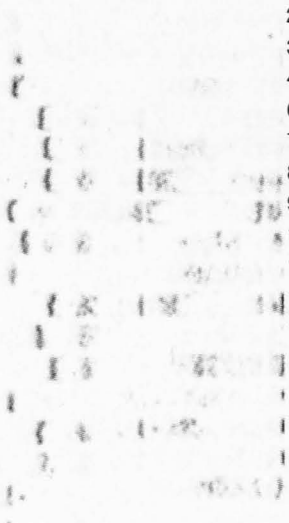
1 streptomycete single spores (LL)
2 streptomycete spore chains (LL)
3 streptomycete spore chains (LL)
4 streptomycete spore chains (LL)
5 actinomycete spores (meso)
6 streptomycete spore chains (LL)
7 streptomycete spore chains (LL)
8 streptomycete spore chains (LL)
9 streptomycete spore chains (LL)
10 streptomycete non-sporing (LL)
11streptomycete spore chains (LL)
12 streptomycete spore chains (LL)
13 actinomycete spore chains (meso)
13 actinomycete spore chains (meso)
15 streptomycete spore chains (LL)
16 streptomycete spore chains (LL)

2 streptomycete spore chains (LL)
32 streptomycete spore chains (LL)
31 streptomycete spore chains (LL)
30 streptomycete spore chains (LL)
29 streptomycete spore chains (LL)
28 actinomycete non-sporing (meso)
27 streptomycete non-sporing (LL)
25 streptomycete spore chains (LL)
24 streptomycete spore chains (LL)
23 streptomycete spore chains (LL)
21 streptomycete spore chains (LL)
20 streptomycete spore chains (LL)
19 streptomycete spore chains (LL)
18 streptomycete spore chains (LL)
17 streptomycete single spore (LL)
1 streptomycete single spores (LL)

Figure 17. DGGE profile of actinomycetes isolated from >251 μm soil aggregates.

Products from separate strains run on 40-70% denaturing gradient gel for 210 minutes.

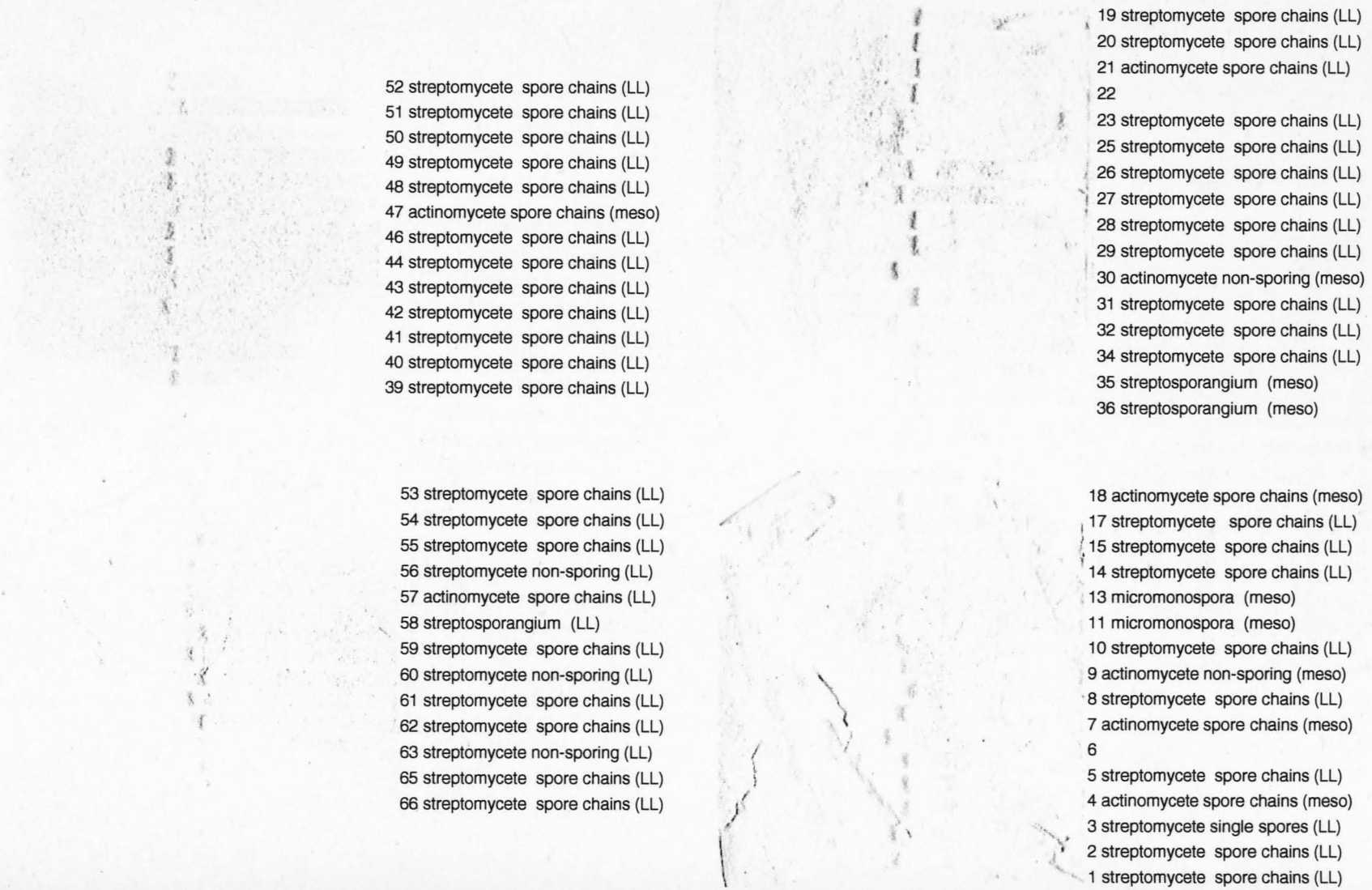
39 actinomycete non-sporing (LL)
38 streptomycete spore chains (LL)
37 streptomycete spore chains (LL)
36 streptomycete spore chains (LL)
35 streptomycete spore chains (LL)
34 streptomycete spore chains (LL)
33 actinomycete non-sporing (meso)
31 microtetraspora (meso)
29 streptosporangium (meso)
28 streptoverticllium (LL)
27 streptomycete spore chains (LL)
26 streptomycete spore chains (LL)
25 actinomycete non-sporing (meso)
23 streptomycete spore chains (LL)
22 streptomycete spore chains (LL)
20 actinomycete non-sporing (meso)



2 streptomycete non-sporing (LL)
3 actinomycete spore chains (meso)
4 streptosporangium (meso)
6 streptomycete spore chains (LL)
7 micromonospora (meso)
8 actinomycete non-sporing (meso)
9 streptomycete spore chains (LL)
10 streptomycete spore chains (LL)
11 streptomycete spore chains (LL)
12 micromonospora (meso)
13 streptomycete spore chains (LL)
14 streptomycete spore chains (LL)
15 streptomycete spore chains (LL)
16 micromonospora (meso)
18 streptomycete spore chains (LL)
19 actinomycete spore chains (meso)

Figure 18. DGGE profile of actinomycetes isolated from 63-251 μm soil aggregates.

Products from separate strains run on 40-70% denaturing gradient gel for 210 minutes.



52 streptomycete spore chains (LL)
51 streptomycete spore chains (LL)
50 streptomycete spore chains (LL)
49 streptomycete spore chains (LL)
48 streptomycete spore chains (LL)
47 actinomycete spore chains (meso)
46 streptomycete spore chains (LL)
44 streptomycete spore chains (LL)
43 streptomycete spore chains (LL)
42 streptomycete spore chains (LL)
41 streptomycete spore chains (LL)
40 streptomycete spore chains (LL)
39 streptomycete spore chains (LL)

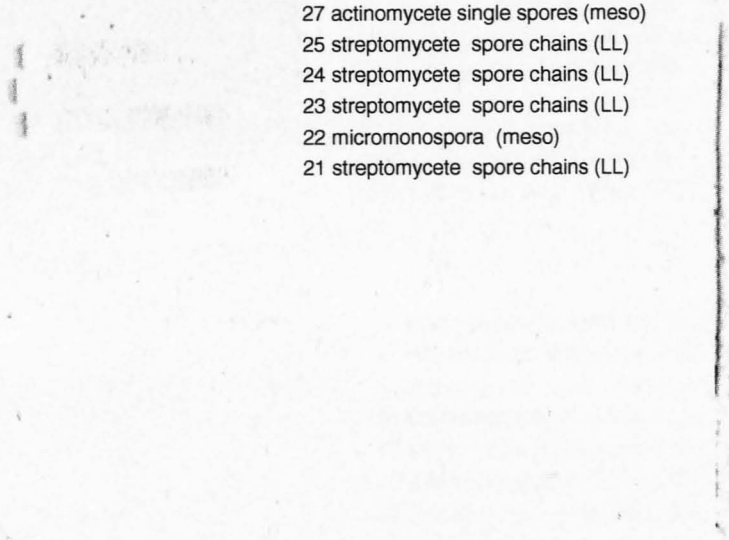
19 streptomycete spore chains (LL)
20 streptomycete spore chains (LL)
21 actinomycete spore chains (LL)
22
23 streptomycete spore chains (LL)
25 streptomycete spore chains (LL)
26 streptomycete spore chains (LL)
27 streptomycete spore chains (LL)
28 streptomycete spore chains (LL)
29 streptomycete spore chains (LL)
30 actinomycete non-sporing (meso)
31 streptomycete spore chains (LL)
32 streptomycete spore chains (LL)
34 streptomycete spore chains (LL)
35 streptosporangium (meso)
36 streptosporangium (meso)

53 streptomycete spore chains (LL)
54 streptomycete spore chains (LL)
55 streptomycete spore chains (LL)
56 streptomycete non-sporing (LL)
57 actinomycete spore chains (LL)
58 streptosporangium (LL)
59 streptomycete spore chains (LL)
60 streptomycete non-sporing (LL)
61 streptomycete spore chains (LL)
62 streptomycete spore chains (LL)
63 streptomycete non-sporing (LL)
65 streptomycete spore chains (LL)
66 streptomycete spore chains (LL)

18 actinomycete spore chains (meso)
17 streptomycete spore chains (LL)
15 streptomycete spore chains (LL)
14 streptomycete spore chains (LL)
13 micromonospora (meso)
11 micromonospora (meso)
10 streptomycete spore chains (LL)
9 actinomycete non-sporing (meso)
8 streptomycete spore chains (LL)
7 actinomycete spore chains (meso)
6
5 streptomycete spore chains (LL)
4 actinomycete spore chains (meso)
3 streptomycete single spores (LL)
2 streptomycete spore chains (LL)
1 streptomycete spore chains (LL)

Figure 19. DGGE profile of actinomycetes isolated from 20-63 μm soil aggregates.

Products from separate strains run on 40-70% denaturing gradient gel for 210 minutes.



27 actinomycete single spores (meso)
25 streptomycete spore chains (LL)
24 streptomycete spore chains (LL)
23 streptomycete spore chains (LL)
22 micromonospora (meso)
21 streptomycete spore chains (LL)

1 streptomycete spore chains (LL)
2 streptomycete spore chains (LL)
3 streptosporangium (LL)
4
5 streptomycete spore chains (LL)
6 actinomycete spore chains (LL)
7 streptomycete spore chains (LL)
8 actinomycete spore chains (LL)
11 actinomycete non-sporing (meso)
13 streptomycete spore chains (LL)
14 actinomycete non-sporing (meso)
15
17 streptomycete spore chains (LL)
18 actinomycete spore chains (LL)
19 streptomycete non-sporing (LL)
20 streptomycete non-sporing (LL)

Figure 20. DGGE profile of actinomycetes isolated from the 2-20 μm soil aggregates.

Products from separate strains run on 40-70% denaturing gradient gel for 210 minutes.

The amplified products from the >251 μm aggregate isolates were run on 3 separate gels (See Figure 17). Most of the bands did not separate that much from each other probably because many of them were streptomycetes. There were four pairs of morphologically identical strains, numbered 2 and 6; 15 and 16; 34 and 35; and 29 and 30 which were all streptomycetes producing buff coloured substrate mycelium and grey aerial mycelium. These comparisons were made immediately after they were isolated from the soil fraction. The PCR product from each pair of these strains ran at identical positions to each other on the same gel.

The amplified products from the 63-251 μm aggregate isolates were run on two gels (See Figure 18). The PCR products separated well from each other exhibiting the high diversity of actinomycetes recovered from this soil fraction. On one gel, the amplified products from strain numbers 9, 11 and 15 ran to identical positions. These strains had yellow substrate mycelium, grey aerial mycelium and were all streptomycetes. On the second gel, another group of morphologically similar strains which were numbered 20, 25, 29, 33, 38 and 39 ran to the same position on the gel. These strains produced buff coloured substrate mycelium, some had either a minute amount of white or otherwise no aerial mycelium, and a meso-DAP cell wall composition. However, strain 38 had LL-DAP cell wall chemotype whilst strain number 29 produced sporangia which were not observed in the other isolates. The amplified products from a pair of streptomycete strains, numbered 22 and 36 with buff substrate mycelium and grey aerial mycelium ran to identical positions on the gel.

The PCR products from the 20-63 μm aggregate isolates were run on 4 gels (See Figure 19). The products separated well but not as much as the those derived from the 63-251 μm aggregate isolates. There were many groups of streptomycete strains with buff or yellow coloured substrate mycelium and grey aerial mycelium whose amplified DNA products migrated together on the same gel. They formed 4 groups of strains, numbered: 1, 2, 3, 5 and 17; 19 and 20; 48 and 49; and 59, 61 and 66 although strain number 3 produced no aerial mycelium. The products from strain numbers 44 and 46 ran to the same position on the gel. These streptomycete strains produced pink substrate mycelium and white aerial mycelium. The 16S rDNA product from strain number 30 ran to a unique position on the gel and it was noticed that this strain exhibited unusual

morphology. It had orange substrate mycelium, white aerial mycelium with a meso-DAP cell wall composition.

The PCR products from the 2-20 μm aggregate isolates were run on two gels and the products separated well from each other (See Figure 20). The streptomycete strains, numbered 1, 2 and 8 possessing buff coloured substrate mycelium and grey aerial mycelium formed PCR products which ran to the same position on the same gel. One product that ran to a unique position on the gel was derived from strain number 6 which showed rather unusual morphological characteristics. It produced buff coloured substrate mycelium, white aerial mycelium and had meso-DAP cell wall chemotype.

The analysis of the positions of the PCR products on the 40-70% denaturant gels, showed that 74% of the bands further up the denaturant gradient were from actinomycetes, with a meso-DAP cell wall composition. Actinomycetes with a LL-DAP cell wall chemotype (92%) formed PCR products which were retarded at a lower concentration of denaturants. These comparisons were made only on gels which showed good separation between the different amplified products. When the sequences of the amplified region were examined, it was found that the GC content for streptomycetes (62%) was slightly lower compared to some other actinomycetes, e.g. *Saccharopolyspora* (64%).

PCR primers

Primers used for DGGE (numbering based on *E. coli* positioning)

High GC microorganism specific forward primer 226-243:

5′ GGATGAGCCCGCGGCCTA 3′

Universal reverse primer from 513-528 including GC clamp (underlined):

5′ CGCCCGGGGCGCGCCCCGGGCGGGGCGGGG

GCACGGGGGGCGCCGCGGCTGCTGGCACGTA 3′

Universal Chemiclamp reverse primer from 513-528 including ECORI cutting site (underlined) and Chemiclamp:

5′ AACGCGAATCCCGCGGCTGCTGGCACGTA 3′

Universal forward primer from 6-27:

5′ TGAGAGTTTGATCCTGGCTCAG 3′

Universal reverse primer from 797-813 and GC clamp (underlined):

5′ CGCCCGGGGCGCGCCCCGGGCGGGGCGGGG

GCACGGCGGGACGGCGTGGACTCACAG 3′

CONTINUED

Universal reverse primer from 701-721 comprised GC clamp (underlined):

5′CGCCCGGGGCGCCCCGGGCGGGGCGGGCACG

GGCGGTGTTCCTCCTGATATCTG 3′

Actinomycete specific forward primers from 307-325 and comprises first 40 bases as GC clamp from Sheffield *et al.*, 1989 (underlined):

5′ CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCC

CGGGCGGCCGGCCACACTGGCACTGGCACTGAG 3′

Actinomycete specific reverse primer from 696-716:

5′ AGTTCCTCCTGATATCTGCGC 3′

7.3.3 Amplification of 16S rRNA Regions Using Other Primers

Two primers were designed which were highly conserved for actinomycetes although they usually differed from other bacteria by 2 base pairs. The forward primer which had a GC clamp synthesised at the beginning of the 5' end of the primer annealed to position 307-325 (*E. coli* numbering) and the reverse primer annealed to a region from position 696 to 716. The region which was amplified was similar to the region of the 16S rDNA amplified using the previous primers from 226 to 513 which showed very little sequence variation between genera. Chromosomal DNA extracted from *Streptosporangium viridiogriseum* subsp. *viridiogriseum*, *Streptoverticillium cinnamomeum* and *Streptosporangium cinnabarium* were individually PCR amplified using these primers and the products were run separately on perpendicular denaturing gradient gels. Each of the products had quite different melting points. The melting points for *Streptosporangium viridiogriseum* subsp. *viridiogriseum*, *Streptosporangium cinnabarium* and *Streptoverticillium cinnamomeum* were 57%, 55% and 51%, respectively. The products were then run on a parallel denaturing gradient gel to determine the optimum time required for separation of the amplified products. This gel had a denaturing gradient from 45-75% and it was found that the products running on this gel began to separate after 270 minutes whilst maximum separation was observed at 390 minutes (See Figure 21a). It was found that running the amplified products for longer led to the bands becoming unfocused and therefore no longer discernible. Sometimes there was nothing visible on the gel when the same conditions were repeated and the amplified products were run for 6.5 hours, perhaps because the products had already melted.

Two forward primers and 3 reverse primers were designed to create different length 16S rDNA products using different combinations of forward and reverse primers in the PCR. The reverse primers had slightly different GC clamps attached to the 5' end. Each of the different products was independently analysed on DGGE to determine whether it was possible to overcome the problems encountered with the previous products amplified from 226 to 513, which had separated poorly and had become unfocused. The largest of the products formed was amplified from position 27 to 797 (*E. coli* numbering) which included the actinomycete hypervariable region at position 180 to 200. It was expected that amplification of this hypervariable region would lead to good separation of products

between different actinomycete species but this did not occur. A sequence of the 16S rDNA was also amplified from 226 to 797 which did not include the hypervariable region. PCR amplification was performed using these 2 primer pairs on the DNA extracts from a selection of actinomycetes which were mixed together after the PCR was completed and run on parallel denaturing gels. It was found that most of the products did not separate and at a particular denaturant concentration the bands became unfocused and finally disappeared.

Using different actinomycete type strains, DNA extracts were recovered and were individually amplified from positions 226 to 701 of the 16S rDNA. These products were found to separate best on a 45-60% denaturing gradient gel, although only the product from *Clavibacter michiganese* separated clearly from the other amplified products (See Figure 21b). The other products were derived from 4 different high GC content actinomycete genera, one low GC content actinomycete and an amplified soil DNA extract. These amplified products ran to the same position on the denaturing gel but running them further resulted in the bands becoming unfocused.

A narrower denaturant gradient was established which ranged from 50-55% denaturant. The products were run for 1, 2, 3 and 4 hours to determine the optimum separation corresponding to distantly and closely related actinomycetes (See Figure 21c). It was found that the products amplified from 226 to 797 separated on the denaturing gradient gel, with the products of the high GC content actinomycetes migrating further compared to the products of the low GC content actinomycetes. However, it was not possible to separate the amplified products derived from actinomycetes which possessed a low GC content, from each other. These particular actinomycetes were *Arthrobacter oxydans* and *Actinomyces* spp. It was also not possible to separate the amplified products derived from the high GC content actinomycetes, *Streptomyces aureofaciens* and *Rhodococcus rhodochrous* from one another.

Another denaturing gel was prepared which had a narrow denaturing gradient ranging from 50-60%. The mixtures of amplified products were run on this denaturing gel at closer time intervals to determine if it was possible to obtain a fleeting separation of amplified products before they became unfocused (See Figure 21d). It was found that in

2 different mixtures there were 3 separate bands, one which migrated furthest relating to the high GC content actinomycetes whereas the other 2 bands were retarded in the denaturant gradient much sooner. This result was unusual because in one of the mixtures there was only one low GC content actinomycete, *Arthrobacter oxydans* and yet there were 2 bands clearly visible corresponding to this group of bacteria.

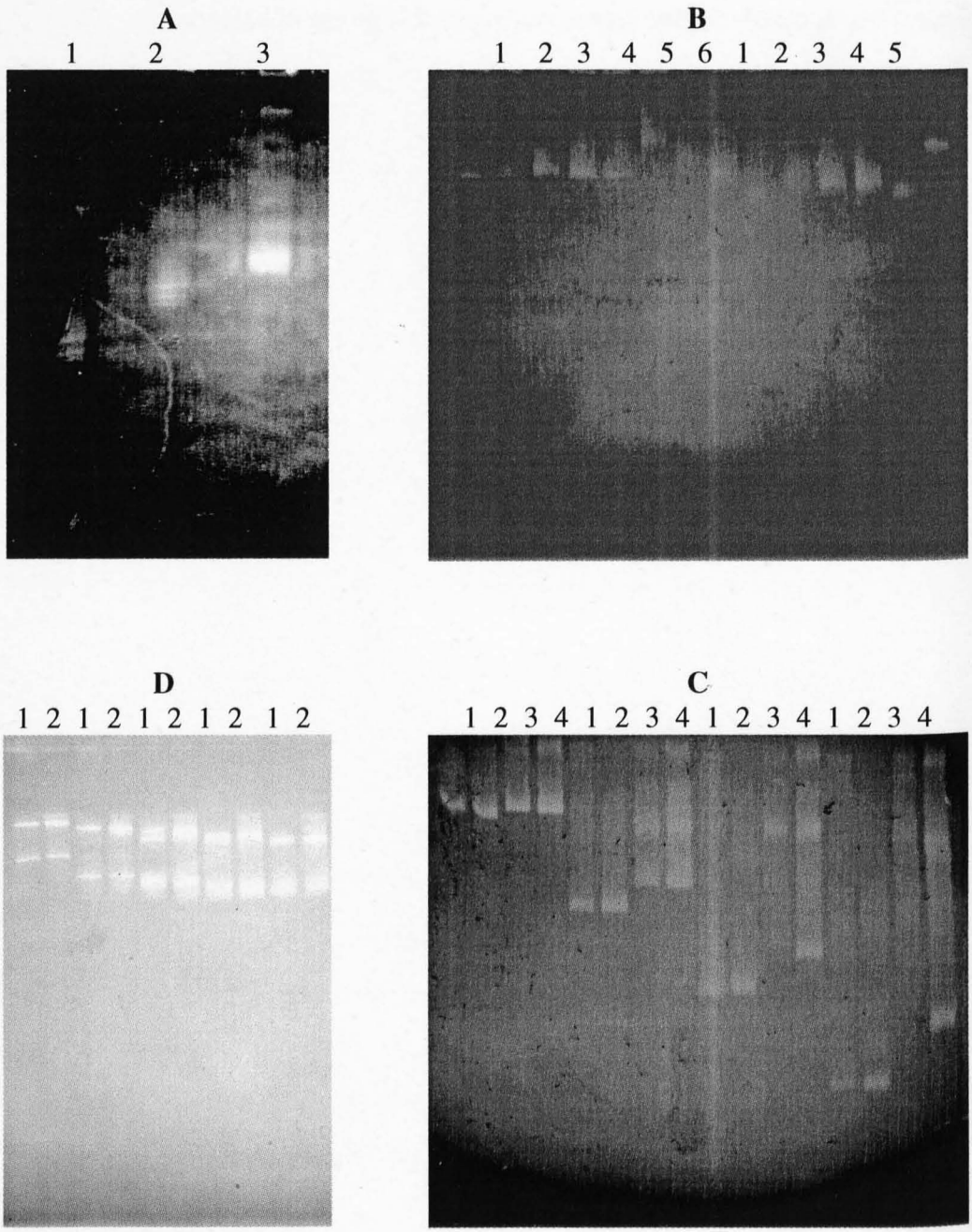
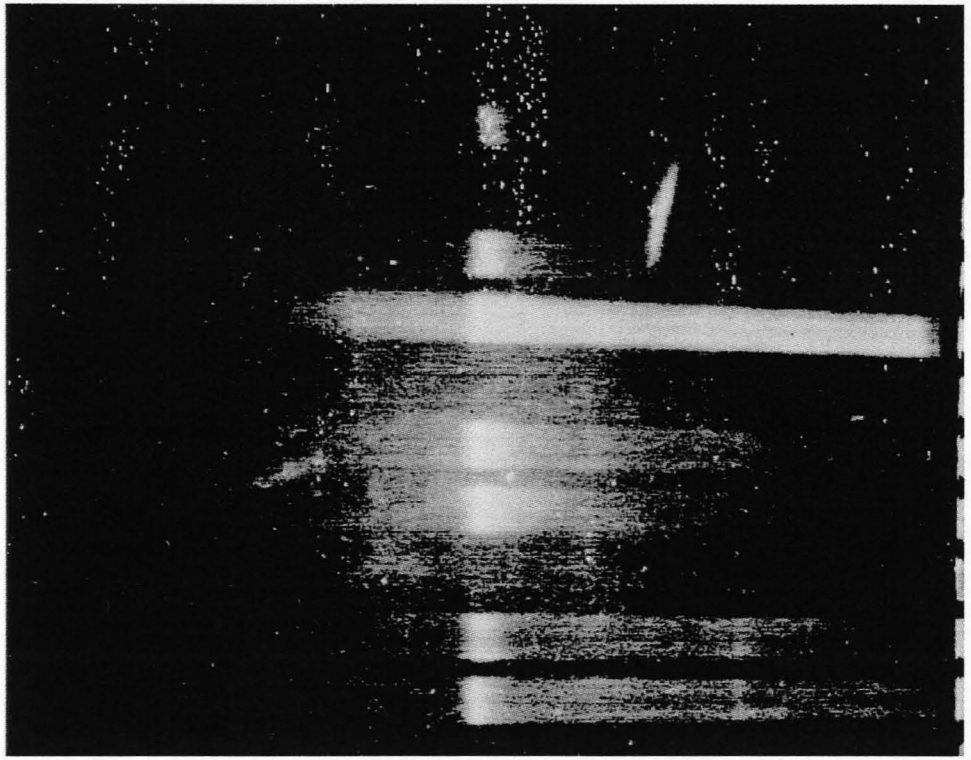
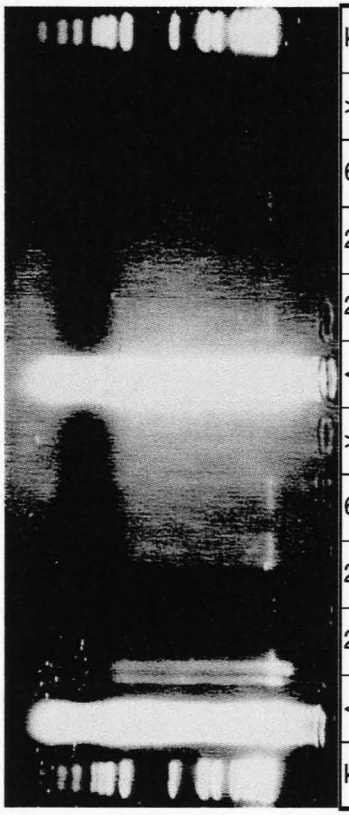

A
1 2 3
B
1 2 3 4 5 6 1 2 3 4 5
D
1 2 1 2 1 2 1 2 1 2
C
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4

Figure 21. Actinomycetes amplified using different 16S rDNA primers.

(Top left) *Streptosporangium virdiogriseum*, *Streptosporangium fragile* and *Streptoverticillium cinnamomeum* amplified from 307-716 and run on a 45-75% denaturing gradient. (Top right) Strains amplified from 226-701 and run on a 45-60% denaturing gradient for 1.5 and 2.5 hours. 1-*Streptomyces aureofaciens*, 2-*Actinomyces* sp., 3-natural *Nocardia* isolate, 4-*Rhodococcus rhodochrous*, 5-*Arthrobacter crystallopoietus*, 6-soil. (Bottom right) Time travel gel on 50-55% denaturing gradient gel for 1, 2, 3 and 4 hours. Lane 1: *Clavibacter michiganese*, *Streptomyces aureofaciens* and *Rhodococcus rhodochrous* amplified from 226-797. Lane 2: *Arthobacter oxydans*, *Streptosporangium roseum* and *Actinomyces* sp. amplified from 226-797. Lane 3: same strains as in lane 1 but amplified from 226-797. Lane 4: same strains as in lane 2 but amplified from 26-797. (Bottom left) Strains amplified from 226-701 and run on 50-60% denaturing gradient gel, for time course starting at 2 hours and then every 10 minutes. Lane 1: *Actinomadura malachitica* and *Arthrobacter oxydans*. Lane 2: *Microbispora rosea*, *Micrococcus luteus* and natural nocardia isolate.





kb ladder
>251 µm soil aggregates
63-251 µm soil aggregates
20-63 µm soil aggregates
2-20 µm soil aggregates
<2 µm soil particles
>251 µm soil aggregates
63-251 µm soil aggregates
20-63 µm soil aggregates
2-20 µm soil aggregates
<2 µm soil particles
kb ladder

Figure 22. PCR products from soil fraction DNA extracts run on DGGE.

The region 243-513 on the 16S rDNA was PCR amplified from DNA extracted from unfractionated soil and soil fractions. (Top) products run on an agarose gel to check amplified products were formed. (Bottom) products run on DGGE with gradient at 40-70%.

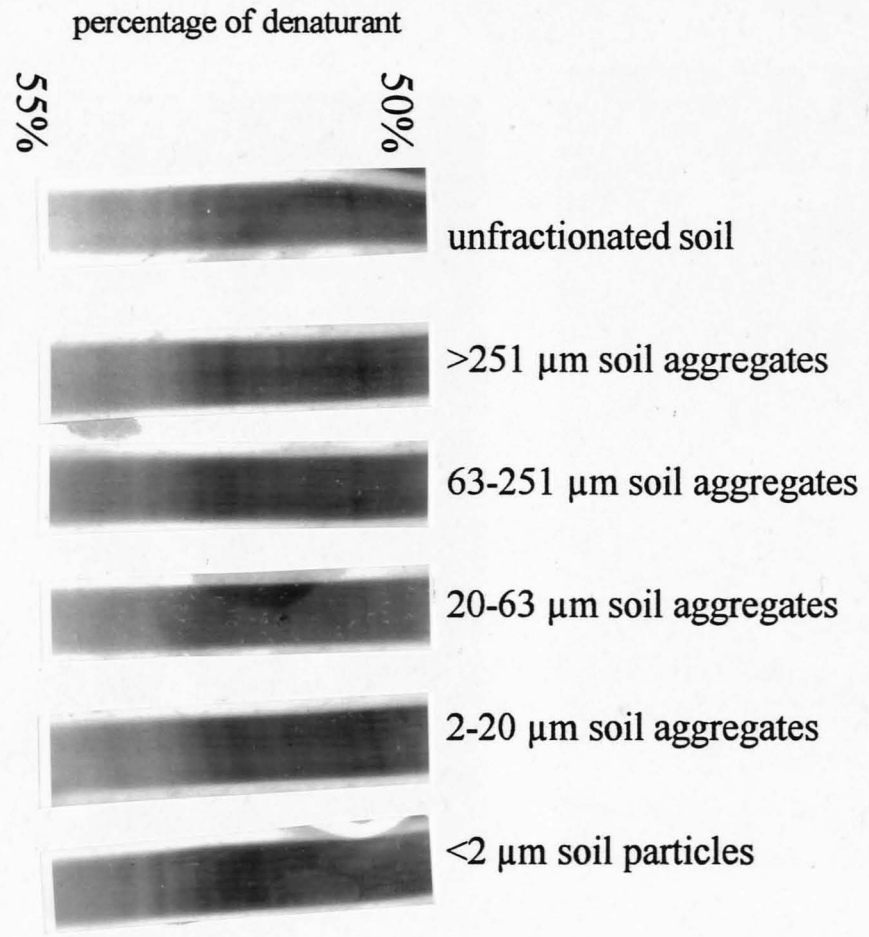

percentage of denaturant
55%
50%
unfractionated soil
>251 µm soil aggregates
63-251 µm soil aggregates
20-63 µm soil aggregates
2-20 µm soil aggregates
<2 µm soil particles

Figure 23. Focused bands of PCR products from soil fraction DNA extracts run on DGGE.

The region 243-513 on the 16S rDNA was PCR amplified from DNA extracted from unfractionated soil and soil fractions. Run on DGGE with gradient at 40-70% (using Fisons acrylamide) at 60°C for 210 minutes.

7.3.4 DGGE analysis of amplified products corresponding to soil fractions

DNA was extracted from unfractionated soil and the soil fractions and was amplified using the actinomycete specific primers. Using a low concentration of primers with BSA and DMSO in the amplification mixture, products were obtained for all the soil fractions except the 20-63 μm soil fraction. They were run on a parallel denaturing gradient gel with a 40-70% formamide and urea gradient and bands were observed only at a position of 51% denaturant, where streptomycete amplified products were presumed to run (See Figure 22). The positions of the bands were determined by estimating the percentage of denaturant, although it was assumed that the gradient was perfectly linear. It was expected that there would be a band running further down the gel corresponding to non-streptomycete actinomycetes amplified DNA from the 63-251 μm and 2-20 μm soil fractions. The amplified DNA of many actinomycetes isolated from these 2 soil fractions ran further down the gel compared to the amplified products of streptomycetes. This indicated that the unusual actinomycetes formed a major part of the total actinomycete population when they were grown on selective medium, whereas the amplified products from DNA extracts of soil fractions showed that the streptomycetes formed the bulk of the population.

Another experiment was carried out to determine whether there was PCR bias for amplification of the streptomycete region. Mixtures of an unidentified streptomycete isolate and an unidentified *Streptosporangium* DNA were amplified using these primers, under the conditions the soil DNA had been amplified, previously. It was found that the streptomycete DNA was amplified instead of the *Streptosporangium* DNA, except when the *Streptosporangium* DNA concentration was 100 fold greater than the streptomycete DNA and then the *Streptosporangium* DNA was amplified preferentially to the streptomycete DNA.

It was hypothesised that use of a low concentration of primers may be causing the bias, so the next experiment was carried out using a 10 fold higher concentration of primers in the PCR of DNA from the soil fractions. Amplified products were obtained for all the soil fractions which were run on denaturing gradient gel using the conditions described previously, except the gel was made with acrylamide supplied by Fisons instead of Biorad (See Figure 23). The bands which appeared on the gel were sharp, instead of the

usual unfocused bands and their positions appeared to have become retarded compared to the normal position of the unfocused bands. There were at least 4 bands in all of the soil fractions except the 20-63 μm soil fraction which had only 2 faint bands. Recent results have shown that actinomycete PCR products run on denaturing gradient gels made using acrylamide supplied by Fisons, yielded sharp bands.

7.4 Conclusions

Within the region of 16S rDNA that was amplified, it was expected that there would only be a few base pair differences between genera. It was anticipated that the addition of the GC clamp at one end of the PCR product would make it possible to detect 95% of single base differences although they would migrate quite close together (Myers *et al.*, 1985a). However, base changes that occur upstream of the 16S rDNA gene may have a complementary base change further downstream which would result in the GC content remaining unchanged. This occurs quite frequently within the 16S rDNA gene. Nevertheless, it may be possible to distinguish these two products from each other on the denaturing gradient gel, as many of the transversions like guanosine to cytosine or adenosine to thymidine, are detectable by a slight separation of the DNA bands (Fischer and Lerman, 1983). It was expected that there would be few base changes between genera for the region that was amplified, and that there would be even less base differences between species belonging to the same genera. Therefore, the amplified products of the different genera would separate more from each other, than species within the same genera on the denaturing gradient gel. The maximum separation of the PCR products on the denaturing gradient gel was no greater than 3 cm, which is what would be expected for the separation of homoduplex DNA. The actinomycetes which were morphologically similar ran to identical positions on the denaturing gradient gel but there were also many PCR products derived from dissimilar actinomycete strains which ran to identical positions. These may either have had identical sequences or very slightly dissimilar sequences which were not detected by DGGE. The amplified products from the 63-251 μm soil aggregate isolates exhibited the greatest diversity on the denaturing gradient gels which corresponded well with the diversity of these isolates based upon partial identification.

Many of the amplified products from the actinomycetes with a meso-DAP cell wall composition ran further along the gel, than the amplified products from actinomycetes with a LL-DAP cell wall content. This result was either due to the products from meso-DAP actinomycetes having a higher GC content or being slightly longer (Fischer and Lerman, 1980). The separated bands on the denaturing gradient gel were often unfocused, indicating that the DNA strands had completely disassociated (Theophilus *et al.*, 1989). To overcome this problem the Chemiclamp which forms a permanent covalent bond at one of the 5' end bases, was tested but this also resulted in unfocused bands on the denaturing gradient gel. It was unlikely that the unfocused bands were a consequence of PCR error since Sheffield *et al.* (1989) showed that a clear band was still discernible despite the some amplification errors. In a previous study, it was shown that there are some unusual differences between where the PCR products are theoretically expected to run and where they actually did run on the denaturing gradient gel (Weber *et al.*, 1991). For example, two $H2K^b$ mutants should have run near to the parental strain but instead did not form any sharp bands.

Actinomycete DNA usually has a high GC content and, based on theoretical calculations, amplified DNA with 75% GC content would not form sharp bands on the denaturing gradient gel (Myers *et al.*, 1985b). However, this actinomycete 16S rDNA product possessed a GC content of 62%. Products containing an equivalent high GC content formed sharp bands on the denaturing gradient in other studies, perhaps as a result of using acrylamide supplied by another company (Wawer and Muyzer, 1995). During amplification studies of actinomycete DNAs, a region of more than 50 bases in the middle of the 16S rDNA product had a GC content greater than 75% although the alogorithm to calculate the melting of the DNA strands showed that there were only slight differences of less than 1°C between these regions. Alternatively, the DNA may dissociate and then form stable stem loop structures at the higher denaturant concentration (Weber *et al.*, 1991).

An investigation into the nature of the diffuse bands was carried out, using the combination of different primers which could be used in PCR to amplify a region within the first half of the 16S rDNA. It was noticed that the longer amplified products melted at a lower denaturant concentration compared to the shorter products. This can be

explained by the probability that the longer amplified products are more likely to contain low melting domains, which would melt first resulting in a retardation of the migrating product in the denaturing gel (Fischer and Lerman, 1980). If there were several melting domains, then it is possible that once the first domain began to melt, the amplified product in the denaturing gel became diffuse and remained in this state until the other domains also started dissociating. Only when all the possible domains have melted, can the amplified product become retarded in the denaturing gel and therefore focused. For this reason it was not surprising that the shortest amplified product, from positions 226 to 513, gave the least diffuse bands compared to the larger amplified products. It was also observed that the unclamped version of this amplified product ran as far into the denaturant gradient as the GC clamped product when the latter was finally retarded. This was an unexpected result since the unclamped amplified product should have completely dissociated and formed diffuse bands on the denaturing gradient gel. This indicated that apart from the GC clamp which never dissociated under normal DGGE conditions, that there were other regions of the amplified product which were still dissociating in the denaturing gradient as they continued to migrate. The addition of the GC clamp or the Chemiclamp may have enhanced the stability of the DNA fragment although it may have induced an unpredictable melting nature on the molecule. The *Clavibacter* product of 800 nucleotides became diffuse after 1.5 hours on the 45-60% denaturant gel, but it refocused after 2.5 hours. This result indicated that one low melting domain began dissociating to cause the diffuse band but after it had completely melted, the other domains melted at a much higher level of denaturants. Calculations to determine the melting behaviour of GC clamped products compared to unclamped products using Macmelt showed that the melting domains were relative equal.

Previous ecological studies using DGGE have compared the amplified products of total direct DNA extract from the environmental sample to the DNA recovered from the combined bacteria grown on media (Muyzer *et al.*, 1993; Wawer and Muyzer, 1995; Ferris *et al.*, 1996; Teske *et al.*, 1996). In these studies, a variable region was usually amplified within the 16S rDNA, which resulted in a high sequence difference between unrelated bacteria. Hence the banding position for each sequence was generally specific for that particular microorganism. Consequently it was found that the banding patterns derived from the amplified total DNA of the environmental sample were greater than the

combined profile from the bacteria grown on media. This indicated that there were many nonculturable microorganisms within the sample. However, in one study the sequence was identified as belonging to streptomycetes but the microorganism had not been isolated from the wall painting. After the sequence had been found, it was possible to isolate the streptomycete on selective medium (Rölleke *et al.*, 1996).

Previous work to investigate MPN-PCR of streptomycete spores inoculated into soil, showed that an amplified product was obtained when the number of spores was no lower than 10^5 per gram soil (Baker, not presented). This poor detection compared to MPN-PCR of other microorganisms may be attributed to a number of factors. These could be poor lysis of spores which would affect DNA extraction, whilst the high GC content and perhaps the length of the PCR product may affect PCR amplification. For this reason, a conserved region was chosen to ensure that bands would appear on the gel, because many variable amplified products running at a separate position on the gel may have appeared undetectable. This may have occurred in the amplified DNA extract for the 20-63 μm soil fraction, as an amplified product was observed on the agarose gel, but when it was run on the denaturing gradient gel no bands were clearly visible. The isolation studies indicated that the streptomycete diversity was highest within this soil fraction compared to the other fractions.

There were many problems in using DGGE to analyse the actinomycete population in soil which resulted in unfocussed bands on the gel. This was overcome by using a cheaper acrylamide product supplied by Fisons although the explanations for this observation are unknown. Nevertheless, in future studies using DGGE, it is important to know whether the high GC microorganisms can be successfully analysed. A set of PCR primers were designed which amplified a region of the 16S rDNA from 226-513. It was found that when the product created from soil DNA extract was run on DGGE, that a band was formed which ran further into the denaturant gradient which was derived from unusual actinomycetes. At a lower denaturant concentration another band was formed which corresponded to streptomycetes. It would appear that using this combination of PCR primers and running the amplified product on DGGE would be useful in discovering soils which contain a high population of unusual actinomycetes. Consequently isolation methods can be developed to recover these isolates.

CHAPTER EIGHT

8. Discussion

This study of streptomycetes in soil was performed using microcosms and one experiment was carried out to discover the distribution of actinomycetes in freshly sampled soil. Soil microcosms attempt to create model homogeneous environments, unlike fresh soils which contain extraneous material such as plants, stones and animals. Stones form an inert part of the soil, but the plants enable bacteria to thrive on the nutrients they exude, whilst animals may be most beneficial in the dispersal of nutrients and microorganisms. When a chemical analysis was carried out it showed that fresh soil was more closely related to mesocosms containing growing plants than microcosms (Teuben and Verhoef, 1992). In the studies presented in this thesis, it is assumed that the lack of plants and animals corresponded with the lower chemical components in microcosms. They also found that soil microcosms had a higher microbial respiration in comparison to fresh soil, perhaps due to microbial degradation of remaining plant material.

In nonsterile soil microcosms inoculated with *Streptomyces coelicolor* spores the viable bacterial counts were enumerated in the soil fractions. In this soil, the majority of bacteria growing as viable colonies on agar plates were in the 2-20 μm soil fraction. This result was expected since other studies which examined continuously cultivated soils showed that the microbial population was highest in the 2-20 μm and <2 μm soil fractions (Jocteur-Monrozier *et al.*, 1991).

However, the microbial population in the 2-20 μm soil aggregates was lower than the corresponding counts in the same fraction of fresh soil perhaps due to the effects of desiccation (Van Gestel *et al.*, 1991). This decrease in viable count would depend on the type of clay particles which contribute to the formation of the 2-20 μm soil aggregates because kaolinite aggregates to form quasicrystals whilst smectite forms interlayers. A comparison by Jocteur-Monrozier *et al.* (1991) of soils composed of mainly kaolinite or smectite indicated that when kaolinite was the primary component, many of the bacteria

were found in the outer pores, which meant that they could be removed during soil fractionation. But when smectite was the major component, it formed a covering layer over fragments of organic debris but once this organic material had been degraded a hollow pore structure remained. This extensive pore structure within soil aggregates was visualised by scanning electron microscopy and bacteria were observed inside as microcolonies. One of the advantages for microorganisms being inside soil aggregates is that water can be retained within the pores by capillary pressure under mild drying conditions. Obviously under much harsher conditions the bacteria within these soil aggregates would suffer the same fate as those in the outer pores of soil aggregates composed of kaolinite. Nevertheless, when the soil was rewetted, the microbial population would be recovered within the 2-20 μm soil fraction because the bacteria would not be removed by soil fractionation.

The association of microbial counts with smaller soil aggregates may have been a consequence of the inversely proportional relationship between surface area and size of the soil aggregates for equivalent weights of soil fractions (Jocteur-Monrozier *et al.*, 1991). It would be anticipated therefore that the smaller soil aggregates would have a higher bacterial population attached to their surface than the larger soil aggregates. This could appear to be true on the basis of previous studies which indicated that many of the bacteria may have arisen on clay particles through attachment rather than preferential growth (Degens and Sparling, 1996). From their results, it was proven that there was no link between clay content in soil aggregates and growth of bacteria determined by the incorporation of radioactively labelled glucose. The relationship between bacterial numbers and aggregate sizes disintegrated for the largest soil aggregates because these aggregates contained a large proportion of organic matter, probably as fine roots (Kabir *et al.*, 1994). These bacterial numbers were based on viable propagules which grew on plates but the total quantity of cells was also counted in each of the soil fractions collected. The total direct bacterial counts were determined by fluorescent staining using acridine orange and it was found that there were no statistically significant differences between any of the counts in soil fractions of the fractionated Warwickshire soil. Similar results were obtained in previous studies using agricultural soil whereas greater differences were observed in the fractions collected from the grassland soil (Kabir *et al.*, 1994).

There were no significant differences between the total and viable bacterial counts in soil fractions collected from Warwickshire soil, probably due to the large errors in determining the total direct counts. In other studies, there were significantly higher total bacterial counts compared to total viable bacterial counts throughout the soil fractions, although it was more marked in the 2-20 μm and >250 μm soil fractions (Richaume *et al.*, 1993). In this original study, the discrepancy was attributed to either the non-culturability of microorganisms or aggregation of cells. In Warwickshire soil, a large proportion of non-culturable bacteria may have been within the larger soil aggregates because it was observed there was no increase in the proportion of total viable bacteria over a 15 day incubation. In the 2-20 μm soil fraction however, the total viable counts increased until their counts were indistinguishable from the total direct counts during the 15 day soil incubation. A possible cause for this increase may have been accredited to the soluble nutrients from dead microbes entering the soil pores of the air-dried soil when the soil was rewetted. These nutrients may have enabled bacteria to grow within the smaller soil aggregates and therefore they appeared as viable colonies on spread plates.

Sterile and nonsterile soil microcosms inoculated with *Streptomyces coelicolor* spores initially showed that the spores were not associated with the soil particles, however with incubation of the soil microcosms, the mycelia from germinating spores became associated with increasingly larger sized soil aggregates. This clearly indicated that the streptomycete spores readily attached to the smaller soil aggregates because these aggregates possessed a higher surface area than an equivalent dry weight of those that were larger.

Once the spores germinated, it was found the streptomycetes grew better in sterile soil compared to non-sterile soil which correlated well with the results of similar previous studies (Wellington *et al.*, 1990). In the sterile soil, the indigenous bacterial population was destroyed, effectively removing any microbial competition to the inoculants. In previous microcosm studies other inoculants such as *Streptomyces lividans* were observed by scanning electron microscopy growing within the crevices of soil particles, probably on the remnants of cell debris (Clewlow *et al.*, 1990). Many of these cells would have been confined to pore spaces but nutrients outside may not have been available for microbial degradation. The pores may be important for maintaining cell

viability because water is held within them, protecting the cells from desiccation. This may have contributed to the significant decreases detected in other studies when mycelial inoculants were used, perhaps because many of them were randomly distributed throughout the soil (Ozawa and Yamaguchi, 1986; Wang *et al.*, 1989). Obviously this would depend on whether the microbes were inoculated before or after the water content of the soil was adjusted (Beare and Bruce, 1993). Other evidence indirectly suggesting that microbes grow in pores was shown by a burst in microbial activity when the soil was mixed causing the nutrients to redistribute so that some may have finished up in the pore spaces (Winding *et al.*, 1994).

It was most likely that there were more nutrients in sterile soil microcosms than the nonsterile ones because dead bacteria would have been available. However, in the nonsterile soil, it was observed that the total viable counts in the 2-20 μm soil aggregates increased during the initial period of incubation. These bacteria would have competed with the streptomycete inoculants for nutrients. Consequently, the streptomycetes were found to be growing better in the 20-63 μm soil aggregates while the majority of microorganisms grew in the 2-20 μm soil fraction. The fact cannot be eliminated that the inoculated streptomycetes were not growing in the smaller soil aggregates with the other microbes. This would perhaps indicate that the streptomycetes were using complex compounds available in both of these soil fractions.

Previous studies showed that the organic content in the 20-50 μm soil fraction was low but because streptomycetes form mycelia, this would enable them to spread over regions which possess few nutrients (Locci, 1988). It appeared that the indigenous bacteria grew in the 2-20 μm soil fraction and to a lesser extent in the larger soil aggregate fractions. This was probably caused by most of the plant material being removed from the soil during the initial pre-treatment by dry sieving. The lower organic contents would perhaps explain why streptomycetes were so slow to proliferate throughout the larger soil aggregates.

An analysis was performed to investigate the indigenous streptomycete distribution in air-dried Warwickshire soil. The results suggested that the streptomycete counts were highest in the dispersible <2 μm soil fraction of the rewetted air-dried soil when the soil

microcosms were initially established. This was expected as the majority of the propagules in the soil would have been spores because any mycelia would have been destroyed. Once the soil was rewetted, faster growing microorganisms within aggregates may have predated on streptomycetes, particularly on spores which did not germinate (Zeph and Casida, Jr, 1986). This may have led to lower streptomycete counts being detected in many of the soil fractions.

The indigenous streptomycete populations significantly increased in the >251 μm, 2-20 μm and <2 μm soil fractions although no differences were observed between the total propagules and spores probably because various species germinated at different periods after the soil microcosms were prepared (Babich *et al.*, 1994). The occurrence of streptomycete growth within these particular soil fractions seemed to reflect their growth on organic matter contained within the >251 μm and 2-20 μm soil fractions, while the <2 μm soil fraction contained propagules that were either once attached to soil aggregates or spores that were dispersed by the microorganism away from the soil aggregates. The lower microbial activity in the 2-20 μm soil aggregates would have enabled streptomycetes to grow. The nutrients within the larger soil aggregates may have been decaying fine roots enmeshed within the aggregates (Kabir *et al.*, 1994).

Streptomycetes within the water stable aggregates should be able to grow through the narrow pore system because they can withstand low oxygen concentrations provided carbon dioxide levels do not accumulate (Williams *et al.*, 1972). These studies indicated that streptomycete mycelia protruded from soil pores when the soil aggregates were examined under the scanning electron microscope. One soil aggregate appeared to have very thin mycelium growing between 2 pores. This would not be impossible since it has been noted that streptomycete mycelium in soil may be much thinner than its growth on laboratory medium (Locci, 1988).

The indigenous streptomycete populations in fresh soil were higher on the roots, in the larger soil fractions and in the <2 μm soil fraction. Previous studies on fresh pasture rhizosphere soils showed that the bacteria were predominantly on the roots and in the >2000 μm soil aggregates in accordance with the high organic matter found within these 2 fractions (Kabir *et al.*, 1994). The soils they used, possessed a significantly higher

proportion of growing roots compared to those found in continuously cultivated soils. Investigations into the microbial populations on roots by De Leij *et al.* (1993) showed that there was a low density of fast growing bacteria on the younger roots whilst there were much higher populations of slower growing microbes associated with the older roots. They postulated that slow growing bacteria may effectively use low quantities of metabolites released by roots and compete with other bacteria in crowded conditions.

It would appear that the streptomycetes were growing on the dead roots particularly on the basis of earlier studies (Mayfield *et al.*, 1972) or they were decaying fine roots which held the larger soil aggregates together (Hattori, 1973). In studies by Kabir *et al.* (1994), it was found that the <2 μm soil fraction contained the lowest proportion of organic carbon compared to the other soil fractions, yet the microbial counts were reasonably high within this fraction. It was postulated that these microorganisms had originally been attached to larger soil aggregates but they were removed during the soil fractionation.

It was probable that this dispersible soil fraction contained spores which could be distributed to new microsites. Surprisingly though, the streptomycete population in the 2-20 μm was outcompeted by the high microbial population detected within the soil fraction. There was also a low streptomycete population in the 63-251 μm soil fraction which may explain why Streptosporangium and Micromonospora strains, which are normally infrequently isolated (Zenova *et al.*, 1995), were readily isolated from this air-dried fraction

The streptomycete isolates from the >251 μm and <2 μm air-dried nonsterile soil fractions were morphologically similar with grey aerial and buff coloured substrate mycelia. These isolates may either represent the most prolific sporulators or the most abundantly growing streptomycetes in this soil. In favour of the latter argument, it was postulated that a particular plant root may possess specific organic compounds which can only be effectively degraded by certain actinomycetes (Xu *et al.*, 1996). Consequently, a large diversity of plants may equally provide a similarly diverse actinomycete population. It can be assumed that the botanical diversity of Warwickshire soil would have been limited because this soil was used for agricultural purposes.

The streptomycete diversity on the basis of colony morphology, denaturing gradient gel profiles and secondary metabolite profiles was much better in the 20-63 μm than in any of the other soil fractions, even though the actinomycete diversity was greatest in the 63-251 μm soil aggregates. These streptomycetes may have grown on the microbial by-products as well as on dead bacteria resulting from dying roots which ceased to exude nutrients. This would have represented a diverse source of nutrients which could have enabled many different types of streptomycetes to grow. Many of the streptomycetes recovered from this soil fraction may also have represented the slow growing forms while those from the larger soil fractions may have been the fast growers, degrading root fragments. Such differences have been observed for other bacterial populations (Ka *et al.*, 1994).

A high diversity of actinomycetes was recovered from the air-dried 2-20 μm soil fraction even though the total actinomycete population was much lower than for other bacteria within this soil fraction. These actinomycetes may have been living on the wide range of either complex, undegraded compounds or soluble nutrients exuded from roots. It is also possible that the high bacterial populations in this soil fraction, as well as on the spread plates, may have enabled slower growing actinomycetes to form colonies, as a consequence of resistance to inhibitory substances produced by other bacteria (De Leij *et al.*, 1993). The diversity of actinomycetes in each of the soil fractions showed equally diverse metabolic profiles which were determined from the HPLC chromatograms.

In the pharmaceutical industry many streptomycetes are screened for the production of novel compounds which may be useful as antibiotics. A major problem in randomly screening streptomycete isolates is to ensure that a diverse collection of strains are recovered. To succeed in this objective, strains are being obtained from unusual soil environments, such as desert sands (Langley *et al.*, 1991). One of the major aims of this work was to determine whether a particular soil fraction, would exhibit a higher proportion and higher diversity of streptomycetes compared to other indigenous bacteria.

Results indicated that the most abundant streptomycetes were found in the largest soil aggregates whilst the greatest diversity was in the 20-63 μm soil fraction. The latter fraction was easily recovered by sedimentation for 10 minutes, after the larger soil

aggregates had been removed by wet sieving. Ideally it would be a useful technique to use in order to enhance the diversity of streptomycetes recovered from any particular soil. These conclusions were determined from the results of soil fractionation using just one type of soil, Warwickshire soil. It cannot be assumed that the greatest streptomycete diversity will be recovered within the same soil fraction for other soils. For instance, most experimental work has shown that the microbial population was predominant in the 2-20 μm and <2 μm soil fractions whilst the 20-50 μm soil fraction possessed the lowest microbial biomass (Jocteur-Monrozier *et al.*, 1991; Van Gestel *et al.*, 1991). In soils which possessed a high clay and silt content, high microbial biomasses were determined in the soil fractions ranging from <2-50 μm in diameter while the 200-2000 μm soil fraction had the lowest microbial population (Kabir *et al.*, 1994). Within this latter soil fraction, it would be expected that the highest streptomycete diversity would be detected.

There are many streptomycetes which are isolated from soils that have an unknown texture. This would have to be determined by completely disrupting the soil structure and then collecting the sand, silt and clay fractions. The problem however does not end there, because different types of clay may combine to form small soil aggregates which differ in microporosity. It was discovered that smectite formed porous aggregates which contained the bacteria whereas kaolinite formed less porous aggregates so that the bacteria were recovered in the dispersible soil fraction (Jocteur-Monrozier *et al.*, 1991). In the latter soil type, it was possible that the bacteria were originally in surface pores from where they could easily be removed during the soil fractionation process. Another factor to consider is that the quantity of clay and silt particles will proportionally determine the amount of microbial degradation products, which in turn may affect the population of streptomycetes (Christensen, 1987). Each of these parameters would have unknown influences on the streptomycete population in soil.

Actinomycete isolates were extracted from each of the soil fractions and each of them was identified to finally produce a profile of the strains in Warwickshire soil. Recovering these strains was laborious so another method was required to analyse quickly the actinomycete populations within the different soil fractions. In previous studies, it was found that a small region of the 16S rDNA could be amplified from soil DNA extracts

and the products could then be run on a denaturing gradient gel (Muyzer *et al.*, 1993). In an ideal system each band on this gel would correspond to a separate microorganism. This means that the product could be recovered from the band and then sequenced to identify the microorganism from which the product was derived.

From each of the soil fraction isolates, DNA was extracted which was separately amplified using specific primers for high GC microorganisms. The patterns which were obtained indicated that the isolates from the 63-251 μm and 2-20 μm soil fractions were the most diverse representing several genera. The region which was amplified ranged from 226-513 (*E. coli* positioning) and previous sequencing studies have shown that the region from 202-482 (*E. coli* positioning) was completely homologous for 14 type strains which included *Streptomyces* and *Streptoverticillium* species (Witt and Stackebrandt, 1990). Therefore the products created using these primers showed almost no variation between different streptomycete species whilst there was probably only a small variation between different actinomycete genera. When comparisons were made between the 16S rDNA sequence data for different genera, it was found that *Frankia* DSM43005 and *Nocardia asteroides* showed 80.5% and 79.2% similarity to streptomycetes, respectively (Witt and Stackebrandt, 1990). Hence it was be expected that species within the same genera would migrate either as a single band or a number of multiple bands quite close together.

A single diffuse band was formed on the denaturing gradient gel when the PCR products were amplified from isolates which had been identified as streptomycetes. These PCR products were observed as single bands on the denaturing gradient gel but they became unfocused and never refocused as described in former studies on microbial populations in soil (Muyzer *et al.*, 1993). It was noticed that as the size of the amplified product increased, that the bands on the denaturing gradient gel became more ill defined.

The bands should have refocused because the PCR product, except the GC clamp, should have become single stranded causing the DNA molecule to become greatly retarded as it migrated through the gel. It would appear that the actinomycete PCR products may have become partially denatured but some portions of the sequence may have reannealed forming much more stable double stranded DNA segments than the

whole original PCR product (Weber *et al.*, 1991). The possibility for this to occur would be higher for actinomycetes due to their high GC content, because it would be more likely that very short sequences could reanneal with another nearby group of bases which possess a complementary sequence. Also the stronger bonding between guanine and cytosine residues would enable the double stranded DNA to remain annealed under high denaturing conditions.

It seemed that these secondary reannealing sequences could not be formed when the acrylamide mixture was changed from the product supplied by Biorad to another product obtained from Fisons. Both of these products showed similar quality characteristics and so it was not possible to elucidate what was causing the diffuse bands.

The region of the 16S rDNA which showed minor variations between different actinomycete genera was amplified because it was expected that the bands for certain genera would group together on the denaturing gradient gel. This would enable actinomycete genera to be identified from the denaturing gradient gel without the need for sequencing. It was observed that products from 90% of streptomycetes were retarded sooner than those from 50% of other actinomycete genera. This was not surprising because most streptomycetes share 91% sequence similarity in their 16S rDNAs (Wellington *et al.*, 1992; Witt and Stackebrandt, 1990). Many products from the other actinomycetes also migrated with the majority of the amplified streptomycete products. It was not surprising to find that the streptomycete amplified products were retarded later than the *Mycobacterium* and *Arthrobacter* amplified products, because in other studies, it was found that the streptomycetes possessed a higher GC content, even in their 16S rDNAs (Cai and Collins, 1994). This result was also observed when longer products were created using primers which amplified a similar segment of the 16S rDNA.

Using the original actinomycete primers, it was anticipated that 3 major bands would be obtained from some soils and that these bands would correspond to arthrobacters, streptomycetes and other actinomycete genera. This could prove to be interesting since most ecological studies have shown that streptomycetes are more readily isolated than other genera of the actinomycetes but these other genera could be identified to be

present by a band which migrates furthest in the denaturing gradient gel. If a significant product was obtained then selective isolation could be concentrated on this soil to recover the unusual actinomycetes. Obviously, sometimes these products may be due to the 10% of streptomycete products which migrated to this position or the amplified products from micromonosporas, *Actinomadura* and streptosporangia which are the next commonly isolated genera on the HV and AV media (Nonomura and Ohara, 1969).

PCR products were obtained from the soil fractions and when these were run on the denaturing gradient gel, it was found that only bands were obtained which corresponded with the position where most streptomycetes commonly migrated. It was expected that some bands would be visualised at a position on the gel where products of some other actinomycete genera were observed, especially for the DNA amplified from the 63-251 μm and 2-20 μm soil fractions. These particular soils fractions showed that there were many different types of actinomycetes when isolation studies were carried out, as described previously. When an experiment was performed to investigate PCR bias, it was found that PCR of the region between 226-513 of the 16S rDNA favoured streptomycetes compared to other actinomycete genera.

The product created from the streptomycetes was retarded sooner than the product from some other actinomycete genera perhaps due to the slightly lower GC content of the streptomycete product. It was therefore possible that the PCR amplification favoured the generation of products with a lower GC content (Suzuki and Giovannoni, 1996). Alternatively, it was also possible that the primers were more specific for the streptomycetes because there were fewer mismatches between the primers and the region of the 16S rDNA (Heuer *et al.*, 1997).

The experiment was repeated but this time the products were run on a denaturing gradient gel using an acrylamide mixture obtained from Fisons. On this gel, there were several bands which were obtained in all of the soil fractions except for the 20-63 μm soil fraction. It was concluded that, because distinct bands were obtained, these bands represented sequences amplified from microorganisms which were at a detectable level. Although a PCR product was formed corresponding to the 20-63 μm soil fraction, no bands were observed on the denaturing gradient gel. It was possible that there were

many different bands but the staining technique was insufficiently sensitive to reveal bands on the denaturing gradient gel.

This would present problems in interpreting the data obtained from denaturing gradient gels because few bands could indicate a low microbial diversity, whereas if clones of the amplified products were obtained which were then sequenced, these may reveal a higher microbial diversity. This problem could be overcome by comparing the densitometric values of the stained products run on a standard polyacrylamide gel and a denaturing gradient gel. If the values obtained from the bands on the denaturing gel equal the value measured for the single product from the standard polyacrylamide gel, then all the observed bands on the denaturing gradient gel would represent the bacterial diversity in the soil. However, if the total values obtained from the denaturing gradient gel were much lower, then these bands would represent only a small proportion of the microbial diversity, since many other bands would not be visible on the gel.

To ensure that the densitometric measurements were equivalent to the population of the bacteria which were represented by the sequences, BSA could be used in the PCR. BSA in PCR enabled products to be formed in proportion to the quantity of templates, whereas without BSA there was no relationship between products formed and the templates (Romanowski *et al.*, 1993). Obviously, it may be quite difficult to gain the exact profile of the microbial diversity by using either culturing techniques (Wellington *et al.*, 1987) or molecular techniques due to bias (Liesack *et al.*, 1991; Reysenbach *et al.*, 1992).

Due to the complexity of the molecular methods, there may be many reasons for bias, ranging from the original DNA extraction to finally analysing the amplified products. For instance, DNA extraction may be affected by clay content of soil which is known to effectively bind DNA molecules (Aardema *et al.*, 1983). The clay content may also act to preserve DNA which would otherwise have degraded much sooner (Romanowski *et al.*, 1993). The DNA recovery may also be affected by whichever DNA extraction method is used since so may have been developed (Trevors, 1992). Unfortunately this can make it difficult to compare the results obtained from different studies.

Once the DNA is extracted, it has to be amplified and previous results have shown that the greater the similarity between two amplified DNA fragments, the higher the probability of chimeric products being formed (Wang and Wang, 1996). Because the amplified 16S rDNA region in these studies showed very high similarity between actinomycetes, some of the bands which were observed on the denaturing gradient gel may have been chimeric products. This could only be verified by sequencing the excised bands.

It would therefore be best to amplify a section of DNA which showed high sequence variation for different bacteria. This strategy has been carried out in some DGGE studies, and sequences were obtained which were matched against those already existing in the databases (Santegoeds *et al.*, 1996). Sequences which showed close similarity were identified to particular bacteria. In these studies and many others, eubacterial primers were used on DNA extracted from microbial environments which are known to have a limited bacterial diversity (Muyzer *et al.*, 1993; Ferris *et al.*, 1996).

The *in situ* molecular diversity of a soil has been shown to be extremely high in comparison with DNA recovered from cultured bacteria of the same soil (Torsvik *et al.*, 1990b). If this soil DNA extract was amplified using eubacterial primers, too many bands on the denaturing gradient gel could be obtained which would make it impossible to determine anything from the banding patterns (DGGE workshop, Braunsweig, 1995).

These results have shown that the DGGE technique could be used to investigate the actinomycete diversity in different soils. More specifically, by using the primers which amplify the 16S rDNA gene between 226-513, and then running the products on the denaturing gradient gel, it should be possible to identify tentatively, the group of actinomycetes from the corresponding bands. It has been shown that infrequently isolated actinomycetes were represented by a product migrating furthest along the denaturing gradient gel. Hence this technique could be used to determine which soils possessed a high population of these interesting actinomycetes.

Once they had been identified, it would then be possible to isolate these actinomycetes. It was surprising that the actinomycete diversity was greatest in the 2-20 μm soil fraction, even though the eubacterial population was much higher than the actinomycete

population. On the basis of these results, this soil fraction should be recovered to obtain the best diversity of actinomycetes. This would appear to offer an opportunity to recover actinomycetes belonging to genera, which have been poorly represented in comparison with streptomycetes. These strains may eventually provide interesting secondary metabolites when they will be analysed by screening programs at pharmaceutical companies.

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10. Appendix

Soil Fraction And Original Number	Strain Number	Genus Predicted
unfractionated-01	HCA00309	streptomycetes
unfractionated-02	HCA00310	streptomycetes
unfractionated-04	HCA00311	rhodococcus
unfractionated-05	HCA00312	streptomycetes
unfractionated-06	HCA00313	unidentified
unfractionated-07	HCA00314	unidentified
unfractionated-08	HCA00315	streptomycetes
unfractionated-09	HCA00316	streptomycetes
unfractionated-10	HCA00317	streptomycetes
unfractionated-11	HCA00318	streptomycetes
unfractionated-12	HCA00319	streptomycetes
unfractionated-14	HCA00320	unidentified
unfractionated-15	HCA00321	streptomycetes
unfractionated-16	HCA00322	streptomycetes
unfractionated-17	HCA00323	streptomycetes
unfractionated-18	HCA00324	unidentified
unfractionated-20	HCA00325	streptosporangium
unfractionated-21	HCA00326	streptomycetes
unfractionated-22	HCA00327	streptomycetes
unfractionated-23	HCA00328	streptomycetes
unfractionated-24	HCA00329	streptomycetes
unfractionated-25	HCA00330	streptomycetes
unfractionated-26	HCA00331	streptomycetes
unfractionated-28	HCA00332	streptomycetes
unfractionated-29	HCA00333	streptomycetes
unfractionated-30	HCA00334	streptoverticillium
unfractionated-31	HCA00335	streptomycetes
unfractionated-32	HCA00336	streptomycetes
unfractionated-33	HCA00337	streptomycetes
unfractionated-36	HCA00338	rhodococcus
unfractionated-37	HCA00339	streptomycetes
unfractionated-38	HCA00340	streptomycetes
unfractionated-39	HCA00341	streptomycetes
unfractionated-40	HCA00342	streptomycetes
unfractionated-41	HCA00343	unidentified
unfractionated-42	HCA00344	unidentified
unfractionated-44	HCA00345	streptomycetes
unfractionated-45	HCA00346	streptomycetes
unfractionated-46	HCA00347	streptomycetes
unfractionated-47	HCA00348	streptomycetes
unfractionated-49	HCA00349	streptosporangium
unfractionated-50	HCA00350	unidentified
unfractionated-51	HCA00351	streptomycetes

unfractionated-52	HCA00352	streptomycetes
unfractionated-53	HCA00353	streptomycetes
unfractionated-54	HCA00354	streptosporangium
unfractionated-55	HCA00355	streptomycetes
unfractionated-56	HCA00356	streptomycetes
unfractionated-57	HCA00357	unidentified
unfractionated-58	HCA00358	streptomycetes
unfractionated-59	HCA00359	streptomycetes
unfractionated-60	HCA00360	streptomycetes
unfractionated-61	HCA00361	streptomycetes
unfractionated-62	HCA00362	streptomycetes
>251 μm aggregates-01	HCA00363	streptomycetes
>251 μm aggregates-02	HCA00364	streptomycetes
>251 μm aggregates-03	HCA00365	streptomycetes
>251 μm aggregates-04	HCA00366	streptomycetes
>251 μm aggregates-05	HCA00367	micromonospora
>251 μm aggregates-06	HCA00368	streptomycetes
>251 μm aggregates-07	HCA00369	streptomycetes
>251 μm aggregates-09	HCA00370	streptomycetes
>251 μm aggregates-10	HCA00371	streptomycetes
>251 μm aggregates-11	HCA00372	streptomycetes
>251 μm aggregates-12	HCA00373	streptomycetes
>251 μm aggregates-13	HCA00374	unidentified
>251 μm aggregates-14	HCA00375	unidentified
>251 μm aggregates-15	HCA00376	streptomycetes
>251 μm aggregates-16	HCA00377	streptomycetes
>251 μm aggregates-17	HCA00378	streptomycetes
>251 μm aggregates-18	HCA00379	streptomycetes
>251 μm aggregates-19	HCA00380	streptomycetes
>251 μm aggregates-20	HCA00381	streptomycetes
>251 μm aggregates-21	HCA00382	streptomycetes
>251 μm aggregates-22	HCA00383	streptomycetes
>251 μm aggregates-23	HCA00384	streptomycetes
>251 μm aggregates-24	HCA00385	streptomycetes
>251 μm aggregates-25	HCA00386	streptomycetes
>251 μm aggregates-26	HCA00387	unidentified
>251 μm aggregates-27	HCA00388	streptomycetes
>251 μm aggregates-28	HCA00389	streptomycetes
>251 μm aggregates-29	HCA00390	streptomycetes
>251 μm aggregates-30	HCA00391	streptomycetes
>251 μm aggregates-31	HCA00392	streptomycetes
>251 μm aggregates-32	HCA00393	streptomycetes
>251 μm aggregates-33	HCA00394	streptomycetes
>251 μm aggregates-34	HCA00395	streptomycetes
>251 μm aggregates-35	HCA00396	streptomycetes
>251 μm aggregates-36	HCA00397	streptomycetes
>251 μm aggregates-37	HCA00398	streptomycetes

>251 μm aggregates-38	HCA00399	streptomycetes
>251 μm aggregates-39	HCA00400	streptomycetes
>251 μm aggregates-40	HCA00401	streptomycetes
>251 μm aggregates-41	HCA00402	streptomycetes
>251 μm aggregates-42	HCA00403	unidentified
>251 μm aggregates-43	HCA00404	streptomycetes
>251 μm aggregates-44	HCA00405	streptomycetes
>251 μm aggregates-45	HCA00406	streptomycetes
>251 μm aggregates-46	HCA00407	streptomycetes
>251 μm aggregates-49	HCA00408	streptomycetes
63-251 μm aggregates-01	HCA00409	streptosporangium
63-251 μm aggregates-02	HCA00410	
63-251 μm aggregates-03	HCA00411	streptosporangium
63-251 μm aggregates-04	HCA00412	streptosporangium
63-251 μm aggregates-05	HCA00413	micromonospora
63-251 μm aggregates-06	HCA00414	streptomycetes
63-251 μm aggregates-07	HCA00415	micromonospora
63-251 μm aggregates-08	HCA00416	unidentified
63-251 μm aggregates-09	HCA00417	streptomycetes
63-251 μm aggregates-10	HCA00418	streptomycetes
63-251 μm aggregates-11	HCA00419	streptomycetes
63-251 μm aggregates-12	HCA00420	micromonospora
63-251 μm aggregates-13	HCA00421	streptomycetes
63-251 μm aggregates-14	HCA00422	streptomycetes
63-251 μm aggregates-15	HCA00423	streptomycetes
63-251 μm aggregates-16	HCA00424	unidentified
63-251 μm aggregates-17	HCA00425	micromonospora
63-251 μm aggregates-18	HCA00426	micromonospora
63-251 μm aggregates-19	HCA00427	unidentified
63-251 μm aggregates-20	HCA00428	pseudonocardia
63-251 μm aggregates-21	HCA00429	streptosporangium
63-251 μm aggregates-22	HCA00430	streptomycetes
63-251 μm aggregates-23	HCA00431	streptomycetes
63-251 μm aggregates-24	HCA00432	unidentified
63-251 μm aggregates-25	HCA00433	unidentified
63-251 μm aggregates-26	HCA00434	streptomycetes
63-251 μm aggregates-27	HCA00435	streptomycetes
63-251 μm aggregates-28	HCA00436	streptoverticllium
63-251 μm aggregates-29	HCA00437	streptosporangium
63-251 μm aggregates-30	HCA00438	micromonospora
63-251 μm aggregates-31	HCA00439	microtetraspora
63-251 μm aggregates-32	HCA00440	streptomycetes
63-251 μm aggregates-33	HCA00441	unidentified
63-251 μm aggregates-34	HCA00442	streptomycetes
63-251 μm aggregates-35	HCA00443	streptomycetes
63-251 μm aggregates-36	HCA00444	streptomycetes

63-251 µm aggregates-37	HCA00445	streptomycetes
63-251 µm aggregates-38	HCA00446	streptomycetes
63-251 µm aggregates-39	HCA00447	unidentified
20-63 µm aggregates-01	HCA00448	streptomycetes
20-63 µm aggregates-02	HCA00449	streptomycetes
20-63 µm aggregates-03	HCA00450	streptomycetes
20-63 µm aggregates-04	HCA00451	unidentified
20-63 µm aggregates-05	HCA00452	streptomycetes
20-63 µm aggregates-07	HCA00453	unidentified
20-63 µm aggregates-08	HCA00454	streptomycetes
20-63 µm aggregates-09	HCA00455	unidentified
20-63 µm aggregates-10	HCA00456	streptomycetes
20-63 µm aggregates-11	HCA00457	micromonospora
20-63 µm aggregates-13	HCA00458	micromonospora
20-63 µm aggregates-14	HCA00459	streptomycetes
20-63 µm aggregates-15	HCA00460	streptomycetes
20-63 µm aggregates-16	HCA00461	streptomycetes
20-63 µm aggregates-17	HCA00462	streptomycetes
20-63 µm aggregates-18	HCA00463	unidentified
20-63 µm aggregates-19	HCA00464	streptomycetes
20-63 µm aggregates-20	HCA00465	streptomycetes
20-63 µm aggregates-21	HCA00466	unidentified
20-63 µm aggregates-22	HCA00467	unidentified
20-63 µm aggregates-23	HCA00468	streptomycetes
20-63 µm aggregates-24	HCA00469	streptomycetes
20-63 µm aggregates-25	HCA00470	streptomycetes
20-63 µm aggregates-26	HCA00471	streptomycetes
20-63 µm aggregates-27	HCA00472	streptomycetes
20-63 µm aggregates-28	HCA00473	streptomycetes
20-63 µm aggregates-29	HCA00474	streptomycetes
20-63 µm aggregates-30	HCA00475	unidentified
20-63 µm aggregates-31	HCA00476	streptomycetes
20-63 µm aggregates-32	HCA00477	streptomycetes
20-63 µm aggregates-33	HCA00478	streptomycetes
20-63 µm aggregates-34	HCA00479	streptomycetes
20-63 µm aggregates-35	HCA00480	streptosporangium
20-63 µm aggregates-36	HCA00481	streptosporangium
20-63 µm aggregates-37	HCA00482	streptomycetes
20-63 µm aggregates-38	HCA00483	streptosporangium
20-63 µm aggregates-39	HCA00484	streptomycetes
20-63 µm aggregates-40	HCA00485	streptomycetes
20-63 µm aggregates-42	HCA00486	streptomycetes
20-63 µm aggregates-43	HCA00487	streptomycetes
20-63 µm aggregates-44	HCA00488	streptomycetes
20-63 µm aggregates-46	HCA00489	streptomycetes
20-63 µm aggregates-47	HCA00490	unidentified

20-63 µm aggregates-48	HCA00491	streptomycetes
20-63 µm aggregates-49	HCA00492	streptomycetes
20-63 µm aggregates-51	HCA00493	streptomycetes
20-63 µm aggregates-52	HCA00494	streptomycetes
20-63 µm aggregates-53	HCA00495	streptomycetes
20-63 µm aggregates-54	HCA00496	streptomycetes
20-63 µm aggregates-55	HCA00497	streptomycetes
20-63 µm aggregates-56	HCA00498	unidentified
20-63 µm aggregates-57	HCA00499	unidentified
20-63 µm aggregates-58	HCA00500	streptosporangium
20-63 µm aggregates-59	HCA00501	streptomycetes
20-63 µm aggregates-60	HCA00502	nocardia
20-63 µm aggregates-61	HCA00503	streptomycetes
20-63 µm aggregates-62	HCA00504	streptomycetes
20-63 µm aggregates-63	HCA00505	streptomycetes
20-63 µm aggregates-64	HCA00506	streptomycetes
20-63 µm aggregates-65	HCA00507	streptomycetes
20-63 µm aggregates-66	HCA00508	streptomycetes
2-20 µm aggregates-01	HCA00509	streptomycetes
2-20 µm aggregates-02	HCA00510	streptomycetes
2-20 µm aggregates-03	HCA00511	streptosporangium
2-20 µm aggregates-04	HCA00512	streptosporangium
2-20 µm aggregates-05	HCA00513	streptomycetes
2-20 µm aggregates-06	HCA00514	unidentified
2-20 µm aggregates-07	HCA00515	streptomycetes
2-20 µm aggregates-08	HCA00516	unidentified
2-20 µm aggregates-11	HCA00517	unidentified
2-20 µm aggregates-13	HCA00518	streptomycetes
2-20 µm aggregates-14	HCA00519	unidentified
2-20 µm aggregates-16	HCA00520	micromonospora
2-20 µm aggregates-17	HCA00521	streptomycetes
2-20 µm aggregates-18	HCA00522	unidentified
2-20 µm aggregates-19	HCA00523	streptomycetes
2-20 µm aggregates-20	HCA00524	streptomycetes
2-20 µm aggregates-21	HCA00525	streptomycetes
2-20 µm aggregates-22	HCA00526	unidentified
2-20 µm aggregates-23	HCA00527	streptomycetes
2-20 µm aggregates-24	HCA00528	streptomycetes
2-20 µm aggregates-25	HCA00529	streptomycetes
2-20 µm aggregates-27	HCA00530	unidentified
<2 µm aggregates-01	HCA00531	unidentified
<2 µm aggregates-02	HCA00532	unidentified
<2 µm aggregates-03	HCA00533	unidentified
<2 µm aggregates-05	HCA00534	streptomycetes
<2 µm aggregates-06	HCA00535	unidentified
<2 µm aggregates-07	HCA00536	streptomycetes

<2 μm aggregates-08	HCA00537	unidentified
<2 μm aggregates-09	HCA00538	unidentified
<2 μm aggregates-10	HCA00539	streptomycetes
<2 μm aggregates-11	HCA00540	streptomycetes
<2 μm aggregates-12	HCA00541	streptomycetes
<2 μm aggregates-13	HCA00542	unidentified
<2 μm aggregates-14	HCA00543	streptomycetes
<2 μm aggregates-15	HCA00545	streptomycetes
<2 μm aggregates-16	HCA00546	streptomycetes
<2 μm aggregates-17	HCA00547	streptomycetes
<2 μm aggregates-18	HCA00548	streptomycetes
<2 μm aggregates-19	HCA00549	unidentified
<2 μm aggregates-20	HCA00550	streptomycetes
<2 μm aggregates-21	HCA00551	streptomycetes
<2 μm aggregates-22	HCA00552	streptomycetes
<2 μm aggregates-23	HCA00553	streptomycetes
<2 μm aggregates-24	HCA00554	streptomycetes
<2 μm aggregates-25	HCA00555	unidentified
<2 μm aggregates-26	HCA00556	streptomycetes
<2 μm aggregates-27	HCA00557	streptomycetes
<2 μm aggregates-28	HCA00558	streptomycetes
<2 μm aggregates-29	HCA00559	streptomycetes
<2 μm aggregates-30	HCA00560	streptomycetes
<2 μm aggregates-31	HCA00561	streptomycetes
<2 μm aggregates-32	HCA00562	streptomycetes
<2 μm aggregates-33	HCA00563	unidentified
<2 μm aggregates-34	HCA00564	streptomycetes
<2 μm aggregates-35	HCA00565	unidentified
<2 μm aggregates-36	HCA00566	streptomycetes
<2 μm aggregates-37	HCA00567	streptomycetes
<2 μm aggregates-38	HCA00568	streptomycetes
<2 μm aggregates-39	HCA00569	streptomycetes
<2 μm aggregates-40	HCA00570	streptomycetes