The Characterisation and Detection of Plant Pathogenic Streptomycetes In the Natural Environment

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Contents

Summary

Streptomyces scabies has been attributed to be the causal agent of common scab, a superficial disease of the potato. Confusion over the taxonomic position of the organism arose as a result of the erroneous designation of a type strain that did not match the original description. This confusion was compounded by the deposition of many taxonomically distinct pathogenic strains in culture collections under the name of *Streptomyces scabies*. These studies attempted to clarify the taxonomic position of this organism. Common scab strains were characterised on the basis of phenotypic variation and hybridization to 16S rRNA probes. Pathogenic strains appeared to conform to three centres of variation similar to the *S.albidoflavus*, *S.rochei* and *S.diastaticus* *Streptomyces* spp. groups. The pathogenicity of putative pathogens was investigated and the pathogenic basis to the taxonomically heterogeneous group confirmed. Further studies focused on the development and application of approaches to the monitoring and detection of these strains in soil. Strain, ISP5078 has been well characterised and was selected as a model strain to pursue these objectives. Monitoring and detection strategies evaluated included: screening ISP5078 for selective phenotypic markers (such as antibiotic resistance) to assist in its selective recovery from soil and attempting to insert the marker genes *xylE* (novel to the *Streptomyces*) and *nptII* (a kanamycin resistance determinant) into the chromosome of strain ISP5078. Studies were also initiated to apply 16S rRNA targeted oligonucleotide probes to the monitoring of streptomycete inoculants in the natural environment. Studies focused on the development and evaluation of a method for the extraction and recovery of 16S ribosomal RNA from soil and the application of 16S rRNA probes to *in situ* hybridizations in the analysis of the lifecycle of scab-causing *Streptomyces* strains *in situ*. The influence of the potato rhizosphere on common scab strain populations was assessed by applying specific strategies to follow the fate of ISP5078 in sterile soil with and without potato plants. The lifecycle and activity of scab-causing streptomycetes in association with potatoes and soil was investigated using scanning electron microscopy and *in situ* hybridization.

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Declaration

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself unless otherwise stated in the text or acknowledgements. All sources of information have been specifically acknowledged by source of reference.

P. A. Bramwell

Abbreviations

ATCC	American type culture collection
BSA	Bovine serum albumin
c.f.u.	Colony forming units
Cont.	Continued
DEPC	Diethylpyrocarbonate
DMF	Dimethyl fluoride
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPS	Deoxynucleoside triphosphates
ds	Double stranded (DNA)
DTT	Dithiothreitol
EDTA	Diaminoethanetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FA	Fluorescent antibody
GEM	Genetically engineered microorganism
GOS	Homogenisation buffer for the preparation of RNA (Hughes & Galau, 1988).
hrs	Hours
ISP	International *Streptomyces* project
J.I.	John Innes Institute, Norwich, UK.
Km^R	Kanamycin resistant
min(s)	Minutes
MPN	Most probable number analysis
M.S.D.	Minimum signficant difference
NIAB	National Institute of Agricultural Botany, Cambridge, UK.
npt	Neomycin phosphotransferase
PBS	Physiological basal saline

PCR	Polymerase chain reaction
PEG	Polyethelene glycol
p.s.i.	Pounds per square inch
PVPP	Polyvinyl pyrrolidine
RASS	Reduced argenine salts agar
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
SEM	Scanning electron microscope
SET	Sucrose-tris-EDTA buffer
SNA	Soft nutrient agar
ss	Single stranded (DNA)
SSC	Sodium salt-citrate buffer
SSM	Simple matching coefficient (Sokal & Michener, 1958)
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TES	N-Tris (hydroxmethyl) methyl-2 aminoethane sulphonic acid
Tm	Melting temperature of DNA/RNA duplex
Tn	Transposon
Tris	Tris (hydroxymethyl) aminoethane
TSB	Tryptone soya broth
UPGMA	Unweighted pair group method with arithmetic averages (Sokal & Michener, 1958)
UV	Ultraviolet light
Vol(s)	Volume(s)
v/v	Volume per volume
w/v	Weight per volume

w/w	Weight per weight
YEME	Yeast extract malt extract media

Chapter 1

Introduction

1.1 The actinomycetes

Actinomycetes are Gram-positive bacteria with a high guanine plus cytosine (%G C) content in their DNA (>55%). The group encompasses genera exhibiting a wide range of morphological complexity extending from the coccus (*Micrococcus*) and rod-coccus cycle (*Arthrobacter*) through fragmenting hyphal forms (*Nocardia*, *Rothia*) to genera with a permanent and highly differentiated branched mycelium (*Micromonospora*, *Streptomyces*) (Goodfellow & Williams, 1983). Over 45 genera have now been validly described (McCarthy & Williams, 1990). Actinomycetes form branching hyphae (usually 0.5 to 1.0 µm diameter) and have been placed in the order of Actinomycetales (Williams & Wellington, 1982). The order has been subdivided into a number of families, which include the family Streptomycetae. The Streptomycetae is composed of aerobic actinomycetes that form a non-fragmenting substrate mycelium, which may bear spores (*Microellobosporia*, *Elytrosporangium*, *Kitasatoa* and occasionally *Streptomyces*) however, most genera produce a well-developed aerial mycelium bearing chains of arthrospores enclosed in a thin fibrous sheath (Cross & Goodfellow, 1973).

1.2 Approaches to the characterization of actinomycetes

1.2.1 Morphology

Morphology has played an important part in the delimitation of actinomycete genera (Williams & Wellington, 1980) and in distinguishing *Streptomyces* from other sporing actinomycetes (Locci, 1988). The distinctive morphology of the *Streptomyces* sporing structures, both in terms of spore chain morphology and spore surface ornamentation have been widely used in the classification of *Streptomyces* species groups (Pridham *et al.*, 1958; Tresner *et al.*, 1961; Williams & Wellington, 1980; Locci,1988).

1.2.2 Chemotaxonomic approaches

The importance of morphological characters to actinomycete taxonomy has been reduced with the development of chemotaxonomic approaches (Lechevalier *et al.* 1981; Minnikin & Goodfellow, 1981; Saddler *et al.* 1987; O'Donnell, 1988). Most schemes for the identification of genera are currently based on morphology, cell wall composition and other chemotaxonomic characters. The importance of diagnostic morphological and chemotaxonomic characters vary according to the particular genera under study. Hence morphological characters have been important in distinguishing streptomycetes from other sporoactinomycetes with a similar peptidoglycan cell wall structure. The actinomycete genera have been divided up into seven cell wall chemotypes (Lechevalier & Lechevalier, 1970). The isomeric form of diaminopimelic acid (meso or LL-DAP) and presence of whole cell sugars has been useful in the separation of certain *Nocardia* and *Actinomadura* from the *Streptomyces* as species of these genera may be morphologically alike (Wellington & Cross, 1983). Streptomycetes are characterised by the L-isomer of DAP and glycine in whole-cell hydrolysates, termed wall chemotype I (Lechevalier & Lechevalier, 1970).

Nocardioform genera are distinguished primarily by their cell wall chemistry as their morphology is variable and often not diagnostic. The distribution of mycolic acids has been used to distinguish between *Mycobacteria*, *Nocardia* and *Corynebacterium* (Minnikin *et al.* 1975; Minnikin & Goodfellow, 1981). Polar lipids, fatty acids and menaquinone patterns have also provided valuable information in the characterization of actinomycetes (Wellington & Cross, 1983).

Manchester *et al.* (1990) obtained polyacrylamide gel electrophoresis banding patterns of whole-cell proteins for selected representatives from the major *Streptomyces* and *Streptoverticillium* cluster groups, in order to determine the potential of this approach in streptomycete systematics. Protein patterns supported phenotypic and chemotaxonomic classification for the phena 1A *S.albidoflavus*, 1B *S.annulatus* and 18 *S.cyaneus*. However other groups including phenon 55, *Streptoverticillium griseocarneceum* appeared electrophoretically heterogeneous.

1.2.3 Numerical taxonomic approaches

Reliance or emphasis on one particular character for identification has repeatedly caused problems in the development of classification systems (Silvestri *et al.*, 1962). Recent contributions to microbial taxonomy have emphasised the need for polythetic as opposed to monothetic classifications (Sneath, 1962), whereby identifications are determined on the basis of many equally weighted characters simultaneously. This approach has been termed numerical taxonomy. Numerical taxonomic schemes require extensive statistical analysis both for their construction and for the assessment of identifications. Much work on the development of these systems has been performed by Sneath and colleagues (Sneath, 1957a;b; Sneath, 1962). Divisions into taxa at various similarity levels were made on the basis of correlated features. The numerical taxonomic approach involves the determination of a large number, at least 100 characters (Sneath, 1962), all are given equal weight with the proportion of characters common to two organisms representative of their overall similarity (expressed as % S values) (Wellington & Cross, 1983).

The principles of numerical taxonomy were applied to the identification of

actinomycetes by Silvestri *et al.* (1962) who assigned 190 streptomycetes to 25 centres of variation. This approach has subsequently been adopted by Williams *et al.* (1983a), Langham *et al.* (1989) & Kamper *et al.* (1991) for the further reclassification of the *Streptomyces* genus. Numerical classification has also been applied to the identification of other genera (reviewed by Wellington & Cross, 1983) and recently included the *Actinoplanes* (Goodfellow *et al.*, 1990).

1.2.4 Nucleic acid approaches

Actinomycetes have a high content of guanine and cytosine (%GC), which may be characteristic for certain taxa and has been of some value in the delimitation of certain genera such as the nocardioform taxa (Wellington & Cross, 1983). *Streptomyces* have a mol % GC of between 69 and 73% (Williams *et al.*, 1989). DNA:DNA reassociation studies have also been applied to the classification of actinomycetes, where they have been applied to the characterization of *Rhodococcus* species (Mordarski *et al.*, 1977). Okanishi *et al.* (1972) studied the DNA homologies within the group of strains described as *S.griseus* on the basis of 11 phenetic diagnostic characters. The authors identified homology values ranging from 36 to 104% with DNA from the type strain (ISP 5236), indicating the great genetic heterogeneity within the group. Mordarski *et al.* (1986) performed DNA homology studies on a larger number of streptomycetes from cluster 1, *S.albidoflavus* (Williams *et al.*, 1983a). Partial congruence was demonstrated between the numerical phenetic classifications of Williams *et al.* (1983a) and the DNA homology studies. The work highlighted problems within *Streptomyces* systematics and that different taxonomic approaches imposed alternative structures on the organisation of strains within the genus.

DNA relatedness has recently been applied to members of *S.ipomoea*, the causative agent for soil rot or pox of sweet potato (Labeda & Lyons, 1992) . A group of 25 strains isolated from the United states and Japan exhibited high average values of relatedness (85%) to the type strain and much lower values of DNA relatedness 39 and 17% to other plant pathogenic species and other morphologically similar groups of *Streptomyces*, thus indicating the distinctness of this group of strains. DNA relatedness analysis has also recently been applied to a diverse collection of *S.scabies* strains (Healy & Lambert, 1991) (Section 3.1).

Stackebrandt *et al.* (1981;1983) have investigated the relatedness of a number of type species representing various actinomycete genera using DNA : DNA homologies and RNA cistron similarities. DNA and RNA homologies have allowed the actinomycete genera to be grouped into three distinct clusters (Stackebrandt *et al.*, 1981). With *Streptomyces*, *Chainia*, *Kitasatoa*, *Streptoverticillium*, *Microbellosporia*, *Elytrospoangium* and *Actinosporangium* forming one tight cluster (Stackebrandt *et al.*, 1981) and now included within the *Streptomyces* genus (Goodfellow *at al.*, 1986 abcd). The grouping of actinomycete genera on the basis of molecular homologies was supported by several physiological and biochemical properties of taxonomic value (Stackebrandt *et al.*, 1983). Gladek *et al.* (1985) used 23S rRNA : DNA pairing studies to investigate the relationships between members of the *Streptomyces* genus (Williams *et al.*, 1983a) with *S.lavendulae* and *Streptoverticillium* strains. High similarity values were obtained between the three groups of strains indicating their high taxonomic relatedness. The highly conserved nature of the rRNA molecules allows the elucidation of more distant relationships than is possible by determination of DNA : DNA homologies. Taxospecies that show no DNA : DNA homology can be assigned to distinct

homology groups on the basis of RNA : DNA pairing studies (Mordarski *et al.*, 1981). This approach has been of value in supporting the integrity of the *Rhodococcus* group and indicating its close relationship with *Mycobacterium*, *Nocardia* and *Streptomyces* (Mordarski *et al.*, 1980). 16S rRNA targeted oligonucleotide probes specific for a region on the 16S RNA molecule diagnostic for the *Streptomyces* genus have been used in conjunction with phenotypic data to demonstrate the proximity of *Kitasatosporia* strains with the *Streptomyces* and to propose the unification of the *Kitasatosporia* strains with the *Streptomyces* genus (Wellington *et al.*, 1992).

Confirmation of suprageneric actinomycete groupings and elucidation of intrageneric groupings has been achieved by more detailed analysis of the 16S rRNA sequence data (Stackebrandt & Charfreitag, 1990; Witt & Stackebrandt, 1990; Stackebrandt *et al.*, 1991). Comparison and alignment of partial 16S rRNA sequences has provided evidence in support of the unification of the *Streptoverticillium* and *Streptomyces* genera (Witt & Stackebrandt, 1990) as well as unravelling phylogenetic relationships within the *Actinomyces* genus (Stackebrandt & Charfreitag, 1990) and amongst specific wall chemotype IV actinomycetes which lack mycolic acids (Embley *et al.*, 1988).

A limited number of DNA restriction fragment length polymorphism (RFLP) studies have been performed on actinomycetes (Hinterman *et al.*, 1981; Crameri *et al.*, 1983; Bloom *et al.*, 1989a). Crameri *et al.* (1983) and Dobritsa (1985) have demonstrated RFLP patterns to be strain specific, while Hinterman *et al.* (1981) have shown RFLP patterns to be stable markers under various growth conditions. Bloom *et al.* (1989a) assessed the value of RFLPs in the analysis of *Frankia* isolates which are not amenable to traditional actinomycete characterization as there appear to be few morphological

differences between isolates and physiological differentiation has proved unsatisfactory. RFLP analysis was correlated with DNA : DNA homology studies. Each isolate produced a unique RFLP pattern and isolates identified to be closely related via DNA homologies appeared to have related RFLP patterns. 16 Isolates were clearly separable into 9 groups on the basis of these patterns. These gel groups could be correlated with specific patterns of carbohydrate and organic acid utilisation, the only phenotypic characters identified to allow classification of isolates into separate groups (Bloom *et al.*, 1989b).

1.2.5 Actinophage host specificity

Wellington & Williams (1981) investigated the limits and relationships of the *Streptomyces* genus with respect to the phage activity spectra. The host specifity of phages isolated to *Streptomyces* strains and other cell wall chemotype I genera were determined. Phage isolated to *Streptomyces* strains appeared able to lyse a range of other genera of the same wall chemotype I. These genera all fell within the phenetically defined *Streptomyces* genus described by Williams *et al.* (1981; 1983a) with the exception of the *Kitasatasoa* and *Streptoverticillium* strains. The latter two genera showed wall chemotype I reactions with the phage but were not included in the *Streptomyces* genus (Williams *et al.*, 1981). Hence while the phenetic delimitation of the *Streptomyces* genus corresponded largely with phage activity spectra it differed for these two wall chemotype I genera. These findings were confirmed by the activity spectra of phage isolated to non-streptomycete wall chemotype I genera. Both sets of phage were only active on wall chemotype I genera supporting the phenetic conclusions of Williams *et al.* (1981) and the inclusion of many of these other genera within the *Streptomyces* genus.

1.3 The ecology of actinomycetes

Actinomycetes are found widely distributed throughout the natural environment. Although they are most often associated with the solid constituents of litter, humus, dung, soil and rock particles (Lacey, 1973), they have also been isolated from aquatic environments (Weyland, 1981). Much controversy has surrounded the origin of actinomycetes isolated from aquatic environments and whether they did indeed have a terrestrial origin and were merely washed into marine and fresh water localities (Weyland & Helmke, 1988; Jensen *et al.*, 1991). Actinomycetes have also been isolated from atmospheric environmental samples, as many actinomycetes produce propagules that serve as air-borne agents of dispersal and survival (McCarthy & Williams, 1990).

However, the soil is considered to be their most important habitat (Williams &Wellington, 1982). Viable counts of several millions per gram are common, with representatives from over 90% of actinomycete genera having been isolated from soil. *Streptomyces* have been attributed to be the most abundant and important genera of the actinomycetes in the soil (Williams, 1978) hence many studies on the ecology of actinomycetes have actually focused on the ecology of streptomycetes. Other genera that have been frequently isolated from soils include, *Micromonospora*, *Rhodococcus* and *Streptosporangium*. Many soil isolates have been found to be prolific producers of antibiotics and other secondary metabolites *in vitro* (McCarthy & Williams, 1990). In contrast *Thermoactinomyces* species are frequently isolated from composts and other self heating materials (Williams & Wellington, 1982). Various approaches to the selective isolation of specific actinomycete genera from terrestrial environments have been described (Williams & Wellington, 1982;

Williams *et al.*, 1983a; McCarthy & Williams, 1990).

Although the majority of actinomycetes that participate in the complex nutrient cycling processes are purely saprophytic microorganisms, actinomycetes may also form parasitic and symbiotic associations with plants or animals (Williams *et al.*, 1983a). Actinomycetes have been implicated in a number of medical conditions including, tuberculosis, leprosy, mycetoma, generalised systemic infections, dermaides as well as allergic pneumonitis reactions such as those of farmer's lung, mushroom worker's lung and bagassosis. In contrast, they have been attributed to cause the plant diseases of common scab of potatoes and rot of sweet potatoes (Kutzner, 1981) and blue berry (Lechavalier, 1981). The symbiotic associations between plants and actinomycetes that have received most attention are those involving the endophyte *Frankia* which forms actinorhizal associations via the formation of root nodules and fixation of nitrogen in over 200 species of angiosperm plants (Lechevalier, 1988). The ubiquity of actinomycetes has been demonstrated by their isolation from a diverse set of environments including: the intestines of certain arthropods, disintegrating rubber, oilfield ground water and decaying wood (McCarthy & Williams, 1990).

1.3.1 The *Streptomyces* lifecycle

1.3.1.1 *Streptomyces* in soil

Homogenisation experiments and scanning electron microscopy studies indicate that the vast majority of streptomycete colonies isolated from soils by viable plating originate from spores (Goodfellow & Simpson, 1985). Furthermore the growth of *Streptomyces* strains in soil is discontinuous, with the microbe residing in soil for long periods as arthospores or chlamydospores only germinating in the presence of exogeneous nutrients. Scanning electron

microscopy studies show that particulate organic substances, such as root fragments and fungal hyphae are rapidly colonised by streptomycete hyphae (Mayfield *et al.*, 1972). Sporulating streptomycete hyphae appear as nutrients are depleted (Goodfellow & Simpson, 1985). Streptomycete spores although not as resistant to unfavourable conditions as *Bacillus* endospores do appear to be important to longevity and survival (Williams *et al.*, 1972). Spores usually have thicker cell walls than hyphal cells and the presence of an outer sheath contributes to make spores highly hydrophobic. The highly hydrophobic nature of the spore has been interpreted to signify that spores are dispersed either by air or on the surface of water droplets, rather than by water percolating through the soil (Williams, 1976). It has also been suggested that the hydrophobic nature of spores may assist in their adherence and consequent transport via the cuticles of soil arthropods (Ruddick & Williams, 1972).

Streptomyces populations like other soil microbes have been observed to be distributed unevenly in the heterogeneous soil environment. Microcolonies have been associated with particles of organic matter. The rhizosphere and earthworm casts have been considered important microsites for *Streptomyces* (Kutzner, 1981).

Comparison of the actinomycete lifecycle in soil and on the surface of artificial media indicate that a morphologically similar process occurs under both conditions. The developmental processes include polarised growth and mycelial organisation followed by sporulation and the aerial dispersal of spore propagules. The filamentous (and metabolically active) growth form appears well adapted to the colonisation of solid surfaces (Locci, 1988). However, such filamentous branching hyphae are severely restricted in their capabilities for dispersion. Hence dispersal is achieved by the fragmentation of mycelium

and the formation of aerial hyphae bearing spores. Hyphal growth has rarely been observed in natural soils, rather actinomycetes are often observed in soils as short spore chains indicative of their recent production (Locci, 1988). When hyphae are observed *in situ* they are often thinner and sometimes reduced to small pads when compared to those observed under artificial *in vitro* conditions (Mayfield *et al.*, 1972). Under the nutrient limiting conditions of soil *Streptomyces* have been observed to participate in microcycle sporulation i.e. the production of spore chains from germinating spores and accompanied by minimal vegetative growth (Locci, 1988). Such microcycle sporulation is considered to be dependent (in part) on the nutrient limited conditions in soil (Lloyd, 1969; Locci, 1988).

A number of reports exist on the poor germination of streptomycete spores in natural non-sterile soils (Lloyd, 1969; Mayfield *et al.*, 1972). Lloyd (1969) hypothesised that in order for spores to serve as propagules for survival, they should not participate in synchronous germination, but rather germinate when conditions are favourable. Favourable conditions might be determined by the availability of particular nutrients and the absence of antagonistic microorganisms. He obtained the following frequencies of germination of spores: garden soil 1 to 7%, pasture soil 2 to 14% and wheat soil 2 to 11%, these compared with 65 to 90% germination in sterilized garden or pasture soil.

Streptomycetes have been widely reported to be active producers of hydrolytic enzymes, however the ecological significance of such production remains uncertain (Goodfellow & Simpson, 1985). However, streptomycetes are considered to have an important role in the degradation of relatively complex, recalcitrant polymers that occur naturally in plant litter and soil (Lacey, 1973).

Streptomycetes have been shown to be involved in cellulose, pectin, starch, xylan, lignin and chitin degradation and certain species have been found to be able to degrade compounds such as herbicides and plastics (Goodfellow & Simpson, 1985).

1.3.1.2 *Streptomyces* associated with plants

Rhizosphere effects, quantified as R : S ratios i.e. the proportion of bacteria isolated from soil in association with plant roots (R) as opposed to the bulk soil (S) have been reported for streptomycete populations and a number of plants. For example, soya and maize plants were found to harbour 10 to 18 times as many actinomycetes in their rhizosphere (Abraham & Herr, 1964). A number of workers have indicated that high R : S ratios of up to 50:1 have been associated with older plant roots (Watson & Williams, 1974; Williams, 1976), supporting the view that streptomycetes do not compete successfully for the single carbon compounds exuded by young roots but grow on senescent root tissues which contain more complex molecules (Goodfellow & Simpson, 1985).

Qualitative differences in the actinomycete populations observed in the rhizosphere as opposed to those in the bulk soil have been observed, hence Abraham & Herr (1964) identified more actinomycetes able to hydrolyse starch in corn and soy bean rhizospheres. In addition Agate & Bhat (1964) found more pectinolytic actinomycetes and glutamate requiring *Nocardia* in the rhizosphere of mature plants of *Calotropis gigantea*.

Some evidence exists for the production of plant growth regulators such as auxins and giberellin type compounds by actinomycetes isolated from rhizosphere samples. Rhizosphere isolates from *Pinus silvestris* were

identified to produce both auxins and gibberellin type compounds (Strzelczyx & Pokoojska-Burdziej, 1984). Auxin production was particularly evident when isolates were grown on tryptophan containing medium. Production of auxin was more frequently observed among rhizosphere isolates. Auxins have been identified to play a role in plant-mycorrhizal relationships, where they have been attributed to affect root morphology and direct the translocation of soluble sugars to the mycorrhizal roots. Furthermore, reports exist on the ability of rhizosphere microorganisms to enhance mycorrhiza formation.

Mycorrhyzal stimulation has also been considered to be influenced by the production of vitamins by rhizosphere isolates. Ectomycorrhizal fungi have been identified to have a requirement for the B group vitamins, particularly thiamin. Actinomycetes derived from the rhizospheres of *Pinus silvestris* were identified to synthesise a greater number and wider range of B vitamins than isolates from a corresponding non-rhizosphere soil (Strzelczyk & Leniarska, 1985).

Vruggink (1976) has looked at the population dynamics of streptomycetes able to incite common scab, *Streptomyces scabies,* in the presence of different crop plants. However, despite some interesting findings, e.g. in certain soils actinomycete populations declined with a beet crop, in general, differences in soil type appeared to influence streptomycete soil populations more than the cover crop planted. Rouatt & Atkinson (1950) also considered the affect of different cover crops on the soil populations of fungi, actinomycetes and other soil bacteria. They obtained marked qualitative and quantitative differences of the influence of crops on microbial populations. Increases in actinomycete populations were evident in scab infested soil planted with soybeans, a slight increase was noted with soil planted with red clover and with no population

differences were observed in the soil planted with rye. A rhizosphere type effect, with elevated streptomycete populations at the potato tuber-soil interface was also reported (Vruggink, 1976).

1.3.1.3 Streptomycetes as agents in biological control

Numerous reports exist of the inhibitory activity of actinomycete isolates against plant pathogens. Kundu & Nandi (1985) found that the addition of cellulosic waste products (rice stubble and water hyacinth biomass) resulted in the reduction of cauliflower 'damping-off' (*Rhizocotonia solani*). Disease control was attributed to the increased numbers of antagonistic actinomycetes and bacteria that grew with the addition of the amendments to the soil. Although much speculation has surrounded the nature of the antagonistic effects reported and the possible role that antibiotic production by actinomycetes may have in them. Many workers have been unable to demonstrate antibiotic production in non sterile soil conditions (Williams, 1982; Williams & Vickers, 1986). Inability to detect antibiotic activity in the natural environment could be attributed to instability, adsorption to soil colloids and lack of sensitive methods for the detection of these compounds (Williams *et al.*, 1983).

Gause *et al.* (1981) speculate that the high tolerance of antibiotic producing strains to the antibiotics they produce (while closely related strains are highly sensitive to their action) supports the view that antibiotic production is of ecological relevance and may contribute to competitive and antagonistic microbial interactions.

Whaley & Boyle (1967) considered the role of the production of heptaene antibiotics in antagonistic interactions in the rhizosphere of desert plants.

Investigations were made in response to the observation that in the Arizona desert, an increase in several root diseases was accompanied by the addition of moisture and amendments. Streptomycetes were isolated from the rhizospheres of desert plants that were antagonistic to *Fusarium oxysporum*, *Rhizoctonia solani*, and *Verticillium albo-atrum* and identified to be producers of heptaene antibiotics. The effect of different nutrient sources on antibiotic production was investigated under *in vitro* conditions. Their findings indicated that antibiotic production varied with the available carbon and nitrogen source and that some carbon and nitrogen sources that sustained minimal levels of growth were excellent for antibiotic production. Thus reflecting the nutrient status of the soil environment under which it is speculated that production may occur. In addition, the authors demonstrated antibiotic production by rhizosphere isolates on carbon and nitrogen sources identified to occur in the rhizosphere *in vitro*. However, they do emphasise that the possible inactivation of these polyene antibiotics by antagonistic microorganisms and adsorbtion to soil particles does require investigation before a role in ecological interactions can be assigned.

A number of researchers have reported lowered disease incidence of certain fungal pathogens, including *Fusarium oxysporum* (Buxton *et al.*, 1965) and *Rhizocotonia solani* (Henis *et al.*, 1967), in chitin amended soils. Chitin amendment has frequently been shown to promote streptomycete populations (Williams & Robinson, 1981) and reduce the incidence of common scab of potatoes (Vruggink, 1970). Indirect evidence of *Streptomyces* soil isolates as agents in the biological control of fungal pathogens, comes from the studies of Williams & Robinson (1981) on the contribution of fungal mycelium to the soil-borne chitin reservoirs (available to streptomycete populations). They speculate that fungal sources may provide the major component of soil chitin

and add support to the argument by citing observations made by Lloyd *et al.* (1965) on the ability of streptomycetes to lyse living fungal mycelium.

1.4 Plant pathogenic streptomycetes

Few streptomycetes are capable of infecting plant tissues and causing disease. The most notable, in terms of their agronomic importance are the *Streptomyces* species able to cause common scab of potatoes. The causative agent has been described as *Streptomyces scabies* (Lambert & Loria, 1989). Current knowledge of plant pathogenic streptomycetes is given in Table 1.

Common scab is an important disease in potato growing regions throughout the world (Davies & Garner, 1978) affecting all underground parts of the potato plant. Infection results in superficial corky lesions on the tuber surface and an unpalatable tasting potato flesh as a result of geosmin production by streptomycetes.

Table 1

Summary of current knowledge of plant pathogenic streptomycetes

Species	Host Plant	Disease	Reference
S.scabies	Potato, Sugar beet Carrot and others	Common scab (severe)	Hoffman, 1958 Lapwood, 1973
S.griseus *S.aureofaciens* *S.flaviolus*	Potato	Common scab (mostly mild)	Hoffman, 1958 Corbaz, 1964
Streptomyces sp.	Potato	Common scab in acid soils	Bonde & McIntyre, 1968
Streptomyces sp.	Potato	Russet scab	Harrison, 1962
S.Ipomea	Sweet potato	Soil rot & pits	Person & Martin, 1962

(Taken from Kutzner, 1981)

Various types of scab have been described according to the severity and type of scab lesions these include, pitted and raised scab (Jones, 1931), ordinary, superficial, deep and elevated scab (Emillisson & Gustafasson, 1953), russet scab (Harrison, 1962) and netted scab (Scholte & Labruyere, 1985).

Common scab is most prevalent in soils that promote optimal growth conditions for streptomycetes, i.e. sandy, calciferous soils that are well drained and neutral to alkaline in pH. A number of agronomic practices have been employed to minimise the incidence of the disease in scab infested soils (Labruyere, 1971). These include the planting of resistant potato varieties, ensuring the crop is well irrigated particularly during the period of tuber initiation and expansion (Lapwood & Lewis, 1967; Lapwood & Hering, 1970) and reducing the pH of the soil through the application of green manures.

Tubers initiate as a swelling of an underground stem (stolon) as a tuber grows, nodes (eyes) separate from the apical bud as internode tissue forms between them. When first formed the tuber internodes have stomata, but as the tissue expands the stomata are transformed into lenticels (Fellows, 1926). The stoma guard cells are gradually raised above the tuber surface, by division of cells beneath them, further cell division and expansion causes the stoma to rupture and expose the inner cells with the formation of a young lenticel. If the soil is wet, cells proliferate from the lenticel opening, however, if it is dry cell division stops and suberin is deposited on the outermost cell walls (Lapwood, 1973). Initiating potato tubers pass through a phase of susceptibility to infection by scab-causing streptomycetes, corresponding to a specific developmental stage of the lenticels. Susceptible lenticels appear to be newly formed with incompletely suberised filling cells, each lenticel is thought to be susceptible for about seven days (Adams, 1975). Hooker & Page (1960)

demonstrated that suberized periderm could not be breached by pathogenic *S.scabies* isolates even after autoclaving. Lenticel development appears comparable for both scab-resistant and susceptible varieties (Adams, 1975). In wet soils prevention of infection has been attributed to the antagonistic and competitive effects of other soil microorganisms (Lewis, 1962 ; Adams & Lapwood, 1978). However, cell proliferation under wet conditions would displace freshly colonised tissue and may also be involved (Labruyere, 1971).

1.4.1 The ecology of scab-causing streptomycetes

Few attempts have been made to study the ecology of scab-causing streptomycetes and much of the early work is contradictory with some workers finding that the incidence of scab is influenced by the pattern of crops grown between the potatoes and others not. Goss & Afansiev (1938) found that common scab was most severe at sites with short rotations or those incorporating sugar beet. Most workers appear have found that the incidence of common scab was often associated with short rotations or continual cropping with potatoes. Hooker (1956) also found that the common scab infections were most severe in peat soils with short rotations and or a continuous potato crop than by the inclusion of other crops in the rotation. Hooker (1956) was unable to correlate plate counts of streptomycete populations with the incidence of scab. Counts appeared highest in plots cropped to corn, followed by potatoes and lastly onions and soybean. Fallow plots exhibited the lowest counts.

Russet scab (or netted scab) caused by *Streptomyces* sp was identified as a disease associated with short rotations (Scholte *et al.*, 1985).

Keniath & Loria (1989) have investigated the population dynamics of melanin

producing *Streptomyces*, a group that has been identified to include many of the scab-causing strains. Population dynamics of the melanin producing group was studied in response to the growth of different potato cultivars. Streptomycete populations isolated from field plots cropped to potatoes were more diverse than those from the control fallow plots. Differences in population diversity were also seen amongst the streptomycetes colonising the potato rhizosphere and tuber surface, indicating that conditions for streptomycete growth differed in both environments. Qualitative and quantitative differences in streptomycete populations were seen with time, probably as a result of the development of plants and seasonal changes.

The authors also demonstrated differences in the relative numbers of different streptomycete species groups in the rhizosphere of the scab susceptible potato cultivar, 'Chippewa' and the scab resistant cultivar, 'Superior'.

In a further investigation Keniath & Loria (1990) studied the influence of the two potato cultivars on the melanin producing streptomycete populations. Populations were monitored in plots planted with the scab susceptible variety, 'Chippewa' and the scab resistant variety, 'Superior' and also in fallow soil, over a three year period. In general, populations of streptomycetes in soils planted with potatoes increased in the soil, the rhizosphere and on the tuber surface throughout the growing season. While populations in the fallow soil remained stable or declined. No significant differences were observed between the population levels of streptomycetes isolated from the soil and rhizosphere samples from plots planted with both potato varieties. However, the population levels on the tuber surface of the scab susceptible cultivar were significantly higher than those on the scab resistant cultivar. Melanin positive streptomycete population densities in the soil corresponded well with scab

incidence and severity. The authors suggest that this correlation may reflect actinomycete reproduction on the tuber surfaces. As the population levels of actinomycetes as opposed to melanin positive streptomycetes also correlated well with the incidence of scab. Less than 6% of the melanin positive isolates appeared to be common scab pathogens. This finding illustrates the need for selective and distinct markers for pathogenic strains in order to study the ecology and epidemiology of pathogenic strains.

1.5 The soil environment

Soil microorganisms constitute one of five interactive forces in the formation of soil, the other four being climate, topography, parent material and time. The physical and chemical breakdown of the parent rock to fine particles with large surface areas and the accompanying release of plant nutrients initiate the soil-forming process. The two major nutrients that are deficient at this stage are carbon and nitrogen. Thus the initial colonisers of soil parent material are usually organisms capable of both photosynthesis and nitrogen fixation and are predominantly the blue green algae. Once higher vegetation has become established a continuum of soil processes support a dynamic mixture of living and dead cells and soil organic matter (SOM), in various stages of decomposition and small mineral particles. Soil gases, water and dissolved minerals complete the soil habitat (Paul & Clark, 1989).

Soil environments constitute four different environments in which microbial activities occur, these are profiles, horizons, aggregates and colloids. The relative dimensions of the components of the soil environment range from metres (profiles), centimetres (horizons) millimetres (aggregates) to microns (bacteria and colloidal particles) (Burns, 1988).

The formation of the clay-organic matter complexes are the key structural features of most soils. The clay minerals are primarily crystalline, hydrous aluminosilicates comprised of silicon oxide tetrahedra and aluminium hydroxide octahedra sheets. The sheets are associated either in a 2:1 (Si-Al-Si) or a 1:1 (Si-Al) ratio, the unit layers are held together through the formation of hydrogen bonds and weaker van der waals forces. 2:1 ratio clays are usually able to expand upon wetting, exposing a significant internal surface area between adjacent silicon layers, while 1:1 ratio clays do not normally hydrate. 2:1 clays also participate in isomorphous substitution, a process by which structural cations (eg Al^{3+} Si^{2+}) may be exchanged for those of a different valency (eg. Si^{2+} Fe^{3+})which may in turn result in a net negative charge. The electronegative charge is compensated by the adsorption of positively charged exchangeable cations from the surrounding soil solution and is termed the cation exchange capacity of the clay (Stotsky & Burns, 1982).

Soil organic matter (SOM), a macroscopic component represented by the particulate plant, animal and microbial debris in the early stages of disintegration, comprises a biochemically defined and generally soluble fraction, that may include carbohydrate and protein products from the degradation of the macroscopic component and a dark-coloured aromatic and polymeric component arising from the breakdown of lignin as well as microbial synthesis. This humic fraction may be further subdivided on the basis of solubility into humic and fulvic acids and humins. The humic material is of equal importance to the expanding clays in soil microbe interactions. This is because humic materials also expand upon wetting revealing an extensive internal surface area that is a reservoir for water and a vast array of organic and inorganic solutes, available for associated microbial

activity (Burns, 1988).

Clay and humic materials in addition to other organic materials are intimately associated to form organomineral complexes (microaggregates) which cluster to form aggregates. Most clays, soil microorganisms and SOM constituents are negatively charged at neutral pHs. Attachments between components that should naturally repel each other are made by ionic bonding via multivalent cations. Microbial polysaccharides and microbial fibrils also bind clay particles together. Aggregate formation is thus a result of the interactive activity of the soil microflora with plant roots producing filaments and polysaccharides that combine with clays to form organic matter-mineral complexes. Soil aggregates retain water, the thickness and permanence of which depend on the type and amount of clay and organic matter within the aggregates. The water may form bridges from one aggregate to another and comprise the microhabitats in soil where the microbes function. The space between the aggregates constitutes the pore space and may be filled with air and other gases and volatiles (Stotsky *et al.*, 1991).

Postma *et al.* (1991) have attempted to investigate the role of certain cell surface properties of *Rhizobium leguminosarum* in the attachment to soil particles. The growth and survival of the unaltered strain and three Tn*5* mutants with altered cell surface properties, that included the absence of cellulose fibrils, an 80 to 90% reduction in exopolysaccharide production and an altered lipopolysaccharide composition were investigated in sterile and non sterile soil. Their findings affirmed the importance of cellular attachment to soil particles, however they were unable to demonstrate a relationship between bacterial adhesion and cell surface properties, rather they speculate that in certain groups of soil bacteria attachment to soil particles may be a result of

entrapment in soil pores.

Soil organisms show their greatest diversity of species and usually their largest populations in productive soils. The size of the microbial biomass being found to correlate well with the amount of plant growth (1^0 productivity) and with SOM levels. Most organisms exist on the surface of soil aggregates or in the pore spaces between them, with microorganisms reported to occupy between 0.4 and 0.2% of the available pore space (Paul & Clark, 1989). Soil bacteria do not exist as unattached particles in soil, nor are they easily washed from the soil matrix. Rather their cells adhere to or are adsorbed on inorganic and organic surfaces. This level is influenced by the water content of the pores. In a study of a number of factors including: electrostatic charge of the cell surface, hydrophobicity, cell size and presence of capsules and flagella and their influence on the transport of bacterial cells through soil. Bacterial cell size was consistently related to the movement of cells through a soil column with the smaller bacterial cells of less than 1.0 μm being retained by the soil least (Gannon *et al.*, 1991). Pore size also imposes limitations on the grazing and movement of soil organisms. Studies of Heijen & Van Veen (1991), indicated that addition of bentonite and kaolinite amendments to a loam, sand soil could enhance the survival of introduced *Rhizobium* inoculants. Inability to maintain *Rhizobium* inoculants in soil has been attributed to protozoan predation. The addition of clay amendments under appropriate moisture regimes appeared to protect *Rhizobium* cells from predation, by reducing the pore size of the soil to allow growth of the bacterial and but not the larger protozoan cells.

1.5.1 Components of the soil biota

The soil biota is composed of bacteria, fungi, algae and fauna. In addition,

there are viruses, which are dependent on other components of the soil biota for reproduction and dispersal. Bacteria are however, the most numerous component of the biota, some of the more abundant and commonly isolated groups include the following: arthrobacters have been estimated to comprise up to 40% of total plate counts from soil (Paul & Clark, 1989). The group is characterised by pleomorphism and Gram variability and the group is able to oxidatively metabolise a wide range of substrates. They are slow growing microorganisms and poor competitors in the early stages of residue decomposition with other genera competing more effectively for the available simple sugar and amino acid molecules. The *Streptomyces*, *Pseudomonas* and *Bacillus* species probably account for the second largest group of microorganisms present in soil. Any of these genera may account for 5 to 20% of a total bacterial plate count. Pseudomonads are Gram negative rods with polar flagellation. Generally aerobic, most species are organotrophs occurring in soils as well as fresh and marine environments. In contrast, the bacilli are Gram positive to Gram variable rods. Most species are motile and produce heat resistant endospores. There is a great diversity within the genus. Other genera commonly associated with soils include *Clostridium*, *Azotobacter*, *Lactobacillus* and various cyanobacteria. The fungal component of soil can be equally diverse containing slime molds, flagellate fungi, zygomycetes, basidomycetes as well as ascomycetes (Paul & Clarke, 1989).

1.5.2 Microbial interactions

Various types of interactions occur among microbial populations, these include interactions between individual cells and other biotic and abiotic components. These interactions are in a constant state of flux, resulting in a dynamic biological equilibrium among the microbes that shifts with changes in the physicochemical status of the environment (Stotsky *et al.*, 1991).

Two of the most important microbial interactions affecting bacterial populations are predation and competition. Postma *et al.* (1990) considered the interaction of a protozoan predator and indigeneous rhizobia competitors in the colonization of sterile soils by a *Rhizobium* inoculant and the affect of both components on the interaction of the inoculant with soil aggregates. In the presence of the protozoan predator, a high proportion of the *Rhizobium* soil population were found associated with soil particles and aggregates, indicating enhanced survival from predation in protective microniches. In contrast when a *Rhizobium* inoculant was introduced into soil, that had been already seeded with competitive rhizobia, the proportion of the inoculant associated with soil particles was considerably lower. Populations of the introduced inoculant in either the presence of the predator or competitor alone, remained fairly constant in soil. However, in the presence of both the predator and competitor, populations of the introduced inoculant decreased dramatically and in excess of the sum of the effects of both predation and competition separately. Numerous reports exist on the bacterial predation by protozoa (Clarholm, 1981; Habte & Alexander, 1987; 1988a;b). Predation of bacteria by bacteria has also reported to occur (Casida, 1988). A number of bacterial genera have been implicated in nonobligate predation, i.e. predation in the absence or depletion of soluble nutrients, these include *Ensifer adhaerens*, *Agromyces ramosus*, *Actinomyces humiferus* and certain *Streptomyces* and *Myxococcus* strains (Casida, 1988). Bacteriophage may also be important in regulating bacterial population densities in natural environments (Kokjohn *et al.*, 1991) though their significance in natural soils remains to be determined (Herron, 1991).

Ho & Ko (1982) report the prevalence of the antagonistic phenomenon of soil

against populations of fungi, actinomycetes and other bacteria, i.e. fungistasis, actinostasis and bacteriostasis. Microbiostasis appeared to be common to all natural soils under study and overcome by the addition of certain nutrient amendments to the soil. Bacteriostasis appeared to be the easiest to overcome by the addition of amendments to the soil. Brown (1972) demonstrated that bacteriostasis was overcome in a number of soils by the addition of wheat root and seed exudates.

Disease suppressive soils may develop as a result of certain antagonistic interactions between microbes for certain combinations of crop plants and phyopathogens (Schroth & Hancock, 1982). Disease-suppressive soils may be induced or natural. With natural soils being disease-suppressive as a result of the particular physical and chemical characteristics of the soil. In contrast, induced suppressive soils occur independent of soil type and as a result of agronomic cultural and cropping practices. A history of monoculture with a susceptible crop has been found to be a prerequiste for a disease-suppressive soil. Disease-suppressive soils have been reported for take-all of wheat (*Gaeumannomyces graminis* var. *tritici*) and common scab of potatoes (*Streptomyces scabies*) (Scroth *et al.*, 1979). The development of a scab suppressive soil is proceeded by a build up of the disease in the soil (a scab infested soil) with long term, continuous cropping with potatoes. Soil components responsible for the disease-suppressive status of the soil appeared to be microbial since they were sensitive to fumigation and autoclaving treatments (Scroth *et al.*, 1979).

1.6 The association of microorganisms with plant roots

Plant root systems occupy the soil horizon richest in SOM where the live, senescent and dead roots provide substrate materials for microbial growth.

The rhizosphere has been termed the region of soil under the influence of the plant roots and in which there is a proliferation of soil microorganisms. In addition, two other areas of the roots that are colonised by microbes, the rhizoplane (the root surface) and the endorhizosphere (the interior of the root). The concept of the root cortex as being part of a microbial continuum extending from the soil associated with the roots to the root cortical tissues has been introduced (Old & Nicolson, 1982).

The number of soil microorganisms in the vicinity of the root has been found to decrease with the distance from the root tissue. Bacterial coverage of the root has been estimated to be in the range of 5 to 10% (Rovira, 1979). Distribution of bacterial cells on the root surface appears to be uneven and related to plant microbe contact rather than microbial mobility (Howie & Cook, 1985; Dijkstra *et al.*, 1987; Misaghi *et al.*, 1992). Bacterial cells have rarely been found associated with the rapidly growing apical tip, with the first area to be colonized being just beyond the zone of elongation. Weller (1984) monitored the distribution of a fluorescent pseudomonad that was suppressive to the causal agent, *Gaeumannomyces graminis* var. *tritici* of take-all of wheat after its introduction as a coating on the seed. A population gradient of bacterial colonisation was demonstrated along the roots with highest populations at the base of the root and the population declining towards the root tip. A number of studies have consistently identified high densities of microbial cells around cell junctions that have been attributed to an abundant supply of root exudates and an association with dead epidermal cells (Rovira, 1979; Bennet & Lynch, 1981).

Howie & Cook (1985) investigated the role of cell motility in pseudomonads with flagella and non-motile mutants in the colonisation of roots. No

significant differences in motile and non-motile populations were found, indicating that bacterial distribution was dependent on root elongation in the absence of the downward movement of water. Dijkstra *et al.* (1987) considered that the differing bacterial growth rates, in association with passive displacement of cells via elongation of the cells at the root surface could account for the differing densities of colonization of wheat roots between *Pseudomonas fluorescens* and *Bacillus subtilis*, which were determined to be 10^7 c.f.u. cm^{-1} root for *P flourescens* and 10^5 c.f.u. cm^{-1} root for *B.subtilis*. Microbial densities in the rhizosphere environment generally increase with the age of the plant roots (Campbell & McDonald, 1989).

Protozoan population levels also increase in the vicinity of the root and in response to elevated numbers of bacteria, R/S ratios of up to 10 have been observed. Their predation activities are not only thought to affect the microbial populations but also to release minerals back into the soil that might be of benefit to the plant. If the plant is nitrogen or phosphorous limited, as it may well be in most natural ecosystems, the photosynthate produced is probably sufficient for both and it may not matter if some is lost through the roots provided this produces a marginal increase in phosphorous and nitrogen uptake (Campbell & McDonald, 1989). Circumstantial evidence of enhanced deposition of the plant photosynthate via the production of root exudates in natural soils as opposed to *in vitro* conditions has been observed (Lynch, 1982).

Microbial interactions in the rhizosphere and on the root surface (rhizoplane)

The root environment has been characterised as a site of intense microbial competition for nutrients (Fravel, 1988; Weller, 1988). Much interest has

focused on the microbial interactions occurring in the rhizosphere and the rhizoplane with a view to the manipulating the root microflora to either suppress phytopathogens (Biological control) (Weller, 1988) or to enhance plant growth by the introduction of Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper *et al*., 1980; 1989). Considerable inconsistency has been associated with experiments on biological control (Howie & Echandi, 1983) and much speculation still surrounds the reasons for a correlation between reduced disease incidence and increased crop yields. Production of antibiotics by inoculants (Fravel, 1988) and aggressive colonization of available niches or rhizosphere competence (Weller, 1988) have frequently been attributed to the successful establishment and activity of inoculants in the rhizosphere. One of the most successful biological control strategies involved the use of a non-tumourgenic *Agrobacterium radiobacter* K84 strain to control crown gall disease, caused by the related but virulent *Agrobacterium tumefaciens* (Moore, 1985). Control operates via the production of Agrocin by K84, this bacteriocin is taken up into the susceptible strains by a permease encoded by the tumour-forming (Ti) plasmid of *A.tumefaciens* (Engler *et al*., 1975), the requirement for this plasmid encoded gene means that the bacterocin is selectively targeted at *A.tumefaciens*.

Enhanced yields in the presence of PGPR have been attributed to the extracellular production of siderophores, which efficiently complex environmental iron making it unavailable to certain components of the soil microflora (Kloepper *et al*., 1980). Increased yields achieved through inoculation of plants with PGPR were mimicked by the action of a yellow-green fluorescent siderophore isolated from *P.fluorescens* and named pseudobactin (Kloepper *et al*., 1980). Many PGPR have been identified to produce plant growth promoting substances such as auxins and gibberellins

(Brown, 1972; 1974). Positive correlations between production of these compounds *in vitro* with enhanced crop yields and altered root or plant morphology have been reported. However, the significance of these plant microbe interactions remains to be established (Brown, 1974).

Analysis of the organic materials found on or in association with the roots reveal an assortment of soluble molecules that include amino, aliphatic and aromatic acids and amides and sugars. In addition to these are a range of complex insoluble structures such as cellulose, lignin, protein and chitin. With the simple sugars and amino and organic acids common to all rhizospheres and the more complex compounds particular to certain plant rhizospheres. A number of these compounds have particular biostatic and biocidal properties. Pathways for the release of plant assimilates from roots include the leakage or diffusion of molecules across cell membranes, root secretions and extrusions. Root caps and tips are sites of active exudation, releasing mucilaginous secretions in addition to root cap and root tip cells. The main root axis predominantly releases soluble and diffusible material and some mucigel (a mixture of polysaccharides). Mucigel has been identified to be the dominant excretory product of the roots and was found to account for 80% of the carbon lost from wheat roots, aside from losses due to respiration. Labelled ^{14}C tracer studies revealed that 11% of plant assimilate is acquired for microbial respiration and 2% ends up as SOM (Paul & Clark, 1989).

In addition to the enhanced amounts of organic matter available in the rhizosphere, other environmental gradients may occur. In general, the water potential surrounding the roots is usually not that different from un-planted soil, provided that there is continuity between the soil water and the plant root, and the water enters uniformly over the root surface. However, if there is a

local water stress in the rhizosphere these assumptions may not be valid and water uptake may be concentrated into a small proportion of the total root which is in direct contact with the soil. Certain groups of bacteria and fungi will tolerate reduced water potentials. There may also be compaction of soil around the roots, generated as the root pushes through the soil and contributing to a restricted flow of water. Gas exchange in the rhizosphere may be different, for instance in well aerated soils there may be a slight elevation of CO_2 levels and depletion of oxygen. However, as the soil becomes waterlogged or even wet, the situation alters as diffusion of O_2 and CO_2 is slow through the films of water surrounding the roots and soil pores.

Chapter 2

Materials and methods

2.1 Bacterial strains

The bacterial strains used in this study are given in Table 2.

2.2 Chemicals

Chemicals used in this study together with their source are given in Table 3.

2.3 Media

All media was prepared using double distilled water and unless otherwise stated sterilised by autoclaving at 121°C for 15 to 20 minutes. A list of media used, together with their constituents can be found in Tables 4 and 4a. Antifungal and antibiotic additions to these media are listed with the concentrations used in Table 5.

2.4 Buffers and reagents

Buffers and reagents were prepared using double distilled water and unless stated otherwise were sterilized by autoclaving at 121°C for 15 minutes. Buffers, reagents and solutions together with their ingredients are given in Table 6.

Table 2

Source and designation of streptomycetes used in this study

Strain	Received as	Strain Source
ATCC 49173	*S.scabies* sp. nov., nom. rev.	Lambert & Loria (1989)
ATCC 3352	*Streptomyces* sp.	Novo Nordisk Industries, Denmark
ATCC 10246	*Streptomyces* sp.	Novo Nordisk Industries, Denmark
ATCC 15485	*Streptomyces* sp.	Novo Nordisk Industries, Denmark
ISP 5078	*Streptomyces* sp.	ATCC 23962 (IMRU 3018)
S46	pathogenic isolate	D Lapwood, Rothamsted Exp. Station, UK
S47	pathogenic isolate	D Lapwood, Rothamsted Exp. Station, UK
8.2	pathogenic isolate	G Jellis, PBI[1] Cambridge UK
8.6	pathogenic isolate	G Jellis, PBI[1] Cambridge UK
8.7	pathogenic isolate	G Jellis, PBI[1] Cambridge UK
8.8	pathogenic isolate	G Jellis, PBI[1] Cambridge UK
8.16	pathogenic isolate	G Jellis, PBI[1] Cambridge UK
8.17	pathogenic isolate	G Jellis, PBI[1] Cambridge UK
ASS8112	pathogenic isolate	Novo Nordisk Industries, Denmark
ISS	pathogenic isolate	Novo Nordisk Industries, Denmark
RA210	pathogenic isolate	Novo Nordisk Industries, Denmark
PD259	pathogenic isolate	J D Janse, PPS[2] Wageningen, NL
PD260	pathogenic isolate	J D Janse, PPS[2] Wageningen, NL
1028	pathogenic isolate	J D Janse, PPS[2] Wageningen, NL
1033	pathogenic isolate	J D Janse, PPS[2] Wageningen, NL
1034	pathogenic isolate	J D Janse, PPS[2] Wageningen, NL
MP2	scab isolate	E M Wellington, Warwick University, UK
MP9	scab isolate	E M Wellington, Warwick University, UK
R1	scab isolate	E M Wellington, Warwick University, UK
R2	scab isolate	E M Wellington, Warwick University, UK
0446	*S.albidoflavus* (1)	KCI-S-0446
598	*S.bacillaris* (1)	ATCC 15855
326	*S.alboviridis* (1)	ATCC 25425

Table 2 (cont)

Source and designation of streptomycetes used in this study

Strain	Received as	Strain Source
236	*S.griseus* (1)	ATCC 23345
ATCC 25481	*S.albidoflavus* (1)	E M Wellington, Warwick University, UK
DSM 40023	*S.albidoflavus* (1)	E M Wellington, Warwick University, UK
632	*S.albidoflavus* (1)	E M Wellington, Warwick University, UK
232	*S.albidoflavus* (1)	E M Wellington, Warwick University, UK
023	*S.nitrosporeus* (1)	E M Wellington, Warwick University, UK
077	*S.nitgersensis* (1)	ATCC 12769
508	*S.nogaensis* (1)	DSM 40508
0233	*S.ceolicolor* (1)	ATCC 23899
422	*S.coeliatus* (1)	E M Wellington, Warwick University, UK
ATCC 27417	*S.annulatus* (1)	E M Wellington, Warwick University, UK
734a	*Streptomyces sp.* (1)	E M Wellington, Warwick University, UK
c463	*Streptomyces sp.* (1)	E M Wellington, Warwick University, UK
454	*S.viridogenes* (3)	ISP 5454, ATCC 3372
962	*S.atroolivaceus* (3)	E M Wellington, Warwick University, UK
968	*S.atroolivaceus* (3)	E M Wellington, Warwick University, UK
978	*S.atroolivaceus* (3)	E M Wellington, Warwick University, UK
975.2	*S.atroolivaceus* (3)	E M Wellington, Warwick University, UK
4.470	*S.atroolivaceus* (3)	E M Wellington, Warwick University, UK
282RB	*S.atroolivaceus* (3)	E M Wellington, Warwick University, UK
135RB	*S.atroolivaceus* (3)	E M Wellington, Warwick University, UK
164	*S.litmocidene* (5)	E M Wellington, Warwick University, UK
727	*S.exfoliatus* (5)	E M Wellington, Warwick University, UK
082	*S.violaceus* (6)	E M Wellington, Warwick University, UK
593	*S.fulvissimus* (10)	E M Wellington, Warwick University, UK
092	*S.alithoticus* (12)	E M Wellington, Warwick University, UK
TK24	*S.lividans* (21)	JI[3] Institute, Norwich, UK
054	*S.fascilutus* (29)	ATCC 19751

Table 2 (cont)

Source and designation of streptomycetes used in this study

Strain	Received as	Strain Source
558	*S.colombiensis* (61)	ATCC 27425
069	*S.lavendulae* (61)	DSM 40069
316	*S.polychromogenes* (61)	ATCC 12595
550	*S.katrae* (61)	E M Wellington, Warwick University, UK
194	*S.sacemochromogenes* (61)	E M Wellington, Warwick University, UK
445	*S.subtilis* (61)	ATCC 27467
216	*S.lavendulocolor* (61)	E M Wellington, Warwick University, UK
0767	*Stv.griseoverticillium* (55)	ATCC 27436
0435	*Stv. cinnamoneum subsp. azacoleta* (55)	DPDU 0074
0547	*Stv.septatum* (55)	E M Wellington, Warwick University, UK
051	*Stv.albireticuli* (55)	ATCC 19721
ATCC 23934	*Streptoverticillium sp.* (55)	E M Wellington, Warwick University, UK
DH5α	*E.coli*	R Spooner, ICRF, London, UK

[1] PBI - Plant Breeding International

[2] PPS - Plant Protection Service

[3] JI - John Innes Institute

Table 3

Chemicals used in this study

Chemical	Supplier
Acetone	Merck, Sharp and Dome Ltd.
Adonitol	Sigma
Agar no.1	Oxoid
Agarose type II medium EEO	Sigma
Allantoin	Sigma
α-aminobutyric acid	Sigma
Ammonium molydate 'analar'	BDH[1]
Ammonium sulphate	BDH[1]
Ampicillin	Sigma
Arbutin	Sigma
L-arginine	Sigma
L-asparagine	Sigma
Aurintricarboxylic acid	Sigma
Bactotryptone	Difco
Bavistin	BASF PLC, Ipswich
Boric acid	Rhone Poulenc
Bovine serum albumin (BSA) fraction V	Sigma
Bromophenol blue	Sigma
Caesium chloride	BDH[1]
Calcium carbonate	Fisons
Calcium chloride	BDH[1]
Casamino acids	Difco
Catechol	Sigma
Cellobiose	Sigma
Chitin (crab shell)	Sigma

Table 3 (cont)

Chemicals used in this study

Chemical	Supplier
Copper chloride	Fisons
Cyclohexamide	Sigma
Diethylpyrocarbonate DEPC	Sigma
Dipotassium phosphate	BDH[1]
Dithiothreitol DTT	Sigma
DMSO	FSA
DNA (Herring sperm)	Sigma
DNase	Sigma
Egg yolk emulsion	Sigma
Ethanol	Hayman Ltd.
Ethidium bromide	Sigma
Ferric ammonium citrate	BDH[1]
Ficoll 400	Sigma
D-Fructose	Sigma
D-Glucose	BDH[1]
Glutaraldehyde	BDH[1]
Glycerol	BDH[1]
Glycine	BDH[1]
Glycogen	Sigma
Giess-Illosray Reagent I and II	BDH[1]
Guanidine	Sigma
Hexadecyltrimethyl ammonium bromide	Sigma
Hydrochloric acid	Fisons
8-Hydroxyproline	BDH[1]
Inulin	Sigma

Table 3 (cont)

Chemicals used in this study

Chemical	Supplier
Isoamylalcohol	Sigma
Isopropanol	BDH[1]
Kanamycin	Sigma
Lab lemco	Oxoid
Lead acetate	BDH[1]
Lithium chloride	Sigma
Lithium dodecylsulphate	Sigma
Lysozyme (egg white)	Sigma
Magnesium chloride	BDH[1]
Magnesium sulphate	BDH[1]
Malt extract	Oxoid
Manganese chloride	BDH[1]
Mesoinositol	Sigma
Methanol	FSA
Murashige and Skoog medium	Flow Labs
Neomycin sulphate	Sigma
Nonidet P40	BDH[1]
Nutrient agar	Oxoid
Nutrient broth	Difco
Nystatin	Sigma
NZ amine-A	Sheffield Products, Morham Ltd.
Paraformaldehyde	Fisons
Pectin (from citrus fruits)	Sigma
Peptone	Difco
Phenol (chromatography grade)	BDH[1]

Table 3 (cont)

Chemicals used in this study

Chemical	Supplier
Phenol (analar)	BDH[1]
Phenol red	BDH[1]
Polyethylene glycol 1,000	BDH[1]
Polyethylene glycol 6,000	BDH[1]
Potassium acetate	BDH[1]
Potassium dihydrogen phosphate	BDH[1]
Potassium nitrate	BDH[1]
Potassium sulphate	BDH[1]
Protease Peptone	Difco
D-Raffinose	Sigma
L-Rhamnose	Sigma
Rifampicin	Sigma
RNase I	Sigma
Sodium acetate	BDH[1]
Sodium azide	BDH[1]
Sodium borate	BDH[1]
Sodium chloride	BDH[1]
Sodium citrate	BDH[1]
Sodium deoxycholate	Fisons
Sodium dihydrogen phosphate	BDH[1]
Sodium dodecylsulphate	BDH[1]
Sodium EDTA	BDH[1]
Sodium hydrogen carbonate	BDH[1]
Sodium hydroxide	BDH[1]
Sodium perchlorate	BDH[1]

Table 3 (cont)

Chemicals used in this study

Chemical	Supplier
Sodium thiosulphate	Fisons
Starch (soluble)	BDH[1]
Storite	Merck, Sharp and Dhome (MSD)
Ltd.	
Streptomycin sulphate	Sigma
Sucrose	BDH[1]
TES buffer	BDH[1]
Thiostrepton	Sigma
Thiourea	BDH[1]
Tris base	BDH[1]
Triton X-100	Sigma
Tryptone soya broth	Oxoid
Xanthine	Sigma
D-Xylose	Sigma
Yeast extract	Oxoid
Zinc chloride	Fisons
Zinc dust	May and Baker[2]

note

[1]BDH are now Merck Ltd. and [2]May and Baker are now Rhone Poulenc

Table 4: Media

Medium	Constituents (per litre unless indicated otherwise)
YT	Bactotryptone 0.8% (w/v)
	Yeast extract 0.5% (w/v)
	NaCl 0.5% (w/v)
SOB	Bactotryptone 2% (w/v)
	Bacto yeast extract 0.5% (w/v)
	NaCl 10 mM
	KCl 2.5 mM
	After autoclaving and prior to use add:
	$MgCl_2$ 10 mM
	$MgSO_4$ 10 mM
L-Broth	Bactotryptone 10 g
	Yeast extract 5 g
	NaCl 5 g
	Glucose 1 g
	Agar (if solid media) 20 g
R5 (RY2E alternative method)	Sucrose 103 g
	K_2SO_4 0.25 g
	$MgCl_2.6H_2O$ 10.1 g
	Casamino acids 0.1 g
	Yeast extract 5 g
	TES buffer 5.73 g
	Agar 22 g
	Trace element solution 2 ml

Table 4: Media (cont)

Medium	Constituents (per litre unless indicated otherwise)
Trace element solution	$ZnCl_2$ 40 mg
	$FeCL_3.6H_2O$ 200 mg
	$CuCl_2.2H_2O$ 10 mg
	$MnCl_2.4H_2O$ 10 mg
	$Na_2B_4O_7.10H_2O$ 10 mg
	$(NH_4)6Mo_7O_2.4H_2O)$ 10 mg
R2 Modified soft nutrient agar overlay (Matsushima and Baltz)	Sucrose 103 g
	$MgCl_2.6H_2O$ 10.12 g
	$CaCl._2H_2O$ (22.2g/L) 100 ml
	0.25M TES (pH 7.2) 100 ml
	Agar 4.10 g
Oatmeal agar (The oats were boiled for aprox. 1 h prior to autoclaving).	Ground oats 20 g
	Yeast extract 1 g
	Agar 15 g
Reduced arginine, starch, salts agar (RASS)	L-Arginine 0.1 g
	Starch (soluble) 12.5 g
	K_2HPO_4 1 g
	NaCl 1 g
	$MgSO_4.7H_2O$ 0.5 g
	Agar 15 g

Table 4: Media (cont)

Medium	Constituents (per litre unless indicated otherwise)
Soft nutrient agar (SNA)	Nutrient broth 8 g Agar 3 g
Tryptone soya broth (TSB)	Tryptone soya broth 30 g Sucrose 100 g Polyethylene glycol 600050 g (Optional dependent on strain)
Yeast Extract-Malt Extract Medium (YEME)	Yeast Extract 3 g Bacto-peptone 5 g Malt extract 3 g Glucose 10 g Sucrose 340 g After autoclaving add: $MgCl_2$ (2.5 M) 2 ml For preparation of protoplasts add: Glycine 20% (w/v) 25 ml
Potato Propagation Mediuim (pH 5.6)	Murashige and Skoog medium Sucrose 3% (w/v) Agar 0.6% (w/v)
Potato Tuberisation Medium (pH 5.6)	Murashige and Skoog medium Sucrose 8% (w/v) Agar 0.6% (w/v)

Table 4a: Media for Identification Tests

ISP 4 Medium

Solution 1	Soluble starch 10 g
	Distilled water 500 ml
Solution 2	K_2HPO_4 1 g
(pH 7.0)	NaCl 1 g
	$(NH_4)_2SO_4$ 2 g
	$CaCO_3$ 2 g
	Agar 20 g
	Distilled water 500 ml

Solutions 1 and 2 were mixed together prior to autoclaving. The agar was dispensed into 9 cm Sterilin petri dishes.

Glycerol Asparagine Agar

(pH 7.0 to 7.4)

L-Asparagine 1 g

Glycerol 10 g

K_2HPO_4 1 g

Agar 20 g

The agar was dispensed into 9 cm petri dishes.

Peptone Yeast Extract Agar

Bactopeptone 15 g

Protease peptone 5 g

Table 4a: Media for Identification Tests (cont)

Ferric ammonium citrate 0.5 g

Dipotassium phosphate 1 g

Sodium trithiosulphate 0.08 g

Yeast extract 1 g

Agar 15 g

The agar was dispensed as slopes in test tubes.

Basal nitrogen source medium

(pH 7.4)

D-glucose 10 g

$MgSO_4.7H_20$ 0.5 g

NaCl 0.5 g

$FeSO_4.7H_2O$ 0.01 g

N-source 1.0 g

Agar 12.0 g

The following test nitrogen sources were added to a final concentration of 1% (w/v): α-aminobutyric acid, L-histidine, and L-hydroxyproline. The positive control was L-asparagine and the negative control no nitrogen source. Agar was dispensed into Sterilin repli dishes.

Egg yolk medium

(pH 7.0)

Peptone 10 g

Yeast extract 5 g

Glucose 1 g

NaCl 10 g

Agar 12 g

Table 4a: Media for Identification Tests (cont)

Egg yolk emulsion 5%

The egg yolk emulsion was added after autoclaving and the agar dispensed into 9 cm Sterilin petri dishes.

Pectin agar
(pH 7.4)

Solution 1.
KH_2PO_4 4 g
Na_2HPO_4 6 g
Distilled water 400 ml

Solution 2.
$(NH_4)_2SO_4$ 2 g
$FeSO_4.7H_2O$ 0.001 g
$MgSO_4.7H_2O$ 0.2 g
Yeast extract 1.0 g
Agar 10 g
Distilled water 400 ml

Solution 3.
Pectin 5 g
Distilled water 200 ml

Solutions 1,2 and 3 were mixed together after autoclaving.

Nitrate Agar
(pH 7.0)
Nutrient Broth 1 L
KNO_3 2 g
Agar 6 g

The agar was dispensed as slopes in test tubes.

Arbutin Agar
Yeast Extract 3 g

Table 4a: Media for Identification Tests (cont)

(pH 7.2)	Arbutin 1 g
	Ferric ammonium citrate 0.5 g
	Agar 7.5 g

The agar was dispensed as slopes in test tubes, negative controls contained no arbutin.

Allantoin agar	KH_2PO_4 9.1 g
(pH 6.8)	$NaHPO_4$ 9.5 g
	Yeast extract 0.1 g
	Allantoin 3.3 g
	Phenol red 0.01 g
	Agar 7.5 g

The agar was dispensed as slopes in test tubes, negative controls contained no allantoin.

Xanthine agar	Yeast extract 1 g
(pH 7.0)	Lab-lemco 0.8 g
	Glycerol 10 g
	NZ amine-A 2 g
	Xanthine 4g
	Agar 15 g

The agar was dispensed into 9 cm Sterilin petri dishes ensuring an even distribution of xanthine.

Nutrient Agar	Nutrient agar 23 g
	Glucose 5 g

Table 4a: Media for Identification Tests (cont)

The agar was dispensed into 9 cm glass petri dishes.

Soft nutient agar overlay

Nutrient agar 16 g

Basal carbon source	$(NH_4)_2SO_4$ 2.64 g
agar (pH 6.8 to 7.0)	KH_2PO_4 2.38 g
	$K_2HPO_4.3H_2O$ 5.65 g
	$MgSO_4.7H_20$ 1 g
	Agar 15 g

The following carbon sources were filter sterilised and added to a final concentration of 1% after autoclaving: adonitol, cellobiose, L-rhamnose, D-raffinose, meso-inositol, D-mannitol, D-xylose, D-fructose and inulin. The positive control was glucose and the negative control no carbon source. The agar was dispensed into Sterilin repli dishes.

Bennet's agar	Yeast extract 1 g
(pH 7.3)	Lab lemco 0.8 g
	Glycerol 10 g
	N-Z amine-A 2 g
	Agar 15 g

Modified Bennet's agar

Phenol agar	Bennet's agar with 0.1% Phenol
NaAzide agar	Bennet's agar with 0.01% NaAzide

Table 4a: Media for Identification Tests (cont)

NaCl agar	Bennet's agar with 7% NaCl

The agar was dispensed into 9 cm Sterilin petri dishes.

Table 5: Antibiotic and antifungal stocks

Antibiotic	Stock solution	Final concentration in media		
	(mg ml-1)		(μg ml-1)	
		Minimal	Complex	Liquid
Ampicillin[1]	100	100	100	-
Bavistin[2]	50	50	50	-
Cyclohexamide[3]	50	50	50	50
Kanamycin sulphate[1]	50	50	50	50
Neomycin sulphate[1]	10	1	10	1
Nystatin[4]	50	50	50	50
Penicillin G[1]	100	100	100	-
Rifampicin[5]	10	10	10	-
Storite[2]	50	50	50	-
Streptomycin sulphate[1]	50	10	50	-
Thiostrepton[6]	50	20	50	20

[1] H_2O soluble and filter sterilised

[2] Taken up in DMF or DMSO

[3] Autoclaved to go in solution

[4] Dissolved in NaOH and made up in SDW - pH readjusted

[5] Rifampicin taken up in methanol

[6] Thiostrepton taken up in DMSO, DMF or chloroform

Table 6: Reagents and Solutions

Reagent	Constituents	Reference
Alkaline SDS	SDS 2 g 0.3 M NaOH 100 ml	Hopwood *et al.* (1985)
Catechol solution	100 mM catechol in SDW	
Cell Fixation buffer	2 g Paraformaldehyde dissolved in 30 ml SDW and 100 µl 2 M NaOH heated to 55 to 60°C, add 5 ml 10 times PBS and adjust to pH 7.2 to 7.4. Final volume of 50 ml made up with SDW.	
Chloroform Solution	24:1 ratio of chloroform to isoamylalcohol	
Chloroform saturated with TE buffer	TE buffer over chloroform solution.	
Darbyshires solution	pH 8.0 Tris-HCl washed phenol chloroform solution stored under TE buffer.	
DEPC treated H_2O	1000 ml distilled water 1 ml Diethylpyrocarbonate (DEPC) DEPC allowed to dissolve by stirring overnight at 30°C, followed by autoclaving.	

Table 6: Reagents and Solutions (cont)

Reagent	Constituents	Reference
GOS Extraction buffer	200 mM Tris-HCl pH 8.5, 1.5% Lithium Dodecyl Sulphate, 300 mM Lithium Chloride 10 mM Sodium EDTA, 1% w/v Na Deoxycholate, 1% w/v NP-40, 5 mM Thiourea, 1 mM Aurintricarboxylic acid, 10 mM Dithiothreitol	
Glycogen (dissolved by warming and phenol chloroform extracted)	20 mg ml^{-1}	
Guanidine HCl	7.5 M Guanidine 1.0 M Tris-HCl pH 7.0	Hahn *et al.* (1990a)
Hexadecyltrimethyl ammonium bromide (used warmed)	1% (w/v) solution	
Hybridization buffer (*in situ*) autoclaved and stored at -20°C	0.9 M NaCl 20 mM Tris-HCl 0.1% SDS	

Table 6: Reagents and Solutions (cont)

Reagent	Constituents	Reference
10 times Ligation Buffer	70 mM Tris-HCl pH 7.4 7 mM $MgCl_2$ 0.2 mM ATP	
Lysozyme solution	2 mg/ ml dissolved in:	
For Protoplasting:	P. buffer	Hopwood *et al.* (1985)
For plasmid prep.s:	SET	Hopwood *et al.* (1985)
For chromosomal DNA preps.:	TE buffer	Hopwood *et al.* (1985)
PBS buffer (5 times)	650 mM NaCl 50 mM Sodium phosphate buffer (pH 7.2)	
PEG 1000 (Freshly made each time)	25% PEG 1000 dissolved and filter sterilised in P. buffer.	Hopwood *et al.* (1985)
Phenol solution (neutral) Allow to separate into 2 layers. Test pH of upper aqueous level, continue to wash with Tris-HCl until neutral.	150 g Phenol, 200 ml 1 M Tris-HCl pH 7.0 0.1% Hydroxyquinoline.	R. Spooner pers com.
Phenol solution (acid)	500 g melted phenol, 0.5 g hydroxyquinoline 65 ml TE buffer with 1 M NaCl	Hopwood *et al.* (1985)

Table 6: Reagents and Solutions (cont)

Reagent	Constituents	Reference
Phenol Chloroform	Mix equal volumes of Phenol solution with Chloroform.	Maniatis *et al.*(1982)
Plant Propagation Medium (pH 5.6)	Murashige and Skoog medium Sucrose 3% Oxoid agar no. 1 0.6%	
Potato Tuberisation Medium	Murashige and Skoog medium. Sucrose 8% Oxoid agar no. 1 0.6%	P.Dale pers.comm.
Prehybridisation Solution	SDS 7% 0.5 M PO_4 1% BSA 1 mM NaEDTA 10 mg ml^{-1} non-homologous DNA	Church & Gilbert (1984).
Prewashing and filter stipping solution Wash at 80°C for 15 minutes with 2 to 3 changes of solution.	0.1% SSC 0.1% SDS	D.Hahn, pers comm.
P. buffer (Made fresh at each time of use)	Sucrose 103 g K_2SO_4 0.25 g $MgCl_2.6\ H_2O$ 2.02 g Trace element 2 ml	Hopwood *et al.* (1985)

Table 6: Reagents and Solutions (cont)

Reagent	Constituents	Reference
	solution.	
	Distilled water to	
	800 ml	
	After autoclaving and prior to use add in order:	
	KH_2PO_4 (0.5%) 1 ml	
	$CaCl_2$ (3.68%) 10 ml	
	TES buffer (5.73%) 10 ml	
RF1	100 mM RbCl	
	50 mM $MnCl_2.4\ H_2O$	
	30 mM Potassium acetate	
	10 mM $CaCl_2$	
	15% w/v glycerol	
	adjust to pH 5.8 with 0.2 M acetic acid, filter through	
	prerinsed (with SDW) 0.22 µm membrane.	
RF2	10 mM MOPS	
	10 mM $RbCl_2$	
	75 mM $CaCl_2$	
	15% glycerol	
	Adjust to pH 6.8 with NaOH, filter through prerinsed	
	(with SDW) 0.22 µm membrane.	
SET	20% Sucrose	Maniatis *et al.*(1982)
	100 mM Tris-HCl pH 8.0	
	50 mM Na_2 EDTA	

Table 6: Reagents and Solutions (cont)

Reagent	Constituents	Reference
20 times SSC	NaCl 175.3 g Sodium citrate 88.23 g	Hopwood *et al.* (1985)
1/4 Strength Ringers solution	NaCl 2.25 g KCl 0.105 g $CaCl_2$ 0.12 g $NaHCO_3$ 0.05 g	
10 times Stop (loading buffer)	15% Ficoll 400 1 mM Na_2EDTA, 0.25% Bromophenol blue	Maniatis *et al.* (1982)
TBE (10 times, 1 Litre)	108 g Tris base 55 g Boric acid 40 ml 0.5 M EDTA pH 8.0	Maniatis *et al.* (1982)
TE buffer	2 M Tris-HCl pH 8.0 5 ml 0.25 M Na EDTA pH 8. 4 ml	Maniatis *et al.* (1982)
Washing Buffer		
1	0.08 M PO_4 1% PO_4	Church & Gilbert (1984)
2	0.04 M PO_4 1% SDS	""
3	1.0 M PO_4	""

2.5 Growth and maintenance of *Streptomyces* strains

Streptomyces strains were grown on plates of oatmeal or R5 agar (Hopwood *et al.*, 1985) at 30°C. Strains were stored on plates at 4°C and subcultured at approx. 3 month intervals. Suspensions of spore and mycelial material were prepared by scraping the surface of a sporulating culture flooded with 10 ml 20% (w/v) glycerol. The glycerol suspension was recovered and stored at -20°C for long term preservation. Liquid cultures of streptomycete strains were grown in 30 to 40 ml either in TSB with 10% (w/v) sucrose (and depending on the strain 50 g L^{-1} polyethelene glycol 6000) or in YEME, together with appropriate antibiotic additives in 250 ml baffled flasks (fitted with springs) on a Gallenkamp Orbital Shaker (180 to 200 r.p.m.) at 30°C for 48 to 72 hours.

2.6 Preparation of spore suspensions as a source of inoculum

Spore suspensions were prepared as above, however the spores were separated from mycelial fragments by passing the suspension through a syringe fitted with a cotton wool filter. The spores were concentrated by sedimentation (3000 r.p.m. for 20 minutes in an MSE bench top centrifuge) and the pellet drained and resuspended in 5 ml of the diluent (1/4 strength Ringers solution).

2.7 Enumeration of spore suspensions

2.7.1. Direct counts

Spore suspensions were counted using a haemocytometer (0.1 mm 1/400 mm depth counting chamber, Weber Scientific International Ltd.) under phase contrast on a light microscope (Standard 14, Zeiss, West Germany).

2.7.2. Viable counts

Dilutions of a spore suspension were plated out on a suitable medium such as R5, in triplicate. Dilution plates with 30 to 300 colonies were counted.

2.8 Preparation of *Streptomyces* protoplasts

50 ml Broth cultures of *Streptomyces scabies* ISP 5078 were grown for 48 hours in YEME medium (containing 34% (w/v) sucrose, 0.005 M $MgCl_2$ and 0.5% glycine) (Hopwood *et al.*, 1985). 2 times 20 ml volumes of the broth cultures were spun down for 20 minutes at 3,000 r.p.m. in Sterilin universals and the pellets taken up in 10 ml 20% (w/v) glycerol for long term storage at -20°C. 1 ml aliquots of the mycelial paste were transferred to Eppendorf tubes and spun at 6,500 r.p.m. for 5 minutes (in a MSE Microcentar microfuge). The supernatants were discarded and the pellets resuspended in 1 ml 10% (w/v) sucrose. The mycelial suspension was respun at 6,500 r.p.m. for 5 minutes (in a MSE Microcentaur microfuge) and the pellet resuspended in 1 ml lysozyme solution and incubated at 30°C. After 15 minutes the mycelial suspension was trituated with a pipette and reincubated. After a further 15 minutes the suspension was trituated again with a pipette and then diluted with 0.5 ml P.buffer. Protoplast formation was detectable when the heavier mycelial colonies that gravitate to the bottom of the Eppendorf changed to a cloudy suspension. At regular intervals the cells were viewed in phase contrast under the light microscope (Standard 14, Zeiss, West Germany). When the vast majority of cells in a field of view appeared to be phase dark protoplasts, the suspension was passed through a pasteur pipette containing glass wool into a fresh Eppendorf tube. The protoplasts were gently sedimented (for 2 minutes at 6,500 r.p.m. in a MSE Microcentaur microfuge), the supernatant discarded and the protoplasts resuspended and washed in 1 ml P.buffer. The protoplasts were counted at 10 or 100 fold dilutions using a

haemocytometer slide (0.1 mm, 1/400 mm depth counting chamber, Weber International Ltd.). At this point the protoplast suspensions were either frozen slowly by placing the tubes in a beaker of ice at -70°C overnight, followed by separation from the ice and storage at -70°C (Hopwood *et al.*, 1985). Alternatively protoplasts were used directly in PEG mediated transformations (Section 2.9).

2.9 PEG mediated transformation of protoplasts

Transformations were performed according to the methods of Hopwood *et al.*, (1985). When frozen protoplasts were used, they were spun (3000 r.p.m. for 7 minutes in a MSE MISTRAL bench top centrifuge) and washed by resuspending in fresh P.buffer. The resulting 1 ml suspension of protoplasts was respun, the supernatant poured off and the pellet resuspended in the remaining drop (or 50 µl) of P.buffer. 1 to 2 µl of plasmid DNA was added and mixed into the tube followed immediately by 200 µl PEG1000 in P. buffer. The suspension was pipetted 4 to 5 times immediately after the addition of The DNA and PEG. The transformed protoplast mixture was spread onto dried R5 plates (plates were dried so that the surface of the agar was no longer smooth but dried and wrinkled). Plates were incubated at 30°C overnight. After 14 to 24 hs the plates were overlayed with SNA or a modified R5 overlay containing selective antibiotics at 10 to 20% (w/v) of the final concentrations recommended by Hopwood *et al.* (1985).

2.10 Small scale plasmid isolation from *Streptomyces*

Small scale plasmid isolation by alkaline lysis was performed according to Hopwood *et al.* (1985). 1.0 ml of a TSB or YEME grown broth culture under selection for the plasmid of interest was transferred to an Eppendorf tube. The tube was spun (at 6,500 r.p.m. on a MSE Microcentaur microfuge) for 3

minutes, the supernatant removed and the cell pellet resuspended in lysozyme solution made up in SET. The tubes were incubated at 37°C for 30 minutes or until lysis occurred. After incubation the cells were mixed gently and 250 µl of 0.3 M alkaline SDS solution was added followed immediately by 1 minute of harsh vortexing. The cell lysis mixture was then placed in a water bath at 55°C for 30 minutes and the water bath allowed to cool to room temperature. 80 µl of acid phenol chloroform was added to the cell lysates and complete mixing of the phases achieved by vortexing. The lysates were spun (at high on a MSE Microcentaur microfuge) for 5 minutes, the upper phase removed and rextracted once with neutral phenol chloroform and once with chloroform isoamylalcohol. The aqueous phase was then transferred to a fresh eppendorf containing 70 µl 3 M unbuffered sodium acetate and 700 µl isopropanol. The tubes were mixed by inversion and placed at -20°C for an hour. The plasmid DNA pellet was recovered by centrifugation (at high speed on a MSE Microcentaur microfuge) for 5 minutes, drained, washed with 70% (v/v) ice cold ethanol, respun, drained and dried under vacuum. The remaining pellet was resuspended in 100 µl sterile distilled water. Plasmid preparations were visualised on a 1% (w/v) agarose gel and their identity confirmed by restriction digestion.

2.11 Chromosomal DNA preparation

Chromosomal DNA preparations suitable for cloning were prepared from *Streptomyces* scabies ISP 5078 according to Hopwood *et al.* (1985), modified by Derek Hood (Derek Hood, pers comm.).

One gram of wet weight mycelium was placed in a Sterilin universal and suspended in 5 ml of 3 to 4 mg ml^{-1} lysozyme solution and placed at 37°C for 10 to 20 minutes. A freshly made 10% (w/v) SDS solution was added to the

cell lysis mixture to a final concentration of 1% (w/v) and incubated in a water bath at 65°C for 10 minutes. The water bath was allowed to cool to room temperature. A 0.2 volume of 5 M sodium perchlorate was added to the lysed solution and mixed thoroughly. The lysed material was then extracted twice with an equal volume of acid phenol choloroform and twice with chloroform isoamylalcohol. The universals containing the cell lysate phenol chloroform extractions were repeatedly inverted by hand for 10 to 15 minutes (to prevent the DNA shearing), followed by 10 minutes centrifugation (at 3,000 r.p.m.in a MSE MISTRAL 1,000 benchtop centrifuge). The DNA in the aqueous phase was precipitated with 2.5 volumes of ethanol and a 0.1 volume of 3 M sodium acetate. The precipitated DNA was spooled onto a glass rod, allowed to air dry and the rod transferred to a fresh universal containing 1 to 2 ml TE buffer where it was allowed to stand for 30 seconds to allow the DNA to dissolve. RNase was added to a final concentration of 50 $\mu g\ ml^{-1}$ and the DNA solution placed at 37°C for 30 minutes. Finally, the DNA solution was reextracted once with neutral phenol chloroform and 2 to 3 times with chloroform isoamylalcohol. The DNA was precipitated at room temperature with a 0.25 volume of 5 M NaCl and 2 volumes of ethanol. The DNA was spooled onto a glass rod, the rod dipped in ice cold 70% (v/v) ethanol and air dried before being resuspended in 1 ml of TE buffer.

2.12 Growth and maintenance of *E.coli*.

E.coli DH5α was streaked out onto LB agar plates and incubated at 37°C overnight. Plates were stored at 4°C for up to a month before further subculturing. Overnight broth cultures of *E.coli* were grown up in 5 ml of LB in Sterilin universals and shaken at 37°C on a Gallenkamp orbital shaker (180 to 200 r.p.m.) for 6 to 18 hours.

2.13 Small scale preparation of plasmid DNA from *E.coli.*

The following method was obtained as a personal communication from R.Spooner, ICRF, London. Cells from a 5 ml overnight culture were harvested at room temperature (at 3,000 r.p.m. in a MSE MISTRAL 1000 bench top centrifuge). The pellet was recovered, resuspended in 180 µl SET and the cell suspension transferred to a 1.5 ml Eppendorf tube. 180 µl of 4 mg ml^{-1} lysozyme solution in SET was added followed by 300 µl of 10% (w/v) Triton X-100 and the contents of the tube mixed completely. The lysing cells were then placed in a boiling water bath for 1 minute, followed by immediate chilling on ice for 1 minute. High molecular weight DNA, RNA and proteins were pelleted (13,000 r.p.m. on a MSE Microcentaur microfuge for 10 to 30 minutes). 300 µl 7.5 M ammonium acetate was added to the recovered supernatant and the samples placed on ice for 20 minutes. Protein debris was removed by centrifugation (maximum speed for 10 minutes on a [MSE Microcentaur microfuge]). The supernatant was recovered, a 0.7 volume of isopropanol added and the sample placed at -20°C for 10 minutes. The nucleic acids were sedimented (maximum speed for 10 minutes [on a MSE Microcentaur microfuge]), the pellet drained, washed with 70% (v/v) ethanol and resuspended in 60 µl TE buffer and 1 µl 10 mg ml^{-1} RNase solution. 5 µl aliquots were used for restriction digestions.

2.14 Large scale preparation of plasmid DNA from *E.coli.*

Suitable *E.coli* strains were grown up overnight in 400 ml LB media with antibiotics selective for the plasmid of interest. The broth culture was transferred to 500 ml Nalgene centrifuge pots and spun (at 5,000 r.p.m. for 10 minutes [in a JA10 rotor on a Beckman model J2-21 centrifuge]). The cells were drained and the cellular pellet resuspended in 7.2 ml of SET. A further 7.2 ml of ice cold 4 mg ml^{-1} lysozyme dissolved in SET was added, followed

by 12 ml of Triton X-100. After mixing, the flask was immediately swirled over the hottest part of a bunsen flame, until the cells appeared yellow, grey, and glutinous. The flask was placed in a boiling water bath for about 30 seconds and then transferred to ice. The lysate was transferred to Oakridge tubes and spun at 20,000 r.p.m. for 30 minutes at 4°C (in a JA20 rotor on a Beckman model J2-21 centrifuge). The supernatant was recovered and transferred to fresh Oakridge tubes, a 0.5 volume of 7.5 M ammonium acetate was added and the tubes placed on ice for 20 minutes to allow precipitation of the proteins. The tubes were spun at 15,000 r.p.m. for 10 minutes at 4°C (in a JA20 rotor on a Beckman model J2-21) and the supernatant recovered. The volume of the supernatant was determined and a 0.7 volume of isopropanol added. The DNA was allowed to precipitate at -20°C for 10 minutes. The DNA was sedimented at 15,000 r.p.m. (in a JA20 rotor on a Beckman model J3-21), the pellet dried under vacuum and resuspended in 4 ml TE buffer. 4.3 g CsCl was added to the DNA solution together with 0.5 ml 5 mg /ml ethidium bromide and the solution transferred to Beckman quick-seal tubes. The tubes are heat sealed and spun in a (VTI 65 rotor on a Beckman L8 Ultracentrifuge) for 18 h at 52,000 r.p.m. The gradient was examined under long wave ultraviolet light and the bands of interest identified. The gradient was secured on a clamp and retort stand, while a syringe needle was used to puncture the top of the tube. The band of interest was recovered by piercing the bottom of the tube and allowing the contents of the tube to pass out slowly. The fraction of interest was recovered in a separate Eppendorf tube. The plasmid DNA appeared as the lower of two bands, the upper band being linear plasmid and chromosomal DNA. The plasmid DNA was cleaned by the addition of an equal volume of isopropanol over TE saturated with CsCl, vigorous shaking and spinning for several minutes (low speed, in MSE Microcentaur microfuge). The lower phase was retained and the isopropanol extraction

repeated until the interphase was clean and the ethidium bromide removed. The volume of DNA was adjusted to 1 ml. The CsCl was diluted with 3 volumes of TE, followed by a 0.1 volume 3 M sodium acetate pH 5.4 and 2 volumes of ethanol. The tube was placed at -20°C for 2 h to precipitate the DNA. The tubes were spun at 15,000 r.p.m. for 10 minutes at 4°C (in a JA20 rotor on a Beckman J2-21 centrifuge). The DNA pellet was dried under vacuum and resuspended in 400 µl TE buffer.

2.15 Quantification of DNA and RNA from pure cultures

A known volume of DNA or RNA was made up to 1 ml with sterile distilled water and placed in a quartz cuvette. Readings were taken against sterile distilled water at 260 nm and 280 nm (on a PU 8720 UV/ VIS scanning spectrophotometer). An OD260 of 1 is equivalent to 50 µg ml^{-1} double stranded DNA and 40 µg ml^{-1} single stranded DNA or RNA. OD260/ OD280 ratios of pure DNA and RNA are 1.8 and 2.0 respectively, a lower reading being indicative of phenol or protein contamination.

2.16 Restriction of DNA (medium salt conditions)

Restriction enzymes were used according to the supplier's instructions with the buffers supplied.

2.17 Restriction mapping of plasmid DNA

Plasmids were restricted with a series of restriction enzymes and these products run on 1% (w/v) agarose gels together the size marker ladders: Lambda cut with *Hin*d III and *Eco* RI and pBR322 cut with *Hinf* I and *Eco* RI. The distance of migration from the wells through the gel was measured for restriction products of the plasmid and plotted along with the migration

distance of the size standards on semi-logarithmic graph paper. From the plot it was possible to calculate the sizes of the restriction products and order them on the plasmid.

2.18 Ligation of plasmid DNA

Ligations were performed in a final volume of 15 µl at room temperature for an hour. The ligation mixture included:

1.5 µl 10 times Ligation buffer

1 µl Ligase

1.5 µl 40 mM Dithiothreitol

1.5 µl 5 mg/ ml Bovine serum albumin

9.5 µl SDW and DNA

The reaction was stopped by the addition of 2 µl 200 mM $EDTANa_2$ and 13 µl of SDW was added to bring the final volume to 30 µl.

2.19 Agarose gel electrophoresis

100 ml 0.5 to1% (w/v) agarose gels were run to visualise DNA, 1.5% (w/v) to visualise rRNA. The agarose was boiled over a flame in 100 ml TBE and allowed to cool before the addition of 5 µl of a 10 mg ml^{-1} ethidium bromide solution. The gel was poured into a tape sealed tray and allowed to set. DNA gels were run at 80 to 100 v for 1.5 to 2 hours or overnight at 15 v in TBE running buffer. RNA gels were run at 80 to 100 v for 30 minutes. DNA or RNA bands were visualised on a short wave UV light box.

2.20 Isolation of small fragments from an agarose gel.

The DNA restriction digestion products were electrophoresed on an agarose

gel. If the fragment to be isolated was similar in size to other restriction products, the fragments of similar size were resolved by running a gel with a low agarose content eg. 0.5%. The fragment of interest was identified under UV light and DE81 paper (Whatman size 2.3 mm) was placed perpendicularly to the orientation of the band. The gel was trimmed and orientated about 90° in the gel rig, so that fragment of interest could be electrophoresed onto the DE81 paper, which was backed by dialysis tubing. The presence of the band on the paper was monitored under UV light. When the fragment had migrated onto the DE81 paper, the excess paper was removed and the paper containing the fragment was placed in 400 µl 1.5 M NaCl in TE buffer. The paper was homogenized by pipetting it slowly into the buffer and the tube was then placed at 37°C for 2 hs. The paper fragments were pelleted at maximum speed for 5 minutes (MSE Microcentaur microfuge) and the supernatant recovered. The DNA was precipitated by the addition of 800 µl ethanol and placed at -20°C overnight.

2.21 Preparation of *E.coli* competent cells.

A 5 ml overnight culture of *E.coli* (DH5α) broth was diluted 1 in 100 with 37°C preincubated SOB with 10 mM $MgCl_2$. The cells were grown up at 37°C for 1 hour 30 minutes or until the OD at 660 nm was approx. 0.48 (equivalent to 10^7 cells ml^{-1}). The cells were placed at 0°C for 10 minutes before being spun gently (2 to 3,000 r.p.m. in a JA20 rotor on a Beckman J2-21 centrifuge). The pellet was drained and placed in a volume of ice cold RF1 equivalent to 0.33 of the initial growth volume of cells. The pellet was resuspended by gently pipetting the RF1 buffer over the pellet. The cells were placed at 0°C for 15 minutes, before a further gentle spin (2 to 3,000 r.p.m. in a JA20 rotor on a Beckman J2-21 centrifuge). The resulting pellet was then resuspended in a 1/ 12.5 volume of ice chilled RF2 (of the initial growth

volume). 200 µl aliquots were placed in prechilled Eppendorf tubes, frozen in liquid N_2 and placed at -70°C.

2.22 Transformation of *E.coli*

A 200 µl aliquot of frozen competent cells was thawed at room temperature and then placed on ice. DNA was added to approximately 50 ng and the cells placed on ice for 10 to 60 minutes. The cells were then heat shocked for 90 seconds at 42°C followed by cooling on ice for 1 to 5 minutes. 800 µl of L-broth or SOB was added and the cells incubated at 37°C for 20 to 60 minutes. 100 µl aliquots were plated out on L-broth plates and grown overnight at 37°C. Colonies of interest were transferred to fresh plates using a tooth pick which was placed in the centre of each colony.

2.23 Identification of *xylE* positive colonies

Colonies expressing the *xylE* gene, encoding production of catechol 2,3 dioxygenase, were identified by spraying with a solution of 100 mM catechol. Positive colonies formed a yellow substrate in the presence of catechol (2-hydroxy muconic acid) within approximately 5 minutes of being sprayed. These colonies were immediately transferred to fresh plates with a tooth pick.

2.24 Extraction of rRNA from *Streptomyces* broth cultures.

2.24.1 Method 1.

TSB grown mycelial broth cultures were harvested after 3 to 5 days by centrifugation at 3,000 r.p.m. for 20 minutes. Cells were lysed by sonication (Jencon sonicator) at 24 microns at 0°C for 2 minutes in a solution of 7.5 M guanidine hydrochloride. Cell debris was removed by centrifugation (at low speed in MSE Microcentaur microfuge) and the aqueous supernatant recovered

and extracted twice with neutral phenol chloroform and chloroform. The rRNA was precipitated with 2.5 volumes of ethanol and placed at -70°C for 10 minutes (Hahn *et al.*, 1990a).

2.24.2 Method 2.

Bacterial cells are suspended in the GOS homogenization buffer. These cells were lysed by sonication (Jencon) for 3 minutes at a frequency of between 18 to 24 μm. 1/3 volume of 8.5 M potassium acetate pH 6.5 was added to the lysed cells and placed on ice for 15 minutes. The lysis mixture was spun at 3,000 r.p.m. (on a MSE Benchtop centrifuge) for 30 minutes and the supernatant recovered. The rRNA was then precipitated by the addition of a 1/ 9 volume 3.3 M sodium acetate pH 6.1 and a 0.5 volume isopropanol and placed at -20°C for 1 hour. The nucleic acids were recovered by spinning at 5,000 r.p.m. for 30 minutes, the supernatant was discarded and pellet resuspended in 800 μl TE buffer. A 0.5 volume of 10 M LiCl was added and the reagents mixed thoroughly and placed on ice for 5 to 12 hours. The precipitates were spun at 10,000 r.p.m. for 30 minutes, (Heraeus Sepatech Biofuge 15) and the recovered pellet resuspended in 200 μl TE buffer. 1.5 volumes of 5 M potassium acetate (pH not adjusted) were added to the RNA solution and the samples placed on ice for 3 to 5 hours. After a second spin at 10,000 r.p.m. for 30 minutes (on a Heraerus Sepatech Biofuge 15) the drained RNA pellet was taken up in 30 to 100 μl TE buffer.

2.24.3 Method 3.

A 50 ml broth culture of a particular *Streptomyces* strain was decanted into 2 X 20 ml universals and spun at 3,000 r.p.m. (on a MSE benchtop centrifuge). The resulting pellets were taken up in 2 ml of Darbyshire solution and 2 ml DEPC treated SDW. The cells were resuspended by mixing well. 200 μl of

0.5 M $EDTANa_2$ and 200 µl of N-laurlysarcosine (10%) were added to the cells and the mixture vortexed for 1 minute to lyse the cells. The lysates were spun at 3,000 r.p.m. at 4°C for 30 minutes (in a MSE bench top centrifuge). The supernatant was recovered and 4 times 500 µl aliquots placed into 4 times 1.5 ml Eppendorf tubes. 1 ml of 100% ethanol was added to each of the tubes and the tubes were placed at -20°C overnight. The tubes were then spun at 13,000 r.p.m. at 4°C (in an MSE microcentaur microfuge). The supernatant was removed carefully with a pipette, the pellets air dried and taken up in 50 µl DEPC treated SDW. Samples were stored at -20°C. Precautions were taken to ensure that all materials used was free from RNase enzymes. Glassware, spatulas etc. were baked at 120°C overnight and all solutions were prepared with RNase free reagents and sterile distilled water that had been treated with 0.1% DEPC.

2.25 Dot blots

100 ng of rRNA was applied to Hybond N nylon filters using a dot blot manifold (GIBCO BRL Ltd., Middlesex). Filters were dried on Whatman paper no.1 soaked with 20 times SSC. RNA was fixed to the filters by exposure to short wave UV light (from a transilluminator, Cambridge, UVP Ltd, San Gabriel, USA) for 2 minutes.

2.26 Microwave lysis colony blot method applied to *Streptomyces*.

Microwave lysis methods were based on the methods of Datta *et al.* (1987) and modified for actinomycetes by P. Baker (pers. comm.). Spore and mycelial material was transferred from sporulating plate cultures using tooth picks to circular cut nylon Hybond N membranes placed on R5 plates. The colonies

were grown for 24 to 48 hrs, before the nylon filter was removed from the plate and placed onto 2 to 3 layers of Whatman No. 1 paper saturated with 0.15 M NaCl and 0.1 M NaOH. Colonies were lysed by placing the tray containing the saturated Whatman paper and nylon filters in the microwave (Cooktronic M710, Phillips) at high temperature for 60 seconds. The filters were placed on fresh Whatman paper to dry and the nucleic acids were fixed to the nylon filter by UV irradiation on a short wave UV transilluminator (UVP Ltd., Cambridge, UK.) for 2 to 3 minutes. The filters were stored prior to hybridization in bags (sealed using an Impulse sealer, E82163(s), Hybaid, Middlesex) at 4°C.

2.27 Hybridization conditions

Hybridization conditions were modified from Church & Gilbert (1984). Filters were placed in hybridization tubes containing prewarmed hybridization buffer in a hybridization oven (Mini hybridization oven, Hybaid, Middlesex), at 50°C overnight.

Oligonucleotide probes were 5' labelled using T4 polynucleotide kinase (BRL) and 10 to 20 μCi of gamma ^{32}P adenosine-5-triphosphate (3,000 Ci mmol^{-1}, Amersham) according to (Hahn, 1990).

End-labelled oligonucleotide probe was added to the prehybridization buffer. Hybridizations were allowed to proceed for 6 to 18 hrs at 50°C. Stringency washes were performed with washing buffers I and II for 2 X 20 minutes followed by 3 times 10 minute washes with washing buffer 3. Washes were performed at 40°C, 50°C and 52°C.

Recovery of intact RNA was confirmed by hybridization with the universal

eubacterial probe primer 1115 (Embley *et al.*, 1988). Duplicate filters were used to determine the specificity of oligonucleotide probes.

2.28 Autoradiography and phosphorimage analysis

The filter sealed in a plastic bag (Impulse sealer E821163(S) Hybaid, Middlesex) was placed in a Harner film cassette together with two intensifying screens (with the shiny sides of the screens facing the film). The cassette was placed at -70°C for a period from several hours to several weeks. The exposed X-ray film was developed using Kodak LX-24 developer (5 minutes) followed by a quick rinse in water and finally 2 to 4 minutes in Kodak FX-40 X-ray fixer.

Alternatively, the sealed filter was exposed on a phosphor screen for a period from several hours to a week. The exposed screen was then scanned and the image analysed and processed on a phosphorimager (Molecular Dynamics Ltd, Sevenoaks, Kent).

2.29 Identification of putative pathogenic streptomycetes.

Putative scab pathogens were identified on the basis of 41 morphological and physiological characters contained in the probabilistic identification matrix of Williams *et al.* (1983b).

2.29.1 Morphology

Spore coat surface, spore chain morphology and fragmentation of mycelium.

These traits were examined from coverslip preparations of strains. Unknowns were inoculated at a 45° angle onto a coverslip embedded into ISP 4 or oatmeal agar plates. The strain was allowed to grow and sporulate for 21 days

at 25°C, before the coverslip was removed and fixed in formaldehyde vapour in a desiccator. The dried coverslips were mounted onto stubs using conductive paint and coated with colloidal gold. The samples were then viewed under the scanning electron microscope (Joel JSM T330A). Categories of spore coat surface morphology include smooth, spiny, hairy, warty and rugose (Tresner *et al.*, 1961). Two of these categories were scored positive i.e. smooth or rugose, other phenotypes were scored negative. The following categories of spore chain morphology (Pridham *et al.*, 1958) were scored positive, Rectiflexibiles (long and flexous), Rectinaculiaperti (long and flexous with the occasional spiral or twist), Spirales (spiral spore chains with more than 1.5 turns per chain) and Verticillati (spore chains in verticels).

Substrate Mycelium

The colour of the substrate mycelium was assessed on glycerol asparagine agar after growth for 14 days at 25°C against a colour chart series. The important colour groups for streptomycete identification being yellow-brown and red-orange. A yellow brown substrate mycelium was scored positive, while orange brown was scored negative.

Colour of aerial spore mass

Inoculated plates of oatmeal of ISP 4 agar were examined after 14 days incubation at 25°C. The colour of the aerial spore mass was compared with colour charts (Tresner & Badkus, 1963). The important colour groups for streptomycete identification being red, green and grey. The category observed was scored positive, while the remainder were scored negative.

2.29.2 Inoculation of test media

All media was inoculated from 20% (w/v) glycerol spore and mycelial

suspensions of the unknown strain. The exception to this being the carbon and nitrogen tests, these were inoculated from SDW suspensions of spore and mycelial material.

2.29.3 Production of melanin pigments

Melanin pigment production was established by growing strains on plates of peptone yeast extract iron agar. Melanin production was detectable after 4 days at 25°C as the production of dark pigmentation in the agar when compared with an uninoculated plate.

2.29.4 Growth on carbon sources

Ability to utilise adonitol, cellobiose, D-fructose, inulin, D-mannitol, meso-inositol, D-raffinose, L-rhamnose and D-xylose was assessed over a period of 21 days at 25°C. Ability to utilise the above carbon sources was scored positive or negative with reference to growth on the positive control D-glucose and a negative control without a carbon source.

2.29.5 Growth on nitrogen sources

Ability to utilise α-aminobutyric acid, L-histidine and L-hydroxyproline was assessed after 15 days growth at 25°C against the positive control of L-asparagine and a negative control without a nitrogen source.

2.29.6 Degradation of xanthine

Ability to utilise xanthine was scored for by the production of a clearance zone after 28 days of growth at 25°C. Xanthine degradation was scored positive.

2.29.7 Utilisation of allantoin

Inoculated slopes were incubated for 28 days at 25°C. Allantoin utilisation

was detectable by the occurrence of a colour change from orange/yellow (acid) to pink/red (alkaline). Inoculated negative control slopes without allantoin were compared with test slopes to avoid confusion over production of pigments by the strain and the acid-alkali colour change. Allantoin utilisation was scored positive.

2.29.8 Utilisation of arbutin

Inoculated slopes were incubated for 21 days at 25°C. Arbutin degradation was detectable as a blackening of the agar. Inoculated negative controls without arbutin were compared against test slopes to avoid confusion over blackening from arbutin degradation and blackening from melanin production. Arbutin degradation was scored positive.

2.29.9 Utilisation of pectin

Inoculated pectin plates were incubated at 25°C for 6 days. Degradation was evident by the appearance of a clearance zone after flooding the plates with a warmed solution of 1% (w/v) hexadecyltrimethyl ammonium bromide for an hour. Pectin utilisation was scored positive.

2.29.10 Inhibition of growth by: *Aspergillus niger, Bacillus subtilis* and *Streptomyces murinus*

Growth inhibition by the above strains was assessed by growing the unknown on 3 glass nutrient agar plates for 24 to 48 hs (the strain was not allowed to sporulate). The unknown strains were killed by exposure to a chloroform atmosphere for 40 minutes and overlayed with a 10 to 15 ml soft nutrient agar suspension of each of the above test organisms. A zone of inhibition was assigned a positive score.

2.29.11 Chemical inhibition tests

Unknown strains were tested for ability to grow on modified Bennet's agar containing: 0.01% sodium azide, 0.1% phenol and 7% sodium chloride. Strains were grown for 14 days at 25°C. Ability to grow on the inhibitory compounds was assigned a positive score.

2.29.12 Growth at 45°C

Ability to grow at 45°C was tested by incubating inoculated plates of Bennet's agar at 45°C for 6 days. Growth at 45°C was assigned a positive score.

2.29.13 Resistance/ sensitivity to: neomycin and rifampicin

Sensitivity to the above antibiotics was tested by placing 5 mm diameter discs of filter paper Whatman No.1 paper treated with a 50 µg/ ml solution of the antibiotic onto a Bennet's agar plate spread with the unknown strain. Antibiotic impregnated discs were produced by soaking discs of filter paper in the antibiotic solution for 2 minutes followed by drying under vacuum Williams (1967). Sensitivity to an antibiotic as indicated by the presence of a zone of inhibition was assigned a negative score, resistance or no zone was scored as positive.

2.29.14 Reduction of nitrate

Inoculated nitrate slopes were incubated for 14 days at 25°C. After incubation 0.2 ml of each of Giess-Illosary reagents I and II were added to the slope. Nitrate reduction is detectable by the formation of nitrite and therefore the formation of a red compound with the addition of the Giess-Illosary reagents. Negatives were confirmed by the addition of zinc powder to the slope, this reacts with the reagents to produce the same red compound.

2.29.15 Production of H_2S

Production of H_2S was detected by placing Whatman No.1 filter paper strips soaked in lead acetate in the top of the nitrate agar slopes. A blackening of the lead acetate strips after 14 days of incubation signified production of H_2S and the reaction was scored positive.

2.29.16 Lecithinase activity

Lecithinase activity was detected on inoculated plates of egg yolk agar incubated at 25°C for 6 days. The enzyme causes production of a opaque creamy yellowish precipitate surrounding the edge of the colony. Lecithinase activity was scored positive.

2.29.17 Identification statistics

Three identification statistics were used to assess the reliability of the identification of an unknown. A Wilcox probability score of 0.8 and above indicated a positive identification. The taxonomic distance was a measure of the distance of the unknown from the centroid strain of the cluster to which it was assigned, scores of 0.4 and below were indicative of a positive identification. The standard error (SE) gives a measure of the variation in aberrant test results. A negative score indicated the unknown was closer to the centroid than average.

2.30 Identification of patterns of antibiotic resistance and sensitivity in plant pathogenic streptomycetes

Strains were screened for uncommon patterns of resistance and sensitivity to antibiotics by placing Mastrings multiple antibiotic discs (Mast Laboratories, Merseyside, UK.) onto the surface of streptomycete inoculated spread plates of RASS and R5.

2.31 Glasshouse pathogenicity tests of putative pathogens.

Pathogenicity of isolates was tested under 2 glasshouse regimes.

2.31.1 Method 1

Pathogenicity of isolates was investigated in the glasshouse with an 18 h day, a daytime temperature of 20°C and a night time temperature of 15°C. Spore and mycelial suspensions (in 1/4 strength Ringers solution) were inoculated into 1 kg pasteurised John Innes Compost (Section 2.43) prepared with additional lime and grit at 10^3 to 10^4 c.f.u. g^{-1}. 20 cm diameter pots were sown with certified potato seed of the scab susceptible variety, Maris Piper and the scab resistant variety, Pentland Crown. Strains were tested in triplicate against both potato varieties and the pots were arranged in a randomised block along with uninoculated control plants.

Plants were watered daily for the first two weeks and then twice weekly via gravel covered capillary matting upon which the pots were placed. Soil moisture content was kept below 50% moisture holding capacity (MHC).

2.31.2 Method 2

Pathogenicity of isolates was investigated according to the method 1 with the following differences: 10 L pots were filled with a mixture of equal volumes of peat and sand. The potato variety used was Desiree with 10 replicate pots per inoculant. The pots were watered carefully from above to 50 to 60% moisture holding capacity of the soil.

Progeny tubers from both pathogenicity tests were assessed for scab symptoms after 20 weeks when the plants had begun to senesce. Pathogenicity of isolates

was assessed against any background infection seen on the tubers of the uninoculated control plants. Pathogenicity of isolates was determined according to the percentage coverage of tubers by scab lesions (Large and Honey, 1955). In addition progeny tubers from pathogenicity trial 2 were weighed and the number of tubers per plant calculated.

2.32 Propagation and maintenance of *in vitro* plantlets.

Axenic *in vitro* plantlets of the scab susceptible potato variety Maris Piper were kindly supplied to us by P. Hirsh (Rothamsted Experimental Research Station) and P. Dale (I.P.S.R. Cambridge Laboratory, John Innes Institute). Plantlets were propagated by making internodal cuttings, removing the leaf at the node and placing the bud of the node facing upwards into glass tubes containing Murashige and Skoog medium with 3% sucrose and 0.6% agar, pH 5.6. This medium was sterilised by autoclaving at 10 p.s.i. for 15 minutes.

2.33 Induction of mini tubers

Mini tubers were induced from internodal cuttings placed into Murashige and Skoog medium with additional sucrose (8% sucrose) at pH 5.6. Plantlets developed after 2 to 4 weeks and mini tubers were induced after 4 to 6 weeks (Fig. 1).

2.34 Induction of scab symptoms on the surface of mini tubers.

While the tubers were small and still developing (4 to 5 weeks), they were inoculated by placing a drop of a plant pathogenic streptomycete spore suspension on the tuber surface. Within 4 weeks scab symptoms were visible on the surface of the tubers.

Fig.1 Induction of *in vitro* minitubers

Minitubers were induced from internodal cuttings placed on Murashige and Skoog complete medium containing 8% sucrose. Minitubers appeared after 4 weeks incubation at 20^{0}C. The potato variety used was Maris Piper.

Figure 1

Fig.1 Induction of *in vitro* minitubers

Minitubers were induced from internodal cuttings placed on Murashige and Skoog complete medium containing 8% sucrose. Minitubers appeared after 4 weeks incubation at 20^0C. The potato variety used was Maris Piper.

Figure 1

2.35 Scanning Electron Microscopy of the tuber and potato root surface.

Mini tuber and root material was fixed by placing in 3% glutaraldehyde overnight at 4°C. This was followed by consecutive rounds of drying in 10, 30, 50, 70 and 90% acetone each for 10 minutes and drying overnight in a desiccator. The fixed samples were attached to stubs using conductive paint and coated with colloidal gold using a sputter coater. SEM preparations were viewed on a Joel Scanning Microscope (JSM) T33OA.

2.36 *In situ* hybridizations

2.36.1 Sample preparation

One gram of soil was fixed in 3 mls of cell fixation buffer at 4°C, from 3 hours to overnight. The cells were removed from the heavier soil particles by 2 times 3 ml extractions of cell fixation buffer. At each time the heavier soil particles were allowed to settle for 1 minute and the supernatants decanted off and pooled. The supernatants were spun for 10 minutes at 5000 r.p.m. and the pellet retained. The pellet was washed by resuspending in 1 times PBS, resedimented and retained. The pellet was finally taken up in 500 µl of 1 times PBS and 500 µl of 96% (v/v) ethanol. The fixed soil was stored at -20°C for up to a year. A 1 µl aliquot of the fixed soil suspension was placed onto a glass window of a slide obtained from Cell Line Associates Inc., New Jersey, that had been coated in 0.1% (w/v) gelatin and 0.01% (w/v) chromium sulphate. The soil suspensions were allowed to air dry for 20 to 30 minutes before the slides were placed consecutively in tubes of 50, 80 and 96% (v/v) ethanol each for 3 minutes. The slide was then allowed to air dry for 10 minutes. The hybridizations were performed in a humid atmosphere at 45°C. A hybridization chamber was prepared containing moistened paper towels and

preincubated at 45°C. A 5 µl aliquot of hybridization buffer was added to the 1 µl of dried soil material on the slide, together with 1 µl of the oligonucleotide probe (50 ng/ µl). The slide was placed face upwards in the sealed hybridization chamber and incubated at 45°C for 1 to 2 hours. The slide was then removed from the chamber and the windows rinsed with hybridization buffer. The slide was returned to the chamber and incubated at 48°C for 20 minutes. The slide was finally removed, rinsed with sterile distilled water and air dried for 10 minutes. The slide was either viewed under a fluorescent microscope at 580 nm (red) or stored for viewing at a later date. In order to minimise photobleaching samples were protected with a drop of Citifluor solution (Citifluor, Ltd., London, UK.) made up in 9 parts glycerol and 1 part PBS buffer.

2.36.2 Fluorescent labelling of the oligonucleotide probe

The labelled probe was prepared by R. Amann and D. Hahn according to Amann *et al.* (1990). An amino linker was attached to the 5' end of the oligonucleotide in the last coupling cycle during the synthesis of the oligonucleotide. The primary amino-group was then coupled to the fluorescent dye, tetramethylrhodamine isothiocyanate. The oligonucleotide probe used in this study was the eubacterial probe primer 1115 (Embley *et al.*,1988).

2.37 Recovery of bacteria from soil

1 g of soil was transferred to 9 ml of 1/4 strength Ringer diluent and shaken (at max. speed for 15 minutes) on a Griffin wrist action shaker. The larger soil particles were allowed to sediment and the supernatant serially diluted and plated in triplicate onto RASS or R5 agar containing appropriate antimicrobial agents.

2.38 Extraction of rRNA from soil.

RNA was recovered from soil according to the method given in section 2.24.2, however, spores in soil were lysed by bead beating continuously for 5 minutes in a Braun homogenizer (B.Braun, Melsungen A.G.). A 2.5 : 1 ratio of 0.1 mm diameter glass beads to soil was used. Hence for a 2 g sample of soil, 7 g of beads were added and the extraction buffer added until just below the neck of the tube or bottle.

2.39 Extraction of DNA from soil

The following method was based on Cresswell *et al.* (1991). Ten grams of soil were placed in a 50 ml bead beating bottle (Braun) with 25 g 0.1 mm diameter glass beads and the bottle filled to the neck with 0.12 M sodium phosphate buffer, (pH 8.0). The bottle was placed inside the bead beater (B. Braun, Melsungen A.G.) and shaken for 5 minutes continuously, while cooled with CO_2. The contents of the bottle was transferred to Oakridge tubes and the large particles of soil and glass beads sedimented (6,500 r.p.m. in a JA20 Rotor on a Beckman J2-21 centrifuge). The supernatant was retained and the pellet rextracted with 25 ml 0.12 M sodium phosphate buffer (pH 8.0). The supernatants were pooled and the DNA precipitated with polyethylene glycol 6,000 (Hopwood *et al.*, 1985) and spun at 3,000 r.p.m. (for 10 minutes on a MSE bench top centrifuge). The supernatant was removed and the pellet resuspended in 5 ml TE buffer and extracted twice with neutral phenol chloroform isoamylalcohol, the neutral phenol chloroform was rextracted with TE buffer and the supernatants combined. The aqueous phase was extracted with chloroform isoamylalcohol and the upper phase recovered. The DNA was precipitated at -20°C overnight, centrifuged and washed with 70% (v/v) ice-cold ethanol, vacuum dried and dissolved in 100 µl TE buffer by heating to 60°C and trituration and stored at 4°C.

2.40 Estimation of pH and Moisture Holding Capacity (MHC) of soil

The pH of the soil was estimated accordingly (Fisons, agriculture catalogue), one part soil was placed in two parts deionised water, mixed vigourously for one minute and allowed to stand for two minutes before the pH of the soil suspension was measured. The M.H.C. of the soil was determined according to Allen *et al.* (1974) by drying a sample of soil to constant weight, then allowing the soil to become saturated with water and calculating the % moisture present in the saturated soil. Both estimates were made in triplicate and the mean value taken.

2.41 Sterile soil microcosms

20 g of air dried John Innes compost was sieved through a 2 mm mesh sieve and placed in glass plant propagation tubes. Where soil was amended the amendments were 1% starch and 1% chitin. Soil was sterilised by three rounds of autoclaving and incubation. The soil moisture content was assessed and the sterile soil inoculated with an enumerated streptomycete spore suspension in 1/4 strength Ringers. The moisture content of the soil was then brought up to 15% with the 1/4 strength Ringers inoculum suspension. Suspensions were not mixed into the soil but instead left to filter through the soil matrix. Sterile potato plants (Maris Piper) were introduced into the microcosms either as mini tubers (experiment 1) or as month old plantlets (experiment 2)[Fig. 2].

2.42 Non-sterile microcosms

Non-sterile microcosms were based on the regime for testing pathogenicity (method 1). One kg of pasteurised John Innes compost was inoculated with

Fig.2 Axenic microcosms with and without plants

20 g of John Innes compost was sterilised by three rounds of autoclaving and incubation. Microcosms with plants were seeded with an axenic, sprouting mini tuber (Section 6.3.2) or a 4 week old plantlet (6.3.3).

Figure 2

streptomycete spore suspensions. The moisture content of the pots was kept to less than 50% moisture holding capacity, with pots watered once a week from below. Potato plants were introduced into the pots as certified Maris Piper potato seed tubers.

2.43 John Innes potting compost

The modified John Innes Potting compost was made up by Matt Busby, senior horticulturist from the Science Education Department, Warwick University. It consisted of:

	7 parts (by volume)	loam
	3 parts	peat
	3 parts	limestone grit
Per bushel	0.25 lb	JI base fertilizer
Per bushel	1.5 oz	ground chalk

2.44 Measurement of water content in the soil

Water content in the soil was estimated by taking a sample of soil of known weight (wet weight) followed by drying to constant weight in an oven (105°C), for 48 to 36 hs. The moisture content of the soil was the difference between the wet and dry weights.

2.45 Statistical analysis

Statistical analysis was performed using the MINITAB software package (Minitab statistical software, State college, PA., USA). Minimum significant differences were calculated from analysis of variance data using the method of Peterson (1985). When the number of replicates for each sample point (day)

was the same, Tukey's H.S.D. was employed. However, if the number of replicates differed between sample dates e.g. because of a contamination problem in one particular dilution series the M.S.D. coefficient was used.

Tukey's honestly significant difference:

H.S.D.= Qa ✓(m.s.e./r)

Minimum significant difference:

M.S.D.= Qa✓[(m.s.e. [1/ni + 1/nj])/2]

Where: Qa = Value from the studentised range (Q) table of (99%) confidence limits. Depending on μ (error degrees of freedom [ANOVA]) and n (no. of sample points [days]).

m.s.e.	= mean square error (from ANOVA)
r	= sample size (no. replicates)
n_i	= the lowest no. of replicates
n_j	= the highest no. of replicates.

The Mann-Whitney test

Non-parametric Statisical methods

The theory upon which the two sample t test is based requires that the two sampled populations be normally distributed. Methods not requiring normally distributed data are referred to as non-parametric procedures. These methods may be applied to situations where a two sample parametric test would normally be applied (Zar, 1984).

In this test as for other non-parametric tests the actual measurements are not used. Instead the data are arranged in order of size and assigned a rank order.

The data may be both ranked from the highest to the lowest values or vice versa. For the pathogenicity data, the lowest values were ranked 1, 2 etc. until N.

The Mann-Whitney statistic was calculated as follows:

$U = n1n2 + n1(n1 + 1) - R1 / 2$

and

$U^1 = n1n2 - U$

Where n1 = the population size of sample 1

and n2 = the population size of sample 2

Where R1 = the sum of the sample ranks from population 1

and R2 = the sum of the sample ranks from population 2

As a check on the assignment of ranks $U + U^1 = n1n2$.

For a two tailed test e.g. the null hypothesis is that the scab coverage produced by a putative pathogenic streptomycete is the same as that found on uninoculated control plants. U and U^1 were calculated and the larger of the values matched up according to n1 and n2 on a table of critical values for the Mann-Whitney U distribution. If U or U^1 is greater or equal to the tabulated figure at the α 0.05 level of significance, the null hypothesis is rejected and the two populations found to be significantly different.

2.46 Cluster analysis of taxonomic data using NTYSIS-pc

Data were recorded in binary form and arranged in a matrix (n x t) n = the number of bacteria and t = the number of tests. Data matrices were input into

NTYSIS-pc (Exeter Publishing ltd, Setauket, New York) software on an IBM 386 PC. Similarity and dissimilarity matrices were created using the simple matching coefficient (S_{SM}) (Sokal & Michener, 1958) which includes both positive and negative matches and the Jaccard coefficient (S_J) (Sneath & Sokal, 1973) which only includes positive matches in the SIMQUAL program. Hierarchical clustering was performed in SAHN using algorithms for Single linkage and the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) (Sokal & Michener, 1958). Dendrograms were obtained using the TreeG program (Section 3.6).

Chapter 3

Characterisation and pathogenicity of scab isolates

3.1 Introduction

3.1.1 Taxonomy of the common scab pathogen

The association of a group of soil-borne bacteria, the streptomycetes, with common scab was first made by Thaxter (1891), when he described the group as *Oospora scabies*. These strains were later transferred to the *Streptomyces* genus and renamed *Streptomyces scabies* (Waksman & Henrici, 1948; Waksman, 1961).

Confusion over the taxonomic status of plant pathogenic streptomycetes developed with the deposition of many different, pathogenic isolates in culture collections throughout the world under the name of *Streptomyces scabies*. Additional confusion occurred as the type strain put forward by Waksman and Henrici differed from Thaxter's original description. This resulted in the species being considered *incertae sedis* (type strain not extant, many taxonomically different strains available) in the latest edition of Bergey's Manual of Determinative Bacteriology (Williams *et al.*, 1989).

3.1.2 Approaches to the characterisation of plant pathogenic streptomycetes

Streptomycetes may be characterised and identified on the basis of phenotypic variation (Williams *et al.*, 1983a;b; Langham *et al.*, 1989). *S.scabies* was assigned to *S.atroolivaceus*, cluster 3, in an extensive numerical taxonomic study (Williams *et al.*, 1983a). A more limited selection of strains were characterised phenotypically by Lambert & Loria (1989) in an attempt to clarify the taxonomic position of scab-causing streptomycetes. Their findings suggest that most pathogenic streptomycetes conformed to a defined group consistent with Thaxter's original description of *S.scabies*. The group being characterised by formation of smooth grey spores, spiral spore chains, melanin

production and utilization of selected sugars used in the International *Streptomyces* Project (Shirling & Gottlieb, 1966). This group was not identified as being closely related to any of the major clusters (Williams *et al.*, 1983a) although most similarity was observed with clusters 18, 19 and 23. In the most extensive study to date, 821 *Streptomyces* and *Streptovericillium* strains were characterised on the basis of 329 minaturized physiological tests, (Kamper *et al.*, 1991). Good agreement was observed with Williams *et al.* (1983a) over the assignment of the major clusters, of interest was the classification of the 13 strains of *S.scabies* included in the study. The *S.scabies* strain ISP 5078 used to represent the group in the study of Williams *et al.* (1983a) was transferred from *S.atroolivaceus* to *S.griseus*, (cluster 1, subcluster 3). Other *S.scabies* strains identified to the groups (assigned by Kamper *et al.*, 1991) of: *S.halstedii*, cluster 1-2, *S. olivaceus*, cluster 1-4, *S.exfoliatus*, cluster 2, *S.rochei*, cluster 6 *Streptomyces* spp. cluster 8, *S.violaceus*, cluster 9, *S.niveoruber*, cluster 13, *Streptomyces* spp., cluster 14 and the single member grouping cluster 45, *S.scabies*. Although the strains from the study of Lambert & Loria (1989) were not included in the study by Kamper *et al.* (1991) the authors speculate that they would probably have fallen in the *S.violaceus*, cluster 9 group together with the majority of *S.cyaneus* and *S.diastaticus* strains.

Phenetic characterisation of certain *Streptomyces* species has been supported by chemotaxonomic analysis. Studies have included analysis of fatty acids (Saddler *et al.*, 1987), proteins (Manchester *et al.*, 1990) and DNA (Okanishi *et al.*, 1972; Mordaski *et al.*, 1986). A recent study on DNA homologies of three groups of plant pathogenic streptomycetes (Healy & Lambert, 1991) indicated that the three groups were genetically distinct. Although the majority of strains from the group phenotypically resembling the type strain

ATCC 49173 showed greater than 70% relatedness to the type strain, other values were as low as 21%. A high degree of relatedness was seen among the *S.acidiscabies* group of strains, however, it is possible that most of these isolates were derived from the same strain (Lambert & Loria, 1989). In contrast the *S.albidoflavus* group of strains exhibit very low values of relatedness both within the group and with the type strain.

The Ribosomal RNA Approach

The highly conserved and universally distributed rRNA molecules have emerged as being particularly useful in the elucidation of phylogenetic relatedness and identification of microorganisms (Woese, 1987).

The concept of using particular highly conserved molecules as 'evolutionary clocks' in the elucidation of bacterial phylogeny stems from the analysis of the amino acid sequences in the cytochrome C molecule and its application to unravelling phylogeny among eukaryotes (Fitch & Margoliash, 1967). However, the extraordinary biochemical and evolutionary diversity of bacteria makes the identification of suitable proteins for phylogenetic comparison difficult (Olsen *et al.*, 1986). Ribosomal RNA molecules arise as suitable candidates for phylogenetic studies from their fundamental importance in the translation of the genetic code and the manufacture of proteins, they are therefore functionally and evolutionary homologous in all organisms. Furthermore, ribosomal RNA molecules are extremely ancient molecules. Consequently, nucleic acid sequences may be highly conserved with certain stretches being invariant across the primary kingdoms. The conserved sequences and secondary structure allow the variable sequences to be aligned and hence the degree of homology ascertained (Olsen *et al.*, 1986).

Molecular chronometers or molecules that may serve as suitable 'evolutionary clocks' should meet certain criteria in order to be of value in defining phylogenetic relationships. Ideally these include clock like behaviour, i.e. changes in sequence should occur as randomly and evenly as possible, secondly rates of change should be proportional to the evolutionary distances measured and finally molecules should be large enough to provide sufficient information. Sequences that exhibit clock like behaviour without selective constraints may change rapidly so that they only provide information on short term evolutionary events. Hence the most useful molecules in the elucidation of phylogenetic relationships are functionally constrained. As such, typical clock like behaviour does not occur as some sequences are highly conserved under selective constraints while others are highly variable and accumulate random changes in the absence of selective constraints. Without correct consideration, the variable sequences might increase phylogenetic differences between organisms, leading to invalid phylogenies, while conserved sequences with few changes may have the tendency to under estimate evolutionary distances between orgainisms (Woese, 1987).

Woese and colleagues used 16S rRNA catalogues to reveal three as opposed to two lines of evolutionary descent and hence in the designation of three kingdoms, i.e. the eubacteria, archaebacteria and eukaryotes (Fox *et al.*, 1980). Support for the use of 16S rRNA sequences in the elucidation of phylogenies is gained from phylogenetic studies of the purple photosynthetic bacteria on the basis of 16S rRNA sequence homologies and that these were largely supported by data from cytochrome C, ferredoxin and 5S rRNA analysis. However, some differences in the phylogenetic patterns were observed with the assignment of certain lower rank taxa using these alternative molecular chronometers (Fox and Stackebrandt, 1987). Fox & Stackebrandt (1987)

address the application of 16S rRNA homologies to the classification of the eubacteria with particular reference to the gram positive groups. They argue that at present, higher rank phylogenetic relationships should be assigned on the basis of phylogenetic data such as 16S rRNA sequences, but that these ranks should not be strictly defined in terms of specific similarity values, but rather should be assigned flexibly with a view to maintaining clusters of strains, species etc. that are already defined by taxonomically valid phenotypic characters (Fox & Stackebrandt, 1987; Stackebrandt, 1988). They argue that some phenotypic characters will undoubtedly be good indicators of phylogeny and that they should be harnessed in the development of a classification system that is both practical to use and phylogenetic (Stackebrandt, 1988).

The majority of phylogenetic studies have focussed on the 16S rRNA species for which substantial sequence information has been generated. Comparisons of 16S rRNA oligonucleotide catalogues have been used extensively to demonstrate intergeneric relationships among the actinomycetes (Stackebrandt *et al.*, 1981; 1983). Confirmation of these suprageneric groupings and elucidation of intrageneric relationships has been achieved by more detailed analysis of 16S rRNA sequence data (Stackebrandt & Charfreitag, 1990). A similar approach was taken to elucidate intrageneric relationships within the *Streptomyces* genus (Witt *et al.*, 1989; Witt & Stackebrandt, 1990). Phylogenetic trees constructed on the basis of 16S rRNA sequence homologies correlated well with clustering patterns derived from phenotypic data (Williams *et al.*, 1983a). Alignment and comparison of partial 16S rRNA *Streptomyces* sequence data highlighted two variable regions of the molecule; designated α and ß (Witt *et al.*, 1989). Certain species-groups were proposed as being identifiable via hybridization with complimentary oligonucleotide probes to either one or both target regions. Two probes were recommended for

S.scabies (ISP5078); probe α4, complimentary to the α region at position 1007 to 1024 (*E.coli* nomenclature) and probe ß1, complimentary to the ß region at position 1130 to 1152. Neither of the sequences were identified as being unique to *S.scabies*. *S.diasticus* and *S.caesius* were identified as sharing the same α sequence and *S.lavendulae* and *Streptoverticillium luteoreticuli* identified as sharing the same ß sequence as *S.scabies*. A recent study by Stackebrandt *et al.* (1991) assessed the distribution of these 16S rRNA target regions among a number of streptomycete and *Streptoverticillium* strains that were sequenced in these regions. Their findings from analysis of 77 strains indicated the wide occurence of these sequences, with 19 variations identifable in the α region and only 12 variations identifable in the ß region. Hence the α4 sequence was common to *S.violaceoruber*, *S.cinereus*, *S. diastaticus*, *S.caesius*, *S.griseorubens*, *S.griseus*, *S.spectabilis*. *S.albidoviridis*, *S. minutiscleroticus* as well as a number of uncharacterised soil and marine isolates. The ß1 sequence was common to a large collection of *Streptovericillium* strains and in addition to *S.griseorubens*, *S.albidoviridis*, *S.griseus*, *S.baldicci*, *S.salomonis* together with a number of soil and marine isolates. This evidence suggests that these particular stretches of the variable regions of the 16S rRNA molecule, though not suitable alone for the identification of isolates may contribute an important adjunct to the existing battery of tools for the identification of streptomycetes (Stackebrandt *et al.*, 1991).

The potential of the gamma region for the development of diagnostic probes for identification remains to be realised. This region contains the largest stretch of variable sequence on the 16S rRNA molecule. It comprises 30 nucleotides situated between positions 150 to 200 (Stackebrant *et al.*, 1991).

3.1.3 Pathogenicity of the common scab interaction

Much is still unknown about the cause of pathogenicity in the potato common scab interaction. Unravelling the mechanism of pathogenicity is complicated because pathogenic isolates may appear morphologically, physiologically and serologically inseparable from related non-pathogenic strains (Labruyere, 1971). In addition, the common scab symptoms seen on the tuber surface are a product of the degree of resistance/ susceptibility exhibited by the host plant, the virulence of the pathogen and the environmental conditions present while the developing tubers are susceptible to infection.

3.1.3.1. Extracellular enzymes

A number of workers have suggested that the production of particular extracellular enzymes might be implicated in pathogenicity. Knosel (1970) identified a high pectic acid transeliminase activity in pathogenic isolates, supporting the observation that the scab pathogen initially attacks the middle lamellae, the pectin component of the cell wall in order to then penetrate the cells of the tuber. In addition, McQueen & Schottel (1987) cite evidence for production of a zinc inducible esterase by pathogenic streptomycetes, that is absent in other non-pathogenic strains. Underground parts of the plant, including the tuber are often covered with a waxy polyester like suberin or cutin, which is thought to protect the plant against moisture loss and pathogen invasion. Hence ability to breach the suberin barrier might confer pathogenicity. The zinc inducible nature of the enzyme is interesting, as enzyme activity was not found to be inducible in the presence of other divalent ions, those tested included: copper, manganese, borate, magnesium and calcium (McQueen & Schottel, 1987). Furthermore, zinc is a normal component of the suberin found on the surface of tubers.

3.1.3.2. Vivotoxins

The most notable development towards an understanding the pathogenicity of the common scab organism comes with the identification of the production of a vivotoxin 'thaxtomin' by virulent isolates (Lawrence *et al.*, 1990). Lawrence *et al.* (1990) demonstrated induction of scab lesions by a diffusable compound by placing dialysis tubing between the surface of scabbed potatoes and aseptic mini tubers. Lawrence *et al.* (1990) were unable to recover streptomycetes from the axenic scabbed tissue. The toxin was only recovered in reasonable amounts from scabbed tissue, since the authors were unable to demonstrate toxin production in broth cultures despite trying a variety of media. The toxin was recovered by running material isolated from scab lesions on silica gel, thin layer chromatography plates and crystallizing the appropriate fractions with acetone and methanol.

3.1.3.3. Physiological and biochemical differences

While pathogenic principles evidently have an important role in the pathogenicity of scab-causing streptomycetes. The interaction is greatly influenced both by environmental factors and the physiology of the host potato plant. The range of resistance/ susceptibility to scab exhibited by different potato varieties infers that there are physical or biochemical differences between varieties. Inspection of the periderm (the external layers of cells on the tuber surface) by Cooper *et al.* (1954) revealed differences in the nature of the periderm among resistant and susceptible varieties. There appeared to be a correlation between the presence of layers of collapsed enucleated cells on the surface of the developing tuber and susceptibility to common scab. In contrast, the periderms of resistant varieties appeared to consist of intact nucleated cells. This phenomenon appears to result from the shedding of collapsed cells by the more resistant varieties and the retaining of enucleated

cells by susceptible varieties. Similar histological patterns of the lenticels (thought to be the avenues for invasion by the pathogen) were also observed. Cooper *et al.* (1954) speculate that the collapsed tissue on the tuber surface may provide a suitable growth medium for the invading pathogen. Differences in the histology of the periderms of resistant and susceptible varieties were confirmed in the pathogenicity studies of McKee (1958). Another reported difference between resistant and susceptible varieties has been the chlorogenic acid content of tubers (Schall, Johnson & Simonds, 1953). Resistance was associated with a high content of chlorogenic acid in the tuber periderm and suceptibility with low levels. The association of chlorogenic acid with resitance was thought to be an effect of pH with scab pathogens being inhibited by environments with a low pH. However McKee (1958) and Emilsson (1953) were unable to demonstrate a correlation between the chlorogenic acid content of the periderm and resistance.

3.1.3.4. Environmental variables

An important element in the common scab potato interaction is the effect of environmental variables (Lapwood, 1972; Jellis, 1977). Numerous reports cite the effect of irrigation, particularly during the initial stages of tuber production has on reducing the incidence of common scab (Sanford, 1923; Lewis 1970; Lapwood & Herring, 1972). Lewis (1970) suggested that lack of scab infection during periods of high moisture could be attributed to bacterial antagonism, since he observed unusually small ratios of bacterial to actinomycete populations under moist conditions. Other workers frequently cite the importance of the pH of the soil (Labruyere, 1965), streptomycetes are thought to flourish in alkaline, limed soils, hence methods of control include the application of green manures to acidify the soil. The level of the inoculum in the soil is also thought to be an important factor, so workers tend to add

inoculum uniformly to the soil to compensate for any population differences attributable to pH or the distribution of antagonistic microorganisms when conducting pathogenicity experiments (Labruyere, 1971). The effects of a number of minerals and micronutrients on the plant pathogen interaction have also been considered (Keinath & Loria, 1990).

3.1.4 Pathogenicity Tests

One of the major obstacles to defining the taxonomic relationships between scab-causing microorganisms has been the lack of reliable and rapid methods for testing pathogenicity. Traditionally, seed potatoes were planted in scab infested soil either in the field or glasshouse and scored for scab symptoms once the plants have died down and the crop lifted. The problems with this type of test include, difficulties associated with maintaining a constant environment, in the field this is virtually impossible with workers reporting huge variations due to differences in annual precipitation. Attempts to overcome these problems have included covering the newly emerged plants for the first 9 weeks and while susceptible to infection with polythene tunnels (Jellis, 1975).

Many workers prefer the more controlled environment of the glasshouse (Booth, 1970; Gunn *et al.*, 1983). However even within the glasshouse it is difficult to eradicate the often large gradients in temperature, light and humidity that occur, even within the pot. The inclusion of sufficient replication is used to compensate for a certain proportion of this variation. It is generally possible to have more replication in the field than in the confines of a glasshouse. For these reasons the use of sterile mini tuber assays are attractive, since they eliminate a number of the problems associated with environmental variables. Lawrence & Barker (1963) reported the development

of such a system of pathogenicity screening, where *in vitro* potato plantlets were induced to produce mini tubers on elevated levels of sucrose (8%). They were unable to reproducibly induce scab symptoms on the tubers in tissue culture conditions, but found that by transferring plants bearing mini tubers to a moist vermiculite environment, reproducible induction of scab symptoms upon inoculation with a spore suspension of a plant pathogenic streptomycete was achieved. Lawrence *et al.* (1990) have subsequently applied this system to their studies of the vivotoxin, thaxtomin.

A number of methods have been devised for scoring virulence of isolates on susceptible tubers, though unfortunately a universal standard has not been adopted and different workers have used different criteria in their assessments. The problem is exaggerated by the number of different types of scab symptoms and confusion generated by the different names assigned to them (McKee, 1958). For instance superficial scab is used to include a number of minor types of infection including those described elsewhere as russet (Harrison, 1962) and netted scab (Scholte & Labruyere, 1985). Generally, the two indices of pathogenicity used are the percentage coverage of the tuber by scab lesions and the severity of the lesions. Many workers have indicated a good correlation between these two criteria, such that badly scabbed tubers are covered extensively with severe lesions (Leach *et al.*, 1938; Stevenson *et al.*, 1942; Jellis, 1977) although a few exceptions were noted. Percentage coverage has been frequently scored using keys (Large & Honey, 1955; McKee, 1963; Lapwood & Dyson, 1966; Dowley, 1972).

An alternative to the keys is the spot sampling method developed from the point quadrat method for determining botanical species composition in grasslands and introduced by Lowlings & Ridgeman (1959). The method

involved placing a marked strip longitudinally around the potato and noting the number of points on the strip that were covered by scab lesions. This estimate expressed as a percentage cover of tubers was found to be quite accurate when compared to the percentage scabbed surface area calculated by measuring the area of scabbed lesions using dividers and expressing this as a percentage of the whole surface area established by measuring the area of the potato peelings. However, the accuracy of this method was found to be off set by its laborious time consuming nature (Langton, 1972). Finally Leach *et al.* (1938) and Lauer & Eide (1963) have considered assessing common scab infection using the 'highest scab' method. This method involved scoring the severity of infection according to the severest lesions on the tubers. Although the method was liable to greater test error (Leach *et al.*, 1938) it was found to be a rapid and effective method for assessing clonal resistance (Lauer & Eide, 1963). Many workers have combined both criteria of percentage coverage and lesion type to produce a scab index (Marais & Vorster, 1988). Bjor & Roer (1980) obtain a pathogenicity score by multiplying lesion severity (identified from a key) by the percentage cover times 100/ 27. The figure 27 relates to the product of 9 categories of percentage cover and 3 categories of lesion severity.

3.2 Aims

3.2.1 To investigate the taxonomic position of a group of putative pathogenic streptomycetes with respect to the numerical phenetic classification studies of the *Streptomyces* genus (Williams *et al.*, 1983a) using a probabilistic identification matrix (Williams *et al.*, 1983b). To establish whether these strains have a common identity which is phenotypically definable as proposed by Lambert & Loria (1989).

3.2.2 To assess the value of 16S rRNA targeted oligonucleotide probes described for *S.scabies* ISP5078 (Witt *et al.*, 1989) in the characterization of plant pathogenic streptomycetes. Furthermore to assess whether the patterns of hybridization from the 16S rRNA targeted probes correlate with the phenotypic identifications.

3.2.3 To establish the pathogenicity of isolates under glasshouse conditions. In order to check pathogenicity of strains received as putative pathogens and assess the degree of virulence of isolates. To relate this data to the identification data, to gain further understanding of the identity of the causal agent of common scab of potatoes.

3.2.4 Preliminary studies to investigate the application of an axenic mini tuber assay for testing the pathogenicity of scab-causing streptomycetes. Problems surround the traditional methods for assessing pathogenicity under field or glasshouse conditions. These include variable environmental factors and the difficulties of working in a non-sterile environment, in terms of establishing whether disease symptoms are a product of the inoculant or other members of the soil microflora. Sterile plant tissue culture systems might offer a definitive and rapid solution.

3.3 The phenotypic characterization of a group of putative plant-pathogenic streptomycetes

3.3.1 Characterization

The aims of this study were to establish whether common scab strains form a definable taxonomic grouping as described by Lambert & Loria (1989) or a taxonomically diverse collection of strains as suggested by Labruyere (1971).

Strains were identified using the computer assisted probabilistic identification matrix of Williams *et al.* (1983b). The raw data from the individual tests may be found in Appendix 1.

Our findings are summarised in Table 7. They indicated that the 24 strains in the study identified to 6 of the 23 cluster groups within the probabilistic identification matrix (Williams *et al.*, 1983b). Strains ISP5078, ATCC 10246, S46 and R2 identified to *S.albidoflavus* (cluster 1). Strain ATCC 3352 identified with *S.atroolivaceus* (cluster 3) and ATCC 15485 identified with *S.exfoliatus* (cluster 5). A group of 6 strains including S47, MP2, MP9, R1, 8.2 and ISS identified with *S.rochei* (cluster 12). 4 strains identified well with *S.cyaneus* (cluster 18), these included *S.scabies* sp. nov., nom. rev. ATCC 49173, PD260, 1028 and 1034. Finally 6 strains identified with *S.diastaticus* (cluster 19), these included ASS8112, 8.6, 8.7, 8.8, 8.16 and 1033. Strains PD259 and 8.17 were considered unidentified as the statistical coefficients used to measure the reliability of the identification were well below the recommended limits. Our identifications were consistent with those obtained for strains ISP 5078, ATCC 3352, ATCC 15485 and ATCC 10246 in the study by Lambert & Loria (1989).

3.3.2 Discussion of results

From Table 7 it is apparent that many of the streptomycetes in this study did not conform to a taxonomically defined grouping as proposed by Lambert & Loria (1989). In contrast, two or three phenotypically defined groups emerge. These included the strains with a phenotype typical of the *S.albidoflavus/ atroolivaceus/ exfoliatus* group. Strains ISP5078, ATCC 3352 and ATCC 15485 were also assigned to the *S.albidoflavus/ S.griseus/ S.antibioticus*

Table 7: Identity of putative plant pathogenic streptomycetes

Strain	Best Identification	Cluster	Wilcox Probability	Taxonomic Distance	SE of (d)
ISP5078	*S.albidoflavus*	1	0.994	0.283	(1.4710)
ATCC 10246	*S.albidoflavus*	1	0.922	0.398	1.6015
S46	*S.albidoflavus*	1	0.992	0.361	0.6160
R2	*S.albidoflavus*	1	0.975	0.394	1.4890
ATCC 3352	*S.atroolivaceus*	3	0.972	0.362	1.4380
ATCC 15485	*S.exfoliatus*	5	0.962	0.364	0.4880
S47	*S.rochei*	12	0.995	0.511	4.624
MP2	*S.rochei*	12	0.969	0.438	2.6930
MP9	*S.rochei*	12	0.951	0.491	4.0920
R1	*S.rochei*	12	0.968	0.476	3.6880
8.2	*S.rochei*	12	0.999	0.354	0.4300
ISS	*S.rochei*	12	0.995	0.348	0.2870
PD259	*S.chromofuscus*	15	0.793	0.462	3.5420
ATCC 49173	*S.cyaneus*	18	0.971	0.427	1.5180
8.17	*S.cyaneus*	18	0.619	0.398	0.7760
PD260	*S.cyaneus*	18	0.804	0.357	(0.2113)
1028	*S.cyaneus*	18	0.897	0.413	1.1430
1034	*S.cyaneus*	18	0.739	0.357	(0.2110)
ASS8112	*S.diastaticus*	19	0.989	0.394	0.8010
8.6	*S.diastaticus*	19	0.999	0.342	(0.4830)
8.7	*S.diastaticus*	19	0.820	0.351	(0.2680)
8.8	*S.diastaticus*	19	0.689	0.441	1.9770
8.16	*S.diastaticus*	19	0.997	0.344	(0.4540)
1033	*S.diastaticus*	19	0.997	0.385	0.5720

group or cluster 1 in the study of Kamper *et al.* (1991). A second group emerged with a *S.rochei* type phenotype. Finally strains with a phenotype closest to that of *S.scabies* sp. nov., nom. rev. fell into the *S.cyaneus* and *S.diasticus* (clusters 18 and 19 [Williams *et al.*, 1983a]).

3.4. The application of 16S targeted rRNA probes to the characterization of plant-pathogenic streptomycetes

3.4.1 Hybridisations with α4 and β1 oligonucleotide probes

Ribosomal RNA isolated from a group of streptomycetes (both common scab strains and non-pathogenic strains) according to method 1 (Section 2.24.1) was probed with radio-labelled oligonucleotides complimentary to the 16S rRNA α and β target sequences proposed for *S.scabies* ISP5078 (Witt *et al.*, 1989).

Strains hybridizing with these oligonucleotide probes under stringent conditions (2^0C below Tm) are given in an autoradiograph (Fig. 3) and Table 8 (this data was obtained in collaboration with D.Hahn).

3.4.2 Discussion of results

The differential hybridisations of the α and β probes with the group of common scab strains support the phenetic data in the assignment of different taxonomic identities. The α probe hybridized with strains from clusters 1, 3, 5 and 12 but not with any of the strains from the cluster groups closest to the recently assigned type strain *S.scabies* nov., nom. rev. i.e. 18 and 19 (Williams *et al.*, 1983a). In contrast, the β probe only hybridized with ISP5078 from this group of strains. These hybridization results were confirmed by probing a range of strains, representative of the major *Streptomyces* species cluster groups (Williams *et al.*, 1983a). This specificity study of the *S.scabies* probes within the *Streptomyces* genus was undertaken by D.Hahn.

Fig. 3 Autoradiograph of dot blot hybridization between 16S rRNA of strains of *Streptomyces* species probed under stringent conditions with A - eubacterial consensus probe 5' AGGGTTGCGCTCGTTG (50^0C);
B - α4 5'GGCACCATCTCTGATGG (50^0C);
C - β1 5'ATCACCCCGAAGCATGCT (50^0C).

Organisms: Ia, ISP5078; Ib, ATCC 3352; Ic, ATCC 10246; Id, ATCC 15485; Ie, ATCC 3372; If, S46; Ig, S47; Ih, ISS.
IIa, 8.7; IIb, 8.2; IIc, 8.6; IId, 8.7; IIe, 8.8; IIf, MP2; IIg, MP9; IIh, 962.
IIIa, R1; IIIb, R2; IIIc, 968; IIId, 975.2; IIIe, 4.470; IIIf, ASS8112; IIIg, RA210; IIIh, 135.
IVa, 282; IVb, 978; IVc, TK24.

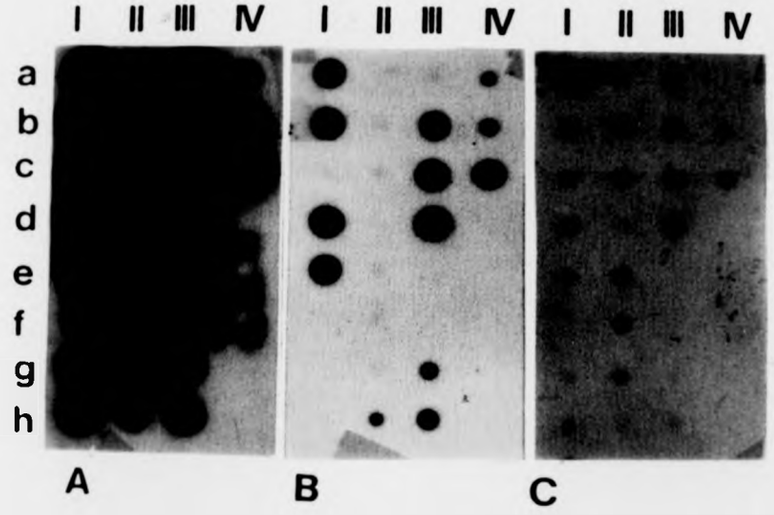


Figure 3

Table 8: Probing results from rRNA probed with the eubacterial primer 1115 (Embley *et al.*, 1988), α4 and β1 (Witt *et al.*, 1990) oligonucleotide probes

Strain	Identification	Eu 1115	α4	β1
ISP5078	*S.albidoflavus* (1)	+	+	+
ATCC 10246	*S.albidoflavus* (1)	+	-	-
S46	*S.albidoflavus* (1)	+	-	-
R2	*S.albidoflavus* (1)	+	+	-
ATCC 3372	*S.viridogenes* (3)	+	+	-
ATCC 3352	*S.atroolivaceus* (3)	+	+	-
962	*S.atroolivaceus* (3)	+	-	-
968	*S.atroolivaceus* (3)	+	+	-
975.2	*S.atroolivaceus* (3)	+	+	-
4.470	*S.atroolivaceus* (3)	+	-	-
135RB	*S.atroolivaceus* (3)	+	-	-
282RB	*S.atroolivaceus* (3)	+	-	-
978	*S.atroolivaceus* (3)	+	-	-
A1TCC 15485	*S.exfoliatus* (5)	+	+	-
S47	*S.rochei* (12)	+	-	-
ISS	*S.rochei* (12)	+	-	-
MP2	*S.rochei* (12)	+	-	-
MP9	*S.rochei* (12)	+	-	-
R1	*S.rochei* (12)	+	-	-
8.2	*S.rochei* (12)	+	-	-
8.17	*S.cyaneus* (18)	+	-	-
8.6	*S.diastaticus* (19)	+	-	-
8.7	*S.diastaticus* (19)	+	-	-
8.8	*S.diastaticus* (19)	+	-	-
ASS8112	*S.diastaticus* (19)	+	-	-
TK24	*S.lividans* (21)	+	+	-

Note: Filters washed at stringent conditions

The results from the hybridisation studies with the β probe, indicated that the β target site was a potential marker for differentiating this common scab strain from other pathogenic strains both in terms of the characterization of isolates and the detection of the strain in environmental samples. This result also confirmed the findings of Lambert & Loria (1989) and others on the atypical nature and hence erroneous designation as the type strain for the *S.scabies* group by Waksman & Henrici (1948).

3.4.3 Colony blot specificity testing of the β probe among strains from clusters 55 and 61

The above findings on the novelty of the β sequence to *S.scabies* ISP5078 were in direct contrast to those of Stackebrandt *et al.* (1991). In their comparison of the variable sequences of 77 streptomycete and *Streptoverticillium* strains, the β target sequence emerged common to a range of strains including streptomycetes and streptoverticillia from cluster groups 1, 3, 5, 10, 12, 29, 55 and 61 (Williams *et al.*, 1983a). The discrepancy between these findings and those of Stackebrandt *et al.* (1991) prompted the rapid testing of the specificity of the β probe against a range of strains from the clusters 55 and 61 in which Stackebrandt *et al.* (1991) had contrary to this data, observed its wide distribution. A summary of the results from the colony blot analysis are given in Table 9. The sequence appeared to be common to the 4 *Streptoverticillium* strains tested as well as a large proportion of the cluster 61 strains probed. These results had important implications for the application of the β targeted probe as an ecological tool. Its widespread occurrence amongst different members of the *Streptomyces* genus prevented its use in the monitoring and detection of specific populations of common scab strains. The β probe did still offer potential for the identification of common scab strains taxonomically related to ISP5078. The colony blot method

Table 9: Summary of colony blot probing results of a range of cluster 55 and *Streptoverticillium* strains

Strain	Identification	Eu 1115	β1
316	*S.polychromogenes* (61)	+	-
550	*S.katrae* (61)	+	+
194	*S.sacemochromogenes* (61)	+	+
558	*S.colombiensis* (61)	+	-
445	*S.subtilis* (61)	+	+
216	*S.lavendulocolor* (61)	+	-
069	*S.lavendulae* (61)	+	-
0767	*Stv.griseoverticillium* (55)	+	+
0435	*Stv. cinnamoneum subsp. azacoleta* (55)	+	+
0547	*Stv.septatum* (55)	+	+
051	*Stv.albireticuli* (55)	+	+
ISP5078	*S.scabies* (1)	+	+

Note: Hybridisations achieved after washing filters under stringent conditions

offered a rapid indication of probe specificity, however, the failure of duplicate filters to agree was indicative of the error associated with non-specific binding of the probe to debris from lysed colonies. While the trend in the hybridization of RNA from a number of cluster 61's and *Streptoverticillium* strains with the β1 probe appeared to be valid. These results could not be considered definitive.

3.4.4 Specificity of the β sequence among strains (pathogenic and non-pathogenic) closely related to those identified to harbour the β sequence (Stackebrandt *et al.*, 1991)

To resolve the confusion over the occurrence of this sequence it was considered important to investigate the specificity of the probe amongst a group of isolates both pathogenic and non-pathogenic that either appeared closely related to ISP5078 or to represent taxonomic groups harbouring the β sequence (Stackebrandt *et al.*, 1991). Ribosomal RNA was extracted according to method 1 (Section 2.24.1) for the group of strains given in Table 10. Strong hot signals were obtained by probing with the universal eubacterial probe 1115 (Embley *et al.*, 1988). However, signals from a duplicate filter probed with the β oligonucleotide probe were substantially weaker. Both blots were resolved by phosphorimage analysis and the results obtained presented in Table 10. This data supported the findings of Stackebrandt *et al.* (1991) on the distribution of this sequence among certain streptomycete and streptoverticillia strains. Hybridization signals were obtained from strains representative of clusters 1, 3, 12, 29 and 61.

One concern over the interpretation and significance of these results was that the rRNA probed and isolated according to method 1 was quite degraded. Probing degraded rRNA has been considered satisfactory using

Table 10: Summary of hybridizations among streptomycete and *Streptoverticillium* strains

Strain	Identification	Eu	β1
326	*S.alboviridis* (1)	+	+
598	*S.bacillaris* (1)	+	+
236	*S.griseus* (1)	+	+
077	*S.nitgersensis* (1)	+	-
0446	*S.albidoflavus* (1)	+	-
508	*S.nogaensis* (1)	+	-
0233	*S.ceolicolor* (1)	+	-
422	*S.coeliatus* (1)	+	-
632	*Streptomyces sp.* (1)	+	+
734a	*Streptomyces sp.* (1)	+	+
c463	*Streptomyces sp.* (1)	+	-
S46	*S.albidoflavus* (1)	+	+
R2	*S.albidoflavus* (1)	+	-
454	*S.viridogenes* (3)	+	+
ISP5078	*S.scabies* (3)	+	+
082	*S.violaceus* (6)	+	-
593	*S.fulvissimus* (10)	+	-
092	*S.alithoticus* (12)	+	+
160	*S.griseorubens* (12)	+	-
MP2	*S.rochei* (12)	+	-
MP9	*S.rochei* (12)	+	-
S47	*S.rochei* (12)	+	+
8.2	*S.rochei* (12)	+	+
ISS	*S.rochei* (12)	+	-

Table 10: Summary of hybridizations among streptomycete and *Streptoverticillium* strains (cont)

Strain	Identification	Eu	β1
R1	*S.rochei* (12)	+	-
054	*S.fascilutus* (29)	+	+
0547	*Stv.septatum* (55)	+	-
550	*S.katrae* (61)	+	-
194	*S.polychromogenes* (61)	+	+
216	*S.lavendulcolor* (61)	+	+

Note: Hybridisations performed under stringent conditions (2°C below Tm) with the eubacterial probe primer 1115 and the α4 targetted probe

oligonucleotide probes (Stackebrandt pers. comm.) with the proviso that the data generated with the specific probes is interpreted with reference to a duplicate blot probed with a universal eubacterial probe. The eubacterial probe was considered to be a reliable control to ascertain whether the RNA is sufficiently intact for probing.

3.4.5 Extraction and probing of intact 16S rRNA

Although the probing results confirmed the findings of Stackebrandt *et al.* (1991) on the distribution of the β sequence within the *Streptomyces*. Reluctance to define its specificity from the probing of degraded rRNA prompted a further study using a similar selection of strains from which intact rRNA was isolated (Method 3, Section 2.24.3).

Attempts to extract intact rRNA from a wide range of streptomycetes illustrated differences in the ammenability of strains to rRNA recovery (Fig. 4). A number of strains required modified growth conditions. Avoidance of cell lysis and hence RNA degradation prior to harvest was essential in the recovery of intact rRNA. ISP5078 was among the strains most susceptible to lysis in broth culture. Strains were handled so that baffles in flasks and the addition of large amounts of PEG 6,000 or sucrose (components that are used to promote diffuse growth and could also promote lysis) were either omitted or minimised. Broth cultures were harvested during log phase and all attempts were made to avoid handling ageing material that was close to lysing. In addition the use of frozen mycelial stocks was avoided, as the freeze thawing step also promoted cell lysis.

The differences observed between strains in the ease and recovery of rRNA stimulated speculation on the selective bias that could be introduced when

Fig. 4 rRNA isolated from a range of streptomycetes

Organisms: 1, 233; 2, 558; 3, R2; 4, 0727; 5, S46; 6, 191; 7, S46; 8, 0547; 9, ATCC 3352; 10, 734A; 11, ISS; 12, ATCC 3352. Intact ribosomal RNA was more readily recovered from strains 558, R2, S46; while degraded rRNA was recovered from strains ATCC3352, ISS and 191 under the same rRNA preparation regime.

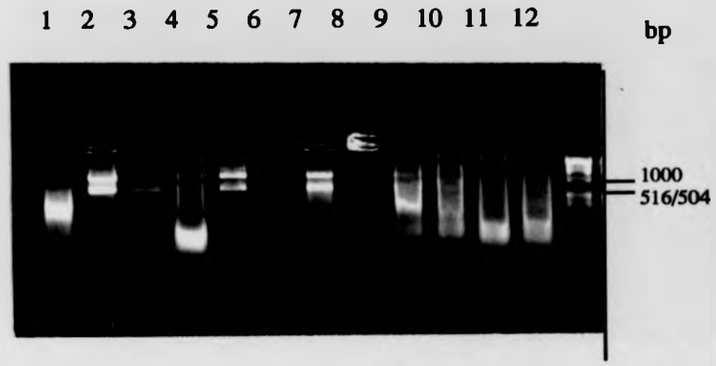


Figure 4

similar techniques are applied to the analysis of environmental communities. In order to extract intact rRNA from *Streptomyces*, growth and lysis conditions had to be optimised on a strain by strain basis. An approach that would not be possible in the recovery of rRNA from environmental samples.

The strains probed, together with the hybridization results obtained are given in Fig. 5 and Table 11. The pattern of differential hybridisation with the α probe observed in Table 8 was confirmed and expanded upon with additional strains. In addition, the confusion associated with the specificity of the β probe seemed to resolve itself. Very faint hybridization signals were obtained from the β probe at the 40°C low-stringency wash (Fig. 5c), however problems of low level contamination on the phosphorimage screen meant that no signals were detectable at the 50°C stringency wash. When the strength of signal from the α and primer 1115 probed blot was compared with that obtained with the β probe it was clear that signals were significantly weaker. These results indicated either poor binding of the β1 probe with the RNA or alternatively that there were problems associated with the probe itself either it had been labelled inefficiently or had undergone some degradation. The fact that this probe was exposed to similar treatment and preparation as the other probes it appeared unlikely that the labelling could be so much more inefficient or the probe be exposed to nuclease degradation. Hence, these basic checks should be performed to establish the integrity of the probe and before drawing conclusions on the ability of the probe to bind.

Poor binding of the probe does appear a probable explanation for the discrepancy between the data described above and Stackebrandt *et al.* (1991). Furthermore the data of Stackebrandt *et al.* (1991) appears to have been largely obtained through the comparison of sequences rather than

Fig. 5 Autoradiograph of dot blot hybridization between 16S rRNA of strains of *Streptomyces* species probed under non-stringent (40^0C) and stringent (50^0C) conditions with: A - eubacterial consensus probe 5'AGGGTTGCGCTCGTTG; B - α4 5'GGCACCATCTCTGATGG; C - β1 5' ATCACCCCGAAGCATGCT (Stackebrant *et al.*, 1991).

Fig.5a Organisms hybridizing with A- the eubacterial consensus probe (40^0C):

Ia, ATCC10246; Ic, 8.7; Ie, ATCC10246; If, 8.2; Ig, 8.2.

IIb, S47; IIc ATCC49173; IId, ISP5078; IIf, 0446; IIg, 233; IIh, 0547.

IIIc, 233; IIId, 727; IIIe, S46; IIIf, 558; IIIg, 598; IIIh, 8.7; IIIj, ISS.

IVa, ATCC3372; IVb, ATCC3352; IVc, R2; IVd, ISP5236; IVe, 326;

IVf, 069; IVg, ATCC15485.

Vd, 023; Ve, 023; Vf, S46; Vg, ISP5236; Vh, 632; Vi, 632.

VIc, 069; VIf, ISP5078; VIh, ISP5236; VIi, 092; VIj, 8.7.

VIIb, ATCC49173; VIIc, ATCC3352; VIId, 164.

Fig. 5b Organisms hybridizing with B- the α4 probe (50^0C):

IId, ISP5078; IIIb, c463; IVa, ATCC 3372; IVb ATCC 3352; IVc, R2;

IVd, ISP5236; Vg, ISP5236; Vh, 632; Vi, 632; VIf, ISP5078; VIi, 092.

Fig.5c Organisms hybridizing with C- the β1 probe (40^0C):

IIf, ATCC3352; IIIg, 558; IIIh, 8.7; IVf, 069; Vg, ISP5236; Vh, 632;

Vi, 632; VIf, ISP5078; VIh, ISP5236; VIj, 8.7; VIId, 164.

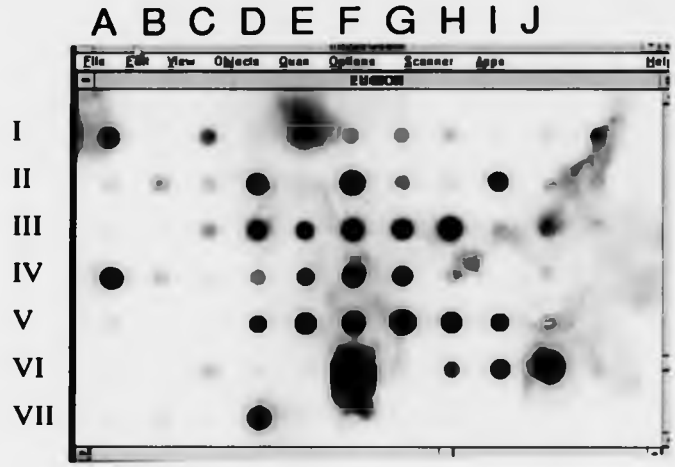


Figure 5a

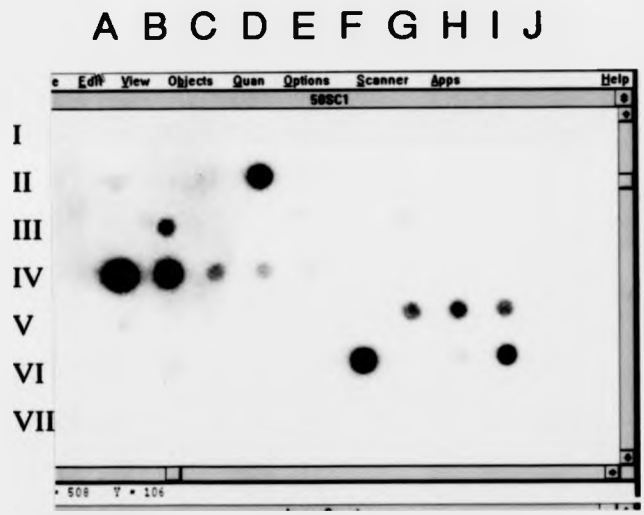


Figure 5b

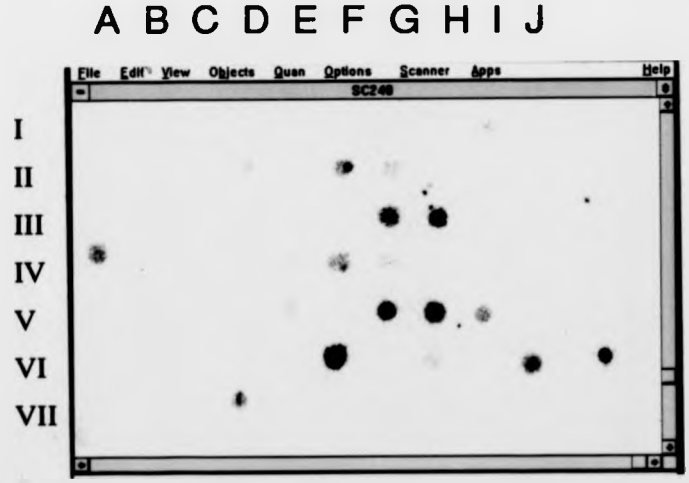


Figure 5c

Table 11: Specificity of α4 probe at stringent hybridisation conditions

Strain	Identification	Eu	α4
ISP 5078	*S.albidoflavus* (1)*	+	+
ATCC 10246	*S.albidoflavus* (1)*	+	-
S46	*S.albidoflavus* (1)*	+	-
O446	*S.albidoflavus* (1)	+	-
598	*S.bacillaris* (1) (ATCC 15855)	+	+
326	*S.alboviridis* (1)* (ATCC 25425)	+	-
ISP 5236	*S.griseus* (1) (ATCC 23345)	+	+
233	*S.ceolicolor* (1) (ATCC 23899)	+	-
023	*S.nitrosporeus* (1)	+	-
632	*S.albidoflavus* (1)	+	+
R2	*S.albidoflavus* (1)	+	+
ATCC 3352	*S.atroolivaceus* (3)*	+	+
ATCC 3372	*S.viridogenes* (3)	+	+
164	*S.litmocidene* (5)	+	-
727	*S.exfoliatus* (5)	+	-
ATCC 15485	*S.exfoliatus* (5)*	+	+
077	*S.nitgersensis* (1) (ATCC 12769)	+	-
O92	*S.althioticus* (12)	+	+
8.2	*S.rochei* (12)*	+	-
ISS	*S.rochei* (12)	+	-
8.7	*S.diastaticus* (19)*	+	-
ATCC 49173	*S.diastaticus* (19)*	+	-
558	*S.colombiensis* (61) (ATCC 27425)	+	-
O69	*S.lavendulae* (61)	+	-
0547	*Stv.septatum* (55)	+	-

* received as common scab strains

hybridization studies. It is difficult to account for the strong signal from ISP5078 observed in Fig. 3, but which has subsequently been unreproducible. Since the eubacterial probed blot indicated that similar amounts of RNA were present in all the samples, it cannot be attributed to an overloaded well of the manifold. However, it can only be concluded that in recent studies with the probe, blank blots (obtained in support of the result in Fig. 3) and initially attributed to be indicative of specificity were indicative of poor binding. The data generated in Table 10 actually appears indicative of the specificity of the probe, with these weaker signals were only observed with the added sensitivity of the phosphorimager. The cause for inefficient binding of the probe may be attributable to regions of 2° and 3° structure on the 16S rRNA molecule that prevent the probe reaching the target sequence. Similar problems of poor binding were also encountered with the β *S.lividans* probe (described by Witt *et al.*, 1989) during attempts to probe recovered RNA from *S.lividans* TK24 in soil (D.Hahn pers.comm.). The *S.lividans* probe is targeted against the same variable region of the 16S rRNA molecule (Witt *et al.*, 1989). Furthermore, the RNA applied to the filters had not been denatured prior to its application with either DMSO, heat or a mixture of formamide and formaldehyde (Hames & Higgins, 1987). Unfortunately, the occasion upon which denaturing the RNA was tested as a means to increase availability of the probe target sites and sensitivity of detection, was while working on the extraction of rRNA from soil using method 2. Denaturing RNA prepared using method 2 made no difference to the signal intensity, probably because the RNA clean up procedures involved several high salt precipitation steps which act to break down RNA secondary structure.

3.4.6 Probing of genomic DNA from selected *S.albidoflavus* strains

Genomic DNA was extracted from a limited selection of strains that included strain ISP5078 and probed with the β1 oligonucleotide probe. The results obtained are given in Table 12. Hybridization signals were detected from a number of *S.albidoflavus* strains and also a *Streptoverticillium* strain under stringent conditions, adding further evidence to the findings of Stackebrandt *et al.* (1991) on the wide spread distribution of this sequence among strains of this taxonomic identity. Furthermore the signals obtained were of a similar intensity to those obtained probing the RNA with this particular probe. According to Suzuki *et al.* (1988) there are six rDNA operons in *Streptomyces* species, assuming that the β1 sequence within each operon is the same it is expected that there would be up to six target sites for the probe per genome. This is in contrast to the vast numbers of rRNA copies that might be anticipated in a cell, numbers of 10^4 to 10^5 have been estimated (Barry *et al.*, 1990). These results illustrate further the problems that were encountered when applying the β1 probe to RNA and support the view that poor hybridization signals could be attributable to poor binding as a result of the ribosomal RNA secondary structure obscuring target sites.

In conclusion, the weak signals obtained in Table 10 appear not to be a symptom of badly degraded RNA but rather a reflection of the actual specificity of the probe. Results from the α probe and eubacterial probe were consistently reproducible and since the β probe did not confer additional specificity, rather more limited specificity (Stackebrandt *et al.*, 1991). Future studies should focus on the application of the α probe to the differentiation of scab isolates of the *S.albidoflavus*/*S.atroolivaceus* phenotype from the *S.cyaneus*/*S.diastaticus* phenotype. Data on the widespread distribution of the α and β target sequences among streptomycetes establish the poor suitability

Table 12: Probing of genomic DNA to establish specificity of β1 target sequence among some *S.albidoflavus* strains

Strain	Identification	Eu (50^0C)	β1 (40^0C)	β1(50^0C)
ATCC 25481	Cluster 1	+	+	+
DSM 40023	Cluster 1	+	+	-
ATCC 23345	*S.griseus*(1)	+	+	-
KCI-S-0446	Cluster 1	+	+	-
ATCC 27416	*S.annulatus*(1)	+	+	+
DSM 40508	Cluster 1	+	+	+
c463	Cluster 1	+	+	+
232	Cluster 1	+	+	+
ISP5078*	*S.albidoflavus*(1)	+	+	+
ATCC 23934	*Streptoverticillium*	+	+	+

* Common scab strain

of probes targeted to these sequences as tools for the detection of strains in environmental samples. These findings also illustrate the difficulties in identifying unique naturally occurring genetic markers within the highly conserved 16S rRNA molecule, for members of the *Streptomyces*. The data from these hybridization studies did however, assist in our overall objective i.e. to clarify the taxonomic position of the common scab strains. It supported the premise that common scab strains are a heterogeneous grouping and that the phenotypic heterogeneity is supported by genetic differences (identifiable on the 16S rRNA molecule).

3.5 Pathogenicity of common scab strains

Putative pathogenic streptomycetes were tested for pathogenicity using two methods in the glasshouse. Recent studies on the taxonomy of the scab-causing organism were largely unsupported by pathogenicity data (Lambert & Loria, 1989; Healy & Lambert, 1991). Pathogenicity of isolates isolated from Maine was established in the study by Lambert & Loria (1989) but not for the other strains that were acquired from culture collections as putative pathogens.

3.5.1 Pathogenicity test 1

Results from the first test are presented in Fig. 6. Maris piper potatoes were arranged in a randomised block and grown in 6 times 1.5 L pots in a modified JI compost. the moisture holding capacity (M.H.C.) of the compost was estimated to be 29% and soil moisture levels were maintained at less than 50% M.H.C.. The compost was inoculated with specific test strains. A randomised block design was adopted to minimise the effects of the environmental gradients in the glasshouse. Strains calculated to be significantly pathogenic, in terms of percent coverage with scab lesions according to the Mann-Whitney coefficients at the α 0.05 probability level were: ISP5078, S47, 8.2, 8.7, 8.8,

Fig. 6 Pathogenicity of scab isolates on scab-susceptible potato variety, Maris Piper.

Fig. 6a Percentage cover of scab lesions on the surface of potato tubers.
Percentage cover was estimated according to Large & Honey (1955), scab isolates 8.2, 8.17, 8.7, & 8.16 appeared highly virulent; while scab strain ISP5078 appeared moderately virulent.

Fig. 6b Pathogenicity of isolates was estimated according to the scab index (Bjor & Roer, 1980).
The scab index is based on the % coverage of tubers with scab lesions as well as the severity of the individual lesions. Good correlation has been observed between these criteria, with a high percentage cover usually corresponding with severe lesions.

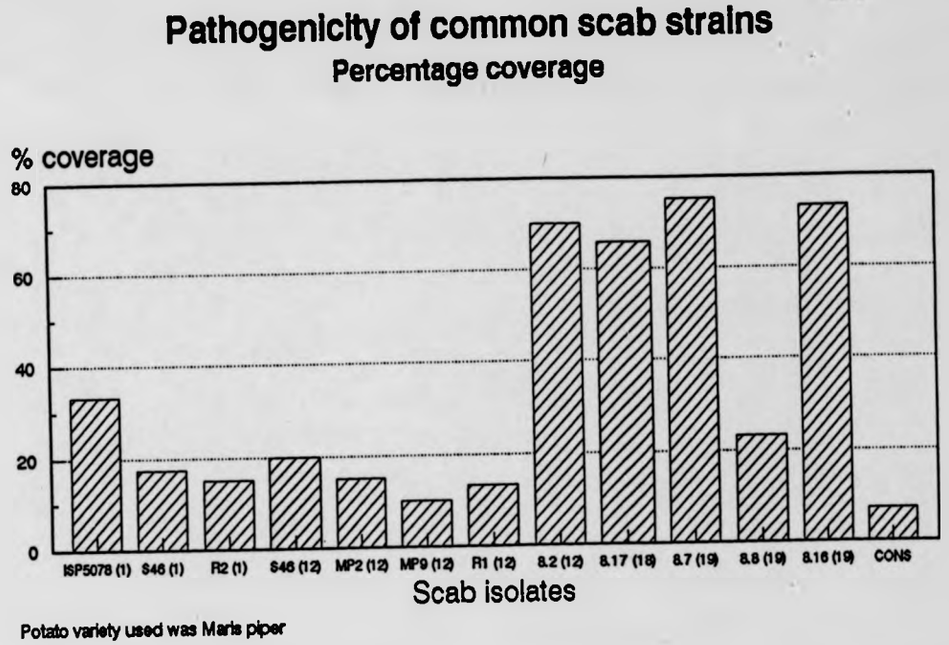


Figure 6a

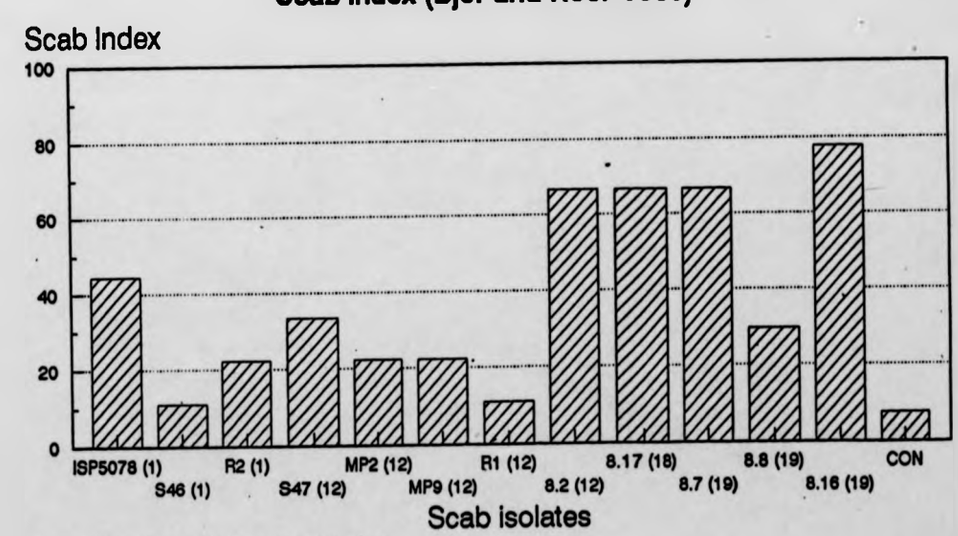


Figure 6b

8.16 and 8.17. The results are given in more detail in Fig. 6 and Table 13. Strains were determined to be pathogenic on the basis of % cover of scab lesion for tubers on each plant. Pathogenicity scores from test pots were ranked together with scores from uninoculated control pots and the Mann-Whitney coefficient calculated. The Mann-Whitney coefficient was used as the data was not assumed to be non-normally distributed and the values assigned were given on an arbitary basis i.e. they were not based on ratio or interval data.

3.5.2 Pathogenicity test 2

This trial was performed in association with the plant pathologists at the Agricultural University of Wageningen. It contained additional replication and larger pots. 10 times 10 L pots were inoculated with each isolate and seeded with the scab susceptible potato variety Desiree. The pots were filled with a well draining compost soil of 50% peat and 50% sand with a M.H.C. estimated to be 32%. Higher levels of soil moisture (in excess of 50%-60% M.H.C.) were adopted, as low moisture levels severely inhibit tuber and root development. Well developed tubers allow better development of scab symptoms (Fellows, 1926). There is a fine balance between sufficient soil moisture to produce good root and tuber development and a dry soil to promote the growth and development of streptomycete populations and hence scab symptoms. The pots were not arranged randomly, but in blocks of 10 with 2 to 3 blocks separated by a row of uninoculated control pots. This compromise on spreading the environmental variables of the glasshouse evenly through the trial was exchanged for better containment of inoculants to blocks of infected pots. One concern with the randomised block lay out was that the most virulent isolates would infect neighbouring pots thus skewing the patterns of pathogenicity among the strains. Obviously the redesign of the trial

Table 13: Pathogenicity data from trial 1 (Maris Piper)

Strain	Identification	%coverage[1]	Lesion type[1]	Scab Index[1]	Pathogenic[2]
ISP5078	*S.albidoflavus*	4	3	44	Yes
S46	*S.albidoflavus*	3	1	11	No
R2	*S.albidoflavus*	3	2	22	No
S47	*S.rochei*	3	3	33	Yes
MP2	*S.rochei*	3	2	22	No
MP9	*S.rochei*	3	2	22	No
R1	*S.rochei*	3	1	11	No
8.2	*S.rochei*	6	3	67	Yes
8.17	*S.cyaneus*	6	3	67	Yes
8.7	*S.diastaticus*	6	3	67	Yes
8.8	*S.diastaticus*	4	2	30	Yes
8.16	*S.diastaticus*	7	3	78	Yes
CONS		1.5	1	5	

[1]values derived from the pathogenicity key of Bjor & Roer (1980)

[2]pathogenicity scores determined to be significant with the Mann-Whitney coefficient

from experiment 1 meant that the potato common scab environment was altered quite significantly and that direct comparison between tests was difficult.

Additional measurements to the percentage cover of tubers were made, these were tuber number and weight. In both experiments all progeny tubers greater than 1 cm in size were assessed for the presence of scab symptoms. Data from the trial is presented in Fig.7 and Table 14. Strains identified to be significantly pathogenic at the α 0.05 probability level were: 8.17, 078, 8.8, MP9, 8.16, ASS8112, R2, R1, A391 and S47. These strains have a mixed taxonomic identity, with strains identifying to *S.albidoflavus, S.atroolivaceus, S.rochei, S.cyaneus* and *S.diastaticus*. A number of strains including 078, S47, 8.8, 8.16 and 8.17 appeared to be pathogenic under both glasshouse regimes. A number of strains were found to be significantly pathogenic in one trial but not in the other these included strains: R2, MP9, R1 and 8.2. The first three strains were not identified as highly virulent strains in either trial and hence it was conceivable that the differences in host plant variety and environmental conditions between the trials could be sufficient to push the pathogenicity score in either direction across the pathogenicity threshold. The differences in the trials seen by strain 8.2 are more difficult to explain as it appeared virulent in the Maris Piper trial with a scab index of 67% and not pathogenic in the Desiree trial (scab index 22%). Again it is plausible that the difference in pathogenicity is attributable to the altered environmental/ plant host variety as these influences have been been documented widely (Lapwood, 1972; Jellis, 1977). Alternatively changes in the pathogenic properties of this strain may have occurred while the strain was maintained in the laboratory for the 18 month/ 2 year period between the trials. These differences do serve to illustrate the difficulties associated with this type of pathogenicity assay and

Fig. 7 Pathogenicity of scab isolates on scab-susceptible variety, Desiree.

Fig. 7a Percentage cover of the surface of potato tubers with scab lesions

Isolates that appeared moderately to highly pathogenic according to the percentage cover criteria (Large & Honey, 1955) include: ISP5078, R2, 8.8 & 8.16.

Fig.7b Pathogenicity of isolates estimated according to the scab index of Boer & Roer (1980).

In general, strains that appeared pathogenic according to the % coverage criteria also appeared pathogenic according to the scab index criteria. However, a few differences were observed; strain 8.17 was illustrated to be a slightly more virulent strain than estimated aaccording to % cover; while strains ISP5078 and R2 appeared less virulent than the estimates obtained in Fig. 7a.

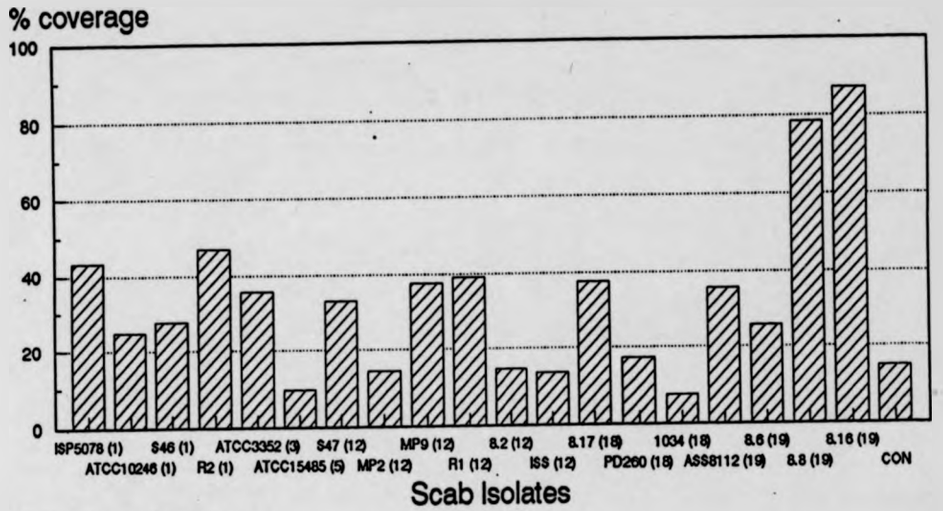


Figure 7a

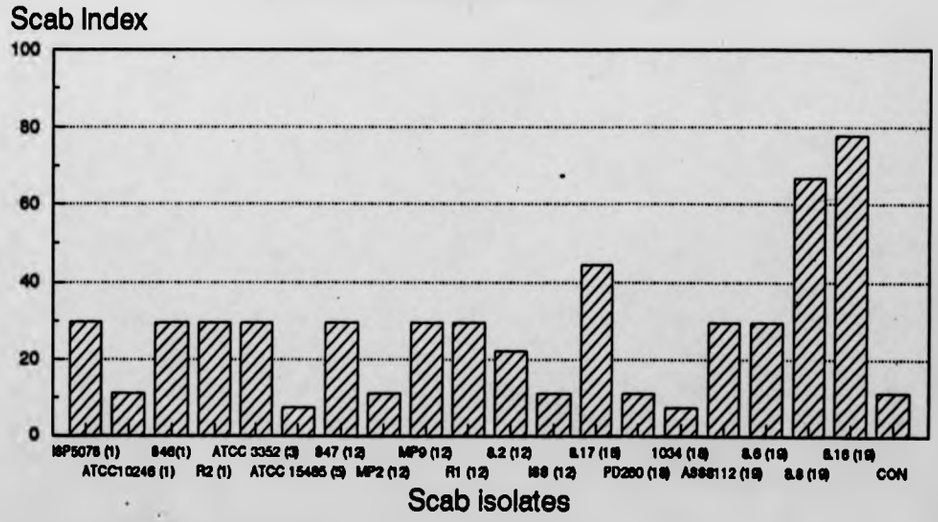


Figure 7

Table 14: Pathogenicity data from trial 2 (Desiree)

Strain	Identification	%coverage[1]	Lesion type[1]	Scab Index[1]	Pathogenic[2]
ISP5078	*S.albidoflavus*	4	2	30	Yes
ATCC10246	*S.albidoflavus*	3	1	11	No
S46	*S.albidoflavus*	4	2	30	Yes
R2	*S.albidoflavus*	4	2	30	Yes
ATCC3352	*S.atroolivaceus*	4	2	30	Yes
ATCC15485	*S.exfoliatus*	2	1	7	No
S47	*S.rochei*	4	2	30	Yes
MP2	*S.rochei*	3	1	11	No
MP9	*S.rochei*	4	2	30	Yes
R1	*S.rochei*	4	2	30	Yes
8.2	*S.rochei*	3	2	22	No
ISS	*S.rochei*	3	1	11	No
8.17	*S.cyaneus*	4	3	44	Yes
PD260	*S.cyaneus*	3	1	11	No
1034	*S.cyaneus*	2	1	7	No
8.6	*S.diastaticus*	4	2	30	Yes
8.8	*S.diastaticus*	6	3	67	Yes
8.16	*S.diastaticus*	7	3	78	Yes

[1] Values derived from the pathogenicity key of Bjor & Roer (1980)

[2] Pathogenicity scores determined to be significant with the Mann-Whitney coefficient

the urgency required in the development and standardisation of an assay to alleviate the problems of environmental variation.

In a number of instances the type of scab lesion seen on infected tubers altered with the susceptible potato variety. This is because the corky scab lesions are a product on the host plant defence mechanism as well as the invading pathogen. The severity of the lesions has been attributed to the degree of resistance or susceptibility of the potato variety to the invading pathogen (Leach *et al.*, 1938), rather than an attribute of the pathogen itself. For instance 078 produced more superficial but far more wide spread lesions on Desiree than Maris Piper (Fig. 8) and 8.16 produced raised scabs rather than pitted scabs on Desiree as opposed to Maris Piper. These differences are consistent with the scab susceptibility ratings given to the varieties by NIAB. Hence Maris Piper is given a score of 2 and Desiree 3, with 2 indicating most susceptibility and 8 most resistance.

3.5.3 The relationship between pathogenicity (determined by % cover of tubers with scab lesions) and affect on crop yield.

Generally common scab is considered to be a superficial disease of the potato affecting primarily the appearance of the potato rather than the yield. However, a number of reports indicate that certain pathogenic strains e.g. those identified to cause netted scab severely attack the root systems and substantially reduce yields (Scholte & Labruyere, 1985). Two indices of yield, tuber number and weight were also correlated against percent coverage with scab lesions. No positive correlations were identified indicating that crop yield was independent of infection. The highest correlation (r=0.642) was observed in a plot of weight of progeny tubers against % coverage by scab,

Fig. 8 Scab infection of Maris Piper, Pentland Crown and Desiree potatoes with ISP5078.

Fig.8a Scab symptoms obtained on Maris Piper and Pentland Crown.
Maris Piper potatoes (NIAB scab susceptibility rating 2) exhibited a moderate to severe scab symptoms with infection with ISP5078. In contrast, Pentland Crown (NIAB scab susceptibility rating 8) was resistant to infection.

Fig. 8b Scab symptoms obtained on Desiree.
ISP5078 infection of Desiree was considerably more superficial than that observed on Maris Piper. Tubers were covered extensively in superficial blemishes rather than penetrating lesions. Desiree has been given a scab susceptibility score of 3 by NIAB.

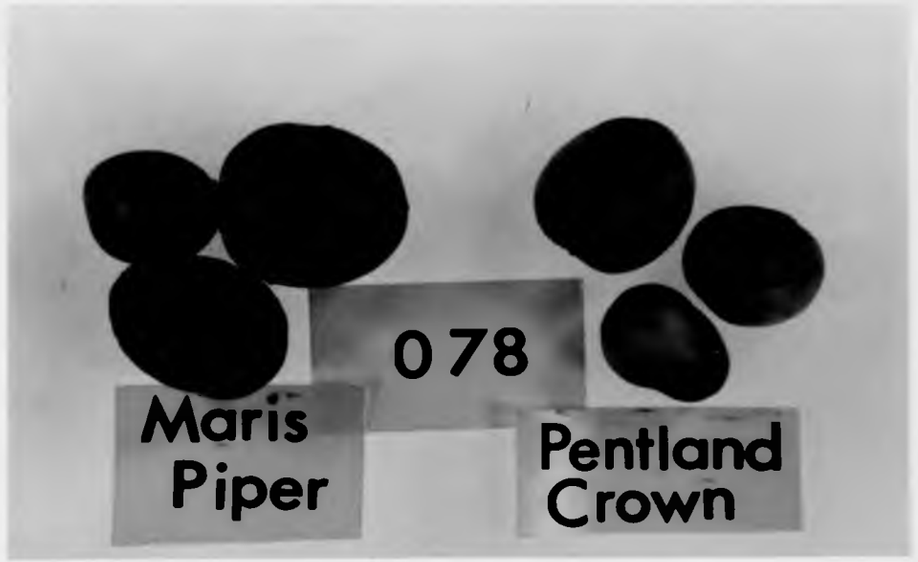


Figure 8a

Figure 8b

supporting a trend in the development of the symptoms with an increase in weight and size of the tuber. A trend observed by Fellows (1926).

3.6 Clustering of putative common scab strains on the basis of phenotype, hybridization with 16S rRNA targeted oligonucleotide probes for *S.scabies* ISP5078 and pathogenicity.

Cluster analysis was performed on the data from the phenotypic tests included in the probabilistic identification matrix (Williams *et al.*, 1983b), hybridization signals obtained with the α4 and β1 probes for *S.scabies* (Witt *et al.*, 1989) and pathogenicity of isolates, determined to statistically significant. Data was organised into matrices in a binary form. The characters of pathogenicity and hybridization with the 16S rRNA probes were weighted to be equivalent of five phenotypic characters. The data has been analysed using several clustering coefficients and is presented as dendrograms (Figs. 9, 10 & 11).

The dendrogram given in Fig. 9 has been constructed by analysing the data using the SSM UPGMA coefficients. The SSM or matching coefficient, estimates similarity on the basis of positive and negative matches, it is the proportion of characters that have the same state (Sneath, 1962). UPGMA or unweighted average linkage indicates that the simple arithmetic average of the similarities across the two groups is taken, equal weight is assigned to each similarity. UPGMA has been recommended for most cluster analysis purposes and has been found to maximise co-phenetic correlation (a measure of hierarchical) (Jones & Sackin, 1980). The strains separate out into clusters accordant with the taxonomic identities obtained with the probabilistic identification matrix (Williams *et al.*, 1983b). Hence the *S.albidoflavus*,

S.atroolivaceus strains cluster at the 66% similarity level and separate out from the *S.cyaneus/ S.diastaticus* which cluster at the 72% similarity and the *S.rochei* strains clustering at approx. 71% similarity. Two strains identified as *S.albidoflavus* fall within the *S.rochei* cluster, indicating the heterogeneity within this cluster (Williams *et al.*, 1983a).

The dendrogram given in Fig. 10 has been produced by analysing the data with the Jaccards and UPGMA coefficients. The Jaccards coefficient does not take the negative matches into account, i.e. in an assessment of similarity two negatives do not count as a similarity (Sneath, 1962). Again the *S.albidoflavus* strains appeared taxonomically distinct from the other scab strains, forming a tight grouping at the 62% level of similarity. The remaining group separated out into two smaller groups one composed of the majority of the cluster 18 and 19 strains at the 55% level of similarity, the other included the *S.rochei* strains and some cluster 1 strains which also clustered at the 55% level of similarity. MP2 and ATCC 10246 appeared as outlying strains separate from the remaining clusters.

The dendrogram given in Fig. 11 has been produced by analysing the data using SSM and single linkage. Single linkage defines the similarity between two groups as the similarity of the two most similar strains, one in each group. The *S.albidoflavus/ S.exfoliatus* strains form a tight cluster at the 68% similarity level, the *S.cyaneus* and *S.diastaticus* strains also cluster together at a similarity level of 60%, with the *S.rochei* strains separating out at the 70% level of similarity. Two outlying strains emerged with this treatment of the data, these strains are MP2 and ATCC 10246 which do not appear similar to the other three clusters. MP2 was identified as *S.rochei* and ATCC 10246 as *S.griseus*.

Fig. 9 Dendrogram to illustrate clustering of scab strains (SSM and UPGMA coefficients).

Scab strains have been clustered on the basis of phenotypic characters, derived from the probabilistic identification matrix (Williams *et al.*, 1983b) pathogenicity and hybridization with 16S rRNA probes. The clusters produced are consistent with the identifications obtained (Williams *et al.*, 1983b). Strains with an *S.albidoflavus*, *S.atroolivaceus* phenotype fall together as do the *S.rochei* strains and the *S.cyaneus*, *S.diastaticus* strains.

Figure 9

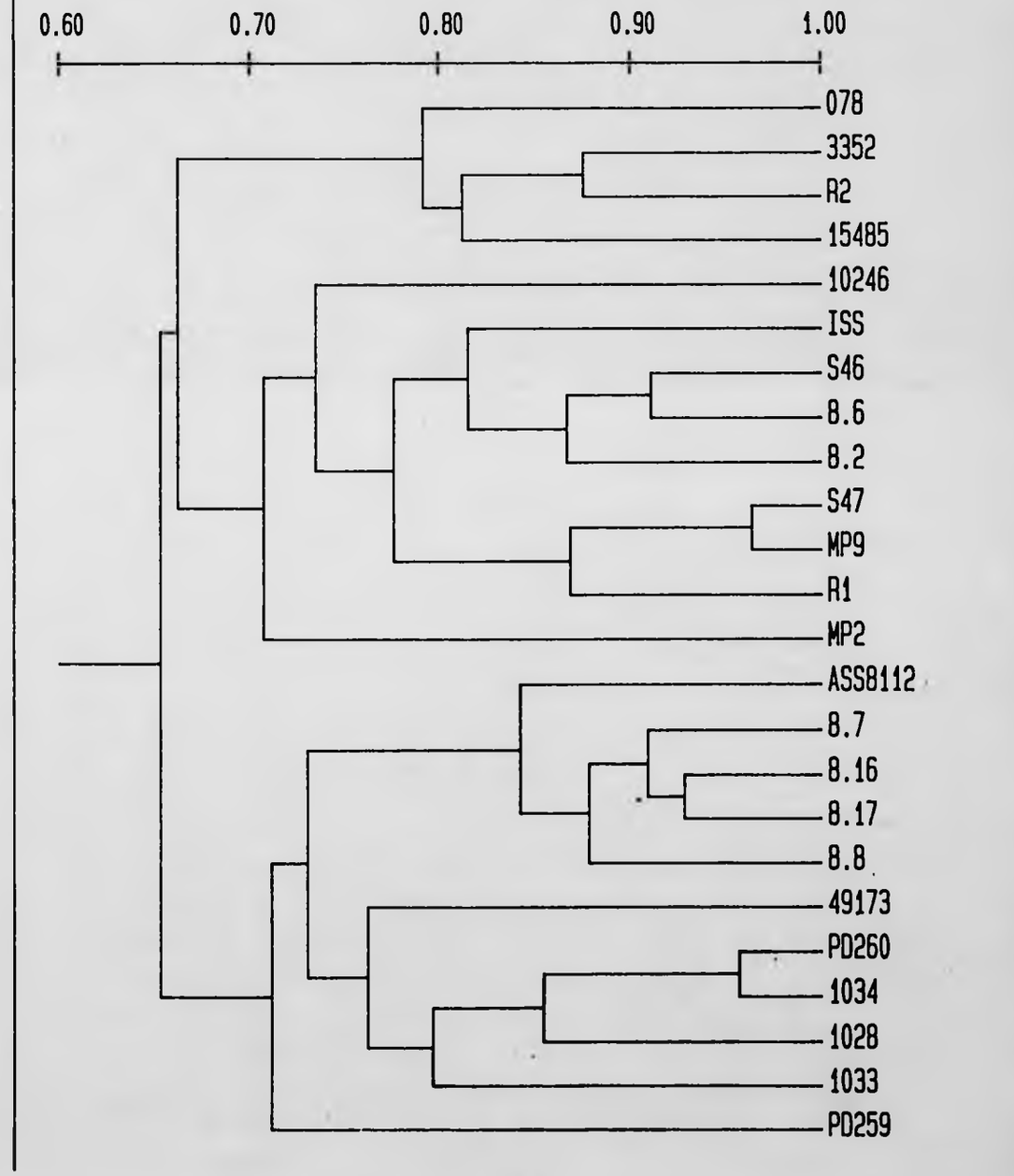


Fig. 10 Dendrogram to illustrate clustering of scab strains (Jaccards and UPGMA coefficients).

Scab strains have been clustered on the basis of phenotypic characters derived from the probabilistic identification matrix (Williams *et al.*, 1983b), pathogenicity and hybridization with 16S targeted rRNA probes. Clusters were produced that were consistent with phenotypic identifications; hence *S.albidoflavus* strains grouped together as did the *S.rochei* and *S.cyaneus*, *S.diastaticus* strains. Two outlying strains ATCC10246 and MP2 did not fall in any of the cluster groups described above.

Figure 10

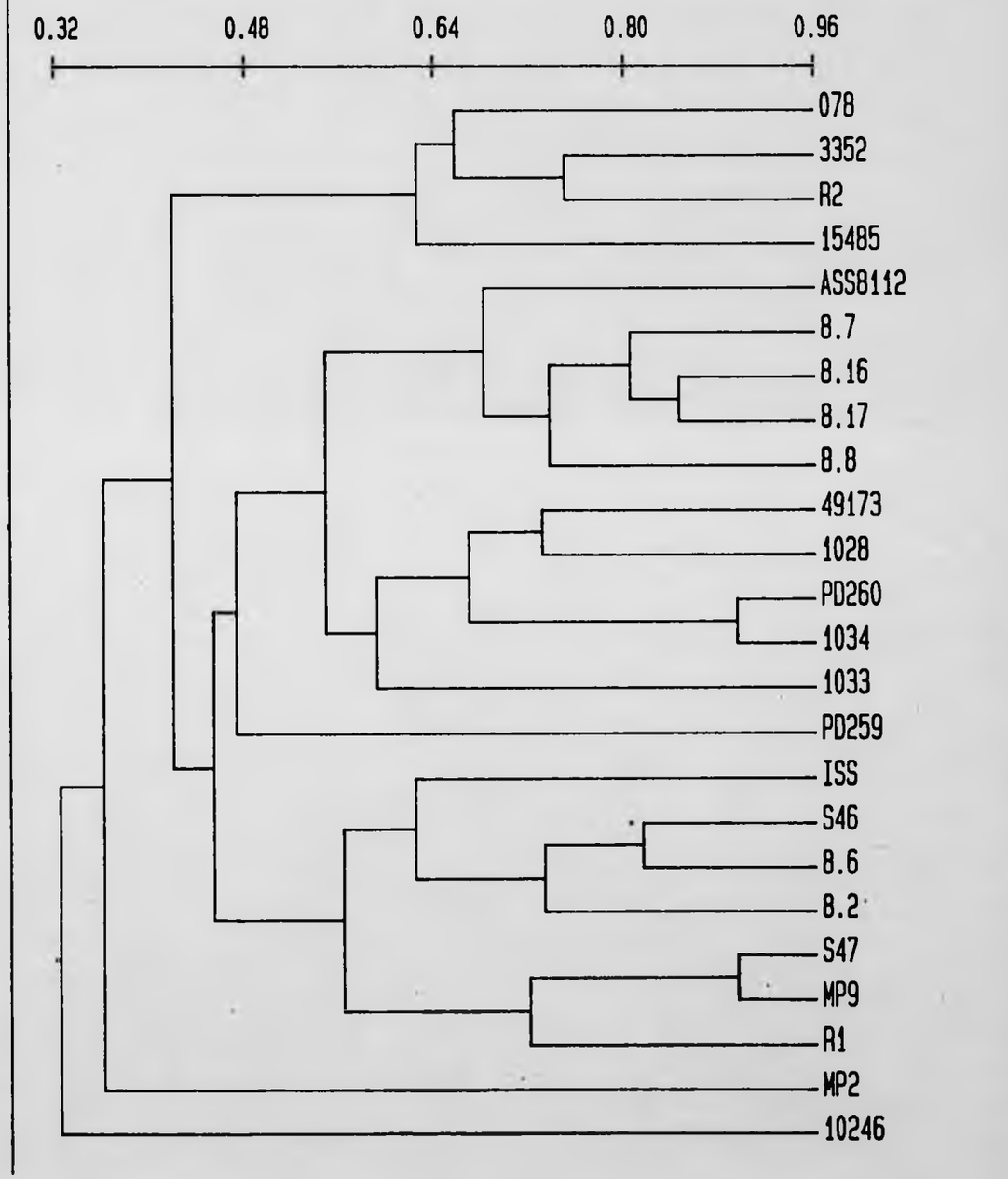
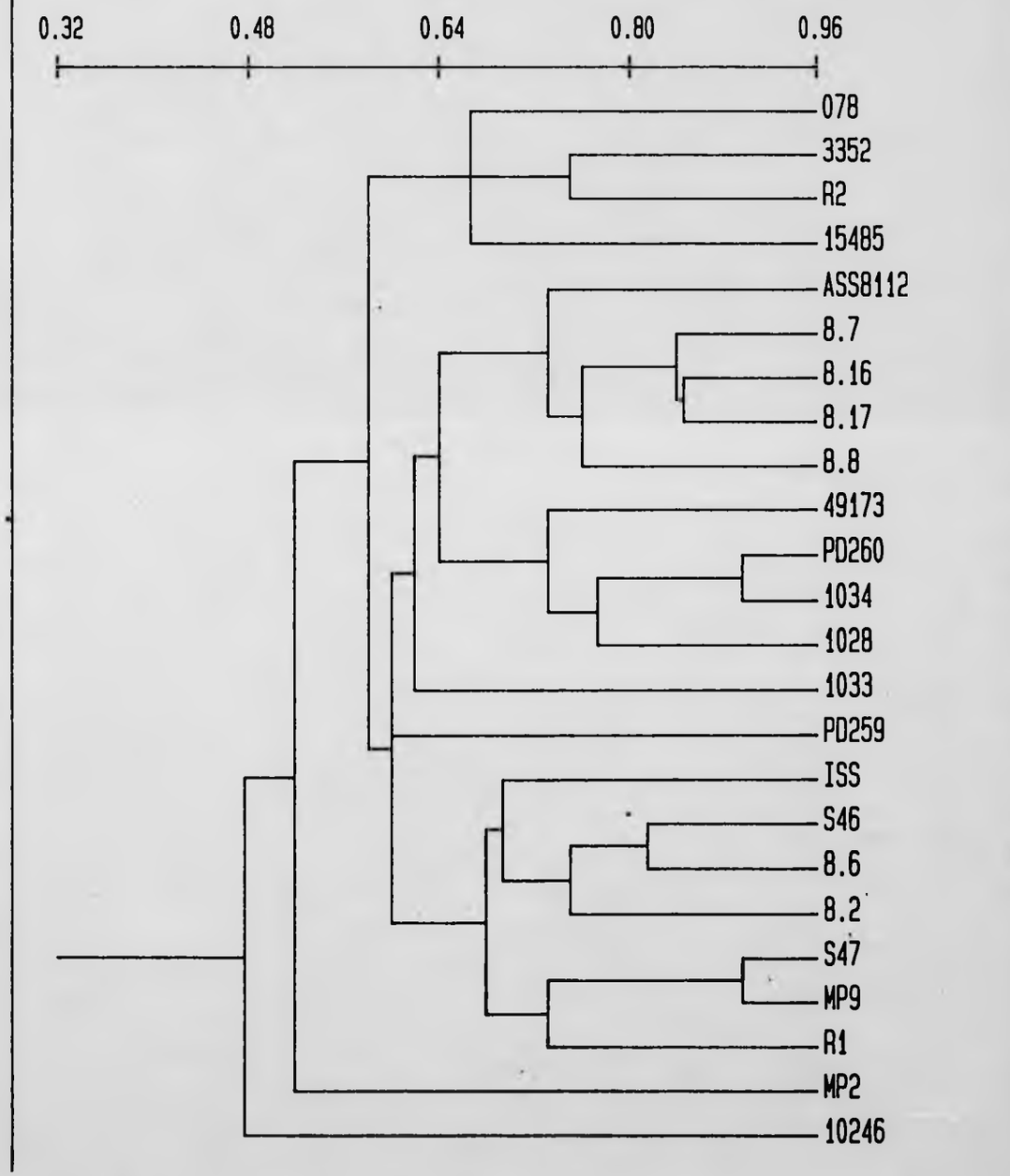


Fig. 11 Dendrogram to illustrate clustering of scab strains (SSM and Single linkage coefficients).

Scab strains have been clustered on the basis of phenotypic characters derived from the probabilistic identification matrix (Williams *et al.*, 1983b), pathogenicity and hybridization with 16S rRNA targeted probes. Good congruence was observed between phenotypic identifications and the cluster groups formed. Strains with a *S.albidoflavus* identity fell together as did, the *S.rochei* and the *S.cyaneus* and *S.diastaticus* strains. Strains MP2 and ATCC 10246 did not group within the clusters described above.

Figure 11



In conclusion, the clustering of the data using a number of coefficients further demonstrated findings of heterogeneity among this group of scab-causing streptomycetes. The fact that these groups appear stable when treated with a number of coefficients emphasised the distinctness of the groups and that the isolates investigated in this study did not have a common taxonomic identity.

3.7 Preliminary studies on the application of a minituber assay for assessing pathogenicity

Developing mini tubers induced on Maris piper plantlets were inoculated with spore suspensions of ISP5078 and MP2. From 6 flasks each bearing 15 to 20 plantlets initiating tubers, the tubers in 2 flasks were inoculated with MP2, 2 flasks with ISP5078 and 2 flasks were left uninoculated. Scab symptoms were visible on the surface of most tubers after 1 month. Symptoms were comparable in severity to those observed on Maris piper in the glasshouse. Hence infection with ISP5078 was more severe with scabbed areas being larger and lesions deeper than those seen on the MP2 infected minitubers. Mini tuber material infected with ISP5078 was viewed under the scanning electron microscope (Fig. 12). A second study was set up to look at the development of a pathogenic strain in association with the developing mini tuber. However, the mini tubers were well developed at the time of inoculation and induction of scab symptoms failed to occur. Furthermore when the surface of the minitubers was examined under the scanning electron microscope the tubers were covered in spores that had not germinated. Mini tuber material was examined from the date of inoculation at days 0, 2, 5, 15 and 30. It was concluded that similar to the findings of workers in the field and glasshouse that mini tubers also pass through a period of susceptibility to infection coincident with the transition of developing stomata into lenticels and that in this later experiment the tubers were inoculated at a stage beyond

Fig.12 Scanning electron micrographs of scab pathogen ISP5078 colonising the surface of axenic minitubers.

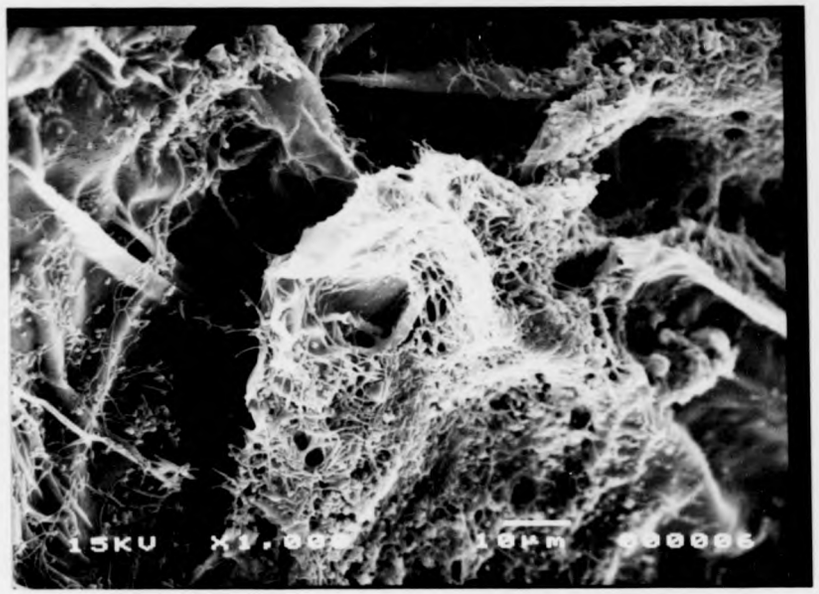
SEM micrograph I (Magnification X 1,000)

SEM analysis of the infected minituber surface (minituber scab lesions) was undertaken at approximately 4 weeks after inoculation of the tuber surface with a streptomycete spore suspension. The micrograph illustrated the minituber surface to be a rich source of nutrients for streptomycete inoculants, as prolific development of the mycelial form of the inoculant was observed. The inoculant was also observed to be sporulating at the tuber surface.

SEM Micrograph II (Magnification X 1,500)

A micrograph at a higher magnification indicated the prolific growth of the mycelial phase of the inoculant, there was little evidence of sporulation at this microsite indicative of an abundance of nutrients.

I



II

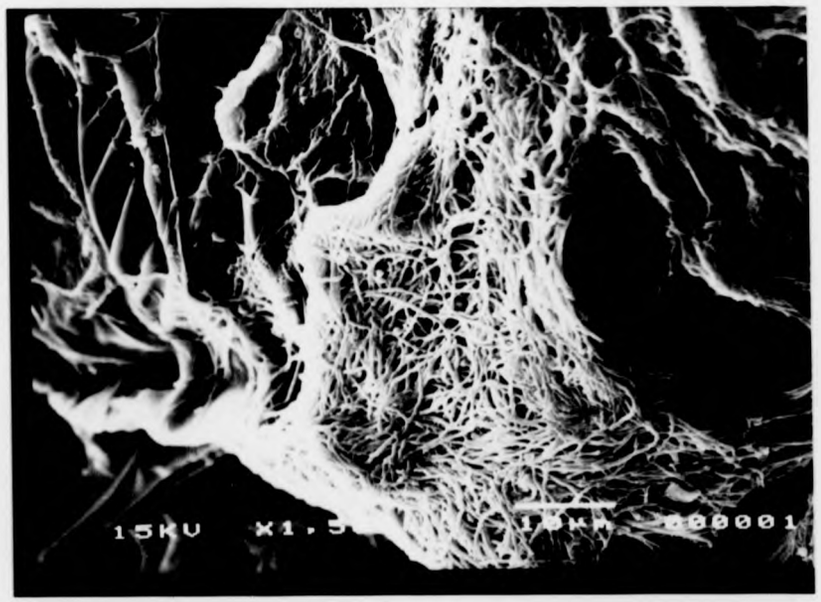


Figure 12

susceptibility to infection. However, this explanation did not account for the lack of germination of the *Streptomyces* spores which while unable to infect the tubers would normally in the absence of competition colonise the tuber surface. Lawrence & Barker (1963) report that they were unable to induce scab symptoms on mini tubers in a tissue culture environment and found it necessary to transfer the developing tubers (approx. 16 days after initiation) into moist vermiculite in a petri dish. The change in growth environment promoted infection and the development of scab symptoms. Hence it is probable that the absence of germination of the *Streptomyces* spores is attributable to the hostile growth conditions of the humid tissue culture environment and possibly the surface of the mini tuber. Mini tubers induced in tissue culture appeared to produce a thicker waxy coating on the tuber surface than is evident on glasshouse or field grown tubers. Lawrence & Barker (1963) discovered that they could promote the severity of infection of mini tubers dramatically by washing the mini tubers with water and soaking in dilutions of methanol, prior to inoculation. They are unable to explain why the washing procedure promoted infection but suggested the dilution of natural compounds on the surface or the possible alteration of osmotic values or the permeability of the tuber surface. These findings suggest that the untreated mini tuber surface may have properties of a physical or chemical nature that may be inhibitory to the growth of *Streptomyces* strains.

3.8 Concluding discussion and future work

Our data support the view of the presence of more than one taxonomic group of plant pathogenic streptomycetes. This is in contrast to Lambert & Loria (1989) who propose that pathogenic strains form a taxonomically defined grouping. However the data does support Lambert & Loria (1989) in their

designation of a new type strain for the *S.scabies* species and their conclusions on the erroneous designation of ISP 5078 by Waksman & Henrici (1948) and its atypical nature amongst other scab strains. It is apparent that a large number of virulent pathogens do appear taxonomically related to the strain assigned by Lambert & Loria (1989) in their revival of the species. However, there are additional virulent strains that do not appear related to *S.scabies* sp. nov., nom. rev. (Lambert & Loria, 1989). Our findings suggest the presence of several centres of variation, identified tentatively as the group of strains around the *S.albidoflavus* cluster 1, *S.rochei* cluster 12 and *S.cyaneus*, *S.diastaticus* clusters 18 and 19. The presence of other pathogenic groups with low values of relatedness to the *S.scabies* sp. nom., nov. rev. has been recently supported by DNA homology studies (Lambert & Healey, 1991). Furthermore the identification of several groups of plant pathogenic strains in this study has been supported by pathogenicity studies. Expertise from plant pathology and actinomycete taxonomy has been combined in the elucidation of the taxonomy of this organism, which has up until now, though deemed essential, largely failed to occur.

The groups of pathogenic strains that have been identified result in part from the source of our strains and any limitations imposed by our methods of characterization. One could speculate that a large scale worldwide study on the isolation and characterization of the causal agents from scabbed material, would reveal further phenotypic and genotypic variation among isolates with pathogenic properties. Further characterization based on phenotypic and genetic markers (eg. 16S rRNA probes) of pathogenic isolates from agricultural centres worldwide will only help to resolve the confused situation. It is critical that this work is underpinned by reliable and standardised assessments of pathogenicity. Essential perquisites are reliable quantitative

measurements, the current measures comprise qualitative keys based on subjective assessments of percentage coverage or lesion type. A quantitative assay e.g. that associated with the production of a toxin [such as that identified by Lawrence *et al.* (1990)] would be invaluable in comparisons of pathogenicity.

The use of the minituber assay used by Lawrence *et al.* (1990) offers a powerful solution to the problems associated with environmental fluctuations in the field and glasshouse. However, one concern over the artificial nature of such assays would be the comparablity of the pathogenicity observed in a minituber assay to that seen in the glasshouse and field. These studies demonstrated comparable infections *in vitro* and *in vivo*. An advantage of the sterile system is the confidence that the inoculant was responsible for the observed symptoms and the absence of any background contamination in the uninoculated controls. In the glasshouse the possibilities for the contamination of treatments with pathogenic isolates that have been either introduced or indigenous are vast. In trial 2 it was possible to see the transfer of aggressive strains into the adjacent uninoculated control pots from the presence of the scab lesion pattern associated with that strain on control tubers. For this reason it was helpful to have the plants in blocks rather than having the individual plants randomised, as it was easier to assess the pattern of a scab infection through a block of ten plants.

It is possible that the mini tuber assay may select for particular pathogenic properties and ignore other factors that have a bearing on pathogenicity such as competitive or antagonistic ability in the tuber/ soil microenvironment. However, these considerations aside, the mini-tuber assay is an important development in the unravelling of the taxonomy and pathogenicity of this

agronomically important group.

Chapter 4

Approaches to the detection and monitoring of streptomycetes in the natural environment

4.1 Introduction

Recent years have seen considerable interest in the development of technology for the detection and monitoring of microorganisms in the natural environment (McCormick, 1986; Pickup, 1991). These advances have in part resulted from the interest in genetically engineered microorganisms (GEMs) which are currently being investigated for use (Stotsky *et al.*, 1991) in areas such as biological control (Lindow, 1985; Pimentel, 1985; Watrud *et al.*, 1985; Entwhistle *et al.*, 1988) the degradation of recalcitrant pollutants (Dwyer *et al.*, 1988), the mining of metals (Brierly, 1985) and the improvement of soil fertility (Amarger, 1988; Hirsh & Spokes, 1988). Interest in the accidental or deliberate release of GEMs into the natural environment has stimulated questions concerning the potential risks to public health and safety and the impact on ecosystems (Domsch *et al.*,1988; Tiedje *et al.*, 1989). Questions about the probabilities of survival, colonisation, and activity of released GEMs and their DNA in established microbial communities may only be answered by applying knowledge derived from the study of microbial ecology and molecular interactions in these habitats (Stotsky *et al.*, 1991). The extremely diverse and versatile nature of microorganisms means that standardised regulations governing release cannot be applied, necessitating regulation on a case by case basis (Colwell, 1986). The development of sensitive detection techniques with which to monitor the survival, growth and genetic transfer by GEMs in the environment is therefore necessary for meaningful ecological data to be obtained. These methods for detection and enumeration of inoculants *in situ* will be dependent on the availability of suitable markers (Colwell, 1986).

Furthermore the development of methodology for the detection and monitoring of microorganisms *in situ* is also pertinent to the study of the ecology and

epidemiology of certain plant, human and animal diseases and will contribute to the elucidation of methods for control and prevention. Ecological data and methodology for detection and monitoring that has begun to emerge in studies of gene transfer between streptomycetes (Wang *et al.*, 1989; Wellington *et al.*, 1990; Cresswell *et al.*, 1992) in the natural environment will thus be of relevance to studies on the ecology of the causal agent of common scab, *Streptomyces scabies*.

4.1.1 Sampling

Obtaining representative samples from soil is particularly difficult because of the non-uniform spatial distribution of bacterial colonies within the soil matrix (Wellington *et al.*, 1990). Nutrient, water and pH gradients may arise from the presence of plants resulting in localised differences in bacterial numbers of 10 to 50 fold in rhizosphere soils (Paul & Clark, 1989). The sampling strategy should therefore consider the variability of the soil material under analysis, the number of samples required can be derived statistically according to the properties being assessed (Bianchi & Bianchi, 1982). However 5 to 10 replicates is considered good practice with as many as 20 to 30 individual samples required from highly variable soil sites (Allen *et al.*, 1974). Errors attributed to the difficulties of sampling a highly heterogeneous environment may be minimized by processing composite samples (Atlas & Baratha, 1981). Spatial variation in field soils can be sufficient to obscure seasonal changes (Ball & Williams, 1968). Samples may be taken with a trowel (cylindrical graduated trowels may be used to sample soil profiles) if they are to be analysed with reference to weight or a graduated metal cylinder or 'corer' if they are to be analysed with reference to volume (Allen *et al.*, 1974). The latter should be designed to avoid compaction of the sample in order to maintain the natural stratification of soil profiles and hence a high degree of

vertical biotic and abiotic heterogeneity (Burns, 1988). Other approaches to studying soil microorganisms *in situ* have involved the burying suitable surfaces for colonisation such as Cholodny-Rossi's glass slide, which is recovered after a given time and examined using microscopy (Atlas & Bartha, 1981). Sampling from plant or animal tissues generally requires removing bacteria from surfaces via washing or scraping and recovery from specific tissues via maceration. Kuchenbuch & Jungk (1982) describe a method for determining concentration profiles at the root surface interface by making thin slices of the rhizosphere soil. Plants are grown in small containers in which the roots are separated from the soil by a screen of nylon cloth, root hairs but not roots penetrate the nylon cloth. The soil block (beneath the nylon sheet) is frozen in liquid nitrogen and then sliced thinly using a refrigerated microtome; the thin slices may then be analysed in terms of distance from the root (Kuchenbuch & Jungk, 1982). Strategies that have been employed in sampling from aquatic environments have usually involved using an evacuated chamber which can be opened at a given depth, while air samples are taken by passing a given volume of air through a membrane filter of a particular pore size (Atlas & Bartha, 1981).

4.1.2 Extraction of bacteria from environmental samples

Traditionally bacteria have been recovered from soils through the mechanical shaking of the soil in an appropriate diluent. Alternatives to mechanical shaking include the application of Waring blenders which are able to disintegrate soil aggregates (Kanazawa *et al.*, 1986). Kanazawa *et al.* (1986) demonstrated 8.9 times enhanced recovery of bacteria from a forest soil using a Waring blender rather than mechanical shaking.

The sensitivity of detection for viable plate count methods has been estimated

to be 10^2 c.f.u. g^{-1} (Trevors & Van Elsas, 1989). However, this may be improved upon by combining methods to extract and concentrate the biomass from environmental material prior to plating (Herron & Wellington, 1990). Detection limits as low as 10 streptomycete spores per 100 g of sterile soil have been achieved (Herron & Wellington, 1990).

Strong chemical and physical associations may form between microorganisms and particulate matter (Stotsky & Burns, 1982). These associations may be of an ionic nature since bacteria have been found to be negatively charged, while clay minerals in soils contain positively charged cations. In addition, bacterial mucigels and fibrillae may contribute to soil-microbe interactions (Paul & Clarke, 1989). Dispersion of soil aggregates has been considered important as entrapment of microorganisms within aggregates is considered to be one of the most important means by which microorganisms are retained in the soil (Hopkins *et al.*, 1991a;b). Attempts to disrupt these soil-microbe associations in order to extract the bacteria have utilised homogenization, chemical dispersants, cation exchange resins and differential centrifugation (Faegri *et al.*, 1977; Bakken, 1985; MacDonald, 1986; Herron & Wellington, 1990; Hopkins *et al.*, 1991a; 1991b).

Faegri *et al.* (1977) recovered 50 to 80 % of soil bacteria using sequential fractionation methods which involved repeated homogenization of the soil and separation by differential centrifugation. The method was developed and evaluated for a soil with a high organic content. The nature of soil aggregation in organic soils differs from that in mineral soils and may account for the reduced recoveries obtained of 30% when the approach was applied to mineral soils (Holben *et al.*, 1988; Steffan *et al.*, 1988). Bakken (1985) combined blending and centrifugation treatments to release bacteria from soil. Further

purification of bacterial cells from clay and organic matter of bacterial cells was achieved by centrifugation density gradients of percoll and ludox where the clay and organic particles were sedimented. However, more than 50% of the bacteria which were probably attached to clay and humic particles also sedimented through the gradient. Baecker & Ryan (1987) have applied hammer-mill comminution followed by high speed liquid homogenization to the recovery of actinomycetes from soil. They obtained results that indicated that the suspension of spores in soil was directly related to the cation exchange capacity and the particle size distribution of the soil. Dispersion of soil aggregates and release of soil microorganisms through the exchange of cations from soil and cell surfaces using an ion exchange resin in conjunction with a non-biocidal detergent is an approach that has been developed by MacDonald (1986), applied to the recovery of streptomycete spores from soil (Herron & Wellington, 1990) and evaluated by Hopkins *et al.* (1991b) for representative sampling of the soil microflora. The cation-exchange approach proposed by MacDonald (1986) for the chemical dispersion of soil particles was established to be the most effective single-step for the disruption of soil aggregates in studies by Hopkins *et al.* (1991a).

Hopkins *et al.* (1991a) have attempted to address the problems of representative sampling of the soil microflora using procedures to disperse soil aggregates and dissociate the soil microorganisms from soil particles. The efficiency of each stage was assessed turbometrically and by measuring the biomass recovered in each fraction using microscopic cell counts, ATP, phospholipid, lipopolysaccharide, ergosterol contents and viable counts. 3 soil types were compared a sandy loam, a clay loam and a peat soil. Microorganisms least contaminated with soil material were recovered from the sandy loam soil, while difficulties were obtained in recovering cells from the

clay and peat soils that were free of the smaller mineral particles or less dense organic particles.

In aquatic environments, water samples are usually filtered and the biomass collected on filters (Sommerville *et al.*, 1989). Donegan *et al.* (1992) demonstrated differential recoveries of bacterial isolates from the phylloplane, the highest populations were recovered from leaf material that had been treated with stomacher blending, rather than with blending, sonication or washing.

4.1.3. Traditional methods of monitoring and detection

4.1.3.1 Viability count

Particular groups of microorganisms indicative of pollution and contamination are monitored in food stuffs, water, activated sludges and environmental samples. Such monitoring is considered essential for public health and safety (Kasper & Tartera, 1990). Viability count methods using particular bacteriological media are used for the routine monitoring of these indicator microorganisms. However, since all media are selective to a certain extent, viable counts are often inaccurate or not indicative of the relative components of the microbial flora sampled (Pickup, 1991). Length and temperature of incubation as well as the particular media components have been found to influence the viability count (Ford & Taylor, 1949). Sorheim *et al.* (1989) compared the populations recovered from soil on three different non-selective media, bacterial isolates exhibiting approximately the same level of diversity were isolated on all media, however each media appeared to select for a different population of isolates with 30% of the population appearing common to all three media. 20% of isolates recovered from 2 of the media were distinct to that particular media and 60% of isolates on the third media were unique to it. Cells extracted from samples may be plated on solid media (spread plate)

or mixed into the molten agar and allowed to set (pour plate). Low melting point agar substitutes have been employed where the pour plate is required and the microbes of interest are sensitive to temperatures of about 40^{0}C (Gardener & Jones, 1984).

Alternatives to the spread and pour plates include the most probable number procedures (MPN) (Alexander, 1982). The MPN technique involves serial dilution of the sample in an appropriate media to an extinction point, (Atlas, 1982). 3 to 10 Replicates of each dilution are made and the pattern of positive and negative scores recorded (i.e. growth or no growth). Statistical tables are used to determine the most probable number of microorganisms present in the original sample. Most MPN techniques employ liquid culture, which may be advantageous when defined media is required and hence in avoiding contamination introduced via the agar in solid media. However, MPN techniques are dependent on growth and reproduction of strains of interest and are therefore subject to the same constraints as the plate count. MPNs may also be less accurate than the plate count as they establish a MPN with confidence limits rather than a number of actual reproductive units. The adaption of microtitre technology to MPN analysis (Rowe *et al.*, 1977) has made the laborious and repetitious technique more accessible to use with environmental samples.

4.1.3.2. Direct count

Methods for the enumeration of microorganisms in environmental samples using microscopy are not dependent on the ability to culture the bacteria in the sample and may therefore be more accurate than viable counts. Total direct counts of bacteria on black membranes, in counting chambers or on natural and artificial surfaces have been achieved using epifluorescence microscopy

(Hall *et al.*, 1990). Epifluorescence is achieved by exciting a range of nucleic acid and protein stains that fluoresce with light of a suitable wavelength. Electron microscopy has also been applied to the direct counting of filtered samples (Hall *et al.*, 1990). Counts of specific components of the microbial community may be achieved where the species or genera of interest are morphologically distinct or distinguishable via alternative means. These may include immunological, biochemical and genetic differences.

4.1.3.3 Immunofluoresence

Fluorochromes, such as fluorescein isothiocyanate (FITC) may be coupled to antibodies (fluorescent antibody or FA) that bind a particular microbial target antigen (Chantler & McIllmurray, 1988). FA may be applied as a natural stain to an environmental sample, if the target microbe is present, the labelled antibodies will bind specifically with the target antigens. When the stained preparation is then viewed under the fluorescent microscope the outlines of the microbes of interest are seen as a result of the light emitted from the FA bound to its surface (Bohlool & Schmidt, 1980). The FA approach makes the study of the autecology of specific microbes possible as it allows them to be visualised in their natural environments (Schmidt, 1974). Immunofluorescence detection has been of value in studying bacteria that are difficult to cultivate and including the methanogens (Conway de Macario *et al.*, 1982). Factors that affect the successful application of fluorescent antibodies to studies in microbial ecology include, specificity, cross reactivity, autofluorescence from environmental materials, nonspecific staining, expression of the antigen encoding genes and stability of the antigen under environmental conditions (Schmidt, 1974; Bohlool & Schmidt, 1980; Ford & Olson, 1988). Difficulties associated with the autofluorescence and non-specific adsorbtion to soil minerals have been overcome by applying a dilute solution of gelatin

hydrolysed at a high pH to the soil preparation prior to staining. The gelatin may also be conjugated with a fluorochrome of contrasting colour to serve as a counter stain in order to highlight the specifically stained bacteria in relation to the gelatin-labelled components of the soil microenvironment (Bohool & Schmidt, 1968). One drawback of the approach is its inherent lack of sensitivity in the enumeration of FA-stained bacteria; in order to count 1 bacterium at a magnification of times 1000, the cell density must be 10^6 to 10^7 g^{-1} soil. In order to improve upon this, it would be necessary to separate the bacteria from the soil and concentrate them prior to staining (Bohool & Schmidt, 1980). Immunofluorescence techniques are; however, unable to distinguish between viable and dead cells.

Immunological detection

In addition to the application of monoclonal antibodies and polyclonal antisera to immunofluoresence microscopy, they may be incorporated in enzyme-linked immunosorbent assays (ELISA). Rather than being coupled to fluorescent dyes, antibodies are linked with enzymes such as alkaline phosphatase, horseradish peroxidase, β-galactosidase and urease (Chantler & McIllmurray, 1988). Such assays have been of great importance in the detection of medically important microorganisms in blood and tissue samples and in the assessment of food contaminants (Brooker *et al.*, 1990). ELISA has been successfully applied to the detection and monitoring of *Rhizobium* strains (Martensson, 1984) and *Pseudomonas putida* (Morgan *et al.*, 1991). Quantitative ELISA has been estimated to be able to produce a positive signal from a lower limit of 10^4 cells. Detection limits of 10 to 10^2 cells /g soil were achieved by Scholter *et al.* (1992) by using an immunoassay with a horseradish peroxidase labelled antibody and a chemoluminesence reaction (rather than the colormetric test used in a standard ELISA procedure) which

was started by adding luminol and measured by scanning with a luminometer.

Monoclonal antibody immunoblot methods have been reported for the detection of *Pedicoccus acidilactici* from foods (Bhuna & Johnson, 1992) and for the rapid identification of *Rhizobium* strains and their quantification in root nodules and preinoculated seed (Olsen & Rice, 1991).

Monoclonal antibodies have recently been applied to the rapid isolation of *P.putida* from lake water samples via immunocapture (Morgan *et al.*, 1991). Magnetic beads were coated with antibodies that specifically bind antigen on the bacterial flagella. The approach served as an enrichment step to be combined with direct detection methods as immunocapture allowed bacteria to be specifically recovered from other microorganisms and environmental components. Wipat *et al.* (1991) have also applied immunocapture to the recovery of streptomycete spores from soil. Page & Burns (1992) have presented preliminary data on the application of monoclonal antibodies targeted to a *Flavobacterium* strain to the enumeration of the strain in soil using flow cytometry. At present the flow cytometry approach appears less sensitive than direct and viable counting procedures.

Cultural vs non-cultural microorganisms

A major limitation to studies in microbial ecology is the inability to isolate and cultivate the vast majority of bacteria. This is confirmed by the discrepancy between plate and direct counts. In lake water samples Jones (1977) was able to obtain direct counts of 10^6 while only obtaining viable plate counts of 10^3 bacteria ml^{-1}. Further studies by Hoppe (1978) and Ferguson *et al.* (1984) on the marine environment estimate the culturable proportion of viable bacteria to be 0.01 to 12.5%. Similar findings have been reported in soil (Atlas, 1982).

Furthermore some bacteria have been shown to become unculturable but retain their viability after exposure to the environment. Bacteria adopting this physiological state are described as non-culturable but viable (NCBV) (Colwell *et al.*, 1985). Microorganisms identified to adopt this state include *E.coli*, *Salmonella typhimurium* and *Vibrio* spp. (Rozak & Colwell, 1987; Colwell *et al.*, 1985). Direct counts do not discriminate between components of the microbial population that are viable and culturable, NCBV and dead. Hence direct counts are inclined to overestimate the viable component of the population. A number of workers have attempted to distinguish between the viable and non-viable component of a direct count. The acridine orange count has been widely accepted for the purposes of obtaining a total direct count. It has been suggested that the technique was able to distinguish between living and inactive cells. The acridine orange intercalates with nucleic acids and fluoresces red-orange in association with RNA and green in association with DNA. However, other factors such as the incubation time, pH and concentration of the dye have also been found to influence the fluorescent colour (Hobbie *et al.*, 1977). Kogure *et al.* (1979) introduced the direct viable count (DVC), whereby cells in environmental samples were incubated with the DNA gyrase inhibitor naladixic acid. The naladixic acid prevents DNA replication and hence cell division; however other synthetic pathways continue resulting in the production of elongated cells. Hence the DVC allows enumeration of cells that are actively growing as well as the proportion that are dormant, but physiologically active. This technique is limited to naladixic acid susceptible Gram negative bacteria. In recent years, molecular methods have been applied to microbial ecology. They present one solution to the problems of investigating components of the ecosystem that are non-culturable. Rather than isolating and culturing the bacterial cells, it is possible to isolate a target

molecule such as RNA or DNA and screen for marker genes or sequences representative of the population of interest. These methods do not discriminate between the viable and dead components of the soil microflora.

Phenotypic Markers

Colony morphology has been used in a number of studies usually in combination with antibiotic resistance determinants for monitoring the fate of inoculants *in situ*. Colony morphology was used to distinguish *Bacillus* inoculants from indigenous populations in mushroom composts in studies of plasmid stability (Amner *et al.*, 1991).

Biomarkers Markers

Microbial biomarkers are chemical components of the microbial cell, which may be analysed both qualitatively and quantitatively in terms of microbial biomass and activities following their extraction from environmental samples (Parkes, 1987). Membrane lipids and their associated fatty acids have received great interest as they are essential cellular components that have great structural diversity and high biological specificity. Furthermore these molecules have been used as important taxonomic markers for the identification of microorganisms (Minnikin & Goodfellow, 1981; Saddler *et al.*, 1987; O'Donnell, 1988) and hence there are large reference data bases of the fatty acid profiles for many bacterial species (e.g. MIDI, Delaware, USA). Certain biomarkers such as the phospholipids are ubiquitous components of cell membranes and as such may be used to provide a measure of the total biomass from both prokaryotes and eukaryotes. Biomarkers that have been used to derive estimates of bacterial biomass include the cell wall constituents peptidoglycan, lipopolysaccharide and teichoic acids (Parkes, 1987; Hopkins *et al.*, 1991a). Other biomarkers are specific to certain genera or species. An

ideal biomarker has both specificity and represents a high proportion of the fatty acid content of the cell. Such specific biomarkers have been used the analysis of the community structure of sulphate reducing bacteria in anaerobic sediments (Taylor & Parkes, 1983; 1985). The successful use of lipid biomarkers to quantify and characterise microbial communities *in situ* relies on the isolation of bacteria which are representative of those *in situ* and their subsequent growth under appropriate conditions to allow determination of their lipid profiles. The isolation of bacteria is not necessarily a prerequisite for the application of fatty acid biomarkers as specific biomarkers may be isolated direct from environmental samples and correlated with particular microbial populations or biological activities (Parkes, 1987). While certain bacteria may be identifiable through the presence of unusual fatty acids, the fatty acid composition of many strains is not a stable characteristic and is susceptible to changes in the growth medium, temperature, aeration as well as growth phase. Hence taxonomic determinations based on fatty acid analysis are carried out under very stringent standardised conditions (Saddler *et al.*, 1987).

Genetic markers : Antibiotic Resistance Mutations

Numerous studies have used antibiotic resistance mutations as selective markers for the monitoring of inoculants *in situ* (Compeau *et al.*, 1988; Fredrickson *et al.*, 1989; Thompson *et al.*, 1990). These markers appear to offer a relatively simple and effective method for monitoring inoculants in complex ecosystems and have been applied to *Rhizobium* nodulation studies (Johnston & Beringer, 1975; Turco *et al.*, 1986), the evaluation of particular biological control agents (Weller & Cook, 1983; Weller, 1984; Fredrickson & Elliott, 1985; De La Cruz, 1992), for investigating the potential for plasmid gene transfer between inoculants (Lacy *et al.*, 1984; Manceau *et al.*, 1986; Van

Elsas *et al.*, 1988) and for investigating the fate of microbes of potential use in genetic engineering (Liang *et al.*, 1982). A number of these studies have highlighted the instability of some spontaneous antibiotic resistances and alterations in fitness and competitive abilities. Turco *et al.* (1986) evaluated the suitability of doubly marked spontaneous antibiotic resistance *Rhizobium* mutants (streptomycin-rifamycin and streptomycin-spectinomycin) in inoculation studies, 93% of the mutants isolated had reduced competitive abilities than their parent strains, while 38% showed reduced N_2-fixation effectiveness. Compeau *et al.* (1988) studied the fate of spontaneous rifampin-resistant mutants of *Pseudomonas putida* and *Pseudomonas fluorescens* in sterile and the live soil from which they were isolated. The *P.putida* mutants showed no differences in terms of growth rate, competitive fitness and membrane protein composition, while the *P.fluorescens* mutants appeared to form two groups, group 1 mutants did not appear to differ from its wild type parent in the traits mentioned above, however mutants from group 2 demonstrated reduced competitive fitness compared to the parent wild type strain.

Thompson *et al.* (1990) employed a triple marked spontaneously antibiotic resistant *Flavobacterium* (streptomycin, rifampicin and kanamycin) and a single antibiotic resistant *Arthrobacter* (streptomycin) in their survival studies of two ecologically distinct bacteria. They report that the approach allowed the accurate determination of the growth and survival of both strains in soil microcosms and that microbial growth rates and biochemical characters of mutants appeared unchanged.

Certain studies have revealed the presence of natural resistances to antibiotics in bacterial isolates from soil and water samples (Kelch & Lee; Cole & Elkan).

Recorbet *et al.* (1992) recently demonstrated the difficulties of using neomycin phosphotransferase (*npt*II) as a selective marker in soil, as 0.1 to 2% of the soil microflora from a population derived from a total viable plate count showed some degree of resistance to neomycin and kanamycin. Colony hybridization methods were used to probe isolates recovered with and without aminoglycoside selective pressure with Tn*5* (a 5.7kb kanamycin-neomycin resistance transposon originally isolated from an R factor transferred from *Klebsiella* to *E.coli* [Berg *et al.*, 1975]) and *nptII* sequences. Sequences homologous to Tn*5* appeared quite common among soil isolates recovered with and without aminoglycosides, however sequences homologous to the *nptII* gene were not detected. Findings of wide spread resistances amongst the soil microflora have reinforced the need to develop alternative and complimentary methods for marking and monitoring inoculants *in situ*. The introduction of novel reporter genes that have not been identified within indigenous populations has been one approach which has received a lot of interest from microbial ecologists.

Genetic Markers: reporter genes

Genetic markers that may be applied to microbial ecology include: (i) oligonucleotide target sequences or signatures, (ii) genetic sequences relating to specific functions such as antibiotic resistance, biosynthesis genes or indeed sequences that relate to morphological characters such as unusual cell wall proteins and (iii) chromogenic markers that encode a particular screenable enzyme activity such as bioluminescence (*lux*) (Rattray *et al.*, 1990; De Weger *et al.*, 1991) and catechol 2,3 dioxygenase (*xyl*E) (Winstanley *et al.*, 1989) or novel substrate utilization *lac*ZY (Drahos *et al.*, 1986). These genetic markers may be introduced into the cell via plasmids or inserted into the chromosome. Although extrachromosomal markers may be more readily introduced into

cells they are inherently more unstable, with the tendency of many plasmids to segregate and be lost at cell division, (Morgan *et al*, 1989; Pickup *et al.*, 1990). Hence, it is preferable to mark strains on the chromosome, but as plasmids are often multicopy, chromosomally encoded genes will probably result in lower expression.

A number of chromogenic or reporter genes have been utilised in the production of marked strains by microbial ecologists. Reporter genes that have been applied to the monitoring of inoculants include *lacZ* (Drahos *et al.*, 1986), the *lux* genes (Rattray *et al.*, 1990; De Weger *et al.*, 1991) and *xylE* (Winstanley *et al.*, 1989; Wipat *et al.*, 1991; Bailey & Thompson,1992). The *gus* reporter gene which encodes production of the *E.coli* enzyme β-glucoronidase has been used extensively in plant systems (Jefferson, 1989). Its potential as a marker in bacterial systems remains to be realised, although attempts are currently being made to assess its use in *Rhizobium* spp. (K.Wilson pers. comm.).

The *xylE* reporter gene encodes the production of catechol 2,3 dioxygenase (C32O) which in the presence of catechol will form a distinctive yellow compound 2-hydroxy muconic semi aldehyde. The *xylE* gene has been used successfully under the control of the lambda pL and pR promoters with thermoregulated C32O expression achieved with the lambda represser *cI*857. These constructs have been applied to studies of plasmid stability and ecology in soil and water samples (Winstanley *et al.*, 1989; Morgan *et al.*, 1989; Pickup *et al.*, 1990; Saunders *et al.*, 1990; Wipat *et al.*, 1991; Macnaughton *et al.*, 1992). Morgan *et al.* (1989) have developed membrane filter methods to detect bacterial cells harbouring the *xylE* gene and its product C23O. They have raised polyclonal antibodies to C23O and applied ELISA technology to

the quantification of C23O direct from water samples. This assay was able to detect up to 10^3 c.f.u. ml^{-1} water. Since C23O activity is destroyed by oxygen, it is rapidly lost outside the cell unless in the presence of protective compounds such as ethanol or acetone, detection of C230 is therefore an indicator of viable host cells (Morgan *et al.*, 1989). Combined use of an enzyme assay for C23O (Sala-Trepat & Evans, 1971) and an immunoassay may provide information on the activity and expression of the C23O phenotype, particularly when used in conjunction with DNA hybridization methods to determine the stability and presence of the gene sequence (Morgan *et al.*, 1989; Saunders *et al.*, 1990). Studies of Pickup *et al.* (1990) focus on the stability of the *xylE* marker in different host backgrounds, within different plasmid constructs in aquatic microcosms. Their findings indicate that loss of the plasmid and hence the *xylE* marker is associated with the multiplication of inoculants *in situ*; they also demonstrate that the marker may appear quite stable in one particular host background and construct when inoculated at a level of 10^5 cells ml^{-1} but is highly unstable when inoculated at concentrations of 10^2 cells ml^{-1} water. Wipat *et al.* (1991) looked at the stability of plasmid constructs harbouring *xylE* in *Streptomyces* strains in soil, while Macnaughton *et al.* (1992) use the plasmid encoded marker in combination with kanamycin resistance to assess the relative ability of *Pseudomonas putida* strains to grow and survive in different soil types, both sterile and live. Populations of the marked *P.putida* declined faster in peat or sandy soils as opposed to soils with high levels of bentonite clay. No background levels of catechol 2,3 dioxygenase positive colonies were detected from the control microcosms.

Bailey & Thompson (1992) have introduced the reporter gene onto the chromosome of a pseudomonad strain isolated from the phylloplane of sugar beet. The *xylE* gene was introduced onto the chromosome by inserting

fragments of chromosomal fragments bearing the *xylE* marker into a colE1 replicon plasmid that is unable to replicate in a non-*Enterobacteriacae* host background. The suicide vector containing the marker was sequenced and a unusual restriction enzyme identified, a kanamycin resistance determinant was introduced at this site and the resulting construct introduced into the pseudomonad host via electroporation. Integration into the chromosome via homologous recombination was confirmed by performing a Southern blot analysis on colonies that were recovered on kanamycin and turned yellow when sprayed with catechol. The authors are currently investigating its use as a marker for studying the microbial ecology of the phylloplane.

A number of workers cite the particular value of the *Pseudomonas putida xylE* reporter gene to *Streptomyces* strains in terms of its stability and good expression (Clayton & Bibb, 1989; Ingram *et al.*, 1989). Alternative reporter genes have also been investigated for use in the *Streptomyces*, particularly with a view to studying the regulation of specific genes during morphological and physiological differentiation (Schauer *et al.*, 1988). The reporter gene *lacZ* appears unsuitable for use in the *Streptomyces* as all the species that have been examined so far have significant levels of endogenous β-galactosidase activity and mutants without this activity were found to have pleoitropic defects, furthermore expression of the *E.coli lacZ* gene in *Streptomyces* is poor as a result of differences in codon usage (King & Chater, 1986; Ingram *et al.*, 1989). The reporter system based on the *Vibrio harveyei luxAB* operon fragment has appeared extremely sensitive and proved useful for investigating the spatial and temporal regulation of developmental genes in *S.coelicolor*, but is inconvenient for screening large numbers of samples (Schauer *et al.*, 1988).

Ward *et al.* (1986) have developed plasmid promoter-probe vectors using the aminoglycoside phosphotransferase gene (*neo*^{R}, *AphII*, *nptII*) reporter gene

derived from the transposon Tn*5* that confers resistance to neomycin and kanamycin. Neomycin phosphotransferase activity may be assayed on antibiotic gradient plates, via a simple enzyme assay or by estimating the amount of *neo* mRNA synthesised. The system appeared highly efficient in *Streptomyces lividans* with expression of the gene correlating well with the transcriptional activity of the promoters being investigated.

The sensitivity of reporter genes like *xylE* in viable plate counts is increased when combined with an antibiotic resistance marker (Trevors & Van Elsas, 1989). Antibiotic resistance markers used in plate counts have an estimated level of detection of 10^2 cfu g^{-1} soil. The ability to produce selective media targeted at the strain under study by the incorporation of antibiotics has meant that antibiotic resistance determinants have and will continue play a important role in studies of microbial ecology (Van Elsas *et al.*, 1986).

Little is known of the metabolic burden that these markers may impose upon the inoculants under study and indeed how alterations to the phenotype and genotype of the strain will in turn affect ability to survive and compete in the natural environment.

Plasmid Stability

Reports vary on the whether plasmids represent a metabolic burden to cells and appear to be dependent on the strain, plasmid and environmental conditions. Chemostat studies of plasmid stability in the absence of selection indicated that plasmid free cell lines have a faster growth rate and maintain higher populations (Godwin & Slater, 1979). However, these effects were found to be dependent on environmental conditions, hence plasmid loss was more frequently associated with phosphorus limited conditions than with

carbon limitation. The effects of environmental influences on R plasmid stability in *E.coli* in soil was also demonstrated by Devanas *et al.* (1986). In addition this study failed to establish a relationship between plasmid size and susceptibility to loss. Alternative studies indicated that certain combinations of compatibility plasmids i.e. TP120 (Inc N) and R1 (Inc FII) are stably maintained in *E.coli* chemostat grown cells preferentially to either one or no plasmids. In contrast, incompatible plasmids TP120 (inc B) and TP113 (inc B) are only maintained in cells in the presence of antibiotic selection (Gowland & Slater, 1984). Schlif & Klingmuller (1983) reported the rapid loss of plasmids introduced from *E.coli* into recipient isolates from soil and water samples, in the absence of selection after reintroduction into soil or water. Plasmid loss was greater at 20^0C than 4^0C and at 4% soil moisture as opposed to 16%. Studies by Jain *et al.* (1987) indicated the stability of large catabolic plasmids in extreme environments (previously identified to support physiologically stressed bacterial populations) such as ground water aquifier environments. These findings are consistent with those of other workers who have been able to observe stably maintained plasmids in environments where it has been difficult to assess the selective pressures operating. Plasmids harbouring tetracycline resistance genes appeared stable in mushroom composts (a microbially dynamic environment) in the absence of any apparent selective pressures (Amner *et al.*, 1991). Levy (1985) suggests that certain groups of bacteria may serve as reservoirs of plasmids bearing resistance to antibiotics and tolerance to heavy metals. It has been speculated that the acquisition of plasmid DNA is a rapid means of adaption to unfavourable environments and that there are strong evolutionary forces acting on the maintenance of plasmids of unknown selective advantage within the gene pool. Cited examples of plasmid reservoirs are *E.coli* for other members of the *Enterobacteriaceae*, *Haemophilus parainfluenzae* for *Haemophilus spp.* and

Staphlococcus epidermis for *S.aureus*.

Studies have also demonstrated that cells bearing Tn*5* or Tn*10* elements showed a survival advantage in chemostats in the absence of any known selective pressure (Biel & Hartl, 1983; Chao *et al.*, 1983). In the later study with Tn*10*, the authors related the survival advantage to the transposition of one of the flanking regions of the transposon. Movement of this element resulted in increased mutation rates and hence greater adaptation of the cells to the growth conditions.

Chromosomal genes may produce less of a metabolic burden on cells than those that are plasmid borne. Constitutive expression of marker genes may also contribute to this problem more than expression that is induced for enumeration purposes (Winstanley *et al.*, 1989; Wipat *et al.*, 1991).

4.2 Aims

4.2.1 To screen for and identify naturally occurring phenotypic markers such as antibiotic resistance and tolerance to toxic compounds in the common scab strain ISP5078 and evaluate the suitability of these markers for the selective isolation of ISP5078 from the indigenous microflora in soil.

4.2.2 To mark ISP5078 on the chromosome with a combination of unique genotypic and phenotypic characters not normally associated with the *Streptomyces*. *The xylE* gene was chosen as it is unique to Pseudomonads harbouring *Tol* plasmids and good expression of the gene has been demonstrated within the *Streptomyces*. This gene was combined with the selective *nptII* reporter gene, which is also expressed efficiently within the *Streptomyces* and allows the selective isolation of strains from soil on

kanamycin and neomycin.

4.2.3 To optimise transformation procedures via protoplasting for strain ISP5078 using plasmid pIJ673. The purpose of this manipulation was twofold: firstly to introduce a cassette harbouring the *xylE* and *nptII* reporter genes with maximum efficiency (and hence to optimise the insertion of the reporter genes into the chromosome via homologous recombination) and secondly to assess the potential of plasmids as markers for common scab strains in order to study their ecology in soil and plant microcosms.

4.3 Selection of ISP5078 for the study of the ecology of a common scab strain

It was felt necessary to focus on the development of methodology for the detection and monitoring of one particular common scab strain from the group that were characterised. Strain ISP5078 was selected for a number of reasons: firstly, the sequence data from Witt *et al.* (1989) included this strain, and hence offered the 16S rRNA target regions as potential markers for the differentiation of this strain from other common scab strains. Secondly the strain appeared pathogenic in both glasshouse trials and it was of particular interest to investigate the ecology of a pathogenic strain. Finally it is one of the few common scab strains to have been included in a number of previous studies (Williams *et al.*, 1983a; Lambert & Loria, 1989; Witt *et al.*, 1990; Kamper *et al.*, 1991). The strain has received attention, because of its initial designation as type strain for *S.scabies* (Waksman et Henrici, 1948) and has therefore been properly described by the International *Streptomyces* Project (ISP) so that there can be no confusion over its identity. A number of studies on scab-causing streptomycetes have involved uncharacterised pathogenic isolates making comparisons between reports difficult.

4.4 Phenotypic markers

In order to look at the ecology of ISP5078 in the soil-potato plant environment, it was necessary to have a number of means available for its detection in soil and plant material. Although problems of selectivity and sensitivity have been associated with the viable and direct count procedures, they represent a base line for the comparison and evaluation of other methodology (Sorheim *et al.*, 1989). Viable plate counts have been an important component of previous studies on the ecology of streptomycetes (Rafii & Crawford, 1988; Rafii *et al.*, 1988; Wellington *et al.*, 1990; Herron & Wellington, 1990).

Naturally occurring phenotypic markers in the guise of resistance to antibiotics have frequently been associated with actinomycetes like the *Streptomyces* (Vining, 1990). The search for naturally occurring phenotypic markers such as antibiotic resistance was therefore an important first approach to the requirement for marked strains. Naturally marked strains offer the potential to study the ecology of a particular isolate without problems of an altered phenotype or genotype that may result from other marking strategies.

ISP5078 was screened for resistance and susceptibility to a range of antibiotics using Mast Rings antibiotic discs on a RASS media (Table 15). RASS was developed specifically for the selective isolation of streptomycetes from soil (Herron, 1991). High levels of resistance were identified to the antibiotics penicillin G, ampicillin and cloxacillin, the sulphonamides trimethoprim and cephalorodine, in addition to resistance to lincomycin and weaker resistance to tetracycline.

Table 15: Summary of Antibiotic Resistance Patterns of ISP5078 on Rass media

Antibiotic	Amount	Resistance/Susceptibility
Ampicillin	3µg	+++
Cephalorodine	5µg	+++
Chloramphenicol	25µg	--
Clindamycin	2µg	--
Cloxacillin	5µg	+++
Erythomycin	5µg	---
Fusidic Acid	10µg	-
Gentamycin	10µg	---
Lincomycin	1 unit	+++
Methicillin	10µg	+++
Novobiocin	5µg	---
Penicillin	1 unit	+++
Streptomycin	10µg	---
Sulphamethaxazole	25µg	---
Tetracycline	10µg	+
Tetracycline	25µg	---
Trimethoprim	1.25µg	+++

Key to scores:

+++ very resistant

--- very susceptible

Note: resistance is determined by placing (Mast Rings, Merseyside) antibiotic discs onto plates spread with ISP5078

In addition to these resistances, analysis of data from the probabilistic identifications indicated that the strain was also resistant to phenol at 0.1% and sodium azide at 0.01%. Resistance to these toxic compounds was investigated alongside the antibiotic resistances for potential in a formulation of a selective medium for ISP5078. Studies focused on the recovery and enumeration of ISP5078 and indigenous streptomycetes from ISP5078 inoculated non-sterile and sterile soils and uninoculated non-sterile soil. Counts from each treatment were compared on RASS with and without selective agents. To test the selectivity of the media under conditions optimal for the growth and activity of the indigenous soil microflora, the dried soil was wetted to 15% moisture with the addition of the inoculum, where appropriate. The soil was incubated at 20°C for 5 days to allow germination and growth of any dormant or resting propagules, before enumeration on dilution plates. The ISP5078 inoculum was added to a final concentration of 10^4 c.f.u.$^{-g}$ soil. This level was below that of the indigenous streptomycete population, so that the medium had to be genuinely selective for ISP5078 for recovery and enumeration.

Since reports exist on the presence of antibiotic resistances among natural isolates (Kelch & Lee, 1978; Cole & Elkan, 1979) and as a result of the selective pressures from their clinical use (Anderson, 1968; Talbot *et al.*, 1980; Hughes & Datta, 1983), initial studies considered the application of phenol and sodium azide as selective agents. Phenol and sodium azide were included in RASS media with and without additional antibacterial and antifungal agents and the selectivity of these media for the isolation of ISP5078 from the background soil microflora assessed. These findings are presented in Table 16. They indicate that certain mixtures of selective agents result in a toxic combination to both the inoculant and background soil microflora. No

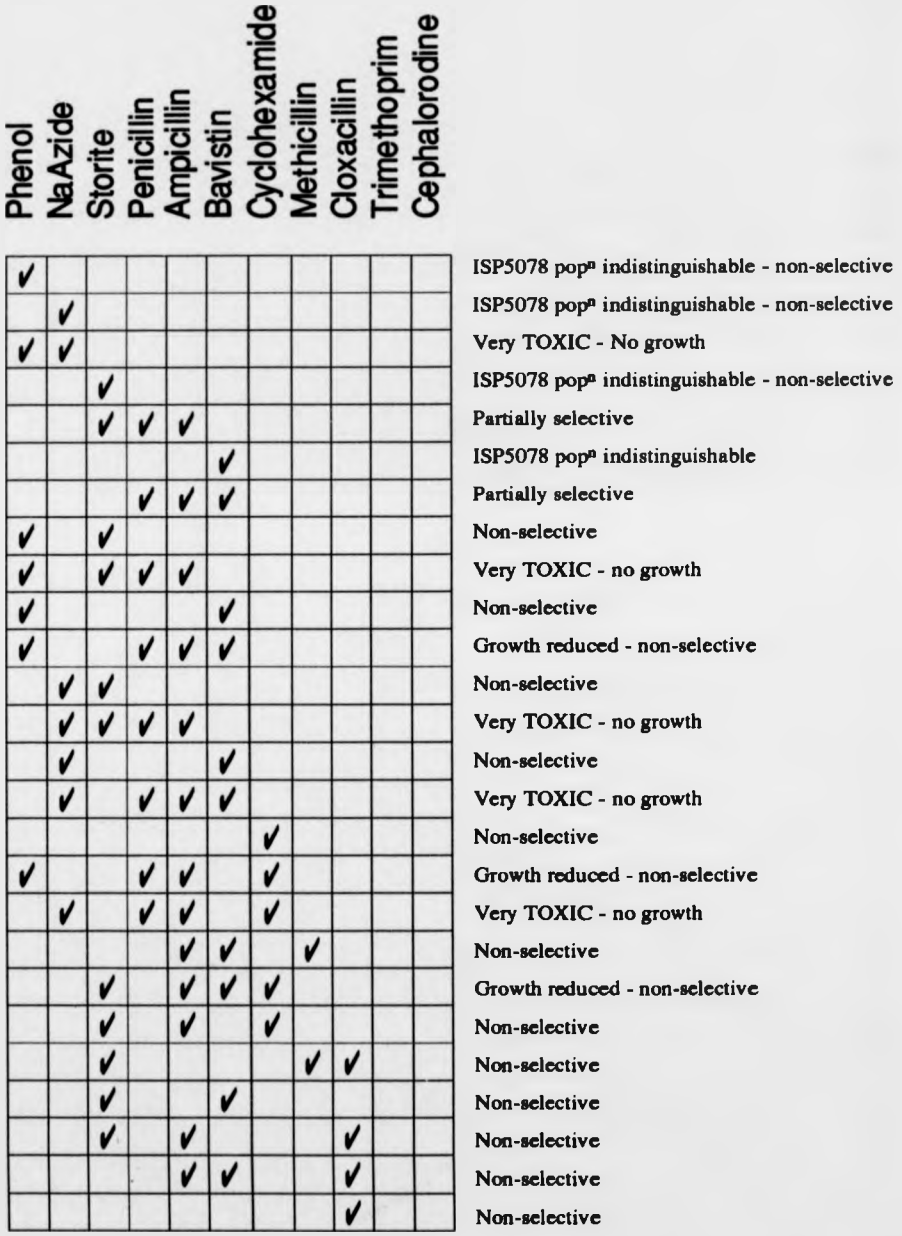


Table 16 Effects of certain combinations of selective agents on the recovery of ISP5078 from soil.

Phenol	NaAzide	Storite	Penicillin	Ampicillin	Bavistin	Cyclohexamide	Methicillin	Cloxacillin	Trimethoprim	Cephalorodine	
		✓		✓	✓		✓	✓			Growth reduced - non-selective
		✓		✓	✓						Non-selective
		✓			✓	✓					Growth reduced - Non-selective
		✓			✓		✓	✓			Non-selective
					✓	✓					Non-selective
		✓				✓					Non-selective
		✓		✓			✓				Non-selective
		✓		✓	✓	✓	✓	✓			Growth reduced - non-selective
				✓	✓	✓					Non-selective
							✓				Non-selective
					✓		✓	✓			Non-selective
									✓		Non-selective
					✓					✓	Non-selective
						✓			✓		Non-selective
					✓			✓	✓	✓	Non-selective
✓					✓				✓		TOXIC - reduced count
										✓	Non-selective
									✓	✓	Non-selective
					✓				✓		Non-selective
					✓				✓	✓	Non-selective
	✓				✓				✓		TOXIC - reduced count

Table 16 (cont) Effects of certain combinations of selective agents on the recovery of ISP5078 from soil.

particular pattern was evident. It was not possible to identify particular combinations that were toxic; rather it appeared that the combination of any 4 or 5 antibiotics and antifungals would result in a count reduced by 1 to 2 orders of magnitude or no count at all when compared to the count obtained on media without selective additives.

Synergistic toxic effects were exaggerated with the incorporation of sodium azide and phenol in the selective medium. Sodium azide appeared to contribute to more toxic combinations than phenol. Complete toxicity was observed (no count was obtained) when Storite was combined with phenol or sodium azide and when Bavistin was combined with sodium azide. However, when phenol was combined with Bavistin the streptomycete count was reduced by an order of magnitude but a fraction of the population was still recoverable. Since the fungisidal activities of Storite and Bavistin are similar and the former was more prone to the production of these toxic effects, Bavistin was included in selective media in preference to storite in further studies using the plate count. None of the combinations of selective agents tested actually allowed the selective isolation of the scab inoculant, ISP5078 from the background streptomycete soil microflora.

A parallel study on the application of naturally occurring lincomycin resistance as a selectable marker for the recovery of *S.avermetillus* from soil also demonstrated similar widespread antibiotic resistance in indigenous streptomycetes (Rhianon Williams, University of Warwick, pers.comm.).

In conclusion, combinations of β-lactams and sulphonamide antibiotics, phenol and sodium azide were inappropriate for the selective isolation of particular scab-causing streptomycetes from soil. Resistance to the antibiotics

and selective agents under study appeared to be distributed widely through the indigenous population of streptomycetes. This was particularly true of the grey sporulating streptomycetes which included ISP5078. In general, isolation plates were free of other bacteria, suggesting that some of these mixtures of selective agents incorporated in RASS may be appropriate for the general isolation of certain groups of streptomycetes. Finally, the synergistic effects identified with specific combinations of selective agents serve to highlight the problems in the development of media that is sufficiently selective to suppress components of the soil microflora that will rapidly over grow dilution plates and obscure the strain of interest but that also allow reliable enumeration of the inocula.

4.5 Cloning strategy for the introduction of the *xylE* reporter gene into ISP5078

The *xylE* gene encodes the production of catechol 2,3 dioxygenase. This enzyme will catalyse the conversion of catechol into 2-hydroxy muconic semialdehyde, a distinctive yellow coloured compound. The enzyme has been reported to be unique to Pseudomonads harbouring *Tol* plasmids and hence not present within the *Streptomyces* genus. Thus strains marked with the reporter gene would be readily distinguishable from indigenous populations of streptomycetes. The objective was to combine this reporter gene with a selectable marker and introduce this cassette onto the ISP5078 chromosome. The selectable marker chosen was the *nptII* gene. This gene encodes the enzyme neomycin phosphotransferase and confers resistance to the aminoglycoside antibiotics including kanamycin and neomycin. It appeared to be a good selective marker for *Streptomyces* since naturally occurring aminogylcoside resistance has been found to be relatively rare among streptomycete isolates (Phillips *et al.*, 1992).

The overall aim of the cloning work was to produce a cassette containing the *xylE* and *nptII* reporter genes that could be ligated to ISP5078 chromosomal DNA fragments and transformed into ISP5078. Since there were no cloning vectors available for the strain, incorporation of the cassette into the chromosome was dependent on homologous recombination.

The *xylE* gene was derived from pCF32 (Spooner *et al.*, 1987) and the *nptII* gene derived from pNEO (a commercially available plasmid), the *nptII* gene was under the transcriptional control of the Tn*5* promoter (these constructs were kindly supplied by R.Spooner, ICRF, London) (Figs. 13 & 14).

4.5.2. Excision of the *xylE* gene from pCF32

The *Bam*HI flanked *xylE* gene was excised from pCF32, by performing a *Bam*HI digestion in a 100µl volume of approximately 5µg plasmid DNA for 2 hrs at 37^0C. The products of digestion were run on a 1% agarose gel until the smaller *xylE* fragment was well separated from the remaining plasmid DNA. The *xylE* fragment was recovered on DE81 paper (Section 2.20).

Fig. 13 Plasmid map pNEO

A derivative of pBR322 as a result of the conversion of the *Hin*dIII/*Sal*I fragment of Tn5 to *Hin*dIII/*Bam*HI and then inserted into the *Hin*dIII and *Bam*HI sites of pBR322. The *nptII* reporter gene (Km^R) is fired by the Tn 5 promoter. The pNEO plasmid contained another antibiotic resistance marker, ampicillin resistance (Ap^R). The plasmid map has been linearised at the unique *Eco*RI site for convenience.

Key to Restriction Sites:

E	*Eco*RI
C	*Cla*I
H	*Hin*dIII
b	*Bgl*II
P	*Pst*I
Sp	*Sph*I
B	*Bam*HI
S	*Sal*I

Fig. 14 Plasmid map, pCF32

The plasmid contained the *xylE* gene flanked by two *Bam*HI sites.

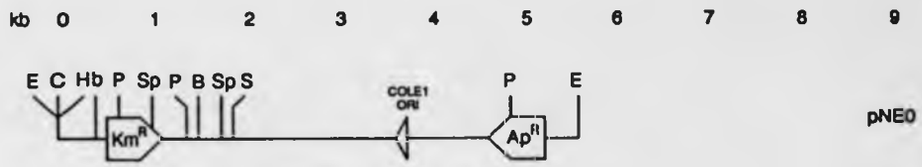


Figure 13

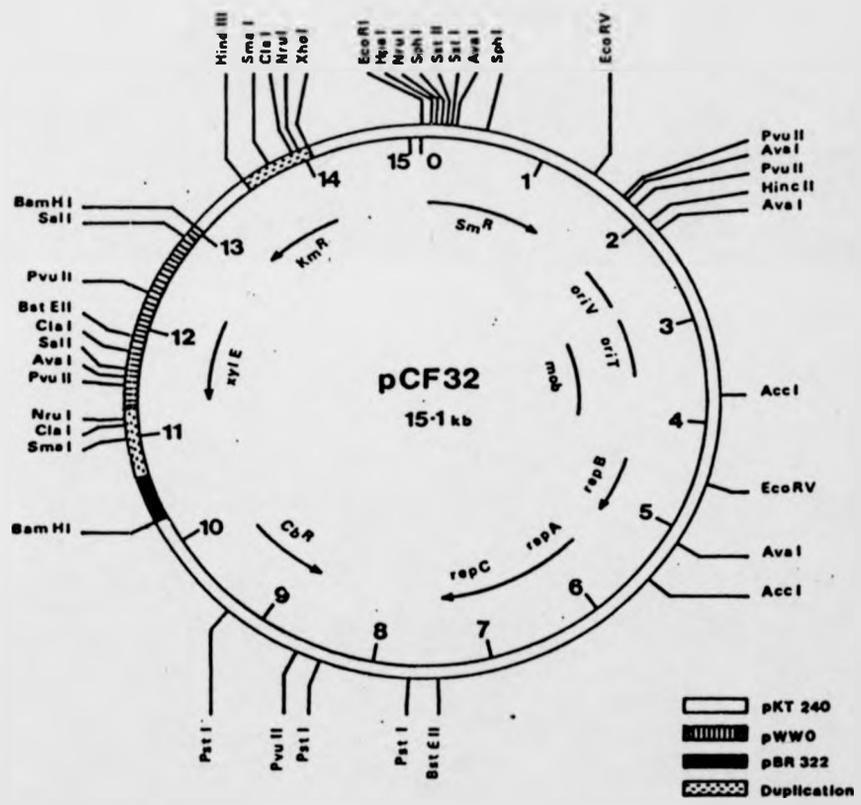


Figure 14

4.5.3 Introduction of the *xylE* gene into pNeo

Since it is possible to ligate a *Bam*HI cut fragment into both a *Bam*HI and *Bgl*II restriction site and the *nptII* gene is flanked upstream by a *Bgl*II site and downstream by a *Bam*HI site, attempts were made to clone the *xylE* gene in at both sites. Hence pNeo digestions were performed with both *Bam*HI and *Bgl*II. The following ligations were set up with these digestion products:

1. 100 ng pNeo/*Bam*HI (negative control)
2. 100 ng pNeo/*Bam*HI and 33 ng *xylE*/*Bam*HI
3. 100 ng pNeo/*Bam*HI and 66 ng *xylE*/*Bam*HI
4. 100ng pNeo/*Bgl*II (negative control)
5. 100 ng pNeo/*Bgl*II and 33 ng *xylE*/*Bam*HI
6. 100 ng pNeo/*Bgl*II and 66 ng *xylE*/*Bam*HI

The ligation products were transformed into *DH5*α competent cells and the transformed cells were spread onto LB plates containing kanamycin and ampicillin. Resultant colonies were sprayed with 100 mM catechol to identify catechol 2,3 dioxygenase activity. 25 positive colonies were recovered from cells transformed with ligation 3 and 1 colony from cells transformed with ligation 6. When sprayed with catechol *xylE* transformants derived from ligation 3 were a mixture of bright and pale yellow colonies. The *xylE* positive colony derived from ligation 6 was bright yellow. The differences in colour were thought to reflect the orientation of the *xylE* gene in the *Bam*HI and *Bgl*II sites, such that the *xylE* cassette in the bright yellow coloured colonies had probably been placed immediately behind and in the same orientation as the *nptII* gene and light yellow colonies probably signifying that the *xylE* gene had been incorporated in the opposite orientation and that transcription of the gene was being fired from the promoter of the ampicillin resistance gene (Ap^R) and

requiring more of the plasmid DNA to be transcribed prior to the *xylE* gene.

4.5.4 Characterization of the *xylE/nptII* constructs

Restriction enzyme digestions were performed on small scale plasmid DNA preparations from all colonies, in order to identify the plasmid constructs of interest. Large scale plasmid DNA preparations were performed on colonies bearing the required plasmid constructs from each of the categories. Constructs designated pPAR 1 and 2 were obtained from ligation 3 (Fig. 14a). Although attempts were made to clone the *xylE* gene into the *Bam*HI and *Bgl*II sites of pNeo, it was considered preferable to have the *xylE* gene cloned into the *Bgl*II site and hence downstream of the *nptII* promoter and upstream of the *nptII* gene. Thus for future experiments any streptomycetes exhibiting neomycin phosphotransferase activity would almost certainly be harbouring the *xylE* gene as well. In contrast, if the *xylE* gene was downstream of the *nptII* gene there would be the possibility of isolating marked streptomycetes that were kanamycin resistant without catechol 2,3 dioxygenase activity. For this reason the single bright yellow colony derived from ligation 6 appeared to be the preferable candidate, with in all likelihood the *xylE* gene would be in the correct orientation, being immediately upstream of the kanamycin resistant Tn*5* promoter and downstream of the *nptII* gene. However, when this plasmid was mapped by restriction analysis it was apparent that it was actually contaminating pCF32. Since no plasmids were obtained with the *xylE* gene inserted at the *Bgl*II site, even in repeated ligations, it is possible that insertion of DNA into the *Bgl*II site inactivates the Tn*5* promoter or interfers with transcription, so that Km^R *xylE* colonies could not be recovered. The pPAR 1 construct appeared to be the next best option, it was analysed by restriction mapping (Fig. 15) and appeared to conform to the predicted construct. Restriction analysis included the following digestions: *Eco* RI, *Eco* RI/*Bgl*II,

Fig. 14a Plasmid constructs

Plasmid maps are presented for constructs pNEO, pPAR1, pPAR2 and pPAR3. pPAR1 and pPAR2 have been generated by inserting the *xylE* gene into the *Bam*HI site upstream from the Km^R gene. The *xylE* gene could be inserted in either orientation with transcription driven by the promoter of the Km^R gene (bright yellow colonies) or the Ap^R gene (light yellow colonies). pPAR3 has been generated from pPAR1 through the removal of the *Bam*HI site between the Km^R and *xylE* genes. Plasmid maps have been linearised at their unique *Eco*RI sites for convenience.

Key to restriction sites:

E = *Eco*RI

C = *Cla*I

H = *Hin*dIII

b = *Bgl*II

P = *Pst*I

Sp = *Sph*I

S = *Sal*I

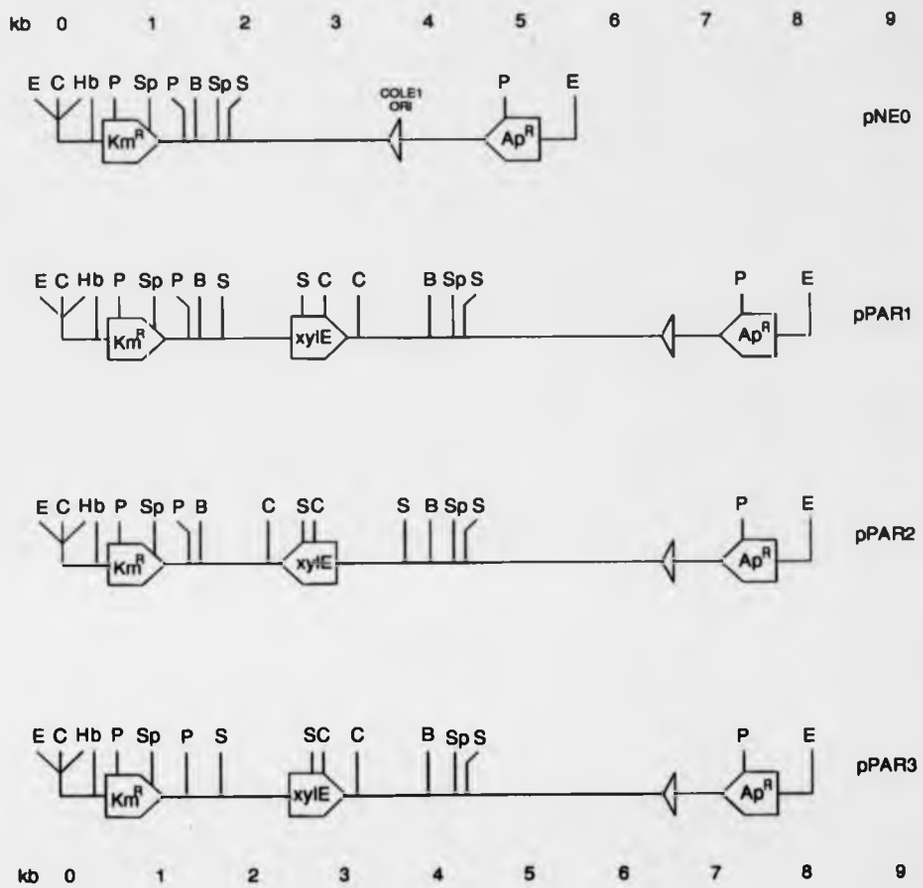


Figure 14a

Fig. 15 Characterisation of pPAR1

Fig. 15a. Restiction analysis included the following digestions : a, *Eco*RI; b, pBR322/*Hin*fI size markers; c, *Eco*RI/ *Bgl*II; d, *Eco*RI/ *Pst*I; e, *Eco*RI/ *Sal*I; f, *Eco*RI/ *Xho*I; g, *Eco*RI/ *Bam*HI; h, *Eco*RI/ *Cla*I; i, λ *Eco*RI/*Hin*dIII size markers; j, *Bgl*II/ *Pst*I; k, *Bgl*II/ *Sal*I; l, *Bgl*II/ *Xho*I; m, *Bgl*II/*Bam*HI; n, *Bgl*II/ *Cla*I.

Fig. 15b Restriction map of the plasmid pPAR1, containing the *xylE* gene was cloned downstream of the Tn5 Km^R of pNEO under transcriptional control of the Km^R promoter.

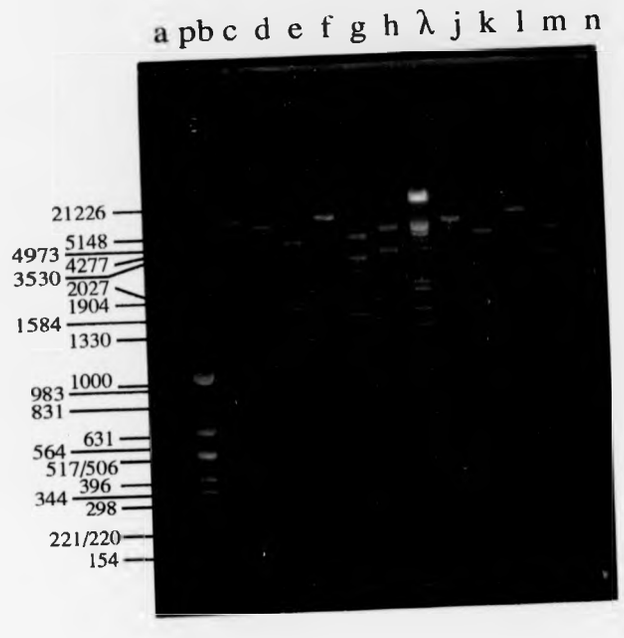


Figure 15a

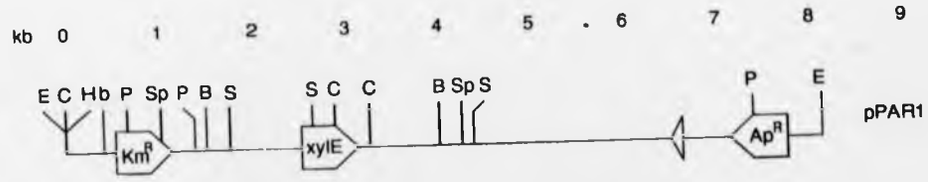


Figure 15b

Eco RI/*Pst* I, *Eco* RI/*Sal*I, *Eco* RI/*Xho*I, *Eco*RI/*Bam*HI, *Eco*RI/*Cla*I, *Bgl*II/*Pst*I, *Bgl*II/*Sal*I, *Bgl*II/*Xho*I, *Bgl*II/*Bam*HI, *Bgl*II/*Cla*I. The products of digestion were run on a 1.5% agarose gel together with lambda and pBR322 size markers.

The pPAR1 construct was used for the development of the *nptII xylE* cassette.

4.5.5 Removal of internal *Bam*HI site by partial *Bam*HI digestion

The *nptII/xylE* cassette in pPAR1 has a *Bam*HI site downstream of it. It would be particularly useful if the cassette was flanked by *Bam*HI sites. This required the addition of a *Bam*HI site upstream of the cassette and removal of the *Bam*HI site between the *nptII* and *xylE* reporter genes. The following procedure was employed for removal of the internal *Bam*HI site.

A *Bam*HI/ pPAR1 digest time course was set up, whereby the reaction was terminated (by removal of a 20 µl aliquot and the addition of 2 µl of 200 mM EDTA) at the following intervals: 0, 1, 2, 4, 8, 15, 30 and 60 minutes. Each aliquot contained approx. 100 ng of DNA. The digestion products were run on an agarose gel, and the band of linear pPAR 1 plasmid DNA identified. The linear fraction was recovered from the gel onto DE81 paper, eluted and ethanol precipitated (Section 2.20).

4.5.6 End filling of *Bam*HI site

Klenow enzyme (the large subunit of *E.coli* DNA polymerase) was used to convert the *Bam*HI ends of the DNA into blunt ends. The precipitated linear pPAR1 was resuspended in a 10 µl volume of:

20 mM Tris-Cl, pH 8.0
7 mM MgCl

10 mM Klenow

and incubated at 37^{0}C for 2 mins followed by the addition of 1 µl of 0.125 mM dNTPs and incubation at 37^{0}C for a further 2 minutes.

4.5.7 Blunt ended ligation

To the end filled linear pPAR1, a 40 µl volume of the following was added.

66 mM Tris-Cl, pH 7.6

6.6 mM $MgCl_2$

5 µl 1 M DTT

100 µg ml^{-1} BSA

1 mM Spermidine

0.2 M ATP

25 U ml^{-1} T4 DNA Ligase

After incubation (6h at room temperature), a 20 µl aliquot was used for the transformation of DH5α competent cells. Cells were spread onto LB plates containing both kanamycin and ampicillin. Transformants were identified after 24 hours by spraying with catechol. Two classes of transformant were expected, corresponding to the loss of the *Bam*HI site either between the *xylE* and *nptII* genes or downstream of the *xylE* gene. Only 3 colonies were recovered. Overnight small scale broth cultures were set up for each in LB with kanamycin and small scale plasmid preparations performed on the harvested cells.

4.5.8 Characterization of pPAR 3

The following digestions were performed on each sample of plasmid DNA: *Bam*HI and *Bam*HI/ *Eco*RI. The products of digestion in addition to pPAR1 *Bam*HI and *Bam*HI/ *Eco*RI digests were run on an agarose gel. One colony appeared to contain the desired plasmid digest pattern. This plasmid was

designated pPAR3. A large scale preparation of the plasmid DNA was performed and analysed by restriction mapping as described for pPAR1 (Fig. 16).

The digestion patterns confirmed the removal of the internal *Bam*HI site. For example, *Eco*RI *Bam*HI double digestion produced a doublet of bands migrating at approximately 4 kb, as do *Bgl*II *Bam*HI digestions. This is in marked contrast to pPAR1 (Fig.15)

4.5.9 Inclusion of a *Bam*HI cloning site upstream of the *xylE/nptII* cassette

To facilitate excision of the *xylE/npt*II cassette from the plasmid DNA, a final manipulation was performed to include a *Bam*HI site upstream of the reporter genes. This was achieved by the introduction of the poly linker cloning sites from M13mp19X replicative form (RF) between the *Eco*RI and *Hin*dIII restriction sites of pPAR3. M13mp19X is a derivative of M13mp19, the *Xba*I site of M13mp19 has been replaced by a *Xho*I site. It was supplied by Prof. J.M. Lord and R. Spooner (Warwick University).

The following digestions were performed:

approx. 500 ng pPAR3 *Eco*RI/ *Hin*dIII double digestion

approx. 500 ng M13mp 19 times RF *Eco*RI/ *Hin*dIII double digestion

These were followed by the following ligations:

8 100 ng pPAR3 *Hin*dIII/ *Eco*RI

9 100 ng pPAR3 *Hin*dIII/ *Eco*RI and 50 ng M13 RF *Hin*dIII/

Fig. 16 Characterisation of pPAR3

Fig. 16a Plasmid pPAR3 was characterised by restriction analysis: a, *Eco*RI; b, lambda size markers; c, *Eco*RI/ *Bgl*II; d, *Eco*RI/ *Pst*I; e, *Eco*RI/ *Sal*I; f, *Eco*RI/ *Xho*I; g, *Eco*RI/ *Bam*HI; h, *Eco*RI/*Cla*I; pb, pBR322 *Hin*fI size markers; j, *Bgl*II/ *Pst*I; k, *Bgl*II/ XhoI;; l,*Bgl*II/ *Bam*HI; m, *Bgl*II/ *Cla*I; n, *Bgl*II/ *Sal*I.

Fig.16b The digestion pattern confirmed the removal of the *Bam*HI site between the *xylE* and *nptII* genes.

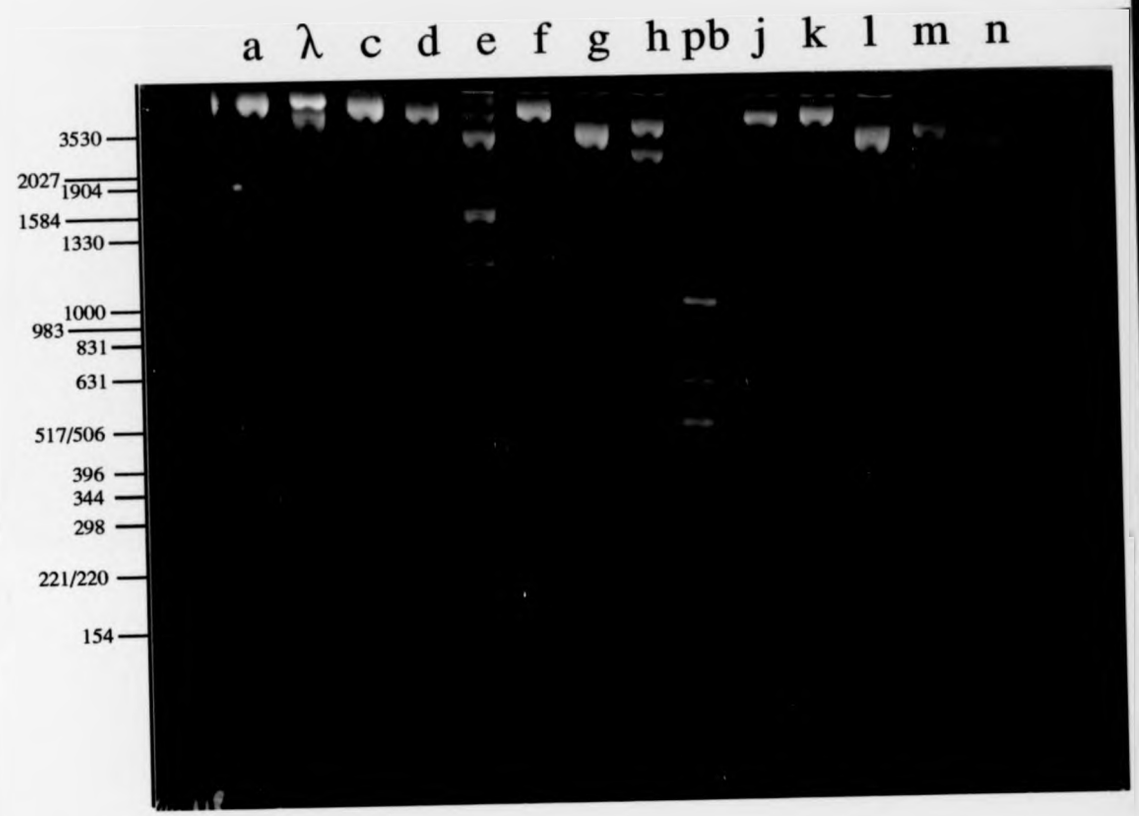


Figure 16a

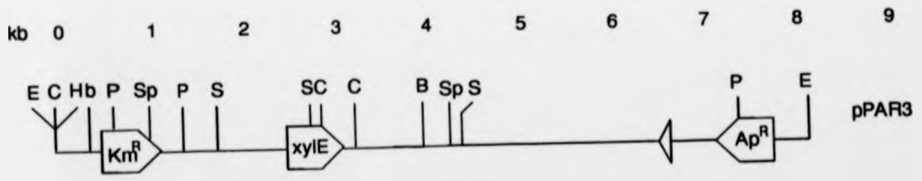


Figure 16b

*Eco*RI

10 100 ng pPAR3 *Hin*dIII/ *Eco*RI and 100 ng M13 RF *Hin*dIII/ *Eco*RI

Ligations were incubated at room temperature for 2 hs. Reactions were terminated by the addition of 2 µl of 200 mM EDTA and 13µl SDW. 6 µl of the final reaction volume was used for a transformation of *E.coli* DH5α.

4.5.10 Characterization of pPAR4

Equal numbers of colonies grew up from all transformation mixtures which turned bright yellow when sprayed with catechol. 18 colonies from cells transformed with ligation 10 were grown up overnight in small scale L-broth cultures containing kanamycin. Small scale plasmid preparations were performed, on the harvested cells and the plasmid DNA digested with *Bam*HI.

Colonies were identified that appeared to contain two *Bam*HI sites. Further confirmation of the introduced *Bam*HI site was achieved by digestion with *Cla*I. *Cla*I digestion patterns confirmed the presence of the additional *Bam*HI site. Large scale plasmid preparations of 4 of these colonies were performed followed by the confirmatory *Bam*HI and *Cla*I digestions (Fig. 17). Two of these colonies were confirmed to have a *Bam*HI site upstream of the *xylE/nptII* cassette and were designated pPAR4.

4.5.11 Excision of the *Bam*HI flanked *xylE/nptII* cassette

Approximately 10 µg of pPAR4 DNA was digested with *Bam*HI, the DNA was loaded into an extended well and run on a 0.6% agarose gel at 20V overnight. The products of the *Bam*HI digestion are very similar in size and required a low % agarose gel and and an extended running time for their resolution (Fig.

Fig. 17 Confirmation of pPAR4

Fig. 17a Confirmation of the construction of pPAR4 and therefore the introduction of the M13 polylinker was achieved through digestion with *Cla*I and *Bam*HI. *Bam*HI digestion generated two fragments of similar sizes (each around 4kb) which were not resolved on this gel and the diagnostic *Cla*I digestion products were a small band of approx. 0.3kb and a larger band of 7.5kb.

Fig. 17b Plasmid map pPAR4

The *xylE/ nptII* genes are flanked by *Bam*HI sites, facilitating excision of this fragment via *Bam*HI digestion.

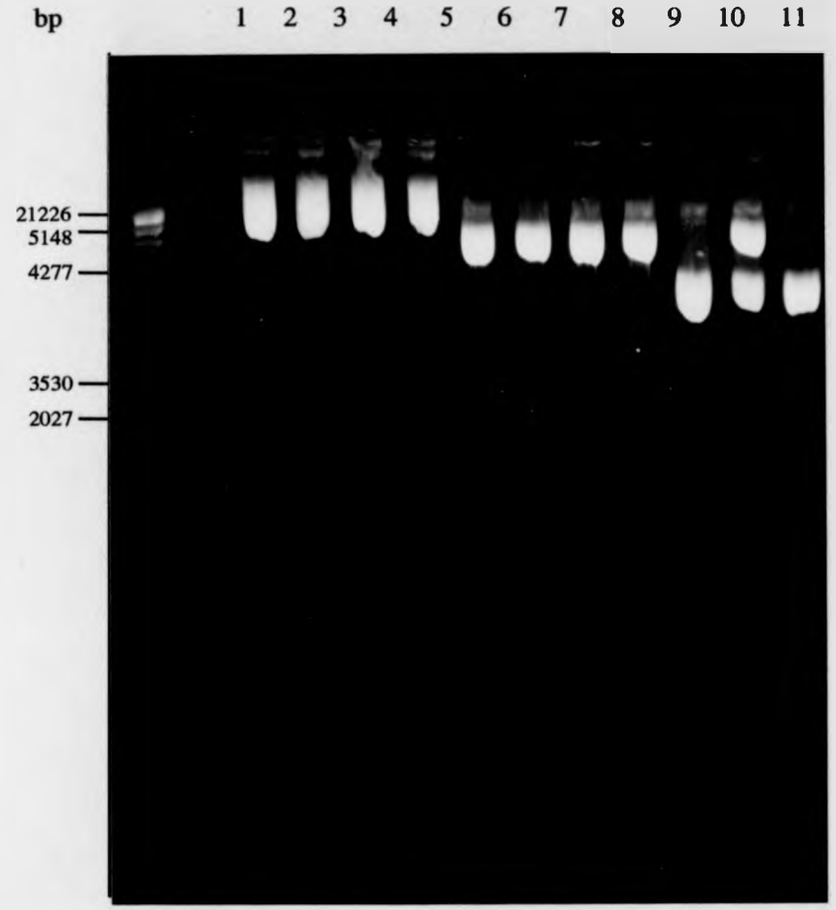


Figure 17a

Figure 17b

18). The larger fragment representing the *xylE/nptII* cassette was recovered onto DE81 paper (Section 2.20). The close proximity of the two fragments meant it was difficult to avoid some contamination from the lower fragment. To reduce this, the lower band was excised prior to the recovery of the upper band onto DE81 paper which was backed by dialysis tubing to prevent loss of the fragment. The fragment was checked for purity on a 0.5% minigel and resuspended in a final volume of 25 µl of which a 1 µl aliquot could be visualised on an ethidium stained agarose gel.

4.5.12 Digestion of ISP5078 genomic DNA with *Bam*HI, *Bgl*II and *Eco*RI

Genomic DNA from ISP5078 was cut with the restriction enzymes: *Bam*HI, *Bgl*II and *Eco*RI in order to assess the frequency of sites for these enzymes. There were many *Bam*HI and *Bgl*II sites giving restriction ladders with a good distribution of different sized fragments (Fig. 19). In contrast, the high GC content of *Streptomyces* DNA makes restriction sites with a high AT content relatively rare, *Eco*RI cut ISP5078 genomic DNA produced restriction ladders containing predominantly high molecular weight fragments (not shown). These observations have been also made by other workers (Chater & Hopwood, 1984). The *Bam*HI and *Bgl*II restriction sites were chosen for the final cloning steps.

4.5.13 Cloning strategy for the incorporation of the *xylE/ nptII* cassette into the ISP5078 chromosome by homologous recombination

The overall cloning approach is summarised in a scheme given in Fig. 20 and proceeded as follows: 40 µl of a saturated solution of ISP5078 genomic DNA solution in SDW was digested with *Bam*HI. The volume was increased to 280 µl with SDW and the products of digestion extracted with phenol chloroform and chloroform isoamylalcohol. The *Bam*HI digested DNA was ligated to

Fig. 18 Excision of the *xylE/nptII* insert

*Bam*HI restricted pPAR4 DNA was run in an extended well of the gel for 18hs on a 0.5% agarose gel to allow separation of the two fragments of similar size. The larger of the two bands contained the *xylE/ nptII* insert and was recovered (according to Section 2.20).

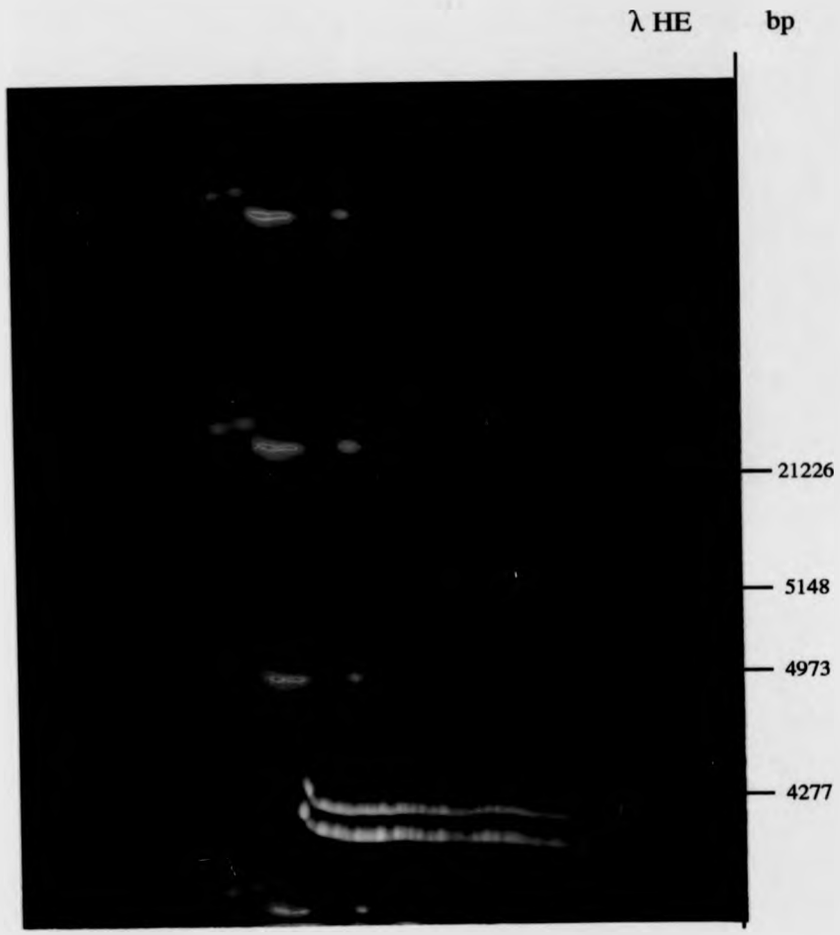


Figure 18

Fig. 19 Restriction of ISP5078 genomic DNA with *Bam*HI and *Bgl*II.

In order to pursue the cloning strategy outlined in Fig. 20 it was important to confirm the presence of significant numbers of restriction sites for the *Bam*HI and *Bgl*II restiction sites. The restriction ladders given in Fig. 19 illustrate the presence of many small bands of DNA indicative of many restriction sites.

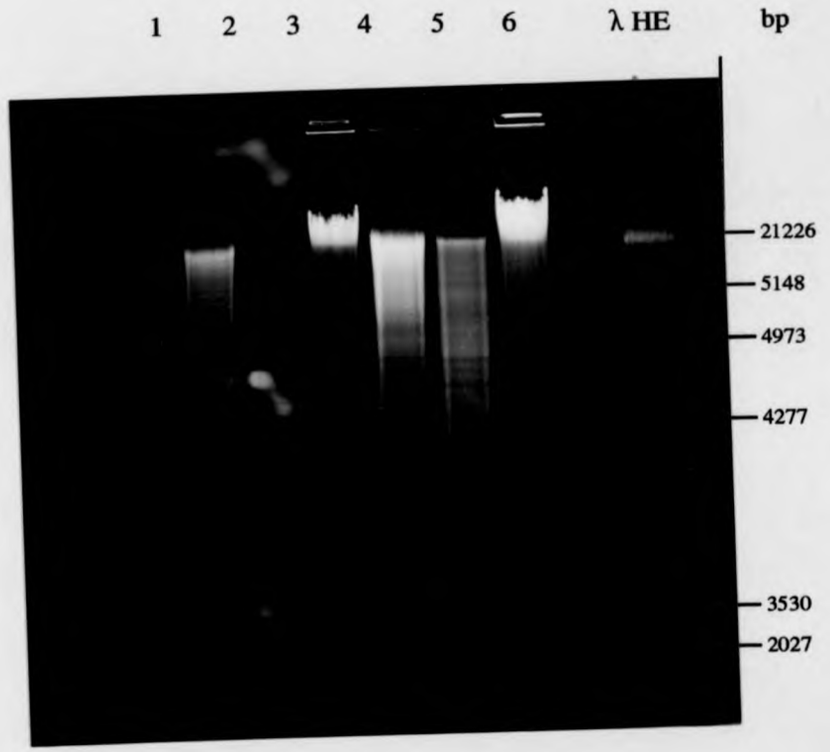


Figure 19

Fig. 20 Overview of ISP5078 cloning strategy

Genomic ISP5078 DNA was digested with *Bam*HI, and the fragments ligated to form closed circles. The genomic circles were then digested with *Bgl*II, the *xylE/nptII* insert was then ligated into the *Bgl*II sites. ISP5078 protoplasts were transformed with these DNA molecules, the low frequency homologous of recombination of the insert into the chromosome was screened for.

Key:

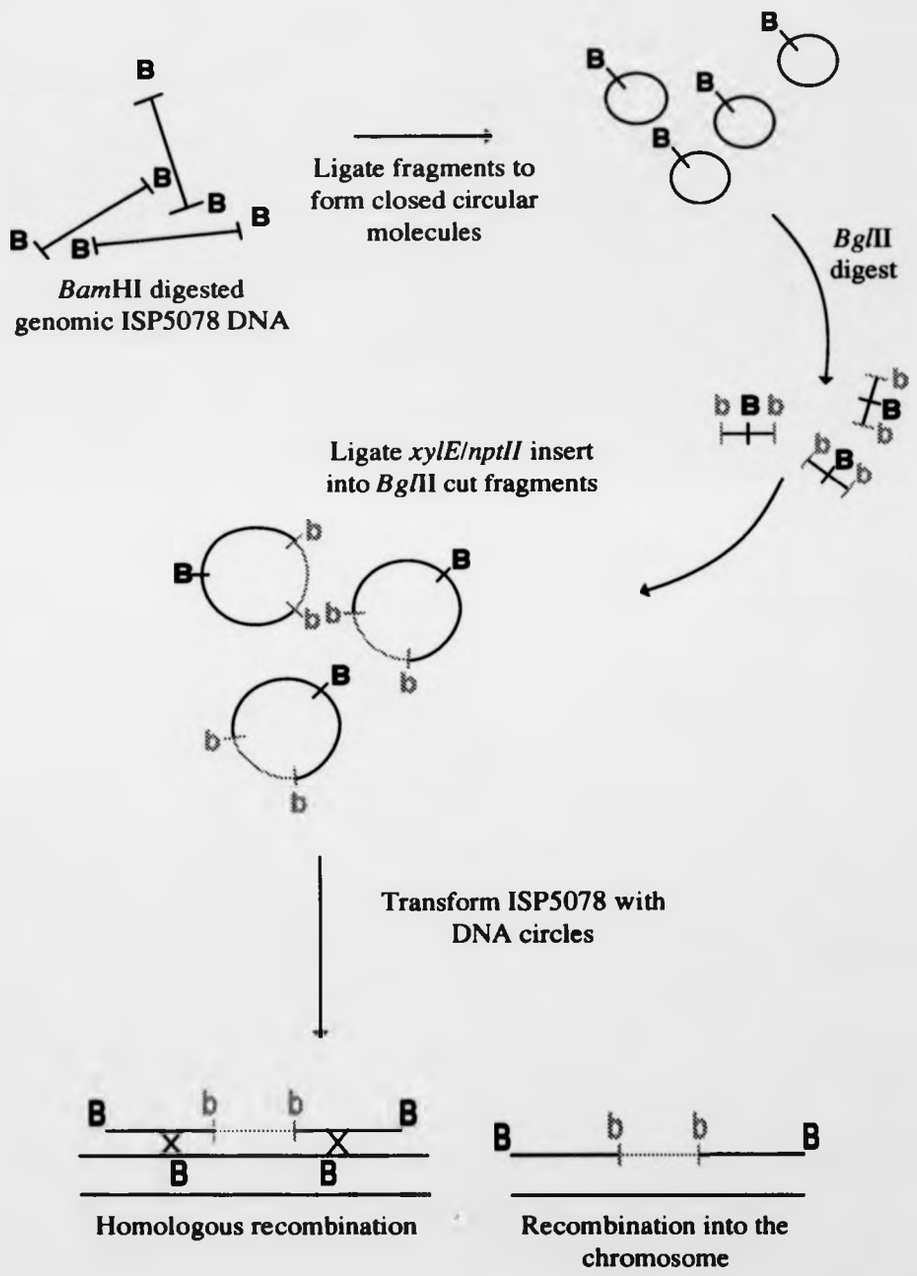
—— = ISP5078 genomic DNA

····· = *xylE/nptII* insert

B = *Bam*HI restriction site

b = *Bgl*II restriction site

Figure 20



form closed circular molecules of DNA in a final volume of 400 µl. A large reaction volume was used to promote intra rather than intermolecular ligation, by the addition of the following:

40 µl 10 times ligase buffer
40 µl BSA (5 mg ml^{-1})
40 µl DTT (40 mM)
3 µl T4 Ligase

The ligation was allowed to proceed overnight at 15^0C. The reaction was terminated by phenol chloroform extraction of the ligation mixture and the DNA precipitated with ethanol. 2 µl of 20 mg ml^{-1} glycogen (a carrier molecule) was added to the ethanol solution to ensure efficient recovery of the ligated DNA.

The precipitated DNA was taken up in a final volume of 30 µl and digested with *Bgl*II in order to cut some of the closed circular molecules at a different point to that of the *Bam*HI site. The DNA volume was increased with SDW and the products of digestion extracted with phenol chloroform and chloroform isoamylalcohol before ethanol precipitation. The *Bgl*II digested DNA was resuspended in 20 µl of the *xylE/ nptII* cassette DNA and ligated overnight at 15^0C with the addition of 2.5 µl 10 times ligase buffer and 25 Units of T4 ligase. The ligation volume was kept small and the concentration of DNA molecules high to promote intermolecular ligation of the insert to the genomic DNA. The ligated molecules were then used in 2 µl aliquots for ISP5078 transformations.

4.5.14 Optimal transformation of ISP 5078

Transformations were based on the methods of Hopwood *et al.* (1985) for the

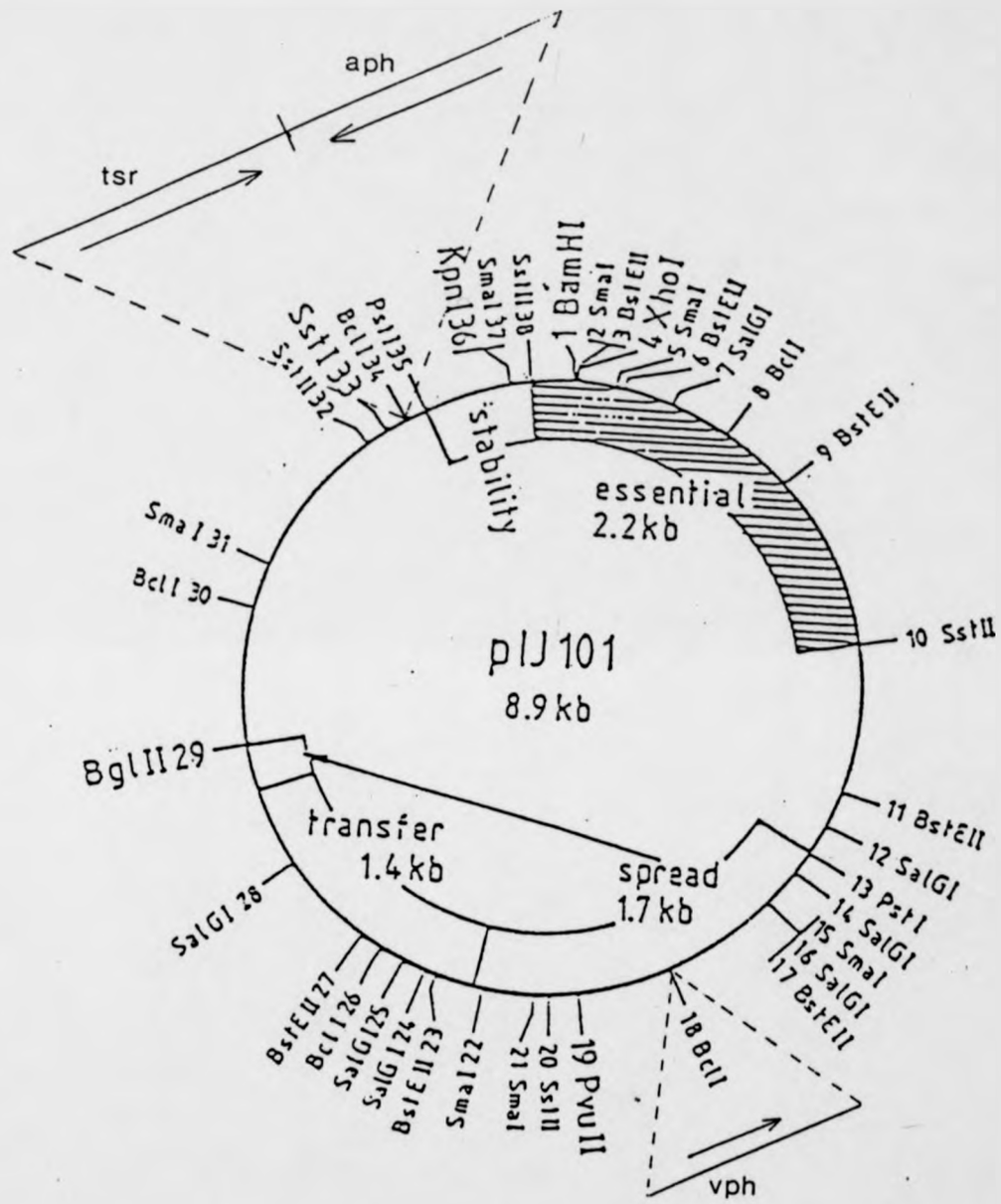
PEG mediated transformation of streptomycete protoplasts. These methods were developed for the transformation of streptomycetes with plasmid DNA. Transformation of protoplasts with chromosomal DNA fragments has been reported to occur at very low frequencies and until recently there were few methods available for the routine insertion of sequences into the chromosome. With this in mind, PEG mediated plasmid transformation methods were used with closed circles of genomic ISP5078 DNA containing the *xylE/nptII* cassette. Transformation conditions were optimised for this strain using plasmid pIJ673. This plasmid is a derivative of plasmid pIJ101 with the spread region disrupted by the inclusion of additional antibiotic resistance determinants (Fig. 21).

ISP5078 broth cultures were grown up in baffled flasks containing YEME medium with glycine according to Hopwood *et al.* (1985). The quality of protoplasts prepared from YEME grown ISP5078 mycelium was better than that achieved from cells grown in TSB with 10% sucrose and PEG. This was probably because the duration of the lysis step was reduced considerably from one hour to 20 to 30 minutes. From the production of 10^{10} protoplasts ml^{-1} the regeneration frequency on dried R5 plates was 10^6 to 10^7, and the number of transformants produced using 1 µl pIJ673 DNA (equivalent to 2.5 µg, 260/280 ratio = 1.9) was 10^3 per plate. A one in 10 dilution was required to produce single colonies. A modified R5 overlay was poured over the developing protoplasts after a 20 h incubation containing antibiotics selective for the plasmid. These were added at 1/10 of the concentration recommended by Hopwood *et al.* (1985). Hence neomycin was added to the overlay to a final concentration of 10 µg ml^{-1} and thiostrepton 25 µg ml^{-1}. 17 of the regenerating transformant colonies were picked, grown up in liquid broth under selection and small scale plasmid preparations performed by alkaline

Fig. 21 Plasmid map, pIJ673

Plasmid pIJ673 was derived from the multicopy plasmid pIJ101 (Kieser *et al.*, 1982). A viomycin gene (vph) has been inserted into the spread region and thiostrepton (tsr) and neomycin (aph) resistance determinants inserted into the non-essential regions.

Figure 21 map of pIJ673



lysis on each of them. Confirmation of pIJ673 was obtained by digestion with *Pst*I to give a typical 5 band pattern given in Fig. 22 and described by Wellington *et al.* (1989). At least 16 of the 17 colonies contained pIJ673, (insufficient plasmid DNA was recovered from the other transformant).

4.5.15 Transformation of ISP5078 with *xylE* cassette and ISP5078 genomic DNA circles.

Using the transformation procedures described above, 2 µl aliquots of the ligated DNA solution were used in PEG mediated transformations of ISP 5078 protoplasts. Regenerating protoplasts were overlayed with soft modified R5 overlay containing kanamycin. However, the result was the production of lawns of kanamycin resistant colonies growing through the agar overlay. This was also the case for regenerating ISP5078 protoplasts that received PEG and SDW but no DNA and were overlayed with kanamycin. To add to the problems of universal resistance to kanamycin after protoplasting, the substrate mycelium of the strain growing on the regeneration agar soon became a very similar bright yellow to that produced by the 2,3 dioxygenase enzyme in the presence of the catechol (Fig. 23). When the colonies were sprayed with catechol and it was not possible to observe a dramatic colour change. Since the colonies exhibited a yellow substrate mycelium, it may not have been possible to detect colonies expressing the *xylE* reporter gene by eye. *xylE* expression in *E.coli* involved a copy of the gene on a multicopy plasmid and so was relatively easy to identify. In contrast a chromosomally encoded gene product was being screened for in ISP5078, hence reduced enzyme product would be expected. Difficulties associated with the colour of the substrate mycelium might have been overcome by spraying the colonies at an earlier stage before the colonies change from a grey-white to a bright yellow or at a later stage of development with production of the white aerial mycelium.

Fig. 22 Confirmation of Transformation of ISP5078 with pIJ673

Plasmid DNA recovered from ISP5078 transformants was restricted using *Pst*I. The 5 band pattern generated was diagnostic for this plasmid (Wellington *et al.*, 1990).

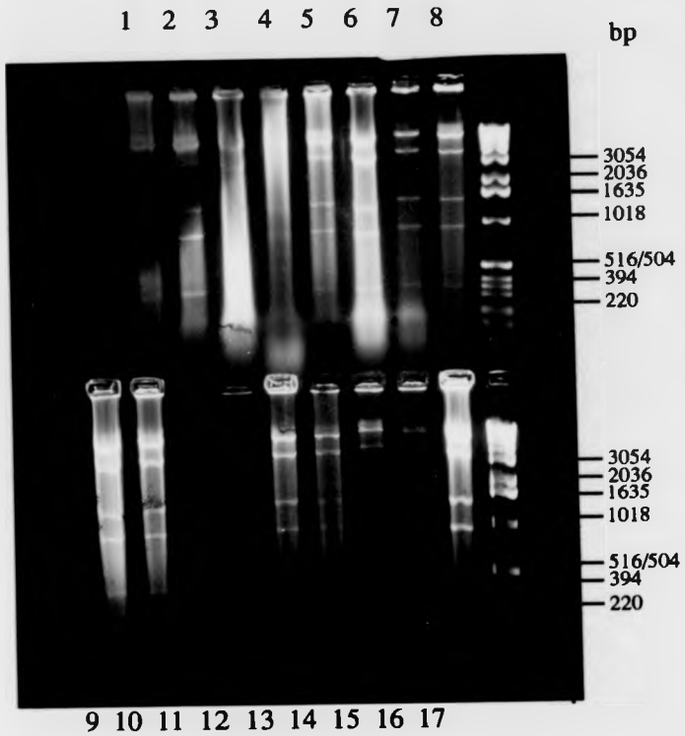


Figure 22

Fig. 23 Comparison of ISP5078 yellow substrate mycelium with the product of the *xylE* gene in the peesence of catechol (2-hydroxy muconic semi aldehyde).

S.lividans 1326 harbouring a plasmid expressing the *xylE* gene is presented alongside the ISP5078 strain. The 2-hydroxy muconic semi aldehyde product was a similar colour to the substrate mycelium of ISP5078, indicating the difficulties in identifying its expression and therefore its use in this strain. Strain MP2 also given in the photograph, has a paler substrate mycelium and may comprise an alternative host strain for this marker.

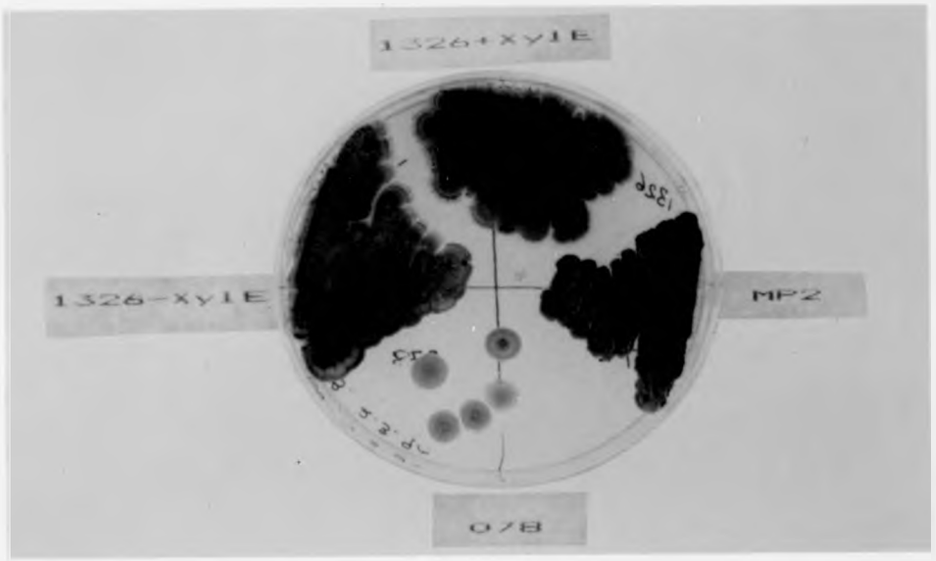


Figure 23

Repeated transformation and spraying of regenerants at different stages in the life cycle did not however, reveal any obvious *xylE* positive colonies.

4.5.16 Induction of cryptic antibiotic resistance genes through protoplasting

ISP5078 was checked for ability to produce catechol 2,3 dioxygenase and resistance to kanamycin prior to beginning the cloning work. The strain proved negative on both accounts and when rechecked the parent strain remained very susceptible to low levels of kanamycin. The induction of kanamycin resistance following protoplast transformation would probably obscure the low frequency homologous recombination event being screened for.

Of particular relevance to these findings are the studies by Hotta *et al.* (1988a;b) on the activation of cryptic aminoglycoside resistance genes by protoplasting. The strains under study belong to *S.griseus*, the group to which ISP5078 was identified. Although little is understood of the influence protoplasting has on the cryptic gene, its activation results in the production of a novel aminoglycoside acetyltransferase AAC (3) that confers high levels of resistance to a range of aminogylcoside antibiotics including: kanamycin, neomycin, gentamycin, dibekasin and paromamycin. Southern blot analysis of a range of streptomycetes probed for the presence of the cryptic gene, indicated that homologous sequences were present in all *S.griseus* strains that have been studied and absent in the other *Streptomyces* species (Hotta & Ishikawa, 1988). The authors speculate that the distribution of this sequence may relate to a specific role of the cryptic gene product in the life cycle of *S.griseus*.

Although the kanamycin resistance mechanism for ISP5078 was not characterised in detail, high levels of resistance to kanamycin (100 μg ml^{-1}) and neomycin (10 μg ml^{-1}) were also obtained. Resistance to aminoglycoside antibiotics amongst actinomycetes has been found to occur as a result of enzymic deactivation either via phosphorylation or acetylation or via ribosomal methylation (Nakano & Ogawara, 1986; Hotta *et al.*, 1988b) or indeed a combination of these mechanisms, resistance spectra are thus determined by the mechanism(s) operating. Streptomycete mutants with resistance to antibiotics may be induced by repeated subculture onto higher and higher levels of antibiotics. Furthermore studies by Phillips *et al.* (1992) indicated that kanamycin resistance of > 5 μgml^{-1} to be rare in natural streptomycete isolates. It is therefore difficult to envisage the dramatic change in resistance to kanamycin by large numbers of ISP5078 protoplast regenerants without the activation of a cryptic antibiotic resistance gene as reported by Hotta *et al.* (1988a). Regenerants grew through a soft agar overlay containing 500 μg ml^{-1} kanamycin. Hotta *et al.* (1988b) report a 200 fold increase in resistance *S.griseus* strains ie. from < 5 μg ml^{-1} to 500 to 1000 μg ml^{-1} resistance with protoplast regeneration. However, further analysis similar to that undertaken by Hotta *et al.* (1988a) would be necessary in order to clarify this.

The kanamycin resistant mutation appeared to be stably maintained in culture both in the presence and absence of kanamycin. Although the kanamycin resistant regenerants did appear morphologically indistinguishable from the parent strain when isolated, after 18 months to 2 years in culture the strain did differ morphologically possessing a characteristic ragged edge compared with its kanamycin sensitive derivative (the altered phenotype was moredistinctive in the absence of kanamycin selection). This is in contrast to Hotta *et al.*

(1988b) who generated kanamycin protoplast regenerants that appeared phenotypically indistinguishable from the parent strain, but which did have distinct RFLP genomic DNA patterns.

4.5.17 The application of kanamycin induced resistance in ISP5078 as a marker for detection and monitoring in soil

Although it was not possible to comply with our initial aims to identify naturally occurring selective resistance markers and to introduce the *xylE* and *nptII* reporter genes into ISP5078, it was possible to generate kanamycin and neomycin resistant strains. Since, aminoglycoside resistance is rare among streptomycete isolates (Phillips *et al.*, 1992) these markers offered potential for the selective recovery of ISP5078 from the indigenous soil streptomycete microflora. However before the ecology of the strain could be studied *in situ*, it was important to assess the stability and the reversion rate of the marker in soil. It would also be important to evaluate whether the induced kanamycin resistance alters the fitness of the strain in terms of its ability to survive and compete in the natural environment (Compeau *et al.*, 1988).

4.5.18 The application of pIJ673 as a selective marker for the detection and monitoring of ISP5078 in soil

The technology for inserting markers into the *Streptomyces* chromosome has developed considerably. Recent developments have seen a sudden increase in the number of delivery vectors utilising integrating plasmids, transposons and bacteriophage (reviewed by Kieser & Hopwood, 1991). Initially these vectors will be evaluated in the genetically well characterised *S.lividans* and *S.coelicolor* strains. Their role in the introduction of markers onto the chromosome of uncharacterised strains remains to be defined. For the short

term the introduction of marked genes into genetically uncharacterised strains may be limited to their introduction on plasmids (Wipat *et al.*, 1991). The disadvantages of using plasmid borne markers have been well documented, namely loss via segregation at cell division, transfer to alternative hosts and the possibility of the additional metabolic burden of the plasmid to the cell. Plasmids have however, been associated with considerable numbers of natural *Streptomyces* isolates (Hopwood *et al.*, 1986) including the common scab strains (McQueen *et al.*, 1985); hence their application as markers would add an important dimension to the study the ecology of the *Streptomyces*. The plasmid pIJ673 (a derivative of pIJ101 [Kieser *et al.*, 1982]) (Fig.21) that has been introduced into ISP5078 confers resistance to a highly selectable combination of antibiotics, particularly suited to the selective recovery of specific *Streptomyces* inoculants from soil (Wellington *et al.*, 1990).

Studies to evaluate the stability of plasmids and their affect on the ecology of the host strain are limited by the requirement for chromosomally marked strains in order to monitor the proportion of the population that are cured of the plasmid but survive under environmental conditions.

4.5.19 Problems and perspectives on the cloning in genetically uncharacterised strains

The majority of heterologous cloning in *Streptomyces* has been performed in *S.lividans*, the reason being the absence of restriction barriers to incoming foreign DNA. With an increased interest in the genetic analysis of the genus, has come the awareness that strains without restrictive systems are rare. The problem is exemplified by the suggestion that *S.fradiae* may have 5 different restriction systems operating (Matushima *et al.*, 1987) and that the *Streptomyces* are an important commercial source for the production of many

restriction enzymes (Chater & Hopwood, 1984). Consequently restriction free mutants of *S.fradiae* have resulted in increased plasmid transformation frequencies in excess of 3 to 4 orders of magnitude (Matushima *et al.*, 1989). *Streptomyces griseus* strains have been suspected to have operational restriction systems (Cox & Baltz, 1984). The potential problem of restriction systems has been emphasised by Hopwood *et al.* (1985) who indicate that the introduction of any DNA from *E.coli* into *S.coelicolor* necessitates prior transformation and recovery from *S.lividans*. Introducing the DNA into a plasmid and passing it through *S.lividans* is an advisable precautionary step in the cloning strategy of uncharacterised strains. Hence this was attempted by ligating the *xylE* cassette into a unique *Bam*HI site of plasmid pIJ486 (a derivative of pIJ101). The success of the ligation was monitored, by analysing the DNA before and after ligation by gel electrophoresis. Although many transformants were produced harbouring pIJ486, none of those screened turned yellow when sprayed with catechol. Plasmids from putative transformants were isolated and restricted however none of the preparations analysed by restriction contained inserts. Although transformations were repeated several times, the approach was not overly pursued. Since reports exist of good expression of the *xylE* gene in *Streptomyces lividans* (Wipat *et al.*, 1991; Ingram *et al.*, 1989) failure to obtain expression is unlikely to relate to the combination of strain and reporter gene. Alternatively, the problems may be attributed to the use of an inappropriate plasmid vector. Hopwood *et al.* (1985) remark that some cloned fragments appear stable in one vector but not another. Hence, future attempts might focus on other vectors and include cloning of the *Bam*H1 flanked reporter cassette into the unique *Bgl*II site in the non-essential regions of pIJ702 (Hopwood *et al.*, 1986). In addition the transformation event being screened for could be quite rare and lack of success related to an insufficient number of transformants screened.

The marker system appeared more problematic than originally envisaged. Production of kanamycin resistant protoplast regenerants were a potential problem with transformation procedures that require a protoplast stage. Furthermore it was felt that chromosomal expression of the *xylE* gene (and hence the reaction with catechol to produce the distinctive yellow compound) may not have been sufficient to be visualised through the yellow substrate mycelium of strain ISP5078. At this point it was decided to abandon this approach and focus on the application of kanamycin and pIJ673 marked strains to studies of the autecology of ISP5078.

4.5.20 Alternative approaches for the transformation of chromosomal DNA that might be applied in further attempts to introduce marker genes onto the chromosome of uncharacterised strains

The low frequency of transformation of chromosomal DNA has been increased by the incorporation of the DNA into liposomes which are then fused with the protoplasts (Makins & Holt, 1981). Rodicio & Chater (1982) report increased transfection frequencies of *S.lividans* protoplasts by ϕC31 in the presence of small DNA free liposomes. However, few reports exist of the application of this approach to cloning in *Streptomyces*. Alternative strategies might also consider electroporation and phage delivery vectors, the latter approach circumventing the need for protoplasting and hence the consequent mutagenesis effect. MacNeil (1987) reported an efficient transformation procedure for plasmid DNA by applying electroporation to *S.lividans* protoplasts. It was also suggested that it might be possible to develop procedures for whole cells such as freshly germinated spores. McHenney & Baltz (1988) report on the broad host range streptomycete bacteriophage (FP43) mediated high frequency transduction of plasmid DNA. The

bacteriophage appeared to be able to transduce some streptomycetes with well characterised restriction systems. A recent report by Hilleman *et al.* (1991) indicated that the frequency of transformation was increased by 10 to 100 times with certain combinations of strain and vector through transformation with single stranded as opposed to double stranded vector DNA.

The Characterisation and Detection of Plant Pathogenic Streptomycetes in the Natural Environment

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Chapter 5

The application of 16S rRNA targeted probes to the detection and monitoring of scab-causing streptomycetes in soil

5.1 Introduction

The pressure to develop suitable methodology for detection and monitoring has resulted in the widespread application of molecular methods to microbial ecology (Ogram & Sayler, 1988; Trevors & Van Elsas, 1989; Pickup, 1991). Molecular approaches are also useful because they offer the opportunity to monitor components of the ecosystem that remain recalcitrant to culture and thus have been poorly studied (Olsen, 1990; Ward *et al.*, 1990; Weller *et al.*, 1991). The shortcomings of traditional methods that rely on the isolation and cultivation of microbes from the natural environment have been illustrated by the findings that only 1 to 5% of soil microorganisms observed by direct counts were actually culturable (Bakken, 1985; Bone & Balkwill, 1986).

In addition molecular techniques allow the possibility to monitor the stability and containment of genetic sequences within microbial populations. Since techniques such as the Southern blot hybridization method allow the detection of genetic rearrangements (such rearrangements might be anticipated to occur with the introduction of genes via transposons or integrating plasmids) as well as the ability to monitor the horizontal transfer of introduced genes into new populations (Jain *et al.*, 1988). Finally since gene probes target gene sequences and not their products these techniques offer the potential to monitor the presence of genes within communities without the prerequisite for their expression (Jain *et al.*, 1988). This latter point is of particular importance, since only a small minority of taxa in an environmental sample are growing rapidly at any time, and thus only a few may be actively expressing genes (Holben & Tiedje, 1988).

The most recent contribution to the battery of detection methods in microbial ecology has been the application of PCR technology. Molecular approaches

encompassing PCR offer highly sensitive levels of detection, without the prerequisite for isolation and cultivation. PCR protocols allow the cloning and sequencing of nucleic acids direct from environmental samples (Zehr & McReynolds, 1989; Giovannoni, 1991; Steffan & Atlas, 1991). The application of molecular biology to microbial ecology has introduced the possibility to analyse the composition of communities of previously undescribed unculturable microorganisms (Giovannoni *et al.*, 1990; Amann *et al.*, 1991).

An initial requirement for the molecular analysis of specific populations is the extraction and recovery of nucleic acids. Generally, this involves extraction and recovery of DNA or RNA from environmental samples. However, it is also possible to extract and culture the cells, (onto selective media) from environmental materials (Pettigrew & Sayler, 1986; Jain *et al.*, 1988). Alternatively gene sequences may be assayed for directly from environmental materials. This approach has been applied to the identification of *Rhizobium* strains from root nodules (Cooper *et al.*, 1987).

The stability of DNA molecules relative to RNA molecules has meant that DNA has often been the target molecule of choice in the application of molecular approaches. The efficiency of recovery of DNA from the environment will contribute to the sensitivity of detection and is dependent on (i) representative sampling and extraction efficiency and (ii) sufficient purification to produce nucleic acids that are amenable to further analysis by probing, digestion by restriction enzymes, ligation and PCR.

The two approaches that have been taken in the recovery of DNA from environmental samples involve the indirect and direct extraction of DNA from

soil. Indirect extraction methods necessitate the prior extraction of the cell biomass from soil, the cell fraction is then lysed and the DNA recovered (Holben *et al.*, 1988). Direct lysis procedures omit a cell extraction step, with lysis occurring *in situ,* followed by the recovery of the DNA from the soil matrix (Steffan *et al.*, 1988).

5.1.2 Indirect extraction of DNA

This approach requiring an initial extraction of the cells from soil was pioneered by Torsvik and colleagues (Torsvik & Goksoyr, 1978; Torsvik, 1980). They used the cell fractionation method of Faegri *et al.* (1977) to separate and recover microbial cells from soil, by rounds of differential centrifugation. However, the DNA isolated was brown in colour and badly contaminated with humic acids. Lengthy purification steps were required to remove the humic acids, these included the addition of 8M urea to the bacterial lysate, the passage of the DNA suspension through an ion exchange column and hydroxyapatite chromatography. These lengthy and exhaustive procedures resulted in substantial losses of DNA and yields of 19 to 28%. Holben *et al.* (1988) took the methodology a stage further, by separating the soil bacteria from the soil in a homogenisation solution containing polyvinyl pyrrolidine (PVPP) and sodium ascorbate. Humic acid contaminants are removed by absorption to the insoluble PVPP polymer. Sodium ascorbate was added as a reducing agent to prevent oxidation of phenolics (these include humic acids). Lysis of the bacterial fraction was achieved by treatment with lysozyme, pronase and sarkosyl. The recovered DNA solution was purified by caesium chloride density gradient centrifugation. Hence Holben *et al.* (1988) were able to shorten the protocol of Torsvik (1980) making it more amenable to environmental samples. The DNA recovered had a purity comparable to that obtained from pure cultures and was readily digested with restriction

endonucleases. The method yielded DNA in the range of 50 to 150 kb, however while applicable to hybridization studies it was considered unsuitable for cloning (Fuhrman *et al.*, 1988).

A procedure for the indirect extraction of DNA from marine planktonic microorganisms was recently described by Fuhrman *et al.* (1988). In the marine environment the problem of low population densities necessitates the filtering of large volumes of water and the collection of cell biomass on filters. DNA was extracted cells collected from water subject to prefiltration (to remove eukaryotes) and concentrated onto 0.22 μm filters. Marine microbes were lysed by incubating the filters in 1% SDS and heating to 95 to 100^{0}C. The DNA recovered was greater than 23 kb in length and sufficiently pure to allow endonuclease digestion with *Sau*3AI and ligation to vector DNA. The efficiency of extraction of nucleic acids from the marine environment was subsequently improved by omission of the prefiltration step (Sommerville *et al.*, 1989). Cell lysis and proteolysis were performed within the housing of the filter unit. This simplicity and containment to the filter unit making the procedure particularly suitable to analysis of environmental samples while on board ship.

5.1.3 Direct extraction of DNA

Much effort has been focused on the development of direct extraction methods for the analysis of environmental communities. The initial cell extraction step utilised for indirect methods assumes complete and representative recovery of cells from the soil. However bacterial fractionation methods may selectively remove cells that are loosely bound and easily dislodged, while leaving those species that are more tightly bound to components of the soil matrix (Ogram *et al.*, 1987). This initial extraction may therefore introduce a bias for a

particular component of the bacterial population. Furthermore, direct extraction methods have been reported to result in higher yields of DNA and shorter protocols (Ogram *et al.*, 1987; Steffan *et al.*, 1988). The first report of this type was described by Ogram *et al.* (1987) for the recovery of DNA from sediments.

Initially soil was washed repeatedly with a sodium phosphate buffer to remove any extracellular DNA bound to the sediment. Cells were then disrupted by heating to 70^0C for an hour in 1% SDS followed by bead beating. The efficiency of lysis was estimated to be as high as 90% in sediments (containing 19 to 44% clay and 3 to 16% organic carbon). Nucleic acids were recovered by 2 times 0.12 M alkaline (pH 8.0) sodium phosphate extractions, followed by precipitation with polyethelene glycol. Recovery of the DNA from sediments containing clay minerals was more efficient at an alkaline pH. DNA is negatively charged at a low to neutral pH and hence is prone to absorption by clay minerals (Ogram *et al.*, 1988). The DNA resuspended from the PEG pellet was subsequently extracted with phenol chloroform and purified by caesium chloride gradient centrifugation and hydroxyapatite chromatography. The DNA yielded ranged from 0.5 to 10 kb and was suitable for hybridization. The low molecular size being attributable to the shearing action of the bead beating step. Purification by both CsCl gradient centrifugation and hydroxapatite chromatography appeared necessary for recovering DNA from sediments containing large amounts of organic carbon. However, purified DNA was recoverable from soils with low to negligible levels of organic carbon with the omission of CsCl gradient centrifugation purification step.

In a comparison of the methodology for direct and indirect extraction of DNA,

particular attention was given to the recovery and purity of DNA extracted from soils high in organic matter and prone to the release of large amounts of humic acids (Steffan *et al.*, 1988). The study indicated that while yields of DNA recovered from the direct extraction methodology of Ogram *et al.* (1987) exceeded those for indirect extraction methods, the problems of humic acid contamination were more exaggerated with direct extraction methods. Humic acids co-purify with nucleic acids, producing a brown coloured DNA pellet. Humic acids may inhibit the action of many restriction enzymes and affect the efficiency of hybridization. Steffan *et al.* (1988) found that the incorporation of PVPP into the homogenisation buffer, followed by caesium chloride density gradient purification and hydroxyapatite chromatography eased the problems of humic acid contamination and promoted the recovery of purified DNA. In addition, while DNA recovered from both procedures was amenable to dot-blot hybridization, humic acid contamination though minimised was able to prevent digestion with the restriction endonuclease *Sal*I. DNA recovered via the indirect lysis procedures was restrictable with *Eco*RI but not when recovered from the direct lysis procedure. Sayler *et al.* (1992) report methodology for the direct extraction of DNA from soils and sediments that is amenable to digestion with a number of restriction endonucleases including *Eco*RI. The method incorporates the lysis regime described by Ogram *et al.* (1987) followed by 3 times 0.12 M sodium phosphate extractions of the soil and bead mixture. The combined supernatants were precipitated with PEG and the recovered DNA extracted with phenol and chloroform isoamylalcohol. The DNA solution was precipitated twice, first with ethanol and sodium acetate and then by the addition of 5 M potassium acetate to a final concentration of 0.5 M and by placing on ice for 2 hours. The resulting brown precipitate was recovered by centrifugation and the DNA solution purified by hydroxyapatite chromatography. The eluted fractions were analysed by

absorption spectroscopy at an optical density of 260 nm; those fractions identifying to contain DNA were pooled and dialysed 4 times against 2 L of 0.010 M Tris-HCl, 0.005 mM EDTA. Following dialysis DNA was concentrated by ethanol precipitation.

5.1.4 Colony and plaque hybridization

The colony blot hybridization technique is probably the simplest application of nucleic acid hybridization and the easiest to integrate with the traditional methodologies of microbial ecology. Bacterial colonies or phage containing plaques may be transferred from an appropriate isolation medium to hybridization filters. The colonies are lysed either enzymatically or under alkaline conditions, and the nucleic acids fixed to the filter. The filter is then used in hybridizations with a suitable probe.

The colony hybridization method has been applied to the detection of specific genotypes in the environment. These have included the study of the stability of catabolic plasmids in ground water aquifier systems (Jain *et al.*,1988), the correlation of naphthalene degradation with the number of naphthalene degrading bacteria in activated sludge systems (Blackburn *et al.*, 1987) and the enumeration of mercury resistant bacteria in contaminated environments (Barkay, 1987; Barkay *et al.*, 1989). The technique has also been applied to the identification of *Rhizobium* strains in legume root nodules (Hodgson & Roberts, 1983).

The sensitivity of the colony blot technique is dependent upon the relative proportions of the target and the background non-target populations that form colonies on the isolation medium. The original protocol (Grunstein & Hogness, 1975) has been adapted for high density screening of catabolic

genotypes in environmental samples (Sayler *et al.* ,1985). Detection limits for colonies harbouring the *Tol* plasmid against a background of non-homologous plasmid bearing *E.coli* strains were established to be 1 *Tol* plasmid bearing colony among 10^6 cells of *E.coli.*

Gene probes may be hybridized to colonies isolated from environmental samples on selective and non-selective medium. Steffan *et al.* (1989) compared the sensitivity of both approaches for the the monitoring of 4-chlorobiphenyl degrading *Alcaligenes* and a 2,4,5-trichlorophenoxyacetic acid degrading *Pseudomonas cepacia* in fresh water microcosms. Non-selective media often failed to detect either inoculants despite their presence at 0.1% of the total population and equivalent to a target population 10^4 viable cells ml^{-1} (determined by other detection methods). In contrast, selective plating even at the lowest dilutions allowed enumeration of both strains, with colony hybridization indicating that 10% of the cells growing on the selective media to be non-target cells.

Selective plating was also necessary for monitoring *E.coli* cells (using colony hybridization methods) seeded into lake water. 48 hrs after inoculation in unfiltered lake water, *E.coli* populations began to decline in the presence of competition from the natural lake microbiota, making detection possible only under selective conditions (Amy & Hiatt, 1989). The colony hybridization technique has been limited to bacteria amenable to the conventional methods of enzymatic and alkaline analysis. However Datta *et al.* (1987) have applied microwave assisted alkaline lysis to *Listeria monocytogenes*, thus making the rapid colony blot hybridization technology applicable to the detection of this medically important group of microorganisms. The methods of Datta *et al.* (1987) have also been applied to the identification of a range of Gram positive

actinomycete genera (P.Baker pers.comm.).

While the colony hybridization technique confers some advantages over traditional methods for detection and monitoring i.e. it may provide information on both the phenotype and genotype of the target microbes. The method is subject to a number of limitations, these include: the selectivity of the isolation medium, the number of target and non-target microorganisms in the environment, the amenability of particular strains to lysis and the inability to cultivate more than a small fraction of the total microbial population from the environment. These limitations illustrate the short comings of a single method for the detection and monitoring of inoculants and highlight the need for an integrated approach based on a number of monitoring strategies. Fredrickson *et al.* (1988) compared a number of monitoring strategies for the detection of a Tn*5* marked *Rhizobium leguminosarum* strain in agricultural soils. Similar counts were obtained using the plate count, MPN plant infectivity, colony blot DNA hybridization, MPN-DNA hybridization and FA direct counts after 5 days of incubation in non-sterile soil. However, after 30 days of incubation plate counts and colony blot hybridisations estimated the *R.leguminosarum* population to be 10^6 CFU g^{-1} dry soil, whereas the FA count and the MPN replica plate count estimated the populations to be 10 fold higher and 10 fold lower respectively.

5.1.5 *In situ* detection

The colony blot hybridization assay has been modified to allow the direct detection of a number of bacteria and viruses from extracts of plants and insects (Owens & Denier, 1981; Keating *et al.* 1989). Such procedures are necessary for the detection of obligate viral pathogens and bacterial symbionts that are not amenable to culture and isolation (Owens & Denier, 1981; Barker

et al., 1985; Flores, 1986; Laksham *et al.*, 1986). The bacterial symbionts *Rhizobium* and *Frankia* have been identified using a crushed root nodule assay (Cooper *et al.* 1987; Simonet *et al.* 1988).

5.1.6 Nucleic acid methods for measuring community diversity and structure

Two relatively rapid approaches to the analysis of community structure and diversity that have been applied to total DNA recovered from environmental samples are firstly, the diversity measurements based on C_0t plots (Torsvik *et al.*, 1990a;b) and secondly the cross-hybridization of total DNA recovered from different microbial communities (Lee & Fuhman, 1990).

5.1.6.1 Community DNA hybridization

In the latter approach total DNA extracted from different marine planktonic bacterial communities was applied in pairwise hybridisations. Hence DNA from one bacterial community was labelled with ^{35}S by nick translation and used to probe DNA extracted from another community that has been bound to nylon hybridization membranes to produce a dot-blot. The intensity of hybridization was calibrated against a control dot containing the same DNA (that was used to make the probe). Differences in intensities of pairwise hybridisations were expressed in terms of percentage similarity after normalisation with the control blot. Reciprocal hybridisations were made for each comparison of pairs. Occasionally reciprocal differences occurred, asymmetric hybridisations were attributed to signify, that either one sample is a subset of the other, or that a species common to both populations has a high GC DNA content. Using this approach the authors were able to establish seasonal and geographical differences in the marine planktonic species composition at a variety of locations (Fuhrman & Lee, 1989; Lee & Fuhrman,

1990).

5.1.6.2 Community reassociation kinetics

DNA reassociation kinetics can be used to provide an estimate of the complexity and diversity of the microbial communities (Ogram & Sayler, 1988). Total DNA recovered from environmental samples was thermally denatured and allowed to reassociate. The reaction follows second-order kinetics, with the rate being dependent on the number of similar sequences, since the greater the number of similar sequences the faster the reassociation. The fraction of reassociated DNA (determined spectrophotometrically) is plotted as a function of the product (C_0t) of DNA nucleotide concentration (C_0) in moles per litre and the reaction time (t) in seconds. Under defined conditions C_0t for a half-completed reaction ($C_0t_{1/2}$) is proportional to the genome size or complexity of DNA. The shape of the curve is indicative of the structure of the community. Hence the presence of a dominant species would be represented by the rapid reassociating fraction of the curve. In contrast a complex community with each species represented equally, would be characterised by a very slow reassociation curve and a high $C_0t_{1/2}$. The preliminary application of the technique to the analysis of the microbial community of an activated sludge system has been discussed by Ogram & Sayler (1988).

Torsvik *et al.* (1990a) have assessed the validity of using $C_0t_{1/2}$ as a diversity index in soil, for the analysis of soil microbial communities. Their findings indicated that the DNA isolated from soil is highly heterogeneous. In addition the reassociation curves did not follow second-order kinetics, indicating the presence of several different fractions of DNA representing both common and very rare biotypes. The rates of reassociation were lengthy, with incubation

times in excess of several weeks required for 50% reassociation. The technique is very sensitive to contaminating impurities with reassociation rates being prolonged in the presence of humic acid contamination and other impurities. A 5% fraction of the total DNA had a reassociation rate equivalent of about a 1/3 of the *E.coli* genome rate and was thought to signify a population of plasmids and or bacteriophage within the sample (Torsvik *et al.*, 1990a). In a comparison of genotypic and phenotypic variation (Torsvik *et al.*, 1990b) used API identification tests and cluster analysis in addition to the C_0t plots. The authors were able to demonstrate good agreement between both phenotypic and genetic indices of diversity. Where the genetic diversity of a community was high i.e. in excess of 90 isolates there was a tendency for the $C_0t_{1/2}$ values to under estimate the number of genomes. Nevertheless, the genetic diversity measurable with C_0t analysis exceeded that obtainable through traditional plating techniques by about 200 times (Torsvik *et al.*, 1990a) and confirmed the findings of others that the strains isolated from natural environments comprise a small fraction of the microbial community.

5.1.7 Genetic probes

A variety of probes varying in sensitivity and specificity have been applied to the detection of different genotypes in the natural environment. A summary of the application of various probes is given in Table 17.

5.1.7.1 Double-stranded DNA probes

Initial hybridization studies with bacteria of environmental importance including *E.coli* (Echeverria *et al.*, 1982), *Salmonella* (Fitts *et al.*, 1983) and viroids (Owens & Diener, 1981) employed double-stranded (ds) DNA probes. The application of ds DNA probes to environmental hybridization studies was

Table 17: The application of genetic probes to environmental studies (cont)

Organism	Gene sequence[a]	Type of Probe Nucleic acid[b]	Hybridization protocol[c]	Reference
Avocado sunblotch viroid	C-virus	cDNA	TE/Dot	Barker *et al.* (1985)
Citrus exocortis viroid	C-virus	cDNA	TE	Flores (1986)
Papaya mosaic potexvirus	C-virus	cDNA	TE	Roy *et al.* (1988)
Lymantria dispar (Gypsy moth) nuclear polyhedrosis virus	C-virus	cDNA	TE	Keating *et al.* (1989)
Bacteriophage UT1	virus	dsDNA	CH	Ogunseitan *et al.* (1992)

Key:

[a] Labelled fragment abbreviations: CG = cloned genomic; TG = total genomic; CF = cloned fragments; C = cloned; DTH = delayed hypersensitivity factor; Vir = virulence. [b] Nucleic acid abbreviations: ds = double stranded; ss = single stranded; Oligo = oligodeoxyribonucleotide. [c] Hybridization abbreviations; CH = colony hybridization; DE = direct extraction; PCR = polymerase chain reaction; TE = tissue axtraction; So = Southern hybridization; Dot = dot blot hybridization.

Note: Table derived from Sayler & Layton (1990)

Table 17: The application of genetic probes to environmental studies (cont)

Organism	Gene sequence[a]	Type of Probe Nucleic acid[b]	Hybridization protocol[c]	Reference
Other Water and Soil Bacteria				
Bacillus subtilis	23rRNA	dsDNA	CH	Kraus *et al.* (1986)
Pseudomonas fluorescens	23rRNA	dsDNA	CH	Festl *et al.*(1986)
P.cepacia AC1100 (2,4,5-T degrader)	1.3 kb repeat	Oligo	DE/PCR	Steffan & Atlas (1988)
4-chlorobiphenyl degrader	pSS50 plasmid	dsDNA	CH	Petigrew & Sayler (1986)
Toluene degrader	TOL plasmid	dsDNA	CH	Jain *et al.* (1987)
Naphthaline degrader	NAH plasmid	dsDNA	CH	Blackburn *et al.* (1987)
Mercury resistant	*mer*	dsDNA	CH	Barkay *et al.* (1985); Barkay and Olson (1986); Barkay (1987)
Mercury resistant	*mer*	dsDNA	DE	Barkay *et al.* (1989)
Viruses and Viroids				
Potato spindle tuber viroid	C-viroid	cDNA	TE	Owens & Diener (1981)
Potato spindle tuber viroid	C-viroid	RNA	TE	Lakshman *et al.* (1986)
Barley yellow dwarf virus	C-virus	cDNA	TE	Habili *et al.* (1987)
Potato virus X	C-virus	cDNA	TE	Baulcombe *et al.* (1984)

Table 17: The application of genetic probes to environmental studies

Organism	Gene sequence[a]	Type of Probe Nucleic acid[b]	Hybridization protocol[c]	Reference
Human pathogens from food and water				
Salmonella	CG	dsDNA	CH	Fitts *et al.* (1983)
E.coli (ETEC)	CF-enterotoxin	dsDNA	CH	Echeverria *et al.* (1982)
E.coli enteric pathogens	*lamB*, *lacZ*	Oligo	DE/PCR	Bej *et al.* (1990)
Listeria	CF-b-hemolysin	dsDNA	CH	Datta *et al.* (1987)
Listeria	CF-DTH	dsDNA	CH	Nortermans *et al.* (1989)
Yersinia	CF-Vir plasmid	dsDNA	CH	Hill *et al.* (1983)
Yersinia	Vir plasmid	Oligo	CH	Miliotis *et al.* (1989)
Legionella	chromosomal	Oligo	DE/PCR	Stambach *et al.* (1989)
Plant Associated Bacteria				
Rhizobium	TG	dsDNA	CH	Hodgson & Roberts (1983)
Rhizobium loti, *Bradyrhizobium*	TG	dsDNA	CH/TE	Cooper *et al.* (1987)
Rhizobium	*nif*, *nod*, *met*	dsDNA	So	Schofield *et al.* (1987)
Frankia	cryptic plasmid	dsDNA	TE	Simonet *et al.* (1988)
Erwinia amylovora	CF-plasmid	dsDNA	CH	Falkenstein *et al.* (1988)

a result of their easy preparation, particularly from poorly studied strains. Double-stranded DNA probes have been prepared from whole genomes, plasmids and subcloned fragments of DNA.

Total genomic DNA probes have been used in identification studies of *Rhizobium* (Hodgson & Roberts, 1983) and Lotus rhizobia (Cooper *et al.*, 1987). Such probes have proved valuable in taxonomic identifications, since they have been found to complement closely related strains, but not however, to be strain specific (Cooper *et al.*, 1987). Genomic probes may also be more sensitive than probing with single copy genes (Roberts *et al.*, 1987).

A number of studies have utilised naturally occurring plasmids (as probes) that have been frequently associated with the strain under study. Simonet *et al.* (1988) have used a cryptic plasmid for the identification of *Frankia* strains in alder root nodules and Steffan *et al.* (1989) and Pettigrew & Sayler (1986) have used a plasmid for the detection of *Alcaligenes* A5 in freshwater microcosms.

Species specific probes have been obtained by screening a library of randomly cloned digested genomic DNA for the presence of DNA fragments that appear unique to the target strain. The approach has been applied to *Bacteroides* spp. (Kuritza *et al.*, 1986), a fungal pathogen of lemon *Phoma tracheiphilia* (Rollo *et al.*, 1987) and for a group of fungal pathogens, *Phytophthora citrophora* able to incite root rot of citrus species. The advantages of cloned fragment probes over genomic probes being that they are easily amplified and more specific. Gene probes for specific phenotypic traits have been applied to the detection and monitoring of particular populations, traits that have been targeted include genes encoding: pathogenicity determinants (Datta *et al.*,

1987), biodegradative capabilities and resistances to antibiotics and heavy metals. Examples include, the formyltetrahydrofolate gene present in the acetogenic bacteria (Lovell & Hui, 1991), the *mer* genes conferring tolerance to mercury (Barkay, 1987; Barkay *et al.*, 1989) and plasmid encoded genes for aromatic hydrocarbon catabolism (Sayler *et al.*, 1985; Blackburn *et al.*, 1987). Such probes may be common to several different species and being specific for a particular function may contribute valuable information about the metabolism or physiology of particular communities.

5.1.7.2 Labelling of double-stranded probes

Nick translation is probably the most common strategy for labelling probes. The substrate for the reaction is double-stranded DNA and may comprise genomic DNA as restricted or sheared fragments or plasmid DNA. A controlled number of nicks (each containing a free 3' OH group) are introduced into the ds-DNA molecule using a small amount of DNase I. Polymerase I initiates a replacement strand synthesis by the removal of preceding nucleotides via the 5' > 3' exonuclease and the simultaneous synthesis of the DNA duplex by the 5' > 3' polymerase activities. The reaction is performed in the presence all four deoxynucleoside triphosphates (dNTPs), generally one of the nucleotides is radio-labelled with P^{32}. Probes of higher specific activity may be produced by incorporating more than one radiolabelled dNTP in the reaction buffer. Hence as the reaction proceeds the labelled nucleotides are incorporated into the probe. The probe is recovered from the unincorporated label and the double stranded form denatured usually by heat, prior to use in hybridizations.

5.1.7.3 Single-stranded DNA and RNA probes

Single stranded DNA or RNA probes may be more sensitive than ds-DNA

probes, since they can only hybridize with the target sequence and cannot reanneal. Single-stranded DNA probes may be produced by cloning ds-DNA into a vector containing a 13-base sequence of the M13 phage universal primer complimentary to the 5' side of a multiple cloning site. The universal fragment is used to initiate synthesis of a single strand of DNA [- strand] from the template by the klenow fragment of *E.coli* polymerase I. Such probes may have a specific activity of 10^8 d.p.m. and do not require denaturation prior to hybridization. Probes for the detection of avocado sunblotch viroids were prepared from single-strand phage M13 DNA clones (Barker *et al.*, 1985).

A similar approach was used by Flores (1986) in the production of cDNA probes complimentary to the single stranded RNA virus CEV. In the latter study, denaturation of the target RNA increased sensitivity of detection significantly. Holben *et al.* (1988) produced single stranded DNA probes complimentary to the *nptII* gene for the detection of *Bradyrhizobium japonicum* in soil. Probes were produced by standard cloning procedures in M13 and labelled by primer extension in presence of radiolabelled dNTPs. To maximise the specific activity of probes, all four dNTPs were ^{32}P labelled.

RNA or ribo probes have been prepared by cloning the DNA of interest into a vector with an SP6 promoter (from *Salmonella* phage SP6) and using SP6 RNA polymerase for the production of RNA transcripts from the vector (Sayler & Layton, 1990). Transcription proceeds in the presence of dNTPs one or more of which are radiolabelled. This approach has been used for the production of RNA probes for the detection of PSTV (Lakshman *et al.*, 1986). Ribonucleotide and deoxyribonucleotide duplex structures differ in relative stability for the same sequence, such that they decrease in stability in the following order: RNA-RNA > RNA-DNA > DNA-DNA. For short RNA-

DNA duplex structures the difference in stability appears negligible, however the use of long RNA transcripts as probes could contribute significantly to an increase in the efficiency of hybridization (Stahl & Amann 1991). RNA and ss-DNA probes have been found to be more sensitive than ds-DNA probes, with the sensitivity of asymmetric RNA probes exceeding that of DNA probes by a factor of 10 (Melton *et al.*, 1984). Despite their reduced sensitivity, double-stranded DNA probes have been used most frequently for the detection of environmentally important microbes (Table 17), this may be due to the additional cloning steps required for the production of ss-DNA and RNA probes (Sayler & Layton, 1990).

5.1.7.4 Oligonucleotide probes

Oligonucleotide probes may be synthesised chemically and comprise short nucleotide sequences (usually less than 40 bp). As such they are highly specific for target sequences with a single miss-match drastically affecting stringency.

In addition to their specificity, oligonucleotide probes targeted against rRNA genes may be highly sensitive because of the high concentration of ribosomal sequences in the bacterial cell (Giovannoni *et al.*, 1988a). Substantial sequencing of the 16S rRNA molecule for the purposes of the elucidation of phylogenetic relationships, allows the design and construction of highly specific probes (Olsen *et al.*, 1986; Pace *et al.*, 1986). A prerequisite of a well characterised genetic system either as an introduced sequence such as a reporter gene or via available sequence data for e.g. the 16S rRNA molecule is required for the design and application of specific oligonucleotide probes.

5.1.7.5 5'-End labelling with P^{32}

Oligonucleotides, restriction fragments and fragmented RNA may be end-labelled with P^{32}. T4 polynucleotidekinase catalyses the transfer of a terminal phosphate group from the gamma-P^{32} ATP to the 5'-hydroxyl group of RNA or DNA. The main limitation of this labelling strategy being the incorporation of a single nucleotide per strand of RNA or DNA. Consequently the specific activity of probes prepared in this way may not be as high as those produced using alternative strategies (Stahl & Amman, 1991). Oligonucleotides may also be used as primers for polymerase chain reaction (PCR) DNA amplification (Saiki *et al.*, 1988).

5.1.8 Polymerase chain reaction (PCR) generated probes

The PCR method can be used to produce unlimited amounts of double-stranded DNA starting from very small amounts of DNA or RNA (if cDNA is synthesised from the RNA using reverse transcriptase prior to amplification). Label can be incorporated during the PCR reaction by the inclusion of labelled dNTPs into the reaction mixture or following amplification by any of the conventional labelling strategies such as nick translation, primer extension and end-labelling (Stahl & Amman, 1991). Recorbet *et al.* (1992) synthesised probes using PCR complimentary to the *nptII* reporter gene that had been introduced into marked *E.coli* strains. The PCR generated probe was labelled by primer extension and used to investigate the genetic basis of kanamycin resistance amongst soil isolates using colony blot hybridization.

5.1.9 Non-radioactive detection

The labelling strategies considered above involve the incorporation of radiolabelled nucleotides into probes. However, interest in the avoidance of these expensive and potentially dangerous radiochemicals has lead to the development of a number of non-radioactive alternatives. Most non-

radioactive methods involve the indirect detection of the target DNA via enzyme-linked antibodies. Initially haptens are incorporated into the DNA probes either chemically or enzymatically. After hybridization, enzyme-linked antibodies are added and a colorimetric assay is used to detect the enzymes attached to the probe : target sandwich. The alkaline phosphatase and horseradish peroxidase enzymes have been used as they allow detection at low levels. Chemiluminescent methods have been developed as an alternative to colorimetric enzyme assays (Scholter *et al.*, 1992).

The original hapten molecules used in these labelling reactions were biotin molecules. Biotin molecules may be incorporated into the DNA molecule via nick translation and the use of biotin-dUTP or via light activation of photobiotin. The glycoproteins avidin and streptavidin have a high affinity for biotin and bind to the biotin labelled probes. Avidin or streptavidin antibodies coated with the enzymes alkaline phosphatase or horse radish peroxidase can be used to assay the amount of DNA hybridized. A number of workers have applied non-radioactively labelled probes to the analysis of environmental (Habili *et al.*, 1987) and food samples (Dovey & Towner, 1989). Labels that have been employed include biotin, fluorochromes, chemiluminescent moieties and enzymes (Urdea *et al.*, 1988).

A number of approaches for amplifying the signal generated from non-radioactively labelled probes, include the attachment of multiple labels to each probe and the design and development of labels with stronger signals (Fahrlander, 1988).

5.1.10 PCR

An alternative strategy for improving the sensitivity of detection of nucleic

acid sequences is the amplification of the target sequence by PCR. The method involves repetitive cycling between high temperatures to melt and denature DNA duplexes, followed a relatively low temperature to allow the anealing of primers with the complimentary region of the target DNA, and an intermediate temperature for primer extension. The temperature cycling is normally attained through the use of a commercially available thermocycler. Initial PCR experiments used the Klenow fragment of the *E.coli* DNA polymerase I to catalyse the extension of the annealed primers (Saiki *et al.*, 1988). The Klenow fragment is irreversibly denatured at the high temperatures required for denaturation of the DNA duplex approx. 94^0C necessitating the addition of fresh enzyme with each cycle. The substitution of the thermostable DNA polymerase isolated from Thermus aquaticus (Taq polymerase) obviates these problems. *Thermus aquaticus* is a thermophilic eubacterium that may be routinely isolated from hotsprings. Taq polymerase exhibits unusually high processing activity however, the enzyme does not possess a 3' exonuclease activity which has been associated with the proof reading functions in *E.coli* (Giovanonni, 1991). This lack of 3'-exonuclease activity may contribute to the low level of premature chain terminations that have been attributed to the enzyme pausing at secondary structure. Consequently, this may result in a relatively high rate of misincorporated nucleotides.

The specificity of the oligonucleotide primers (20 to 30 nucleotides) is important, since failure of a primer to hybridize with the DNA template will result in no PCR product. In addition, hybridization conditions should be optimised to promote specific binding, since DNA oligomers will hybridize with sites containing mismatches under non-stringent conditions and result in non-specific amplifications.

Increased sensitivity of detection results from the production of large amounts of target sequence and therefore an increase in the target : non-target sequence ratio. PCR provides two levels of stringency, primer anealing and probe hybridization.

This latter point has allowed the development of several new techniques with the potential for the rapid screening of environmental samples. Primers may be labelled with biotin, horseradish peroxidase or fluorescent dyes, so that following amplification each copy of the amplified product has the specific label (Chehab & Kan, 1989; Sauvaigo *et al.*, 1990).

5.1.10.1 The application of PCR to the detection of microorganisms in environmental samples

Without amplification gene probes have been reported to detect DNA in the range of 10^3 to 10^4 cells per gram soil (Holben *et al.*, 1988; Steffan & Atlas, 1988). Using PCR technology Steffan & Atlas (1988) were able to detect as few as a 100 *Pseudomonas cepacia* cells per 100 g sediment against a background of 10^{11} non-target microorganisms, representing a 3 fold increase in sensitivity upon hybridization of unamplified DNA.

Chaudhry *et al.* (1989) also used PCR as a means for detecting genetically engineered microorganisms. *E.coli* cells were transformed with 2,4-dichlorophenoxyacetic acid-degradative plasmids harbouring an insert of 0.3 kb eukaryotic DNA derived from napier grass. The napier grass insert was selected as no hybridization was found to occur with it from DNA from a number of microorganisms screened as well as DNA isolated from microbial communities from soil, sewage and lake water. Plasmid bearing *E.coli* cells

seeded into lake water and sewage were monitored using plate counts in addition to PCR amplification and probing. PCR allowed detection of the seeded inoculant after 10 to 14 days incubation, whereas the plate count only allowed detection up to 6 and 10 days incubation.

Pillai *et al.* (1991) combine a cell extraction procedure based on the use of sucrose density gradients with two rounds of PCR amplification for the detection of bacterial sequences in soil. The sensitivity of detection for *E.coli* cells bearing introduced Tn*5* (*nptII*) sequences was determined to be 1 to 10 c.f.u. g^{-1} soil. Detection being an order of magnitude higher for the sandy soil as opposed to the clay loam soil used in this study.

In contrast, Tsai & Olson (1992) combine direct DNA extraction methods with PCR amplification for the detection of *E.coli* 16S rRNA genes. They are able to obtain detection limits of 3 cells g^{-1} (calculated on the basis that there are approximately 7 rRNA genes $cell^{-1}$) this is equivalent to 21 gene copies g^{-1} soil. Inhibitory effects of humic acid contaminants on the polymerase chain reaction resulted in less efficient amplification of nucleic acids from soil samples when compared to pure bacterial cultures. Inhibitory effects of humic acids in contaminated samples were overcome by a 1/ 32 dilution of the DNA extract.

5.1.10.2 The application of PCR technology to the detection of indicator and plant pathogenic microorganisms

Coliform bacteria are used for monitoring the biological safety of water supplies. Their detectable presence in water samples is considered to be indicative of potential faecal contamination, and hence the possible presence of enteric pathogens. Bej *et al.* (1990) used PCR amplification and

hybridization to detect the presence of coliform bacteria in water samples. Amplification of the *lacZ* region of the *E.coli* genome allowed amplification of *E.coli* and other coliform bacteria, including *Shigella* spp. but not *Salmonella*, whereas amplification of the *lamB* region allowed detection of certain coliform spp, but including *Salmonella* and *Shigella* spp. Using amplification and hybridization directed at both gene sequences the authors were able to detect as little as 1 to 10 fg of genomic *E.coli* DNA and as few as 1 to 5 viable *E.coli* cells in water.

Brauns *et al.* (1991) have employed PCR technology for the detection of culturable and viable nonculturable *Vibrio vulnificus* cells, a human pathogen implicated in primary septicemia. The organism is indigenous to estuarine environments and primary septicemia generally results from the ingestion of raw oysters harbouring the bacterium. Traditional monitoring techniques only allow detection of the bacterium during the warm summer months when the bacterium is in a culturable state. Using a region of the cytotoxin-hemolysin gene from *V.vulnificus* identified to be specific to this strain, the authors were able to detect as little as 72 pg of DNA from culturable cells and 31 ng of DNA from nonculturable cells.

The cereal disease take-all has been traditionally diagnosed visually or by culturing fungi from diseased plants. However, unambiguous visual diagnosis is complicated by the fact that many of the disease symptoms may also be attributed to other diseases or environmental factors. Furthermore the selective medium used to culture the strain has been found to be inhibitory to *G.graminis*.

Schesser *et al.* (1991) have used PCR amplification for the detection of the

take-all fungus, *Gaeumannomyces graminis*, in infected wheat seedlings. Primers were designed against a 4.3 kb DNA fragment cloned from the mitochondrial genome of *G.graminis* var. *tritici*. Two rounds of PCR amplification were used, employing nested primers in the second round. The application of two rounds of PCR and the use of nested primers (primers located within the amplified region) promoted highly sensitive and specific amplification avoiding the production of non-specific amplification products.

5.1.10.3 The application of PCR for the cloning and sequencing of nucleic acids

Historically genes have been cloned from organisms of interest by the generation of a gene library in either a lambda phage or cosmid vector (Maniatis *et al.*, 1982) followed by screening of the library for a desired phenotype or genotype. However, the development of PCR technology allows the amplification of specific regions of DNA for sequencing, obviating the need to produce gene libraries, that require exhaustive screening.

Two cloning strategies have been utilised with amplified PCR products, the first referred to as 'forced cloning'. This approach requires the design of primers incorporating restriction sites, that will be introduced into the terminal ends of the amplified product. The advantage of forced cloning techniques being that the vector having been cleaved at two different polylinker restriction sites cannot religate without the insertion of the amplified product. Thus, a large proportion of transformants contain cloned PCR DNA. The disadvantage being that the amplified genes may be cleaved at internal restriction sites and furthermore restriction at terminal sites is frequently less efficient than restriction at internal sites (Giovannoni, 1991).

Wimpee *et al.* (1991) used PCR employing forced cloning techniques for the development of species specific probes for different groups of luminous marine bacteria. Primers were designed to be complimentary to two conserved regions of the *luxA* gene. The primers were constructed to contain different restriction sites, facilitating the cloning of the amplified products into the bacteriophage M13 for sequencing. The specificity and hence cross reactivity of the products of amplification were tested against a range of luminescent bacterial species. Under conditions of high stringency three probes appeared specific for three different species. Initial results also demonstrated the potential for a mixed *lux* probe able to detect all luminescent species under non-stringent conditions.

An alternative strategy for the cloning of PCR products is the application of blunt-ended ligation techniques. DNA molecules generated by PCR have synthetic 5' termini, lacking 5' phosphates. Hence if ligated into a blunt ended vector they generate circular molecules with a single nick in each strand. These molecules are highly transformable.

5.1.10.4 Degenerate primers

Degeneracy in the genetic triplet code, means that the exact DNA sequence of a protein cannot be inferred from its amino acid sequence. However, this may be overcome in approaches involving the application of PCR for cloning and sequencing by employing degenerate primers. Degenerate primers can be produced to cover every single nucleic acid combination for a defined amino acid sequence. The mixture of primers is then used for amplification of particular sequences. Optimal results are achieved with primers of less than or equal to 20kb and for regions with relatively low degeneracy ie. < 64-fold

(Steffan & Atlas, 1991).

Degenerate PCR procedures have been applied to sequence analysis of DNA from environmental samples. Zehr & McReynolds (1989) have used degenerate primers to amplify nitrogen fixation genes (*nif*) from the cyanobacterium *Trichodesium thiebautii*, an organism that has never been maintained *in vitro*. The authors analysed known amino acid sequence data for *nif* gene products of other nitrogen fixing microorganisms and identified regions of highly conserved sequence. They selected regions of less than 200-fold degeneracy and designed mixed primers (17 mers) to cover all possible combinations. DNA recovered from *T.thiebautii* bundles was isolated and subjected to PCR with a degenerate primer mixture of 126 and 96 oligomers for the up and downstream primers, respectively. The products of PCR were introduced into M13 cloning vectors for consequent sequence analysis via blunt-ended ligation. Kirstein *et al.* (1991) extended this work, by cloning and sequencing regions of other marine, nitrogen-fixing microorganisms in an attempt to elucidate their taxonomic relatedness. The study included a heterotrophic isolate from the root/rhizome of the seagrass *Ruppia maritima*, and the heterocystous fresh water cyanobacterium *Anabena oscillaroides*.

The wealth of sequence data available for the 5S and 16S rRNA genes, has allowed selection of oligonucleotide primer sites for the amplification of desired rRNA genes. Giovannoni *et al.* (1990) have exploited the available data, in their design of primers for the sequencing and characterization of Sargasso sea bacterioplankton rRNA genes.

5.1.11 RNA extraction from soil

An alternative target molecule to DNA for molecular detection and

measurement of community diversity are the ribosomal RNA molecules. Targeting RNA as opposed to DNA offers increased sensitivity over DNA as a result of the vast number of ribosomes and hence ribosomal genes per cell. This number has been estimated to be up to 10,000 per cell (Olsen *et al.*, 1986).

A number of methods have been reported for the extraction of rRNA species from soil. Hahn *et al.* (1990a) were able to recover rRNA from a sandy loam and peat soil without detectable humic acid contamination, by lysing the cells *in situ* in a 7.5 M guanidine hydrochloride homogenisation solution using sonication. The supernatant was recovered by centrifugation and extracted with phenol and chloroform before precipitation with ethanol. Any residual humic acid contamination was removed by washing the RNA bound nylon filters at high temperatures, prior to hybridization.

An alternative strategy has been reported for the rapid extraction of mRNA (Tsai *et al.*, 1991). This procedure also involves the direct extraction of the nucleic acids from soil, as the cells are lysed *in situ* by vigorous shaking in a 4 M guanidine thiocyanate solution containing sarkosyl and mercaptoethanol. The pH of the nucleic acid solution was reduced to 4.0 and the solution extracted with phenol and chloroform before precipitation of the RNA with isopropanol. The method yields a 60% recovery of RNA from inoculants seeded into soil. Humic acid contamination occurred in soils and sediments with a high-cation-exchange-capacity.

5.1.12 Analysis of microbial communities using rRNA molecules.

Of the three RNA species present in cells, the 16S and 23S subunits have emerged as the most useful molecules for phylogenetic studies. This has been

as a result of their larger size and higher information content. Most of the studies have focused on the 16S rRNA molecule as a larger data base of sequence information has been accumulated for this subunit.

5.1.12.1 The application of phylogenetic probes to the analysis of microbial communities

Alignment and comparison of 16S rRNA sequences has revealed sequence domains that are unique to particular taxa (Giovanonni *et al.*, 1988a). 16S targeted oligonucleotide probes complimentary to taxon-specific sequences have been used in the analysis of microbial communities (Stahl *et al.*, 1988). The probes have been applied both to the detection and quantification of target organisms within environmental samples by the extraction and probing of recovered nucleic acids (Stahl *et al.*, 1988) and also for the identification of fixed cells in mixed cultures via *in situ* hybridization and fluorescent microscopy (Giovanonni *et al.*, 1988a).

5.1.12.1.1 Hybridization

Stahl *et al.* (1988) used species and group specific 16S rRNA targeted oligonucleotide probes for the enumeration and monitoring of bovine rumen bacterial populations. Using dot-blot hybridization with densitometric analysis, populations of *Bacteroides succinogens* and *Lachnospira multiparus* were monitored in ruminal samples, prior to, during and after perturbation of the ecosystem by the addition of an ionophore antibiotic, monensin. Monensin has been routinely incorporated into cattle feeds to promote their efficient utilisation. However, whether the increased efficiency in the conversion of plant-based feeds into useful compounds to the animal was attributable to a change in ruminal populations or to a change in their metabolism was unknown. Three 16S rRNA targeted oligonucleotide probes were used to

monitor the ruminal bacterial population dynamics: the 'signature' probe hybridized with the RNA from all but one strain of *B.succinogenes*, the other probes identified either of two natural groups within the ruminal assemblage. Although the two genetically defined groups of *B.succinogenes* did respond markedly and differently to the addition of monensin, population changes were transient and were soon restored to their premonensin levels. The bacterial composition appeared well buffered to change by monensin, thus supporting the hypothesis that monensin acts primarily by altering the metabolism of a relatively stable population.

5.1.12.1.2 *In situ* hybridization

Giovannoni *et al.* (1988) identified regions that were kingdom specific for the three primary lines of evolutionary descent: the eubacteria, archaebacteria and eukaryotes. Oligonucleotide probes were synthesised that were complimentary to these diagnostic regions and the specificity of the probes was confirmed by dot-blot hybridizations. The authors were able to demonstrate the application of the kingdom specific probes (labelled with ^{35}S phosphothionate) in the identification of whole glutaraldehyde-fixed cells. The probes also appeared phylogenetic in these *in situ* hybridizations, and cells of a particular kingdom could be identified from mixed cultures using microautoradiography. DeLong *et al.* (1989) applied fluorescently labelled phylogenetic 16S rRNA targeted probes in the identification of formaldehyde fixed cells. The abundance of ribosomes (10^4 to 10^5 per cell) allowed the fixed cells to be readily visualised using fluorescent microscopy. The simultaneous application of multiple probes labelled with different fluorescent dyes permitted the identification of different cell types within the same microscopic field. Fluorescently labelled phylogenetic probes were developed to discriminate between the three kingdoms. In addition the potential for the

technique to distinguish between the two closely related bacteria, *Proteus vulgari* and the son-killer bacterium was illustrated. Finally quantitative microflourimetry demonstrated that the amount of rRNA-specific probe that binds to *E.coli* varies with ribosome content and hence is indicative of activity (DeLong *et al.*, 1989).

5.1.12.2 rRNA sequence analysis for the analysis of microbial communities

RNA isolated from environmental samples may serve as a template for synthetic primers and direct sequencing reactions utilising reverse transcriptase. This approach was taken by Stahl *et al.* (1985) in the characterization of 5S rRNA gene sequences from a yellow hot spring microbial community. This approach was valuable in demonstrating the microbial complexity of the spring since an archaebacterium species affiliated to the sulphur metabolising branch and two eubacterial species that appeared distantly related to *Thermus* spp. comprised 50% of the 5S rRNA sequences analysed.

An alternative strategy to the direct sequencing of rRNA sequences has been the analysis of sequences via the cloning of rRNA genes (Weller *et al.*, 1991). Weller *et al.* (1991) initially isolated ribosomes from environmental samples. Ribosomal RNA was then recovered from the small subunit of the ribosome and randomly primed with dNTPs. This approach eliminated the requirement for primers anealing to the universally conserved sequences and hence the dependence on the use of conserved sequences which may bias the recovery of 16S rRNA sequence data. This approach has also been shown to yield longer sequences (Weller *et al.*, 1991). This method was applied to the analysis of 16S rRNA sequences from a cyanobacterial mat located in Octopus spring.

The study revealed the presence of 3 16S rRNA sequences from three formerly uncultured community members. One sequence was indicative of a cyanobacterial strain, another a green non sulphur bacterium and the third was possibly a novel phylogenetic type. These findings have been confirmed by others who also were able to identify microbial diversity previously undescribed in marine environments (Giovannoni *et al.* 1990; Ward *et al.*, 1990).

Amann *et al.* (1991) combined the recovery and analysis of rRNA sequence information via polymerase chain reaction procedures with the development of specific 16S rRNA targeted oligonucleotide probes for use in *in situ* hybridizations. This approach has been taken to study the *Holospora* bacteria, a highly infectious nuclear endosymbiont of cilliates. 16S rRNA sequence analysis was used to determine the phylogenetic position of *Holospora obtusa* within the *Proteobacteria*. The sequence information was then used for the design of probes for the detection and differentiation of *Holospora* cells within the nuclei of *Paracmecium* species. The fluorescently labelled probes allowed direct visual identification of the bacteria *in situ* and demonstrated colonisation of food vacuoles as well as the macro- and micronuclei of infected paramecia by the endosymbionts . In addition *in situ* hybridization provided confirmation that the amplified and cloned sequences originated from the endonuclear symbionts and were not artefacts from contaminating bacteria used for feeding the ciliates.

5.2 Aims

5.2.1 The primary aim was the development of a method for the recovery of intact ribosomal RNA from soil that is amenable to hybridization with 16S rRNA targeted oligonucleotide probes. Reproducible extraction of nucleic

acids from environmental substrates would allow quantification (via the measurement of radioactive disintegrations or via the analysis of autoradiographs using densitometric analysis) and enumeration of the inoculant under investigation by the calibration of hybridization signals to known amounts of spiked mycelium and spores in soil.

5.2.2 A secondary consideration was to exploit differences in the susceptibility of the streptomycete spore and mycelial components to lysis and refine the methodology for the selective and differential recovery of RNA from spores and mycelium. The development of methodology, selective for different components of the microorganisms life-cycle, offers the potential to investigate the activity and ecology of the inoculant in the natural environment.

5.2.3 To establish the limits of detection for spores and mycelium within soil. The data generated from any method of monitoring is only meaningful within the context of detection range for a particular organism in a particular environmental sample.

5.2.4 To compare the sensitivity of detection by probing with a 16S rRNA targeted oligonucleotide probe to RNA and DNA recovered from soil. The abundance of RNA molecules within the cell, estimated to be 10^4 to 10^5 means that ribosomal-borne sequences are naturally amplified within the cell and thus more amenable to detection in environmental samples. However, this advantage may be counter balanced by the additional care required to recover intrinsically less stable RNA as opposed to DNA molecules.

5.3. Results

All method development work involved the use of soil (wetted to 15% moisture content w/w) sterilised by two rounds of autoclaving and incubation then spiked with known amounts of wet weight mycelium or a specific number of spores. Although difficulties arise in the enumeration of mycelial microorganisms, for the purposes of method development the following loose correlation was used in the conversion of wet weight mycelium into cells, 1 mg wet weight mycelium releases 10^9 protoplasts (D. Hahn pers. comm.).

5.3.1 A comparison of methods for the extraction of rRNA from soil.

The first experiment concerned the comparison of the guanidine hydrochloride method (Hahn *et al.* 1990a, Method 1, Section 2.24.1) with a lithium chloride/ GOS extraction [(Hughes & Galau, 1988) Section 2.24.2].

Ribosomal RNA was recovered from *Streptomyces lividans* TK24 mycelial spiked soils and pure cultures using both methods. Two modified protocols based on these methods were also included in the comparison. In one SDS was added to the guanidine hydrochloride homogenisation buffer to a final concentration of 0.5%. The presence of SDS has been reported to improve the purity of the initial RNA precipitate, its addition after the initial homogenisation avoiding problems of excessive foaming. In the second, the cells were lysed in the guanidine hydrochloride homogenisation buffer, the supernatants recovered and precipitated. The recovered pellet was then taken up in the GOS homogenisation buffer and the RNA recovered according to method 2. The four approaches to the recovery of RNA are illustrated in a scheme in Fig. 24. *E.coli* pure cultures and spiked soils were used as positive controls and uninoculated soil as a negative control. Lysis of the mycelium was achieved by sonication at 0°C for three minutes.

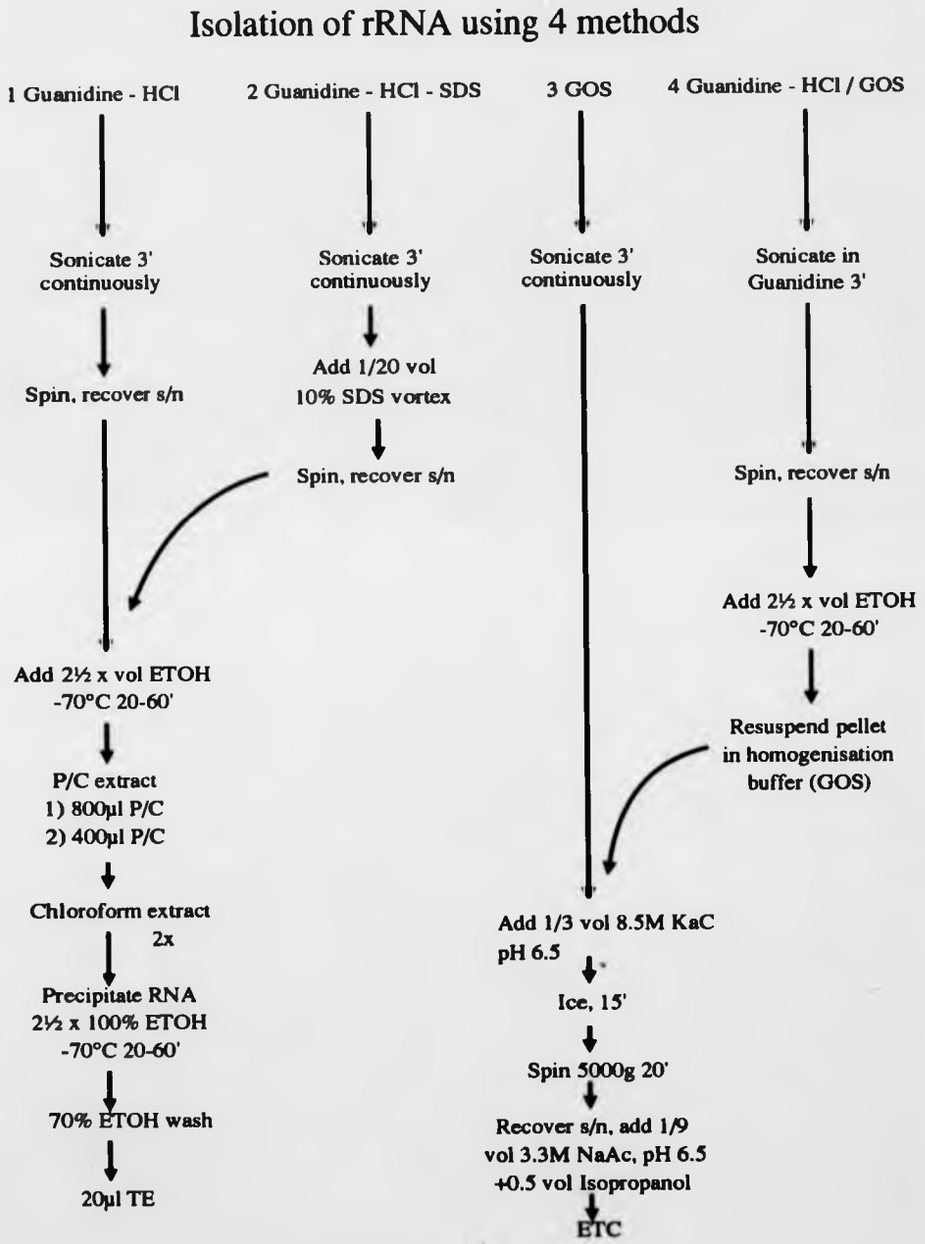
Fig. 24 Isolation of rRNA from soil using 4 methods.

4 approaches to the isolation of rRNA from pure cultures and soil were compared, 2 were based on the Guanidine-HCl method (Hahn *et al.*, 1990a) and 2 on a procedure that was developed for the extraction of RNA from cotton plants (Hughes & Galau, 1988). The soil was a heavy clay.

Key to abbreviations:

ETOH	Ethanol
Gos	Homogenisation buffer (Hughes & Galau, 1988)
HCl	Hydrochloric acid
KaC	Potassium acetate
P/C	Phenol Chloroform
SDS	Sodium dodecyl sulphate
s/n	Supernatant
vol	Volume(s)

Figure 24



Only the unmodified GOS method resulted in reasonable RNA preparations from soil. The other three protocols produced faint smears that were barely discernible on a 1.2% agarose gel. RNA recovery from pure cultures was reasonable for all the methods except where guanidine homogenisation proceeded extraction with method 2 (Hughes & Galau, 1988). Yields of RNA from pure cultures did appear to be greater by gel electrophoresis analysis from the unmodified GOS/ lithium chloride method.

Hence further work focused on the application of the RNA extraction method described by Hughes & Galau (1988) to the recovery of RNA from soil.

5.3.2 The differential extraction of rRNA from spores and mycelium

Streptomycete spores are highly resistant to disruption and lysis. This is in contrast to the mycelial growth form that is readily lysed by treatment with lysozyme or methods of physical disruption, including sonication. Since streptomycetes are characterised by a life-cycle with two distinct growth forms, it is possible to monitor the activity of the inoculants in soil by estimating the relative proportions of spore and mycelium inoculum *in situ*. This approach was taken by Herron & Wellington (1990) in their development of methodology for the differential extraction of spores and mycelium from soil. Differential methods of lysis for the two components of the microbes life-cycle have been exploited by Cresswell *et al.* (1991) for the selective extraction of plasmid DNA from spores and mycelium from soil. In the latter approach DNA was extracted from spores using 0.1 mm diameter glass beads and a Braun homogeniser, in contrast 70^0C heat and SDS were combined to lyse mycelium in soil.

5.3.2.1 Optimal conditions for the physical disruption of spores

Unfortunately, a Braun homogeniser was not available in Wageningen, NL to carry out this experimental work, hence preliminary investigations were made to establish alternative conditions for the lysis of spores using different ratios of 0.1 mm diameter glass beads and periods of sonication.

A *Streptomyces lividans* spore suspension (enumerated by the viable plating) was resuspended in GOS homogenisation buffer and aliquoted into microfuge tubes in 400 µl amounts and equivalent to 5 times 10^7 spores per tube.
Tubes were treated as follows:

1. 0.4 g glass beads and 3 minutes continuous sonication
2. 0.9 g glass beads and 3 minutes continuous sonication
3. 0.4 g glass beads and 2 times 3 mins continuous sonication
4. 0.9 g glass beads and 2 times 3 mins continuous sonication
5. 0.4 g glass beads and 3 times 3 mins continuous sonication
6. 0.9 g glass beads and 3 times 3 mins continuous sonication

Ordinarily the efficiency of cell lysis would be monitored by light microscopy, before, during and after cell disruption. However, the small size of streptomycete spores approx. 1 µm in diameter make them barely discernible under the light microscope. Hence for this investigation the efficiency of lysis was assessed in terms of the viability of spores after treatment and with regard to the yield of recovered rRNA.

The ribosomal RNA was prepared according to Hughes & Galau (1988) and was analysed by gel electrophoresis and dot-blot hybridization by probing with the eubacterial probe, primer 1115. Very little rRNA could be visualised from the spores on an agarose gel other than a faint smear detectable from treatment 4 , however RNA was detectable by dot blot hybridization (Fig. 25).

Table 18 & Fig. 25 Optimisation of conditions for the lysis of streptomycete spores

Various ratios of 0.1 mm glass beads to sample buffer and different lengths of high frequency sonication were experimented with to obtain lysis. Lysis was assessed in two ways, 1. the viability of the cells after the lysis treatment (Table 18) and 2. rRNA recovery after lysis (Fig. 25).

Table 18

Optimal conditions of lysis, established from the viability data corresponded to a viable count of no c.f.u ml^{-1} supernatant or g^{-1} cell debris pellet. No viable colony forming units were obtained with a 2.5 : 1 ratio of beads to homogenisation buffer and 3 times 3 minutes sonication. This was taken to be indicative of efficient lysis.

Fig. 25

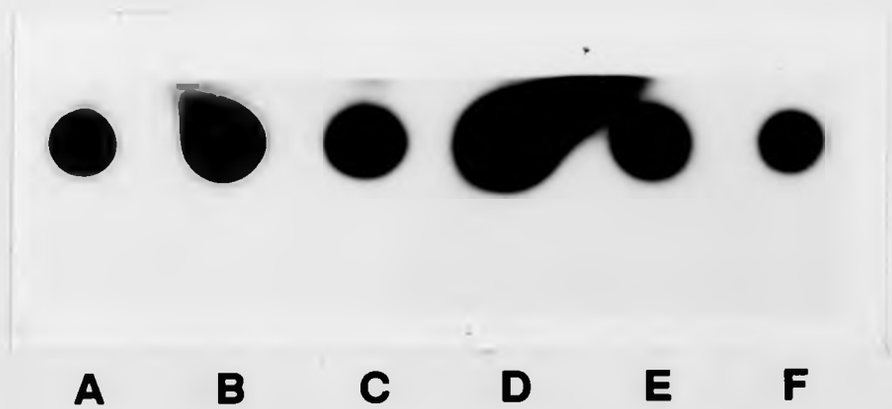
Dot blot hybridization of rRNA recovered from *S.lividans*, TK24 spores using lysis treatments: A- 1:1 beads, 1 X 3 mins sonication; B- 2.5:1 beads, 1 X 3 mins sonication; C- 1:1 beads, 2 X 3 mins sonication; D- 2.5:1 beads, 2 X 3 mins sonication; E- 1:1 beads, 3 X 3 mins sonication; F- 2.5:1 beads, 3 X 3 mins sonication. Maximum recovery of rRNA was achieved with treatment D (2.5:1 beads, 2 X 3 mins sonication), the corresponding viable counts were 4 and 2 c.f.u. ml^{-1} and g^{-1} respectively. Increased sonication reduced viability marginally and contributed to increased rRNA degradation, resulting in a smaller hybridization signal.

Table 18: Viability data from experiment to determine optimal conditions for the lysis of spores

	Treatment		c.f.u.
s/n	1 times 3' sonication	0.4 g beads	$>10^5$
pellet	1 times 3' sonication	0.4 g beads	1500
s/n	1 times 3' sonication	0.9 g beads	117
pellet	1 times 3' sonication	0.9 g beads	353
s/n	2 times 3' sonication	0.4 g beads	35
pellet	2 times 3' sonication	0.4 g beads	16
s/n	2 times 3' sonication	0.9 g beads	4
pellet	2 times 3' sonication	0.9 g beads	2
s/n	3 times 3' sonication	0.4 g beads	45
pellet	3 times 3' sonication	0.4 g beads	21
s/n	3 times 3' sonication	0.9 g beads	0
pellet	3 times 3' sonication	0.9 g beads	0

Key: s/n = supernatant

Figure 25



The cell debris pellet was washed by resuspending in 400 µl of liquid R5 broth, the debris was spun down again and taken up in fresh R5 medium. Samples from the supernatant and pellet were plated out onto solid R5 medium. The number of colony forming units derived from a 100 µl aliquot are given in Table 18. and are a measure of the efficiency of lysis.

In conclusion, the blot and viability counts indicated that a 2.5:1 ratio of 0.1 mm diameter glass beads to buffer produced more efficient lysis than a 1:1 ratio. The disruption of spores increased with longer exposures to sonication, however, the prolonged period of 3 times 3 minute bursts of sonication promoted rRNA degradation balancing out the more efficient spore lysis observed in the viability count data. Hence optimal conditions for spore disruption and recovery of rRNA were considered to be a 2.5 : 1 ratio of beads to buffer and 2 times 3 minute rounds of sonication.

5.3.2.2 Differential extraction of rRNA from spores and mycelium

Using a three minute sonication treatment in the absence of beads together with the conditions established for the lysis of streptomycete spores (2 times 3 minutes on sonication in the presence of a 2.5 : 1 ratio of beads) attempts were made to differentially extract rRNA from spores and mycelium.

Duplicate 3 g samples of soil (using a soil with a high clay content, originating from Germany) were spiked with the following : 1) a *S.lividans* TK24 spore suspension, 2) a TK24 mycelial suspension, 3) TK24 spores and mycelium and 4) uninoculated negative control.

The number of spores inoculated per 3 g sample was estimated to be 1.2 times 10^7 and 100 mg ml^{-1} wet weight mycelium per 3 g soil sample.

The samples were treated with 3 minutes sonication for the lysis of mycelium *in situ*, followed by extraction and recovery of the RNA. Glass beads were then added to the soil to produce a 2.5 : 1 ratio and the samples were sonicated for 2 rounds of three minutes to bring about lysis of the spores. Ribosomal RNA extracts from all samples were compared by gel electrophoresis.

From the gel it was apparent that insufficient RNA was recovered from the quantity of spores used in the experiment to be seen on the gel. Hence only ribosomal RNA from mycelium was visualised on the gel. Secondly, only a fraction of the rRNA was recovered after the initial homogenisation and extraction. Furthermore, more mycelial borne RNA was actually recovered after the second homogenisation treatment i.e. sonication with beads and further extraction. Several extractions would therefore be required to recover all the rRNA released from the mycelium and before the soil could be treated with bead beating to recover the spore rRNA. The finding that more mycelial rRNA was recovered after bead-beating, is consistent with the findings of Cresswell *et al.* (1991) that the recovery of mycelial DNA was more efficient by bead-beating with a Braun homogeniser than lysis by SDS and heat. The bead-beating action serves to break down the soil matrix which may explain in part, the more efficient extraction obtained with this procedure.

Thus, the availability of methodology for the differential lysis of spores and mycelium appeared to be only the first step in the development of a protocol for their differential extraction. Recovering all the mycelial RNA from soil, especially those high in clay colloids with a high affinity for nucleic acids was particularly difficult. Furthermore since spores may have small amounts of

rRNA than mycelial cells (Quiros *et al.*, 1989) only a small fraction of residual mycelial RNA would be required to distort the determinations of the relative abundances of spore and mycelial borne nucleic acids.

5.3.3 An investigation into the frequency and efficiency of washing for the recovery of RNA from soil

This experiment was designed to establish how efficient washing the soil with the homogenisation buffer was in the recovery of RNA from the mixture of soil and lysed mycelium. Two soils were compared the soil used in the above experiment with a high clay content and a soil based potting compost, with a high organic carbon content.

Two volumes of homogenisation buffer were compared, the 3 ml volume that was routinely used in rRNA extractions and a 6 ml volume. Soils were extracted 1, 2, 3 and 4 times with either volume of sample buffer, the supernatants were decanted off after centrifugation to bring down the soil and combined after each extraction. The nucleic acids were then precipitated and the amount of RNA recovered compared on a 1.2% agarose gel.

Our findings indicated that with a 3 ml extraction volume from John Innes potting compost, that the majority of RNA was recovered in the first extraction, however RNA did also accumulate with each subsequent extraction. In addition, the more extractions used the greater the problem of humic acid contamination. Humic acids bind with ethidium bromide and can be discerned to run ahead of the RNA on an agarose gel. When a 6 ml volume of homogenisation buffer was used for the recovery of RNA from 3 g of soil, there was no apparent difference between 1, 2, 3 and 4 extractions in the quantity of RNA recovered. However as before, problems of humic acid

contamination were exaggerated with more than two extractions.

Using both extraction volumes, significantly more RNA was recovered from the John Innes potting compost than the German soil. This supports the findings of others that soils with a high clay content do absorb nucleic acids making their recovery difficult despite repeated extraction (Ogram *et al.*, 1987). Optimal recovery of RNA from the German, clay soil was achieved with either one 6 ml extraction or 2 times 3ml extractions, with further extractions not resulting in significantly improved yields of RNA but in increased humic acid contamination.

Since humic acid acids can contribute enormous problems in nucleic acid analysis by hybridization, two extractions were considered a good compromise in obtaining the bulk of the recoverable RNA from soil without extracting too many contaminating humic acids. Likewise a 3 ml extraction volume appeared more efficient than one of 6 mls. This was probably due to a reduced efficiency with larger volumes at the precipitation stage. Hence, the 2 times 3 ml extraction step described in the method was retained for the recovery of RNA from 3 g of soil. The recovered ribosomal RNA was applied to a hybridization membrane using a dot-blot manifold and probed with the eubacterial probe primer 1115 (Embley *et al.*, 1988). Humic acid contamination may affect both the binding of nucleic acids to the hybridization membrane and the efficiency of hybridization. Initial hybridizations revealed that the RNA samples from this experiment were contaminated with humic acids and resulted in very weak hybridization signals. A final extraction of the RNA with chloroform saturated with TE buffer, purified the RNA sufficiently to obtain strong hybridization signals (Fig. 26).

Fig. 26 Extraction efficiency and reproducibility of rRNA extraction from two soils.

Ribosomal RNA was recovered from duplicate samples of John Innes potting compost, A & B and a heavy clay soil, C & D seeded with *S.lividans*, TK24 mycelium. I; The RNA that was recovered from soil after one extraction, II; RNA recovered and combined from the first two extractions, III; RNA recovered and combined from three, IV; the RNA recovered and combined from four extractions.

The study indicated that the majority of rRNA was recovered from the first extraction. The 2 soils were seeded with equal amounts of mycelium from which RNA recovered, however, the clay soil appeared to retain more of the rRNA than the compost, resulting in reduced hybridization signals. In addition, the extraction of nucleic acids from the John Innes compost appeared more reproducible than those from the clay soil.

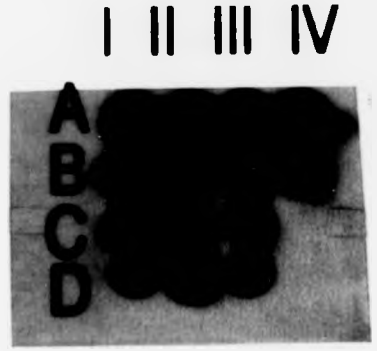


Figure 26

In a review of detection methodology, McCormik (1986) illustrates the problems facing environmental microbiologists in recovering target sequences from soil, as he describes the search and development of improved ways of 'washing dirt' as a fundamental and necessary step towards increased sensitivity in detection and monitoring strategies.

5.3.4 A comparison of the sensitivity of probing RNA and DNA recovered from soil with 16S rRNA targeted probes

RNA was recovered according to method 2 (Hughes & Galau, 1988) while DNA was recovered according to the methodology of Cresswell *et al.* (1991). Nucleic acids were extracted from John Innes potting compost spiked with ISP5078 *Streptomyces scabies* mycelium (35 mg wet weight mycelium g^{-1} soil). Lysis of the mycelium was achieved by beadbeating soil samples for both RNA and DNA extraction.

The samples were analysed by gel electrophoresis and dot blot hybridization. Dot blots were probed with the β probe under stringent conditions. The hybridized blot was resolved on a Betascope (Fig. 27). RNA and DNA extractions were performed on duplicate soil samples. From the blot it was apparent that more rRNA was extracted from one sample than the other, as one set of hybridization signals was marginally stronger than the other. These differences may be due to the heterogeneous nature of the environmental substrate and possible heterogeneity within the inoculum seeded into the soil. However, the data does indicate the variation that may arise in the application of this methodology to environmental samples.

Recovered nucleic acids from the method of Cresswell *et al.* (1991) produce hybridization signals comparable to those obtained by probing RNA.

Fig. 27 Comparison of target sequence for 16S oligonucleotide probe hybridized with ribosomal RNA and genomic DNA recovered from mycelium seeded in soil.

Dot blot hybridization of target nucleic acid recovered from *S.lividans* mycelium spiked into autoclaved John Innes compost.

Hybridization signals correspond to : 1, A 100% rRNA; 1, B 50% rRNA; 1, C 25% rRNA; 1, D 50% denatured rRNA;

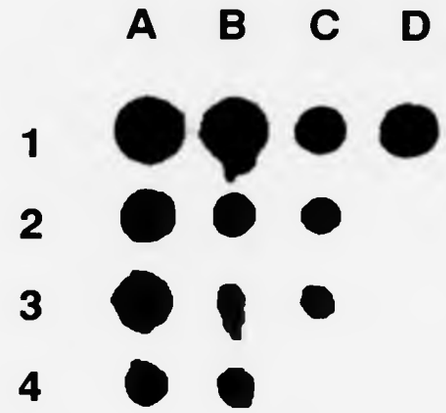
2, A 100% rRNA; 2, B 50% rRNA; 2, C 25% rRNA; 2, D 10% rRNA

3, A 100% DNA; 3, B 50% DNA; 3, C 25% DNA;

4, A 100% rRNA DNase; 4,B 100% DNA RNase; 4, C -ve con;

Signals generated were enumerated in terms of radioactive disintegrations, these counts (given below Fig. 27) corresponded well with size of the hybridization signals.

Figure 27



	A	B	C	D
	counts	**counts**	**counts**	**counts**
1	55789	49581	18888	24053
2	23429	13979	10763	5329
3	20046	9158	9372	2818
4	12280	10799	3264	3044

Radioactive counts measured in each spot of the dot blot above.

However, when these samples were treated with either DNase or RNase the intensity of the signal was reduced by approximately 50%. This indicates that the nucleic acids extracted were actually a mixture of RNA and DNA. When the DNA component (after RNase treatment) was compared with the RNA extracted from the same amount of streptomycete mycelium even with the inefficiencies employed in the RNA extraction method the signal obtained by probing the RNA was at least twice as intense as that achieved by probing DNA. Finally, a comparison was made in signal intensity between RNA that had been denatured with formamide and formaldehyde and untreated RNA. No difference could be observed in the intensity of the signal indicating either that the secondary structure of the RNA prepared according to these procedures had already been removed by the high salt precipitation steps involved in the procedure or alternatively, that the target site for the probe was not obscured by the secondary or tertiary structure of the ribosomal RNA molecule.

5.3.5 Optimal lysis of spores by bead beating

Cresswell *et al.* (1991) established that bead beating was an efficient method for the recovery of nucleic acids from spores. However, since the spores represent the resting stage of the streptomycete life-cycle and as the ribosomal RNA content of cells has been found to correlate with active metabolic activity (Giovanonni *et al.*, 1988a; De Long *et al.*, 1989) it might be assumed that spores would have smaller quantities of ribosomes per cell than vegetative mycelium. Quiros *et al.* (1989) have investigated the ribosomal RNA content of streptomycete vegetative mycelium and spores. Estimates of ribosome content from vegetative mycelial cells and spores at three different stages of germination i.e. dormant, dark and swollen were calculated per mg protein. Their studies indicated that the number of ribosomes almost doubled in the

transition from a dormant spore to a dark spore (a spore in the early stages of germination), the ribosomes in swollen spores increased further to exceed that of vegetative mycelial cells and to be approx. 3.5 times the number associated with dormant spores.

Streptomycetes survive for long periods in the soil as spores, only germinating to produce mycelial colonies in the presence of moisture and nutrients. It was therefore considered necessary to be able to recover ribosomal RNA from spores in soil as efficiently as possible with respect to the detection and monitoring of streptomycete inoculants *in situ*. Although the developmental stage of the spores was not characterised (dormant, dark and swollen), the spores used were freshly harvested from plates prior to freezing and use in these studies. Hence the spores used were probably spores in the dormant or earliest stages of germination and should be a reasonable indicator of the degree of sensitivity that this approach would have for resting spores in soil.

The ratio of 2.5 : 1 0.1 mm diameter glass beads to buffer and soil was retained in these experiments. 3 g samples of soil were spiked with 10^9 spores. Bead beating was substituted for sonication as the facility became available and the technique had been demonstrated to be highly efficient for the lysis of spores and recovery of nucleic acids (Cresswell *et al.*, 1991).

Lysis using the standard GOS buffer and protocol (Hughes & Galau, 1988) was compared with a buffer comprising a potent chelating agent, aminsalicylic acid and an efficient detergent, trisopropylnapthalene (M.Hartley, Warwick University, pers. comm.). In the latter treatment, after homogenisation by bead beating the supernatant was recovered by sedimenting the soil and beads and extracting the recovered supernatant with neutral phenol chloroform, prior

to precipitation of the nucleic acids. The ribosomal RNA was then prepared as usual according to Hughes & Galau (1988).

A third treatment was introduced to assess the effect of the phenol chloroform extraction on the recovery of ribosomal RNA. In this treatment, the GOS buffer was used for the bead-beating homogenisation step, followed by a phenol/ chloroform extraction, precipitation of the nucleic acids and then preparation according to method 2.

Ribosomal RNA extracted from spores from duplicate samples of all treatments was intact and comparable in quantity (Fig. 28). It was apparent that the lysis of spores was already fairly efficient as alterations to the composition of the homogenisation buffer made no difference, hence inefficient yields could probably be attributed to other steps in the protocol. However, of interest was the marked affect that phenol chloroform extraction had on the purity of the RNA recovered. From Fig. 28 it can be seen that the samples that were extracted with phenol chloroform were considerably more contaminated with humic acids than those prepared according to the unmodified procedure. The phenol/ chloroform extraction caused the humic acids and nucleic acids to bind irreversibly. The RNA extraction method (Hughes & Galau, 1988) deliberately avoids the use of phenol and guanidium salt extractions. This is as a result of the irreversible binding of RNA with homogenized leaf tissue when these methods were applied to certain crop plants such as cotton. Presumably these plants were high in phenolic compounds, which include the humic acids that are commonly associated with soils and sediments.

It is possible that the close association of humic acids and nucleic acids

Fig. 28 Optimisation of homogenisation for the lysis of streptomycete spores

Homogenisation buffers: A & B- aminosalicyclic acid and trisopropylnapthalene; C & D- GOS buffer (Hughes & Galau, 1988) with phenol chloroform extraction after bead-beating treatment; E & F, Unaltered GOS treatment.

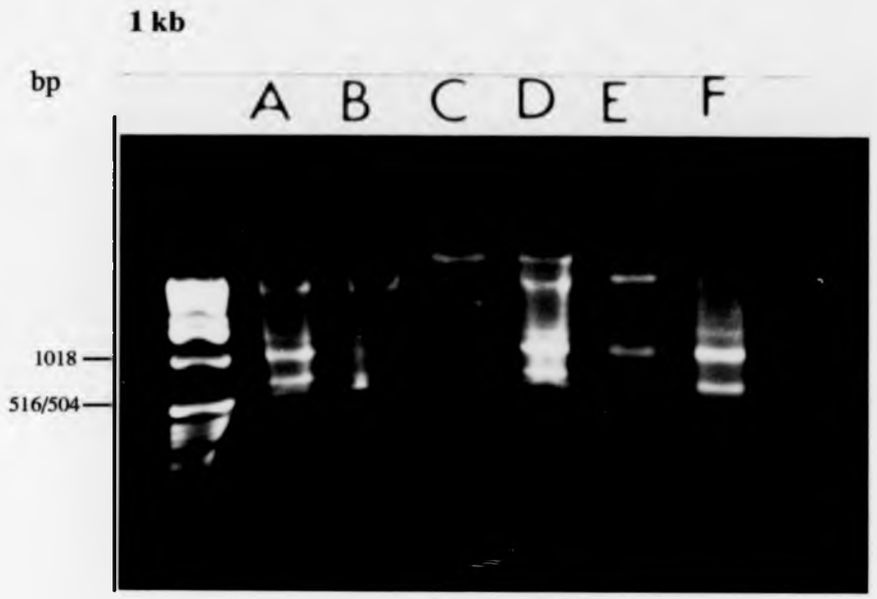


Figure 28

brought about with the neutral phenol chloroform extraction step could be influenced by changes in pH and therefore by altering the ionic state of the RNA molecules. It would be interesting to try an alkaline phenol extraction. Torsvik (1980) attempted to purify DNA from soil using phenol extraction at a pH range from 6.0 to 9.0, purification of the DNA was most effective using phenol at pH 9.0 and with the consequent recovery of the more humic acids in the phenolic phase.

A number of researchers have considered the importance of the pH of extraction buffers on the recovery of nucleic acids from soils. Findings of Ogram *et al.* (1987) indicated that DNA was recovered from the soil matrix of a soil with a high clay content more readily at an alkaline pH. This has been attributed to the fact that at a low to neutral pH the DNA is neutral and moves into the interlamellar space where it is absorbed; in contrast at a higher pH DNA is in the ionic form and excluded from interchelation (Ogram *et al.*, 1987). However, the study was unable to demonstrate any correlation between the content of organic carbon and absorption of DNA to the soil particles (Ogram *et al.*, 1987). Torsvik (1980) reported improved recovery of DNA from soils through the use of an alkaline buffer. Although these studies have not been done with RNA, similarities in chemical structure and behaviour will mean that these findings are pertinent to the extraction of RNA. However, the soil used in this study, was not a heavy clay soil but a sandy loam with a high organic carbon content. In the protocol of Hughes & Galau (1988) the homogenisation buffer was alkaline with a pH of 8.5 indicating the RNA would be in an ionic form. Normally alkaline extraction would be followed by an acidic potassium acetate precipitation step, which the authors indicate promoted the removal of impurities and the recovery of a white nucleic acid pellet.

5.3.6 Detection limits for rRNA recovered from spores

In order to estimate the sensitivity of the ribosomal RNA extraction methodology for the detection of inoculants *in situ*. It was considered important to base such an estimate on the number of spores in soil. The reasons being two fold: firstly, *Streptomyces* survive for long periods in soil as spores and conceivably would be present as such in most soils in which their ecology or presence would be required to be monitored. Secondly, enumeration of mycelial microorganisms is particularly difficult, since estimates based on colony forming units c.f.u. provide no information on the number of cells contributing to a colony.

Thus 3 g samples of John Innes potting compost were spiked with a dilution series of streptomycete spores ranging from 3 times 10^{10} to 10^{3}. Ribosomal RNA was recovered from all samples by bead-beating and the RNA prepared according to Hughes & Galau (1988). Samples of rRNA were suspended in a final volume of 30 µl and a 10 µl aliquot run on a gel. Ribosomal RNA could be visualised from 10^{7} spores or more (Fig. 29). Although the dot-blot hybridization failed, the sensitivity of detection would conceivably be improved by 1 to 2 orders of magnitude depending on the type and specific activity of the probe resulting in a detection limit of the order of 10^{5} to 10^{6} spores per sample.

When compared to the sensitivity of the plate count 10^{2} c.f.u. g^{-1} soil, the RNA method appears disappointingly insensitive. However, the plate count method is selective for microorganisms that are amenable to cultivation, while nucleic acid based detection methods do not impose the constraints and biases of culturability on sampling and extraction.

Fig. 29 Ribosomal RNA recovered from ISP5078 streptomycete spores in soil.

Amount of spores g^{-1} soil: 10, 10^{10}; 9, 10^{9}; 8, 10^{3}; 7, 10^{8}; 6 10^{7}; 5, 10^{6}; 4 10^{5}; 3 10^{4}; +, 10^{8} TK24 spores; -, no spores, control.

Ribosomal RNA from 10^{8} *S.lividans* or ISP5078 spores could be visualised on an agarose gel

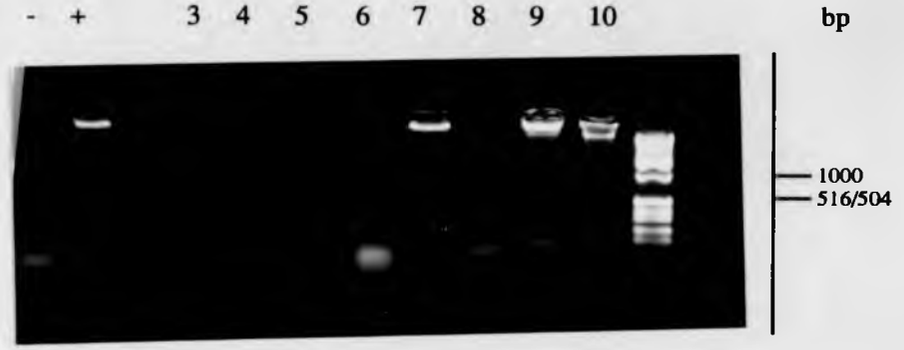


Figure 29

While establishing the efficiency of lysis was considered an important first step to improving extraction efficiencies, there are other stages worthy of some attention. In particular this rRNA extraction procedure relies on repeated high salt precipitation steps for the removal of contaminating molecules such as humic acids. Preliminary assessment of each stage indicated that purification via precipitation was accompanied by substantial losses in yield (M.Brigila, Agricultural University, Wageningen, NL. pers. comm.). The incorporation of components such as PVPP and sodium ascorbate into the homogenisation buffer have been found to ease problems of humic acid contamination from DNA extracted from soil (Holben *et al.*, 1988; Steffan *et al.*, 1988). Since RNA molecules behave similarly to DNA molecules, the incorporation of agents to assist in the recovery of RNA with reduced humic acid contamination early on in the preparation might warrant the omission of the later precipitation step and hence promote enhanced yields. Whether or not there is a possibility of reducing the number of purification steps, the methodology used here was not without problems of humic acid contamination. Without a final chloroform (saturated with TE buffer) extraction the nucleic acids were rarely amenable to dot-blot hybridization. Another manipulation exploited for the purification of DNA from humic acids has been PEG precipitation as an alternative to ethanol and isopropanol which serve to co-precipitate the nucleic acids with humic acids (Ogram *et al.*, 1988; Cresswell *et al.*, 1991). There are a number of developments that have been made while improving methodology for the extraction of DNA from soil that have not been and should be evaluated in protocols for the extraction of RNA from soil. Thus future studies on the development of extraction methods for RNA from soil should be concerned with testing those purification steps most amenable to routine sampling that will be required in environmental investigations.

Increased sensitivity may be obtained by optimising the labelling of probes and the hybridization conditions under which they are used. These studies utilised end-labelled oligonucleotide probes. This labelling system is most efficient when used with γ P^{32} of a high specific activity. In addition, there is a relationship between the efficiency of labelling and the more P^{32} added to the reaction (Maniatis *et al.*, 1982). Stackebrandt *et al.* (1991) emphasise the need to test all probes empirically and ascertain optimal conditions for their use. Optimal conditions of use may be determined by varying the ratio of probe to target sequence bound to the membrane (Williams & Mason, 1987). Furthermore, conditions may be optimised by using replicated dot blots and hybridizing at the same temperature, varying the washing conditions with one set and on the second set varying the hybridization conditions, keeping the washing conditions constant and monitoring the affects under both regimes (Anderson & Young, 1987). The Tm of the probe may also be estimated empirically by hybridizing as appropriate, performing successive washes at increasingly stringent temperatures and monitoring the stability of the duplex after each wash. Such precautionary measures are advised as the Tm of hybrids formed between nucleic acids bound to filters have been estimated to be lower than predicted from solution hybridization (Anderson & Young, 1987).

Improved sensitivity of detection for extraction of nucleic acids might also be achieved by either concentrating the spores from large volumes of soil using methodology of the type described by Herron & Wellington (1990) or through the amplification of RNA or DNA target sequences via PCR (Giovanonni, 1991). With the introduction of PCR methodology, the need to exploit the natural amplification in the cells of ribosomally encoded sequences is

considerably reduced. Furthermore since more care is required for the preparation of rRNA target sequences (as they are more prone to nuclease degradation), it is conceivable that this approach may be superseded by DNA based methods.

However, one significant difference between RNA and DNA molecules is that RNA molecules are indicators of activity. The cellular content of ribosomal RNA molecules has been correlated with metabolic activity (Giovanonni *et al.*, 1988a; DeLong *et al.*, 1989) and ribosomal degradation may occur with a starvation response in some groups of bacteria (Kramer & Singleton, 1992). Furthermore extraction and analysis of mRNA molecules may provide information on the expression of particular genes. Hence, it is conceivable that when the methodology has been established for the sensitive detection and monitoring of strains in soil using DNA based methods, questions will be directed more specifically towards understanding the activity of inoculants *in situ* and their particular role in the ecosystem. The RNA molecules will undoubtedly have an important contribution to play in the elucidation of these answers.

Chapter 6

The application of various approaches to the detection and analysis of the life cycle of common scab causing streptomycetes within soil microcosms

6.1 Introduction

6.1.1 Microcosms

Microcosm model systems were developed with the recognition that it was legally and environmentally unacceptable to test potential pollutants in the field without some prior knowledge of their rates and routes of degradation and/ or transfer through the various components of the biosphere (Burns, 1988). Microcosms have since been adopted to study the fate of microorganisms and their interactions such as the potential for gene transfer in environmental samples (Trevors, 1988). By definition microcosms attempt to mimic certain characteristics of the natural environment and allow predictions to be made about the fate of the specific chemicals and microbes under study, while at the same time allowing some of the many environmental variables to be tightly controlled or even manipulated. They offer more reproduciblity and are more amenable to experimental analysis than studies under environmental conditions. In the design and choice of microcosms there is a conflict between the reductionist approach, which allows the reproducible study of comparatively simple and carefully controlled systems and the holistic approach which acknowledges that environmental processes are much more than the sum of their component parts and attempts to measure these processes in a relatively undiusturbed system (Burns, 1988). Hence while a model system may be easy to design and apply experimentally, the data generated may be of little informative value with regard to the events in the natural environment. In contrast, a complex model may be a valid reflection of a natural habitat, but difficult to interpret. Regardless of the complexity of the microcosm its credibility can only be judged on the basis of whether the data produced simulates the situation in the field (Burns, 1988).

Two of the most common approaches to the design of soil microcosms in

microbial ecology have been either to remove much of the inherent heterogeneity of the soil environment through the combined actions of drying, sieving (through a 2mm gauze) and mixing to create a uniform substrate. The activity of the microbe is then studied with reference to physical and chemical properties of the soil. The second approach maintains the stratification of the soil profile and hence the biological, physical and chemical gradients via the excision of intact soil cores or blocks. Microcosms of both types may be autoclaved and reinoculated with specific members of the soil biota, bacteria, fungi, protozoa, nematodes, earthworms and plants. Under these circumstances recolonisatation is being studied and these microcosms are termed gnotobiotic (Burns, 1988).

6.1.1.2 Microcosm models with plants

Morel *et al.* (1989) have studied the fate of two genetically modified bacteria an *Esherichia coli* and a *Pseudomonas putida* in the corn rhizosphere under hydroponic and sterile conditions with and without other rhizosphere microorganisms. Both bacteria grew well in the presence of root exudates as a sole carbon source. However, the introduction of rhizosphere microorganisms into the microcosm affected the survival patterns of *E.coli* more than *P.putida,* with *P.putida* showing greater resilience to competition than *E.coli.*

Bennet & Lynch (1981) have studied bacterial growth and development in the rhizosphere of gnotobiotic cereal plants. Tubes of autoclaved sand were seeded with wheat, barley and maize plants spiked with specific bacterial inoculants (a *Curtobacterium*, a *Mycoplana* and a *Pseudomonas* sp.). Colonisation of the roots was monitored using total viable plate counts of root sections and light microscopy of analine blue stained sections of root material. Colonisation patterns were the same for the three microbes in the rhizospheres

of wheat and barley. However, the development of the *Curtobacterium* and *Mycoplana* were markedly different in the maize rhizosphere. Both microbes exhibited an initial lag period of about 28h before the introduced populations increased and neither bacterium reached population levels as high as those in the wheat or barley rhizospheres. Interesting patterns of growth were observed in co-inoculation studies, hence when the *Curtobacterium* and *Mycoplana* were both inoculated into the maize rhizosphere, the *Mycoplana* population remained similar to that observed in the single bacterial inoculations but the *Curtobacterium* populations were enhanced by a factor of 10. Observed differences of bacterial isolates in the maize rhizosphere may be attributed to differences in the nature of root exudates, lack of growth stimulating compounds or presence of growth inhibitory compounds. When all three bacteria were co-inoculated the *Pseudomonas* growth rate was stimulated and populations reached similar levels to those attained with single inoculations. In contrast the *Mycoplana* growth rate was reduced and final populations a 1/10 of those observed under single inoculations while *Curtobacterium* populations increased slightly in the first 24h but then disappeared completely. This set of experiments indicates how increasing the complexity the microcosms through the introduction of more that one inoculant affects the growth and survival patterns of individual inoculants.

Armstrong *et al.* (1987) report on the development of a microcosm method to assess the survival of recombinant bacteria associated with plant surfaces (radishes) and a plant-feeding insect (the variegated cutworm, *Peridrom saucia*). Radish seedlings were exposed to cutworm larvae and sprayed with recombinant *Pseudomonas cepacia* harbouring a plasmid with antibiotic resistance determinants and non-recombinant *P.cepacia* strain. Background populations of epiphytic bacteria increased throughout the duration of the

experiment, while *P.cepacia* populations declined. Both *P.cepacia* strains were acquired in the digestive tracts of the cutworm larvae, but did not appear in frass samples. Transconjugation between *Pseudomonas* strains although identified to occur on plates did not appear to occur under these microcosm conditions.

6.1.1.3 Intact soil-core microcosms

Bolton *et al.* (1991) studied the population dynamics of a rifampicin resistant *Pseudomonas* sp. in intact soil-cores planted to winter wheat that were placed under 4 different environmental regimes. These included the laboratory at ambient temperature, in a growth chamber with temperature fluctuations simulating those in the field, field lysimeters and field plots. Although some differences in population size between the laboratory and growth chamber microcosms were identified when compared to populations in the field, in general, colonisation of the roots at the three leaf developmental stage and at the final boot stage of plants was similar under the four microcosm systems. The study demonstrated the validity of using intact soil core microcosms either in the laboratory or a growth chamber to simulate field conditions and the need to calibrate them to field conditions before making predictions on the survival of inoculants.

Bentjen *et al.* (1989) have also assessed the validity of using intact soil-core microcosms to investigate the fate and ecological impact of genetically engineered microorganisms. Soil cores were planted with wheat and maize seeds and inoculated with *Azospirillium lipoferum* Tn*5* mutants. Microcosm leachate, rhizosphere soil, plant endorhizosphere, insects and xylem exudates were sampled and enumerated for *A.lipoferum* Tn*5* mutant populations using an MPN-DNA hybridisation method. Variation between replicates was

observed and attributed to the inherent variability of the soil-core microcosms and therefore an accurate reflection of the heterogeneity of the complex field environment.

Fredrickson *et al.* (1989) used intact soil-core microcosms to investigate the fate of a root growth inhibiting *Pseudomonas* sp. in soil planted with spring wheat. Pseudomonads were marked using Tn*5* mutagenesis and inoculated into the surface layers of the soil-cores. The fate of mutants producing the root-inhibiting toxin (tox+) and unable to produce the toxin (tox-) was studied. Samples were taken from the surface layers of the soil, with reference to depth in the bulk soil, the rhizosphere, the rhizoplane, soil leachates and also from earthworms colonising the soil cores. Mutant populations were enumerated using dilution plating with selective media. Tn*5* mutants had been transported through the colomn with percolating water and were identified in soil-core leachates, they were also present in the gut of earthworms. Population levels in the surface layers of the soil declined with time, however the mutants colonized the wheat rhizosphere and rhizoplane throughout the soil-core. The presence of Tn*5* mutants in the digestive tracts of earthworms demonstrated their potential as vectors in the dessemination of introduced microbes.

6.1.1.4 Aquatic microcosms

Awong *et al.* (1990) describe an aquatic microcosm that utilises survival chambers in a flow through or static renewal system, allowing exchange of materials between chambers and the surrounding water. Other workers have utilised sediment soil cores with an aqueous interface (Burns, 1988).

6.1.2 The application of soil microcosms to the study of streptomycete ecology

Soil microcosms have been used to study the growth, lifecycle and survival of *Streptomyces* strains in sterile and non-sterile soils. The studies of Wellington *et al.* (1990) utilised 200 g soil microcosms in which the soil had been mixed, dried and sieved (4mm sieve) in treatments that were sterile and non-sterile, with and without 1% starch and chitin amendment. Inter and intraspecific transfer of the multicopy plasmid pIJ673 was demonstrated between *Streptomyces* strains. Populations were sampled and enumerated by mechanically shaking soil in 1/4 strength Ringers diluent and plating on selective media. Estimates from viable counts were correlated with scanning electron microscopy observations of the soil to investigate the activity of the strain at different time points throughout the experiment. Initial detection of transconjugants coincided with the observed mycelial stage of the streptomycete lifecycle. Differences in the interspecific plasmid transfer frequencies were observed between inoculants in soil and on agar plates. These differences were attributed to inhibitory interactions occurring between strains on the plates which may be due to antibiotic production and sensitivity by the strains. These antagonistic effects were not observed in soil.

Bleakley & Crawford (1989) considered the effect of a number of environmental factors on the survival and transfer of a conjugative plasmid between *Streptomyces* strains. A number of nutrient regimes were investigated in 51.0 g sieved, sterile silt-loam soil microcosms. These included, amendment with $CaCO_3$, $CaCO_3$ and cotton seed flour, chitin and cellulose and moisture regimes of 20, 40 and 60% water-holding capacity. Viable counts were determined after a month at 30^0C by plating on media selective for the plasmid recipient combination. Heterotrophic microbial

activity was assayed with fluorescein diacetate. Nutrient amendment resulted in two to three times more fluorescein diacetate activity than unamended, limed treatments of an equivalent moisture content. Nutrient amended, low moisture regimes provided optimal conditions for mycelia development and hence conjugative plasmid transfer between *Streptomyces* inoculants in soil.

Wang *et al.* (1989) studied the survival and effect on soil organic carbon mineralisation of a number of wild type, mutant and genetically enhanced lignin decomposing *Streptomyces* strains. The microcosms consisted of a silt, loam soil, which were sterile and non-sterile and with and without lignocellulose amendment. Evolution of CO_2 was monitored in respiration cabinets. All strains survived either as spore or mycelial inoculants for at least 30 days, with selected strains lasting as viable spores for up to 10 months. Oniy two inoculants were able to significantly alter the short term rates of carbon mineralisation. One *S.lividans* recombinant showed enhanced rates of carbon mineralisation particularly in non sterile soil amended with lignocellulose, however this affect was transient, with the strain appearing genetically unstable in soil. Another *S.lividans* strain temporarily reduced carbon mineralisation rates but only in non-sterile soils in the first few days.

6.2 Aims

6.2.1 To assess the influence of potato plants on plant pathogenic streptomycete populations in soil, and establish whether rhizosphere effects occur.

6.2.2. To monitor the growth, survival and lifecycle of plant pathogenic streptomycetes in sterile and non-sterile soil microcosms.

6.2.3 To compare and apply a number of different strategies for the detection and monitoring of plant pathogenic streptomycete inoculants in soil.

6.3 Results

6.3.1 Experiment 1. considered the growth and survival of two streptomycete strains, TK24 a laboratory strain and streptomycin resistant mutant of *S.lividans* and a putative common scab strain MP2 (isolated from Maris Piper) harbouring the plasmid PIJ673 which confers resistance to neomycin, thiostrepton and viomycin. The plant soil microcosm conditions were the same as those adopted in the Maris Piper pathogenicity trial (Section 2.31.1). Hence it comprised 1 Kg of pasteurised John Innes compost seeded with Maris Piper certified seed potatoes in treatments with potato plants. The rationale to work under near environmental conditions being that the regime was a natural progression from the work already established with marked strains in non-sterile soil microcosms (Wellington *et al.*, 1989). Soil in pots with and without plants was kept below 50% moisture holding capacity.

Inoculants were recovered from soil on R5 medium containing selective additives. Selective media for TK24 included streptomycin, rifampicin and tetracycline together with the antifungal compounds nystatin and cyclohexamide. MP2 harbouring pIJ673 was isolated on media containing thiostrepton, neomycin, nystatin and cyclohexamide. Soil samples from pots inoculated with both strains were also spread onto plates containing streptomycin, rifampicin, tetracycline, neomycin, thiostrepton and antifungals for the identification of any TK24 inoculants that may have acquired the plasmid pIJ673 from MP2 via conjugation. The aim was to evaluate the use of

plasmids as markers in terms of their stability in the host strain over time and rates of transfer to a potential recipient strain, *S.lividans* TK24.

The selectivity of the various media were tested on the other strains, thus MP2 harbouring PIJ673 would not grow on R5 with streptomycin, rifampicin and tetracycline or R5 containing streptomycin, rifampicin, tetracycline, neomycin and thiostrepton. The same checks were applied to TK24, which was unable to grow on selective medium except that containing streptomycin, rifampicin and tetracycline. Finally TK24 + pIJ673 grew on R5 with streptomycin, rifampicin, tetracycline, neomycin and thiostrepton, hence this medium did support the growth of TK24 transconjugants. In a previous study (Section 4.4) synergistic and toxic effects were demonstrated between various antibiotic and antifungal combinations in an effort to develop selective media. These effects were demonstrated on RASS (Herron, 1991), a minimal medium that in the absence of selective additives may impose certain nutrient stresses on microorganisms. In contrast, R5 is a rich complete medium (Hopwood *et al.*, 1985) designed for the optimal growth of *Streptomyces* and may therefore allow growth of resistant strains in the presence of various combinations of antibiotics and antifungals.

Pots without plants were sampled by mixing the 1 kg of soil thoroughly with a large, sterile spatula and then removing 3 times 1 g samples. In contrast pots with potato plants, were sampled by blending the subteranean parts of the plant in a Waring blender until homogeneous and evenly distributed before recovering 3 times 1 g samples. 1 g samples were treated as described earlier (Section 2.37) and dilutions plated out in triplicate on the appropriate selective media.

Pots were inoculated with spore suspensions enumerated by viable plating to contain approximately 1 times 10^9 spores ml^{-1}. One ml of a spore suspension was added with the 1/4 strength Ringers diluent at the time of initiation of the experiment soil moisture was added to 15% (w/w) with the inoculum and diluent solution. The inoculum was mixed into the pot to give a final spore inoculum level of 10^6 spores g^{-1} soil. Pots were then seeded with potatoes if required.

The following treatments were set up in pots with and without potato plants:

(1) TK24

(2) MP2 +pIJ673

(3) TK24 and MP2 +pIJ673

(4) Uninoculated

Pots were sampled at the following time intervals: days 0, 2, 8, 16 and 120.

The histograms show the logged viable plate counts (c.f.u.) data for the single inoculations of TK24 and MP2 +pIJ673 with and without potato plants (Fig. 30) and the viable count data from the double inoculation of MP2 +pIJ673 and TK24 with and without the plant (Fig. 31). Pots were sampled and populations enumerated at days 0, 2, 8, 16 and 120. The study focussed on the establishment of inoculants in the early part of the time course and at population levels after 4 months and hence tuberisation and senesence of the parent plant. Potato root and tuber material was examined by scanning electron microscopy at day 120.

6.3.2 Results and conclusions

Some of the difficulties that emerged from running the experiment were the

Fig. 30 Histogram bar charts of single inoculations of *S.lividans* TK24 and MP2 + pIJ673 with and without plants in non-sterile soil.

Difficulties of enumerating the inoculants under non-sterile conditions meant that it was not possible to assess the influence of the plant on ISP5078 populations. Population differences would have to have been considerable to overcome the large error margins (M.S.D. values) associated with the variable non-sterile environment.

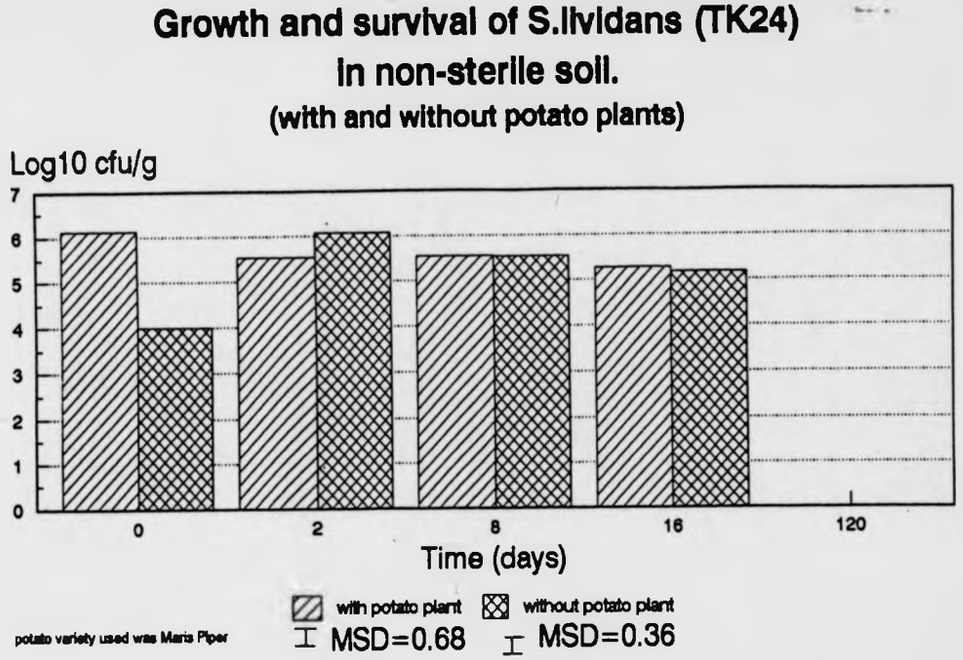


Figure 30a

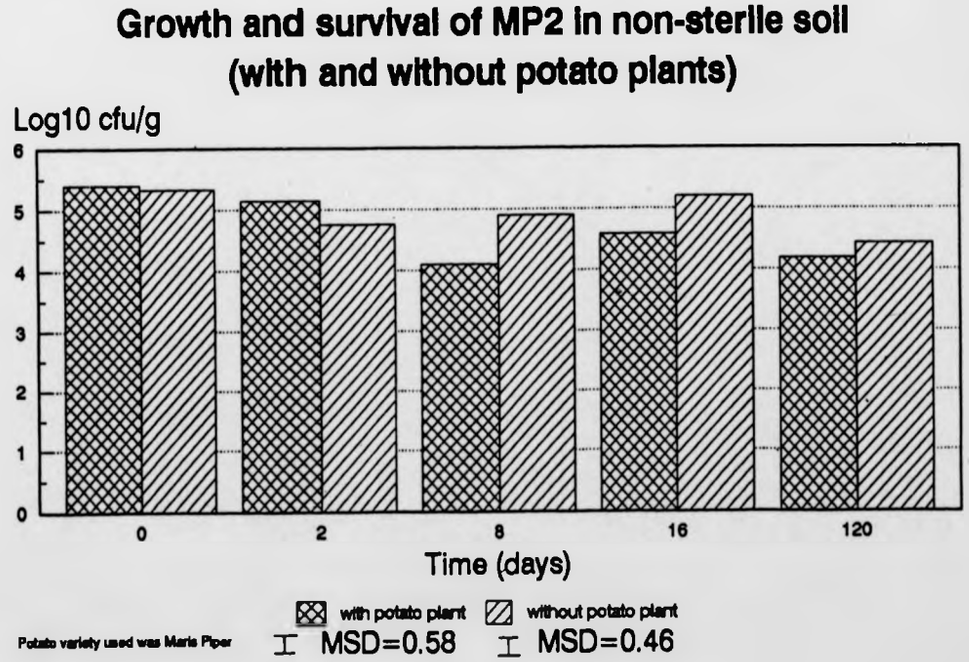


Figure 30b

Fig.31 Histogram bar charts of the double inoculation of *S.lividans*, TK24 and MP2 + pIJ673 in the presence and absence of a potato plant.

Analysis of population trends and dynamics was compromised as a result of enumerating inoculants under non-sterile conditions. However, at day 120 a significant discrepancy between the MP2 + pIJ673 and TK24 populations was observed. The TK24 population was no longer detectable, while the MP2 + pIJ673 was easily recovered and enumerated. The differences in the inoculants relative abilities to grow and survive was attributed to reduced ecological fitness of TK24 as a result of the streptomycin resistant mutation.

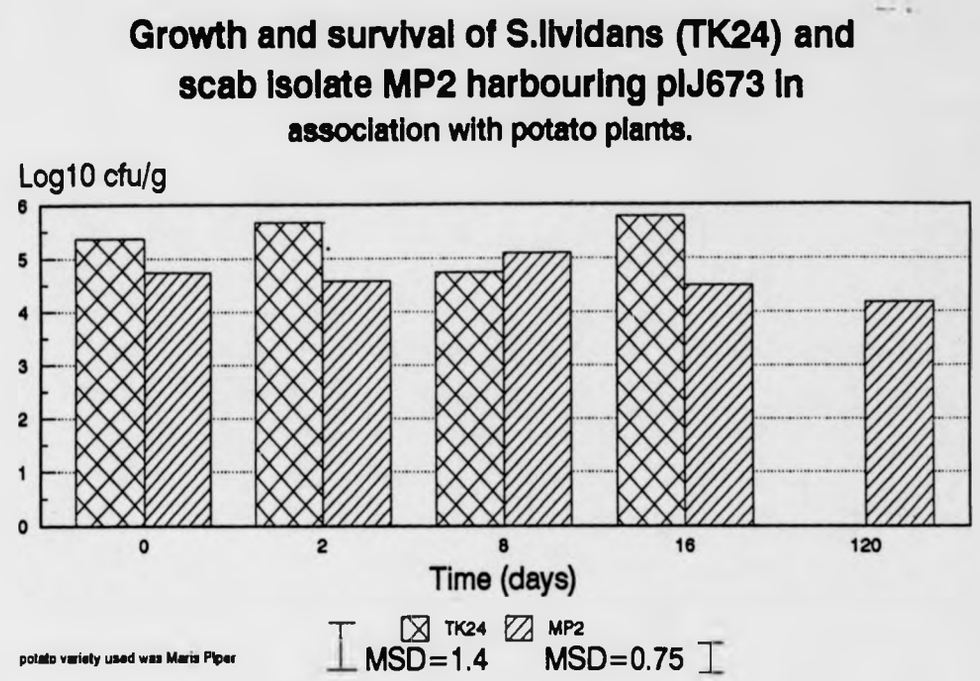


Figure 31a

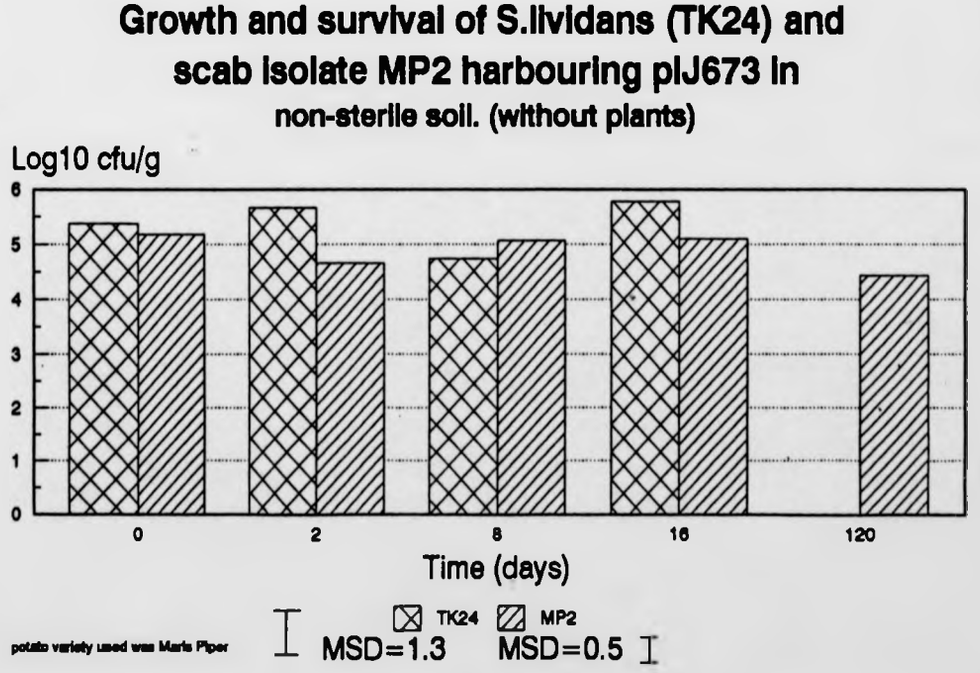


Figure 31b

problems of obtaining reliable viable count data under near environmental conditions. While the levels of inoculum were realistic for infection studies, they were also at similar levels to other indigenous soil microorganisms, of which some appeared to be able to compete more successfully on the selective media. Contamination problems were compounded by the presence of the potato plant, and a decomposing mother tuber. This was illustrated in the scanning electron micrographs (Fig. 32) which show the prolific development of fungi at the tuber surface. This together with the relatively modest amount of replication that was possible to include in the experiment with regard to the volume of soil under study resulted in high M.S.D. values. The analysis of trends in the data was therefore compromised and conclusions have only been drawn over quite dramatic differences in the viable count data. The choice of media confounded the problems, as R5 is a rich, complete medium which supported the growth of many microorganisms.

Uninoculated control pots were monitored for indigenous background actinomycetes that might affect enumeration of the TK24 and MP2 + pIJ673 populations. The selective combination for TK24 appeared to eliminate the growth of all background soil actinomycetes, and the red-blue pigmentation of the strain assisted in its discrimination from the majority of soil-borne actinomycetes. The selective media for MP2 +pIJ673 allowed the growth of a small number of of actinomycetes of varied morphology, at the lowest dilutions. The populations of MP2 + pIJ673 were discernible from these indigenous actinomycetes, as they were generally isolated in higher numbers and were phenotypically uniform.

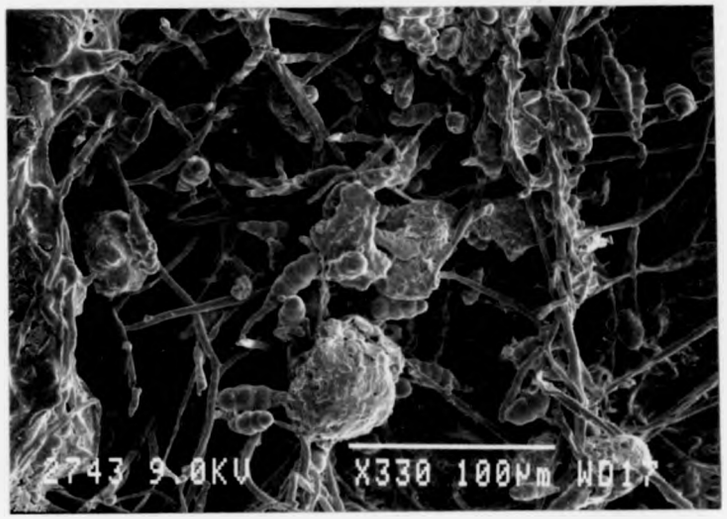
No significant differences were observed in the soil population levels of streptomycete inoculants in the presence or absence of the potato plant. Of

Fig. 32 Scanning electron micrographs of the non-sterile potato tuber surface.

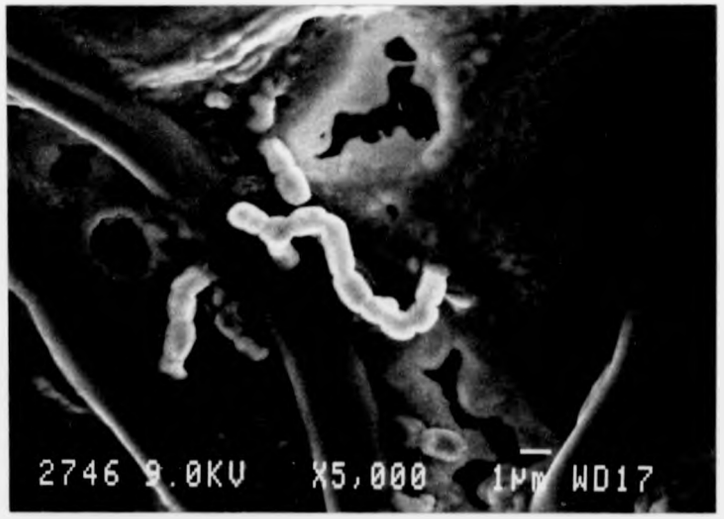
Micrograph I (X 330) visually illustrated the problems of applying viable plating procedures to the enumeration of inoculants in a highly dynamic natural environment. Hence this section of tuber material was covered in a carpet of fungal mycelium and conidia. Fungi rapidly overgrew the dilution plates making the enumeration of inoculants (via cultivation) difficult.

Micrograph II (X 5,000) illustrated the presence of actinomycetes (chains of spores) at the tuber surface despite intense microbial competition for nutrient resources.

Scanning Electron Micrographs of the potato tuber surface
(Day 120) Non-sterile Microcosm



I Magnification X 330



II Magnification X 5,000

Figure 32

interest is the apparent difference in the long term survival of the mutant, laboratory strain TK24 and the natural isolate MP2 + pIJ673 that was observed at day 120. Our results indicated the dying off of the TK24 population with time. However, since the detection limit for the experiment was of the order of 10^3 c.f.u. g^{-1}, it is possible that the inoculant was still present beneath the levels of detection. A decrease in the population levels of TK24 in soil over time has also been shown by Cresswell *et al.* (1992) in microcosm studies over a 60 day period. These results suggest a reduced ecological fitness of this strain as a result of the mutation. They highlight the difficulties of using strains marked by mutations to determine the behaviour and ecology of inoculants under environmental conditions. Such studies are of value in predicting the fate of inoculants planned for release in the natural environment (Thompson *et al.*, 1990) and determining the suitability of specific inoculants as agents in biological control (Weller, 1984). Other workers have also experienced reduced competitive fitness, growth rates and alterations in membrane biochemistry with antibiotic resistant mutants. Compeau *et al.* (1988) identified decreased competitive fitness in certain *Pseudomonas fluorescens* rifampin-resistant mutants and Turco *et al.* (1986) observed reduced competitive nodule-forming ability in doubly antibiotic resistant marked *Rhizobium* species. Both studies, emphasised the need to evaluate the behaviour of mutants alongside the wild type strain in sterile soil microcosm assays before applying antibiotic resistant mutants as marked strains in ecological studies. Until alternative detection strategies become available, comparisons of the behaviour of marked and unmarked strains are not possible under non-sterile conditions. Compeau *et al.* (1988) therefore recommend comparing the fitness of both the mutant and wild type isolates by the co-inoculation of both strains in sterile soil and assessing their relative growth rates and abilities to compete against each other. The population of MP2

+pIJ673 was detectable at day 120, in pots seeded with and without potato plants. Although we were unable to assess plasmid loss, it is of interest that a detectable proportion of the MP2 population retained the plasmid for so long in a highly dynamic environment. Particularly since the plasmid may represent an added metabolic burden on the strain in the nutrient limited soil environment. It is conceivable however, that there may be positive, selective forces acting on the maintenance of the plasmid that we are unable to measure. Alternatively these findings may support others unable to identify selective forces acting on the stable maintenance of specific plasmids in certain host backgrounds (Levy 1985). Amner *et al.* (1991) were able to demonstrate the stable maintenance of plasmids encoding antibiotic resistance determinants in mesophilic and thermophilic *B.subtilis* strains in the absence of selection in a non-nutrient limiting compost environment. The feasibility of using plasmids as markers in *Streptomyces lividans,* TK24 in soil has been investigated by Wipat *et al.* (1991). Plasmids NW4 and NW5, derivatives of pIJ486 that contain a *xylE* gene insert, appeared 95% stable over an 80 day period within streptomycete spores in soil, with 78 to 89% retaining the C23O phenotype. Plasmids were maintained equally well in the *S.lividans* host regardless of whether the *xylE* gene was thermoregulated or expressed constitutively. When *S.lividans* strains harbouring pIJ486 were compared with those harbouring NW4 and NW5 in soil, over time, reduced spore counts and sporulation were observed from the strains carrying NW4 and NW5. These differences were attributed to be a result of the additional metabolic load of the *xylE* insert. Larger co-integrate plasmids appeared highly unstable in *S.lividans*, TK24 in soil.

Since many members of the *Streptomyces* (including many uncharacterised strains) are amenable to plasmid transformation and many natural isolates

harbour plasmids, they may serves as suitable markers with an assessment of the of the rates of instability and metabolic burden to the cell.

Transconjugants were not recovered throughout the course of the experiment. It is possible that they may have occurred at some point before the TK24 population declined and remained below detection levels. However, given the reduced fitness of *S.lividans*, TK24 under these conditions (the count was below detection levels at day 120) it is possible that if transfer was occurring that the additional metabolic burden of the plasmid would result in a strain of reduced ecological fitness.

6.3.2 Experiment 2

To investigate the growth and survival of *S.scabies* ISP5078 in sterile amended and unamended soil microcosms with and without potato plants.

6.3.2.1 Rationale for the experiment: As a result of the difficulties encountered in obtaining meaningful data under non-sterile conditions a study of the lifecycle of the scab-causing strain, ISP5078 was conducted in sterile soil. Such a study under standardised conditions was also considered necessary for the comparison of a number of approaches to detection and monitoring.

6.3.2.2 Experimental design

Four different soil-plant regimes were compared:

(1) Sterile soil unamended

(2) Sterile soil amended (with 1% starch and chitin)

(3) Sterile soil unamended with an axenic potato plant

(4) Sterile soil amended with an axenic potato plant

Duplicate 20 g microcosms were set up for each sample day. The experiment was sampled at days 0, 2, 5, 15, 30, 60, 90 and 120. Soil (John Innes compost) was pretreated by sieving it through a 4mm filter and three rounds of autoclaving and incubation.

The inoculum was added to a final concentration of 3.5 times 10^3 spores g^{-1} soil. Inoculum was added with the 1/4 strength Ringers diluent to 15% soil moisture content (w/w). Plants were introduced into the soil microcosms as sprouting axenic mini tubers.

Three approaches for monitoring and detection of inoculants were compared throughout the course of the experiment:

(i) Viable plate count

(ii) Probing of recovered 16S rRNA from soil

(iii) *In situ* hybridisations

Viable plate counts

Three 1 g samples were removed from the duplicate microcosms after thorough mixing and were suspended and agitated in 1/4 strength Ringers solution. Serial dilutions were plated onto RASS media containing penicillin, ampicillin and cyclohexamide.

Probing of recovered 16S rRNA from soil

RNA recovered from soil microcosm samples according to the procedure of Hughes & Galau (1988) was hybridised with the β1 oligonucleotide probe (Stackebrandt *et al.*, 1991).

***In situ* hybridisations**

Were performed with the eubacterial probe primer 1115 (Embley *et al.*, 1988).

6.3.3 Results

6.3.3.1 Viable count data

Counts from duplicate microcosms were combined to obtain mean values for each treatment. The data was transformed to produce log values and M.S.D.s calculated (Section 2.45). Histograms show the mean logged counts for the ISP5078 populations in sterile amended soil (Fig. 33) and also the population data for ISP5078 in unamended soil (Fig. 34). Figs. 33 & 34 compare the growth of ISP5078 in the presence and absence of a potato plant in both sterile amended and unamended soils. M.S.D values were small, as a result of no background contamination and reduced environmental variation. Significant population differences were observed between the ISP5078 populations in amended and unamended soils (Figs. 35 & 36). Final population levels in amended soils of 10^9 c.f.u. g^{-1} were obtained, exceeding those in unamended soils by 1.0 to 1.5 orders of magnitude. In contrast, no significant differences were apparent between the treatments with and without plants in amended and unamended soils (Figs. 33 & 34).

The data has also been plotted as scaled graphs that indicate the population changes relative to day 0. The scaled graphs (Fig. 33b & 34b) illustrate the differences between ISP5078 populations in the presence or absence of potato plants in amended and unamended soils. The plant exerted little influence over the population under either of the regimes. The growth rate of inoculants in association with plants may be marginally faster, however any differences are small and do not appear significant. The scaled graphs (Fig. 35b & 36b) show the ISP5078 populations with and without amendments in the presence

Fig.33 Growth and survival of ISP5078 in sterile amended soil with and without potatoes

Fig.33a The histogram bar chart indicated that the axenic potato plant did not have a significant influence on the ISP5078 population in sterile amended soil.

Fig.33b The scaled graph indicated an increase in the inoculated population relative to day 0. The graph confirmed previous findings (Wellington et al., 1990) of germination and growth within the first 2 to 15 days. The population reached a threshold by day 15 which was retained throughout the duration of the study. Trends in the population growth patterns were similar with and without the potato plantlet.

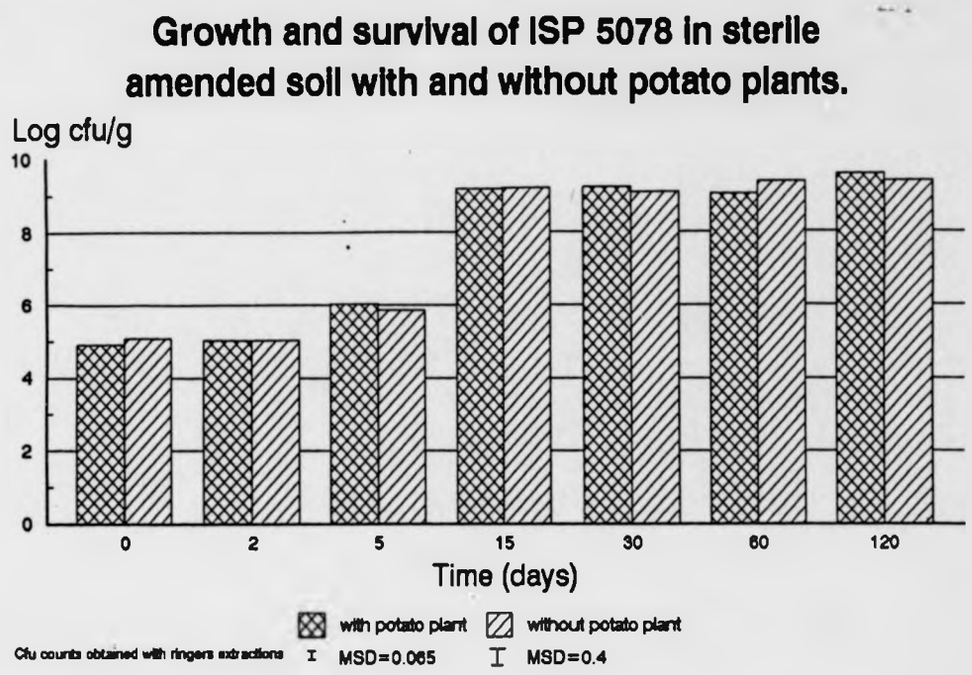


Figure 33a

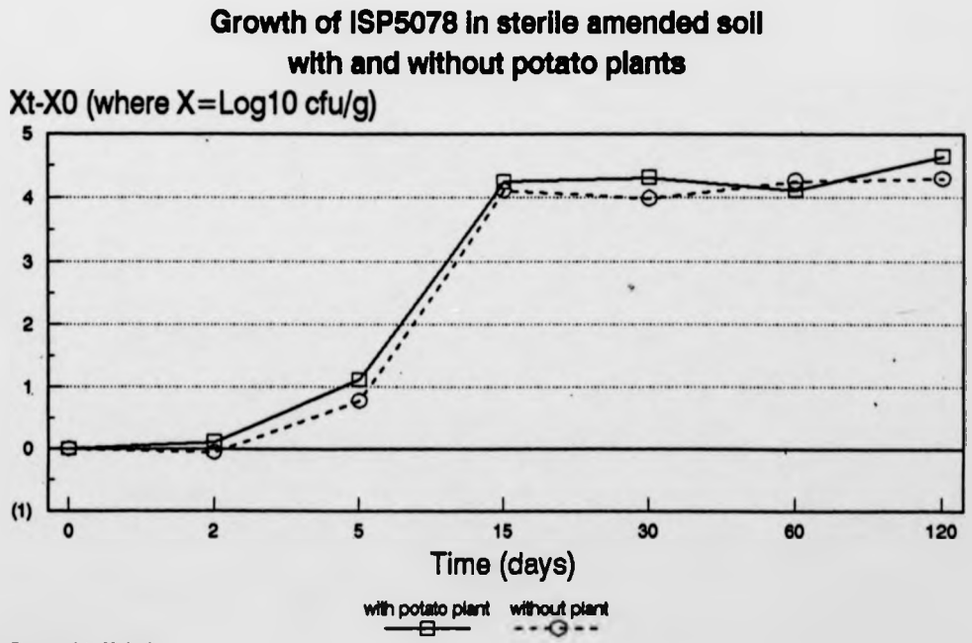


Figure 33

Fig.34 Growth of ISP5078 in sterile amended soil in the presence and absence of plants.

Fig.34a Histogram bar chart. No significant difference in the level of the ISP5078 population was observed in the presence and absence of the potato plant.

Fig. 34b Scaled graph. Population levels of ISP5078 have been illustrated in terms of initial populations at day 0. Similar to findings in Fig.33b, most growth was observed between days 2 and 15. By day 15, the populations had reached a threshold which was maintained or which reduced slightly throughout the duration of the experiment. The growth rate of the inoculant in the presence of the plant appeared marginally faster than in its absence; however these differences were not statistically significant.

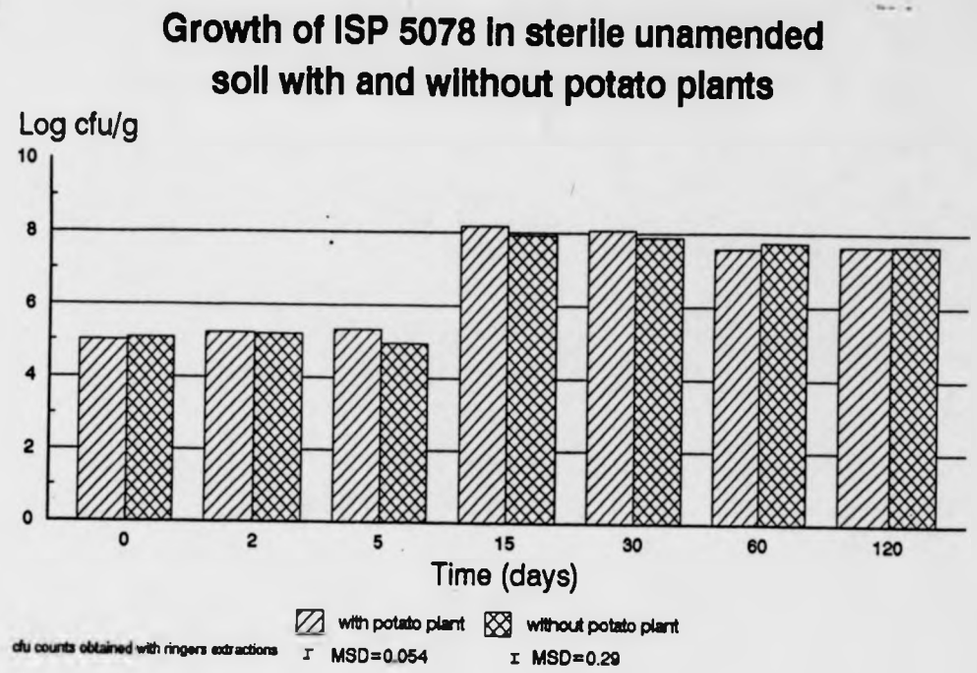


Figure 34a

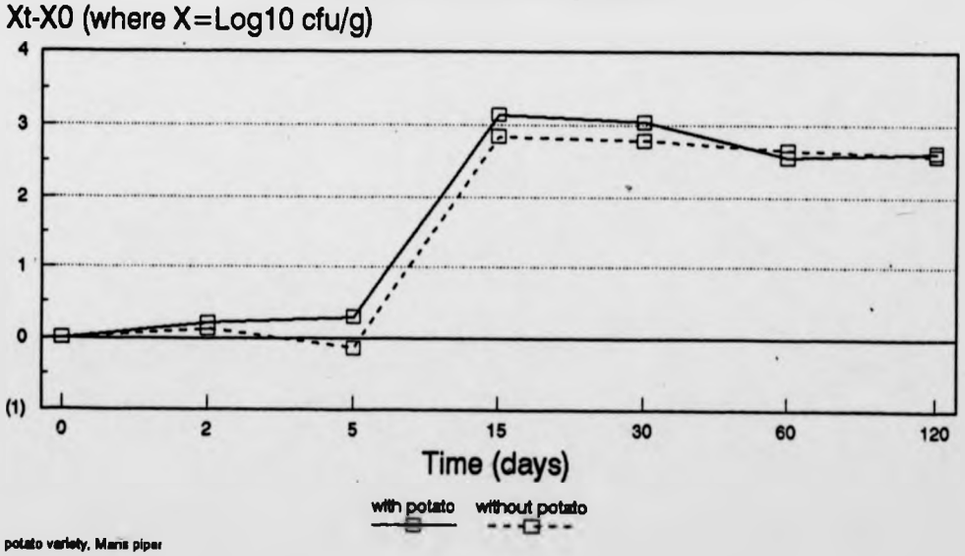


Figure 34b

Fig.35 Growth of ISP5078 in sterile amended and unamended soil in the presence of potato plants.

Fig. 35a Histogram bar chart. Considerable differences in the level of the ISP5078 population was observed in the presence and absence of amendments. The final population in the presence of amendments (1% starch & 1% chitin) exceeded that in the absence of amendments by an order of 1 to 1.5 $\log^{10}$ c.f.u. g^{-1}.

Fig. 36b The scaled graph illustrates the population of the ISP5078 inoculant against time, relative to its initial level at day 0. Between the sample days 2 and 5, the growth rate of the inoculant appeared to be faster in the presence of amendments. From day 5 onwards, the population increases in amended and unamended soils are parallel indicating similar growth rates.

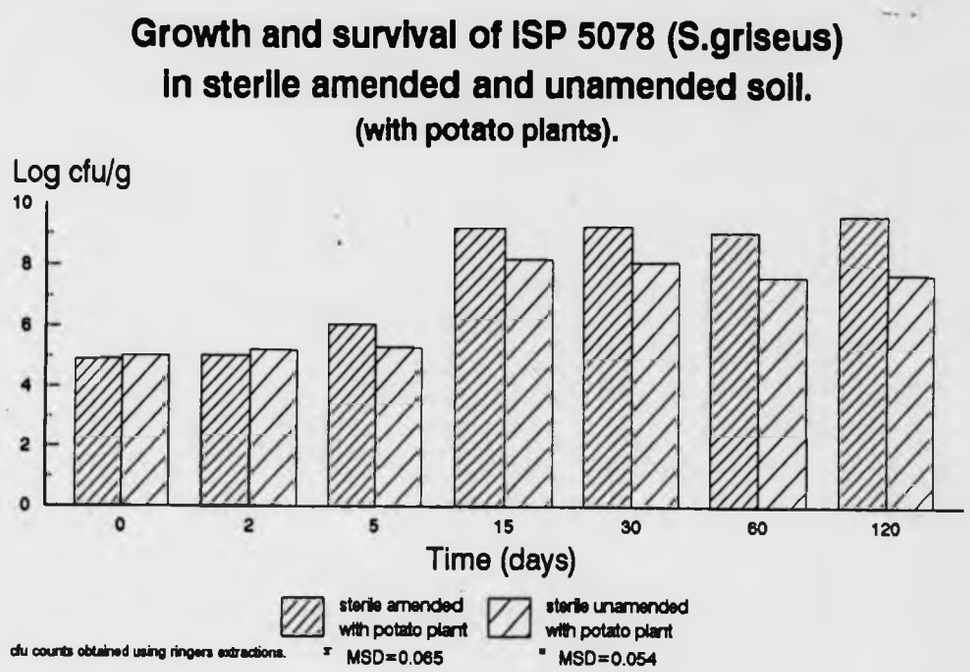


Figure 35a

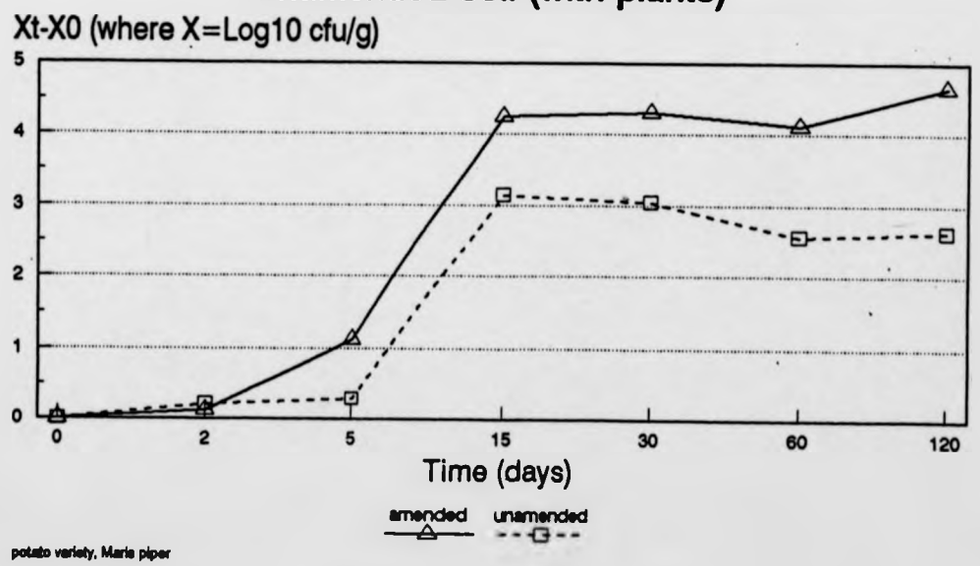


Figure 35b

Fig.36 Growth of ISP5078 with and without amendments in the absence of plants.

Fig.36a Histogram bar chart. Similar trends were observed to those in Fig.35, hence populations of ISP5078 were enhanced 1 to 1.5 Log10 c.f.u. g^{-1} in the presence of amendments.

Fig.36b Scaled graph. Similar to the trends observed in Fig.35 a lag or initial difference in the growth rate was observed between the growth of ISP5078 in amended and unamended soil.

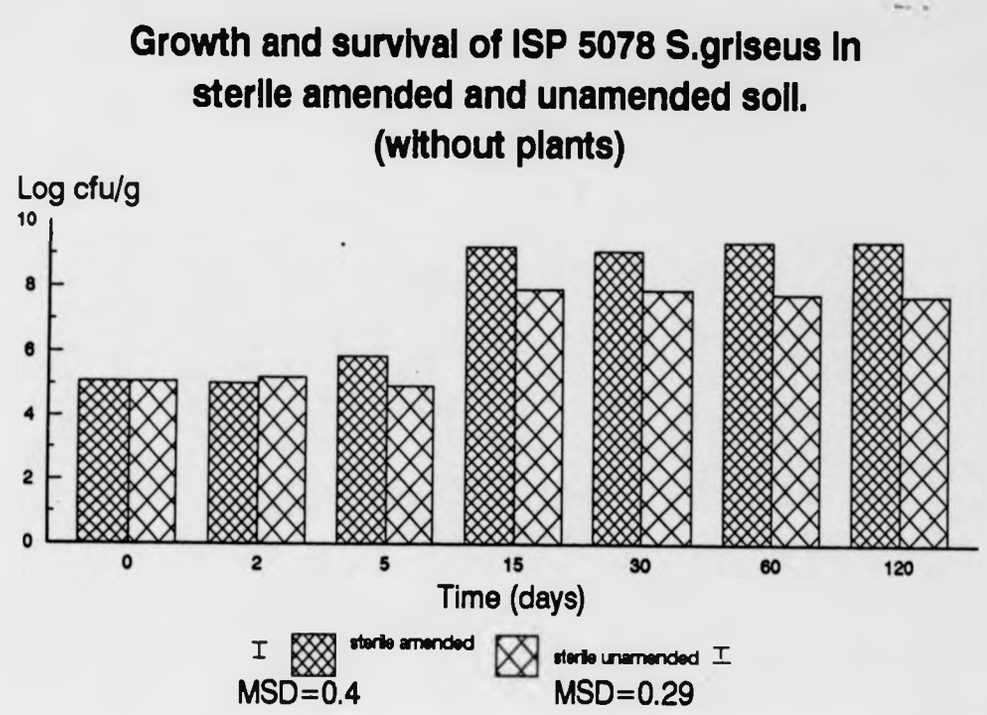


Figure 36a

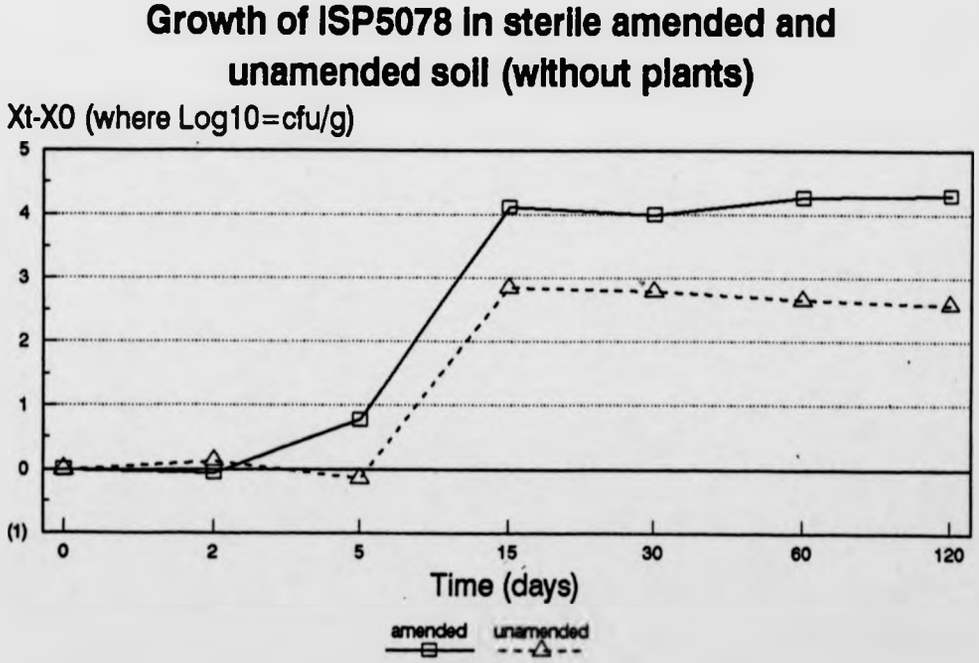


Figure 36b

(Fig. 35) or absence (Fig. 36) of plants. They indicate that the growth rate of ISP5078 was faster (at least initially), in the presence of amendments than without.

The population ratios for the four different regimes, including R : S and amended : unamended ratios are summarised in Table 19. While they illustrate the population differences particularly with the amended and unamended treatments, the ratios are small when compared to those reported as a result of enrichment by the rhizosphere in natural soils. However, this study was performed in sterile soil rather than a natural soil and as such the background soil populations were very high. The autoclaving process released nutrients and minerals into the soil for which there was no competition, hence the growth and development of a greater population of streptomycetes was supported by the bulk soil. Natural soils are also nutrient limited so that exudates from the rhizosphere encourage prolific microbial growth relative to the bulk soil. The 20 g volume of soil, was mixed and sampled uniformly assuming that as a result of the small soil volume, all the soil would be under the influence of the rhizosphere. However, this sampling approach was found to be too insensitive to detect enhanced microbial populations as a result of the presence of a plant. This experiment was run as a batch system, it was set up at 15% (w/w) soil moisture content at day 0 and incubated at 20°C for 120 days. As the system was contained, but did not receive additional moisture and nutirents the plants germinated and grew well for a month, but after this time began to dry out and die off. The interaction between the plant and the streptomycete population was therefore only studied in the early stages of plant development. In a further experiment it was decided that it would be interesting to investigate the plant microbe interaction as the plant developed and at later stages of plant development as enhanced populations have been

Table 19 Streptomycete population ratios with and without amendment and with and without plants

	Ratio	% increase
AM/UA	1.10	11
AM/UA + potato	1.20	20
R/S UA	1.04	4
R/S AM	1.03	3

Note: Lambert and Loria R/S ratios for field populations of streptomycetes 26 in 1986 and 63 in 1987.

associated with older plant roots (Watson & Williams, 1974; Williams, 1976). Furthermore, since the widely reported rhizosphere effect was not detected in the present experiment, it was decided to sample and enumerate the microbial community from the bulk soil and the root surface in a future experiment.

6.3.3.2. Hybridization data

The 16S rRNA was extracted from a 5 g sample of soil (from the sterile amended treatments without plants). Unfortunately, the blot required a long period of exposure to recover the autoradiograph (Fig 37b). The weak hybridization signals were probably a function of poor binding of the probe and contaminating humic acids. However, the relative signals from the blot (and photograph of the gel, Fig. 37a & 37b) support the trends observed in the viable count data. Thus rRNA was barely discernible from soil samples from days 0 and 5 and not discernible at all at day 2, the corresponding counts from these days were the lowest in the experiment. The hybridization signals from days 15, 30, 60 and 90 were much stronger and correspond to a two log increase in the streptomycete c.f.u. counts. A negative control, uninoculated sterile soil was set up in duplicate and gave no hybridisation signal, indicating that the signals were attributable to the RNA rather than soil constituents.

The extractions appeared to be reproducible, as duplicate samples agreed for days 0, 5 and the negative control. Duplicate samples were also prepared for day 90, but unfortunately the first extractions of the soil from both samples were combined in one duplicate and the second extractions combined in the second duplicate. Since most of the RNA comes out with the first extraction the blot has one blank spot and one spot twice as dense for this sample day.

This unfortunate error reflects the great number of manipulations in the

Fig.37a Ribosomal RNA recovered from ISP5078 growing and surviving in sterile amended soil over a 90 day period.

Ribosomal RNA was extracted from the inoculant in soil according to Hughes & Galau (1988) at the following sample times, days: 0, 2, 5, 15, 30, 60 & 90. An uninoculated negative control was also included, gel lane -. Duplicate rRNA preparations were made from soil samples from days 0, 5, 90 and the negative control. In general, the preparations were similar and the technique appeared reproducible. Unfortunately, the first rRNA extractions from both duplicates from day 90 were combined, resulting in one lane with alot of RNA and another without any visible rRNA.

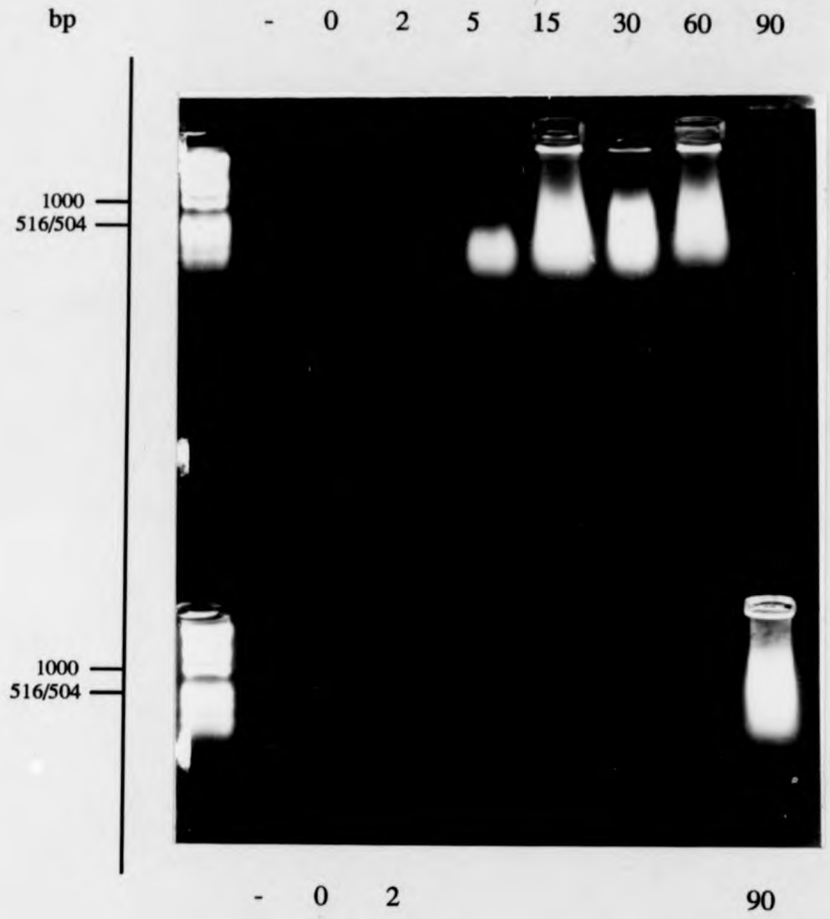


Figure 37a

Fig.37b Dot-blot hybridization of rRNA recovered from ISP5078 growing and surviving in soil over a 90 day period.

Ribosomal RNA recovered from soil (Fig. 37a) was hybridized with the β1 targeted oligonucleotide probe (Stackebrandt *et al.*, 1991) under stringent conditions (50^0C). Hybridization signals were obtained from RNA recovered from soil samples from days: 0, 5, 15, 30, 60 and 90. Duplicate samples were prepared for days 0, 5, 90 and the uninoculated negative control. The signal for day 90 was twice as intense as it should have been, as extractions for both duplicates were inadvertently combined. The relative intensity of the rRNA signals reflected the viable plate count population data. Hence signals were weakest between days 0 to 5 corresponding to lower plate count populations. Where as the increased viable count from days 15 onwards was reflected by a stronger hybridization signal.

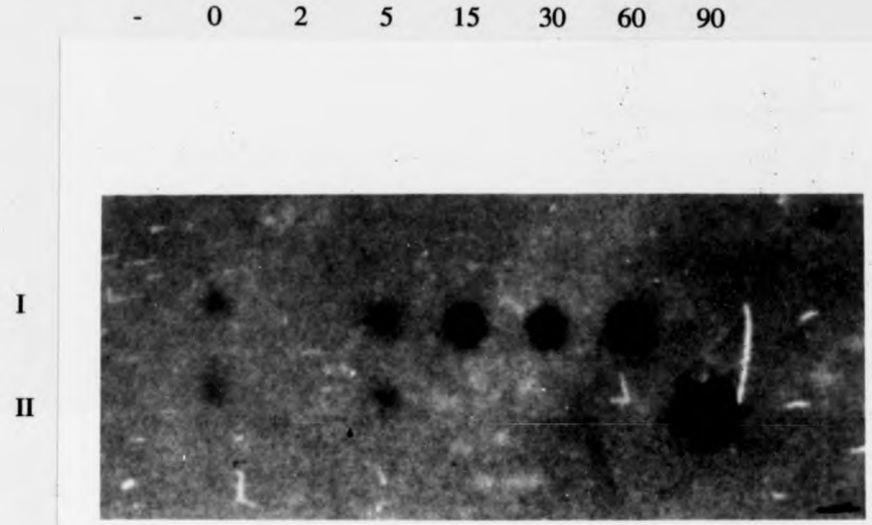


Figure 37b

extraction protocol, that can extend over several days. For example if 5 g of soil is to be analysed 12.5 g of glass beads are added together with 20 to 25 ml homogenisation buffer for beadbeating and consequent extraction. An equal volume of extraction buffer is required to wash the soil and beads to recover any remaining RNA, producing volumes of about 50 ml per sample. Procedures for the precipitation of the nucleic acids amplify the volumes further, 100 to 120 ml. Such volumes generally have to be divided into 4 separate tubes for centrifugation. This experiment involved 13 samples.

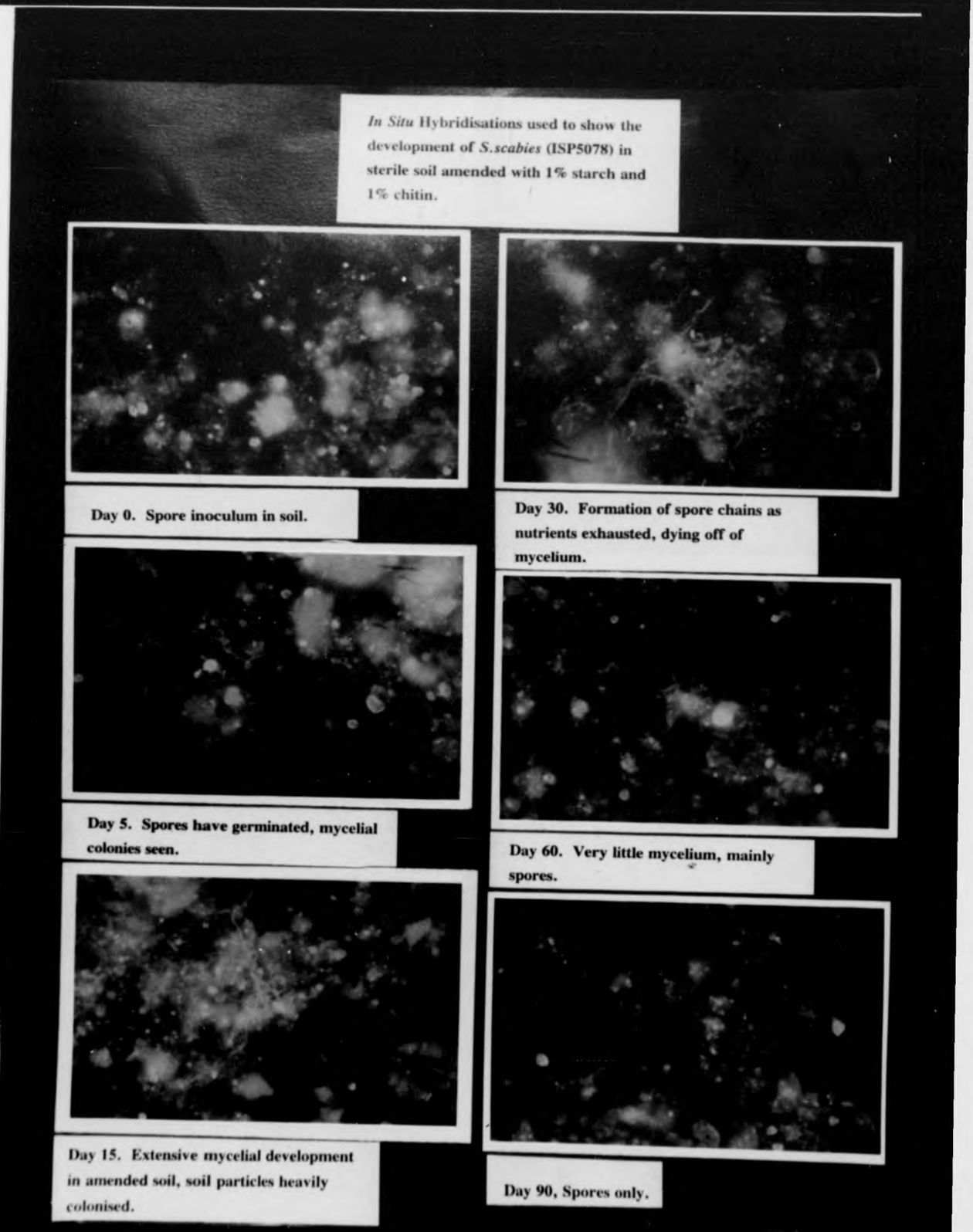
6.3.3.3 *In situ* hybridisations

In situ hybridisations were performed on samples of soil from all microcosms. Hybridisations were attempted with the β1 probe that initially had appeared to be specific for ISP5078, however, the technique was not amenable to this combination of strain and probe. Since the technique depends upon the probe penetrating the cell, aligning and hybridizing with the target sequence, the 2^0 and 3^0 structure of the molecules remain largely intact and may therefore obscure certain target sequences. In addition there are proteins associated with the ribosome, which may also block binding sites. It might be possible to make the target sequence accessible to the probe by proteinase and other denaturing treatments. However, for the purposes of illustrating the approach under sterile conditions we used the eubacterial probe primer 1115, which consistently hybridizes well.

Flourescent light micrographs were taken of the sterile amended (no plant) series of treatments. These photographs also reflect the development of inoculants in sterile amended soil with potato plants (Fig. 37c). In contrast, very little mycelial development was seen in the unamended soil treatments, only one or two very small microcolonies were observed in the day 15 samples

Fig.37c *In situ* hybridization studies of the growth and survival of ISP5078 in sterile amended soil over 90 days.

The *in situ* hybridizations were performed with a rhodamine labelled, primer 1115, Eubacterial oligonucleotide probe. The growth and development of the ISP5078 inoculant was observed in sterile amended soil. At day 0, the spore inoculum could not be discerned from the background autofluorescence of the soil minerals. By day 5, the spores have germinated and small mycelial microcolonies were observed. At day 15, prolific mycelial development and colonisation of the soil substrate was apparent. While at day 30, the transition from the mycelial stage of the life cycle to the production of spore chains was observed. By day 60, partial fragmentation of the spore chains had occurred, as only small residual chains of spores could be observed. Day 90, total fragmentation of spore chains had occurred and single spores could not be discerned from the background autofluorescence.



which, in contrast to the amended soils, revealed prolific, ramifying mycelial growth. In unamended soil, the inoculants appeared to reside in the soil as single spores or small fragmented spore chains not unlike the photographs of day 0 and day 90 from the amended series. This vast difference in the colonisation of the soil matrix was reflected in a 1.0 to 1.5 log difference in the c.f.u. viability counts.

This approach allowed direct visualisation of the lifecycle of *Streptomyces* in soil. Findings indicated that spores germinated to produce ramifying mycelial networks that colonise soil particles and crevices in the presence of nutrients and moisture (days 5 to 15). As the nutrients and moisture were depleted spore chains were produced and the vegetative mycelium died off (day 30), the spore chains fragmented to become shorter and fewer (day 60) until single spores were produced that were no longer discernible from the background autofluoresence of the soil minerals (day 90).

Thus in a nutrient rich environment, either artificially amended or in association with plant root amendments, this approach in conjunction with specific probes offers the possibility to study inoculants *in situ*. The technique is particularly powerful when applied to microorganisms like the *Streptomyces* with a distinctive lifecycle that is indicative of activity and can be analysed by microscopy. In addition to information on the presence and abundance of the strain *in situ*, the technique may be used to provide information on the activity of the strain. As ribosomal content and hence the strength of fluorescent signals may be correlated with biosynthetic activity (Giovanonni *et al.*, 1988).

Conclusion

The three detection approaches applied to the study of ISP5078 in soil were

complimentary and in agreement with regard to the information generated about the inoculants. The viable plate counts, provided numerical population data of c.f.u. in soil that was amenable to statistical analysis. The dot blot approach allowed detection of the culturable and non culturable components of the soil population. It may be calibrated with known quantities of cells and or RNA together with densitometric analysis or quantification of the radioactive disintegrations if probes are radio-labelled, to give estimates of the population density in soil. In the present study the streptomycete inoculant was culturable and as this approach was less sensitive than the traditional viable plate count, it provided least information about the inoculant in soil. If however, it was not possible to deselect the inoculant from a non-sterile soil microflora, but a highly specific probe was available this approach would yield more information. The combination of methods for the direct extraction of nucleic acids from soil with amplification via PCR promises to reduce detection levels from those achievable with viable plate counts (Steffan & Atlas, 1988; Steffan & Atlas, 1991). These approaches have and are likely to continue to focus on the amplification of DNA rather than rRNA (via a reverse transcriptase step) as DNA is more inherently more stable and therefore more amenable to environmental analysis.

Finally, the *in situ* hybridisation technique allowed the interactions between the microbes and environmental substrates to be observed and patterns of growth and activity to be visually recorded. This approach offered insight into the heterogenity of the terrestrial environment, the non-uniform distribution of microbes and colonisation of microsites. It provides information that is obscured by the two previous approaches. This technique is also applicable to non-culturable microorganisms and will no doubt be of value in unravelling their contribution to certain ecosystems (Amann *et al.*, 1992).

6.3.4 Experiment 3

Aim: To study the influence of the potato plant on the growth and activity of strain ISP5078 in sterile soil.

6.3.4.1 Experimental approach

This experiment was a development of the proceeding one, thus the microcosms were set up as before with the following amendments. Four week old plantlets were introduced into the microcosms at day 0, rather than as sprouting minitubers. The aim was to establish mature plants within the microcosms, with extensive root systems so that all bulk soil would be under the influence of the rhizosphere. The water content in the soil was monitored at each sample date and adjusted to 15% moisture holding capacity throughout the course of the experiment.

Microcosms were sampled at days 0, 2, 5, 15, 30 and 60. In addition to the soil samples taken, the roots were recovered, chopped finely and divided into 5 equal and comparable portions. One was used to determine the dry weight, three were weighed and added to 2 ml 1/4 strength Ringers solution for the determination of viable plate counts. The final sample was fixed in 3% glutaraldehyde and used in scanning electron microscopy studies.

Inoculants were monitored by viable plate count procedures as before and via scanning electron microscopy. These approaches were chosen as they offered maximum information without exhaustive manipulations. The information that we obtained with *in situ* hybridisation being comparable to that of scanning electron microscopy (while under sterile conditions). *In situ*

hybridisations are of value for determining the identity of particular inoculants from mixed cultures or non-sterile environmental samples when used in conjunction with specific probes. Furthermore, the higher magnification afforded by scanning electron microscopy offered enhanced resolution.

6.3.4.3. Results

6.3.4.3.1 Viability data

The plate count data has been illustrated as a histogram (Fig. 38a) and a scaled graph (Fig. 38b). Two of the three sets of data consider the populations of ISP5078 in soil, both in the presence and absence of the potato plant. Interestingly, despite the presence of mature plants and extensive root systems, no differences in population levels was discernible. Hence any impact that the rhizosphere or plant had was lost within the 20 g microcosm and must therefore be localised. The population levels of streptomycetes isolated from root material were significantly higher, by an order of magnitude. Root material did appear to support a higher streptomycete population than the sterile soil. Undoubtedly streptomycetes will be concentrated in certain areas of the root tissue, depending on age, the amount or quality of exudates and absent in other areas. This figure may an underestimate as the efficiency of recovery was not assessed.

6.3.4.3.2 Scanning electron microscopy data

Scanning electron micrographs were taken of soil and root samples from each of the sample days (Fig.s 39 to 47). Similar to the *in situ* hybridisation data, they emphasised the difference (that is suggested in the viability count data) in the numbers of streptomycetes associated with the root and those in the soil. While, visualising the organism on the root surface was easier than in soil, fragmented spores were difficult to find in soil, but obvious on the root, the

Fig.38 Growth and survival of ISP5078 in sterile soil with and without axenic potato plants.

Fig.38a A histogram of the viable plate counts for c.f.u. g^{-1} soil/ root of ISP5078 growing and surviving in sterile soil, both with and without a potato plant and in association with the potato root. Elevated population counts of the ISP5078 inoculant were observed in association with the root material. The c.f.u. counts g-1 root material were 1 to 1.5 log10 higher than those recovered from sterile soil (with or without an axenic potato plant). This difference was of the same magnitude to that observed between sterile amended and unamended soil. Counts at the root surface were significantly higher at day 0 than those obtained from soil samples. This was probably as a result of the procedure in which the microcosms were seeded with inoculant. It was added to the soil surface and allowed to percolate through the matrix; since the plant was placed in the surface layers of the microcosm it would come into contact with more inoculant at day 0 than would be distributed in the bulk soil.

Fig.38b Scaled graph. No population differences were observed in the viable plate counts obtained from soil planted with potatoes compared to unplanted soil. The elevated counts observed in association with the roots indicated that the influence of the rhizosphere is localised and can only be detected by sampling the root rather than the soil material.

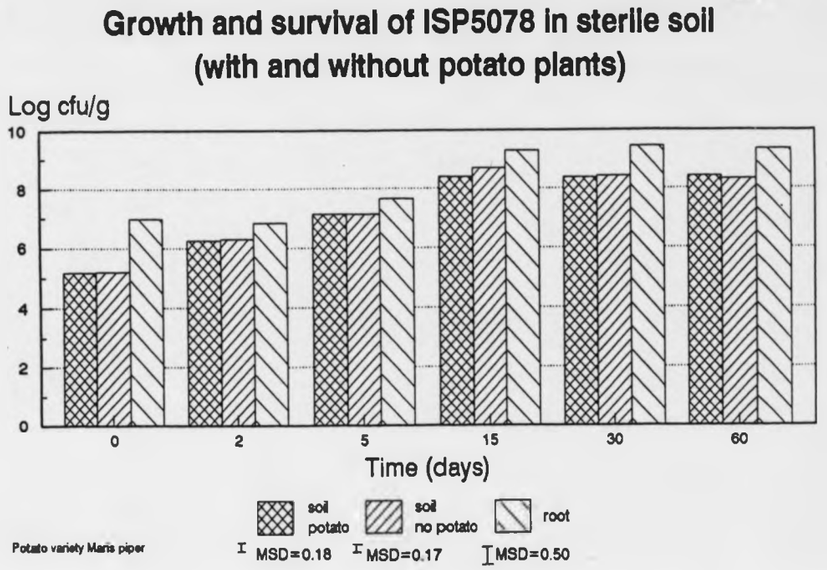


Figure 38a

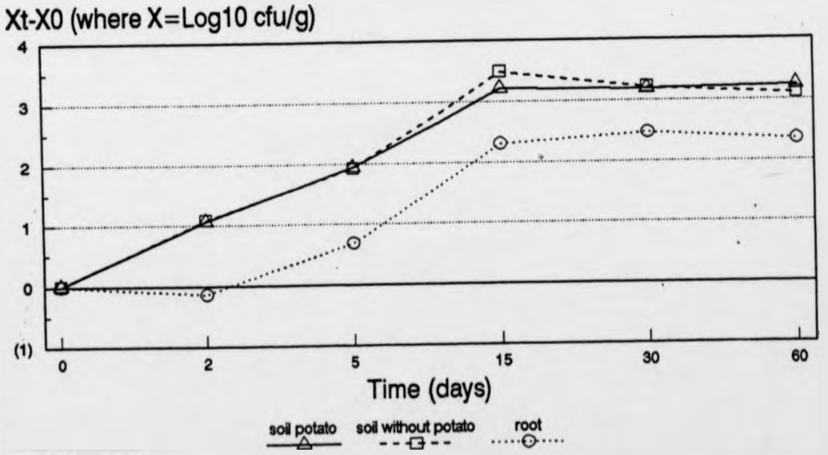
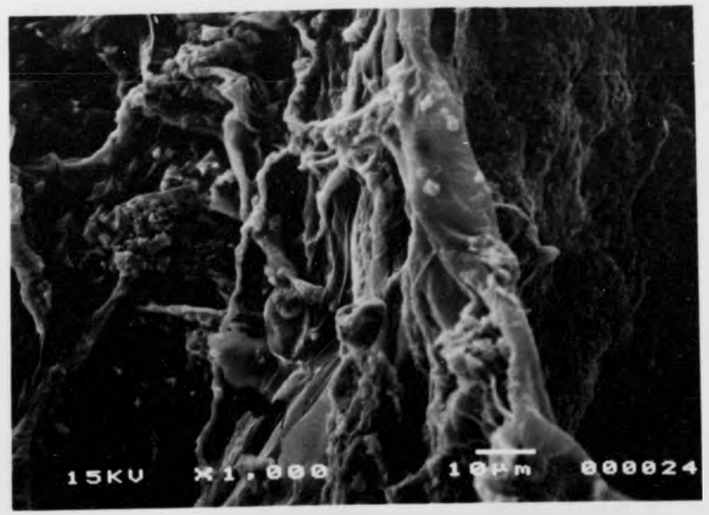


Figure 38b

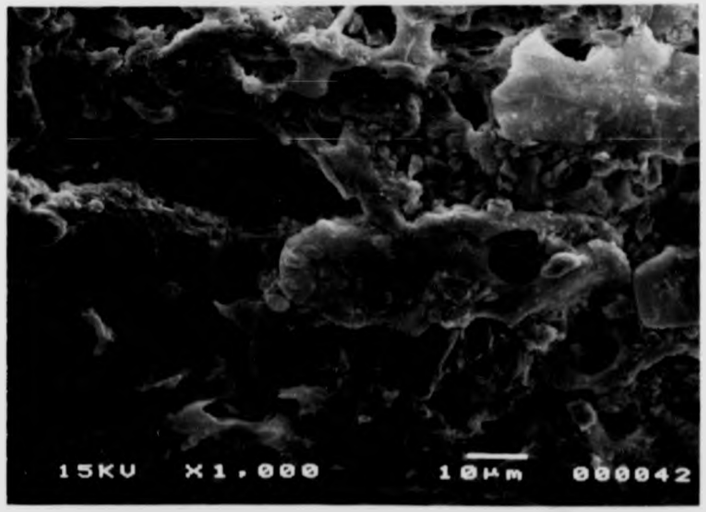
Fig.39 Scanning electron micrograph ISP5078 colonising the potato root surface.

Magnification X 1,000. Day 0, soil and root material. No evidence of the streptomycete spore inoculum.

Day 0



Potato root

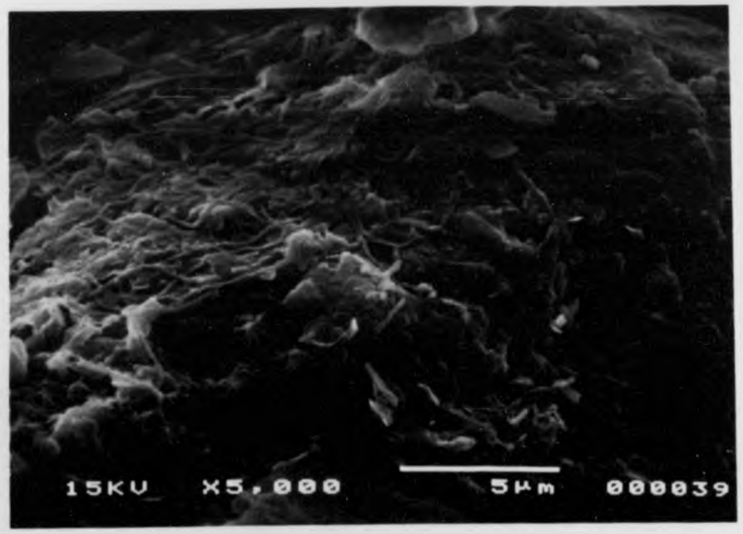


Soil

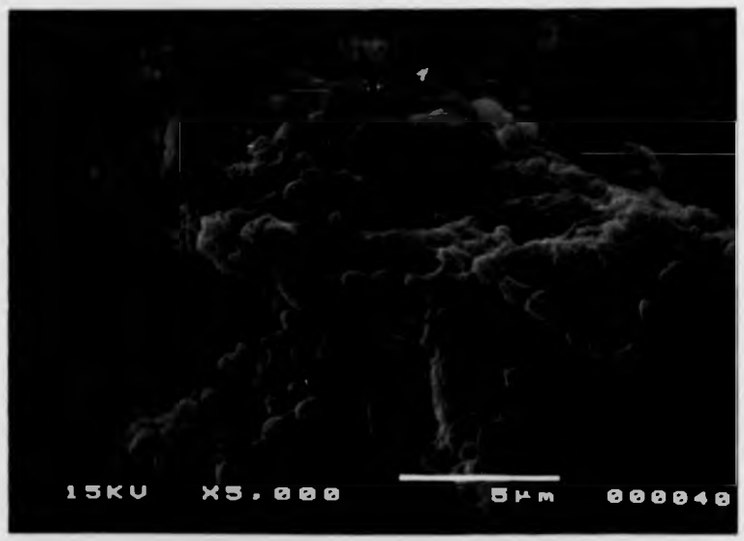
Fig.40 The development of streptomycete inoculants at day 60.

Magnification X 5,000. Inoculants were present in the mycelial and spore growth form at day 60.

Day 60 Potato root



Mycelial development

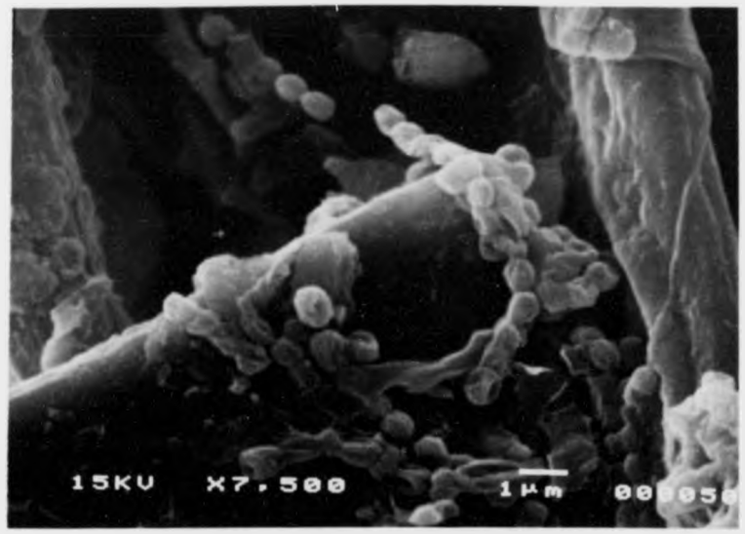


Sporulation

Fig.41 Fragmentation of spore chains (day 60)

Magnification X 7,500

Day 60 Potato root



Fragmentation of spore chains

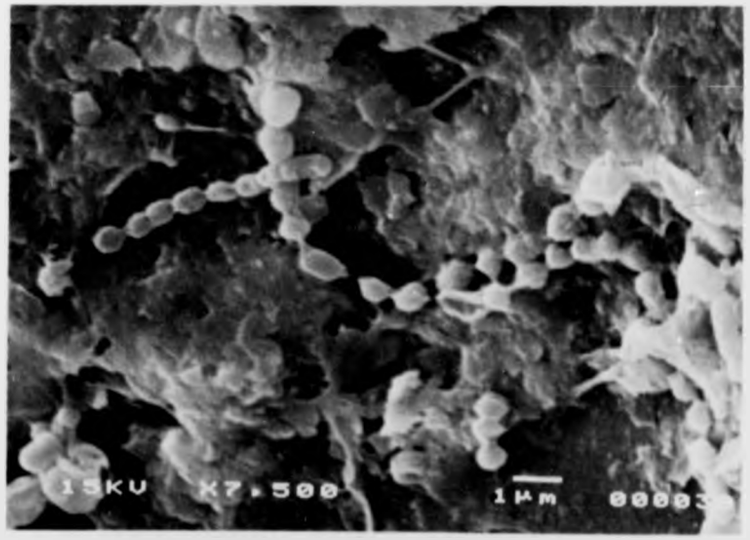
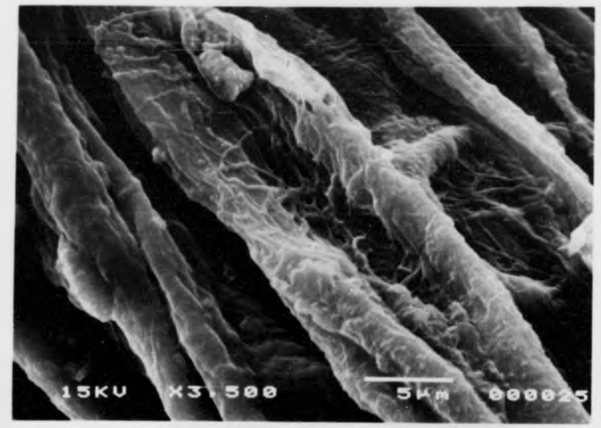


Fig.42 Germination and mycelial development of ISP5078 at the potato root surface at day 3.

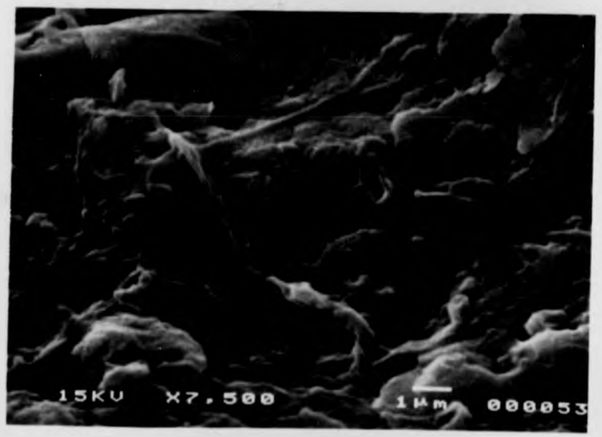
Magnification X 3,500, low power view of the colonisation of the potato root by the ISP5078 inoculant. Spores could not be observed.

Magnification X 7,500, higher power view of collapsed mycelial filaments on the potato root surface, day 3.

Day 3. Potato root



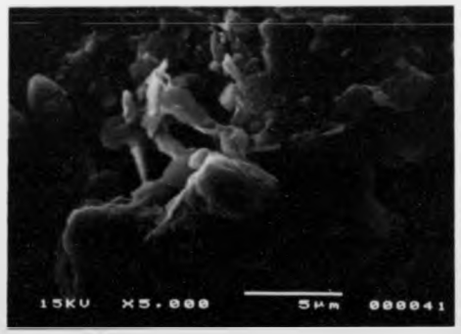
Mycelial development



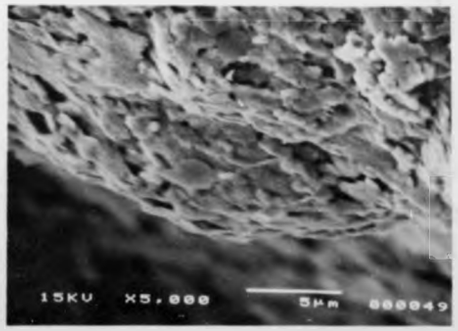
Germinating spores

Fig.43 Scanning electron micrograghs of sterile soil colonised by ISP5078.

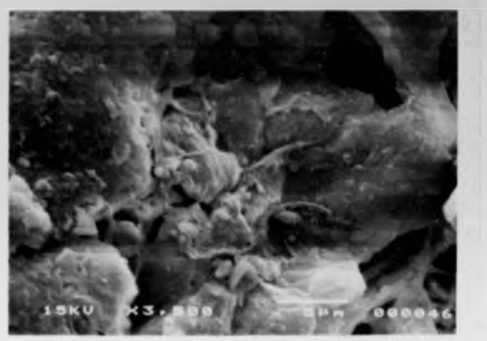
The surface of soil particles was photographed from samples at days 3, 5, 15 and 60. No evidence of the inoculant was observed in any of the samples of soil examined. The soil environment provided a stark contrast to the nutrient rich environment of the potato root, which supported abundant growth of ISP5078.



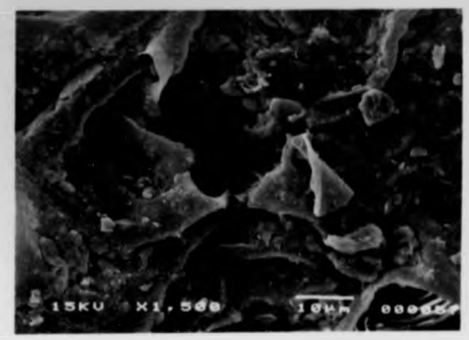
Day 3 Soil



Day 15 Soil



Day 5 Soil

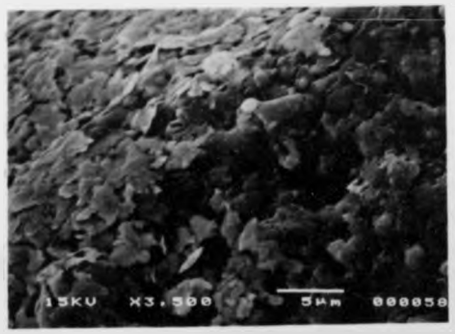


Day 60 Soil

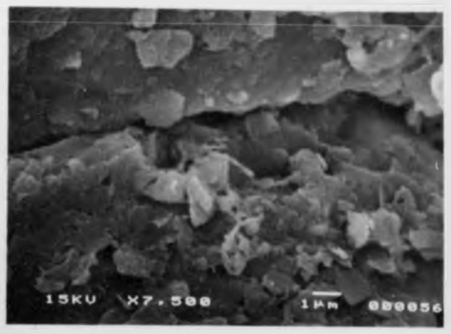
Figure 43

Fig.44 A comparison of the soil and potato root environments at days 15 and 60.

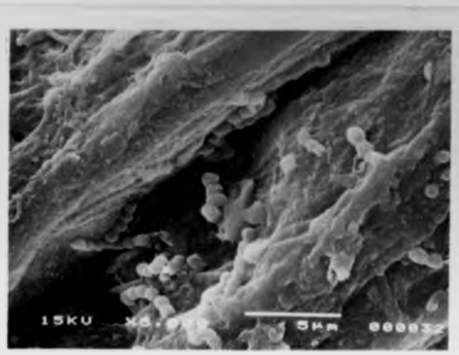
No colonisation of the soil was apparent at days 15 and 60. In contrast the root surface exhibited some mycelial development and sporulation at day 15 and prolific sporulation was observed at day 60.



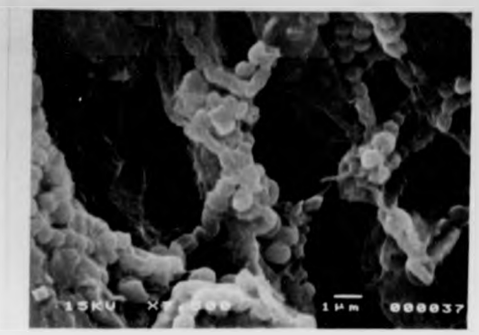
Day 15 Soil



Day 60 Soil



Day 15 Potato root



Day 60 Potato root

Figure 44

Fig.45 Time course of the development of the ISP5078 inoculant in association with a potato root.

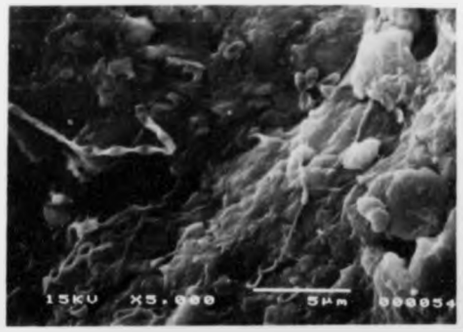
Day 3, germinating spores, some mycelial development.

Day 5, early transitional stages of differentiation from mycelium into spore chains.

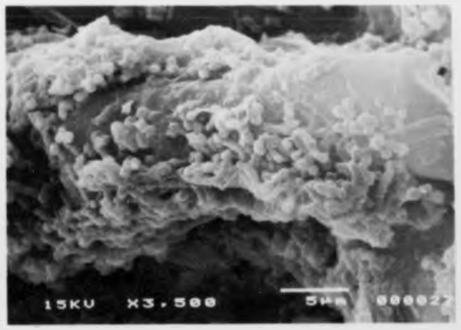
Day 30, prolific sporulation.

Day 60, further sporulation.

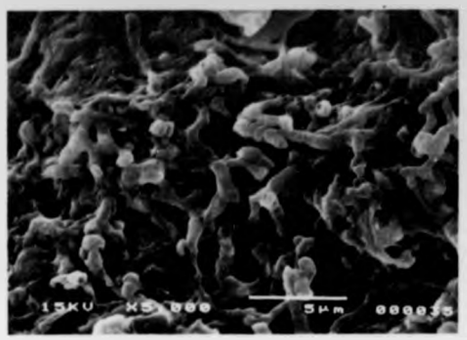
Potato root



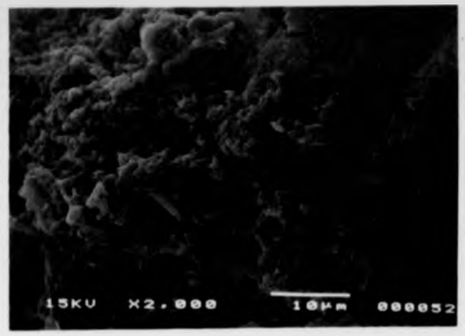
Day 3 Germinating spores



Day 30 Sporulation



Day 5 Differentiation of mycelium into spores



Day 60 Further sporulation

Figure 45

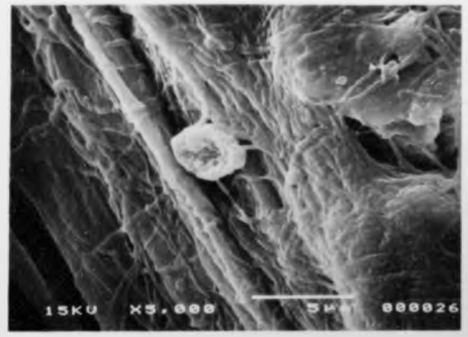
Fig.46 Time course of the ISP5078 inoculant in association with the potato root.

Day 3, mycelial development, colonisation of the root surface.

Day 5, mycelial development and some early sporulation.

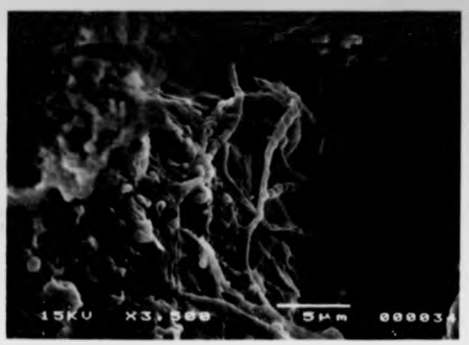
Day 15, spore chains

Day 30, prolific sporulation



Day 3 Potato root

Day 15 Potato root



Day 5 Potato root

Day 30 Potato root

Figure 46

differences in the colonisation of the two substrates (soil and root) appeared considerable. No mycelial development could be observed in the bulk soil. The development of inoculants from germination through the different stages of the lifecycle were visualised at the nutrient enriched root surface. The growth of inoculants at the root surface appeared more dynamic as a result of the developing plant and introduction of the water regime (than seen in experiment 2). At day 60 (Fig. 40) it was possible to observe both the production of spores and the mycelial development of inoculants on different sections of root material, while in experiment 2 the moisture and nutrients had been exhausted at this point and inoculants were present mostly as spore chains. The scanning electron micrographs demonstrated the heterogeneity of the environmental substrate and its non-uniform colonisation by the inoculant.

6.3.5 Experiment 4

6.3.5.1 Aim: The evaluation of a kanamycin resistant mutant and plasmid pIJ673 in sterile soil as selective markers for the detection and monitoring of ISP5078.

6.3.5.2 Experimental design

20 g sterile soil microcosms were set up in duplicate and single inoculations made with the ISP5078 kanamycin resistant mutant, ISP5078 harbouring plasmid pIJ673 and the original ISP5078 strain.* The water content of microcosms was monitored at the times of sampling and adjusted to 15% soil moisture (w/w). Soil from the first two treatments was plated out on the appropriate selective media i.e. R5 with kanamycin for the mutant strain and R5 with neomycin for the strain harbouring the plasmid. Mutation reversion and plasmid loss, or inability of the strain harbouring the plasmid or mutation to grow on the selective media was monitored by plating on R5 without

selectives.

R5 media was used in these studies, as a number of problems were encountered with the combination of the highly selective RASS medium, certain selective agents and these strains. Despite routine subculture and the production of spore inocula from both strains on the RASS antibiotic media, after a cycle of freezing and thawing the strains appeared unable to grow on this selective combination. A number of attempts were made to obtain growth on RASS with selective antibiotics including a transfer to RASS without antibiotics, on which the strain grew but still would not grow when transferred back to RASS with selective additions. The strain was also introduced into soil and incubated for 48 h but again could not be recovered on RASS with antibiotics, even with reduced antibiotic concentrations. These problems were overcome completely by using the R5 media in combination with antibiotics. While this did not affect the present study under sterile conditions, it did make the application of these markers to non-sterile conditions difficult as a minimal and selective media like RASS is essential for the deselection of indigenous microbes and therefore in obtaining reliable viability data from non-sterile soils.

6.3.5.3 Results and conclusions

Viable c.f.u. count data is given in the form of histograms and scaled line graphs for the kanamycin resistant strain (Fig. 48) and strain containing the plasmid (Fig. 49).

6.3.5.3.1 The kanamycin resistant mutant

At day 0, comparable counts were obtained for ISP5078 and the Km^R strain both on the selective and non-selective media, indicating that at the outset the

Fig.48 Stability of ISP5078 kanamycin resistant mutation in sterile soil.

Fig. 48a The stability of the mutation was monitored in soil by plating on R5 + kan and R5 without selection. The growth of the mutant strain was also compared with the ISP5078 parent strain. The c.f.u. viable plate counts g^{-1} soil for the ISP5078 control strain and the Km^R strain in the absence of selection were very similar. The counts obtained for the Km^R strain in the presence of selection were however, approximately 1 log10 less at day 2. Counts obtained on kanamycin continued to be lower than the counts obtained in the absence of selection for the remainder of the experiment.

Fig. 48b Scaled graph. This further illustrated the initial drop in the c.f.u. count of the Km^R strain at day 2 and which was maintained throughout the duration of the experiment. The loss of Km^R phenotype was probably co-incident with the initial burst of germination, growth and cell division.

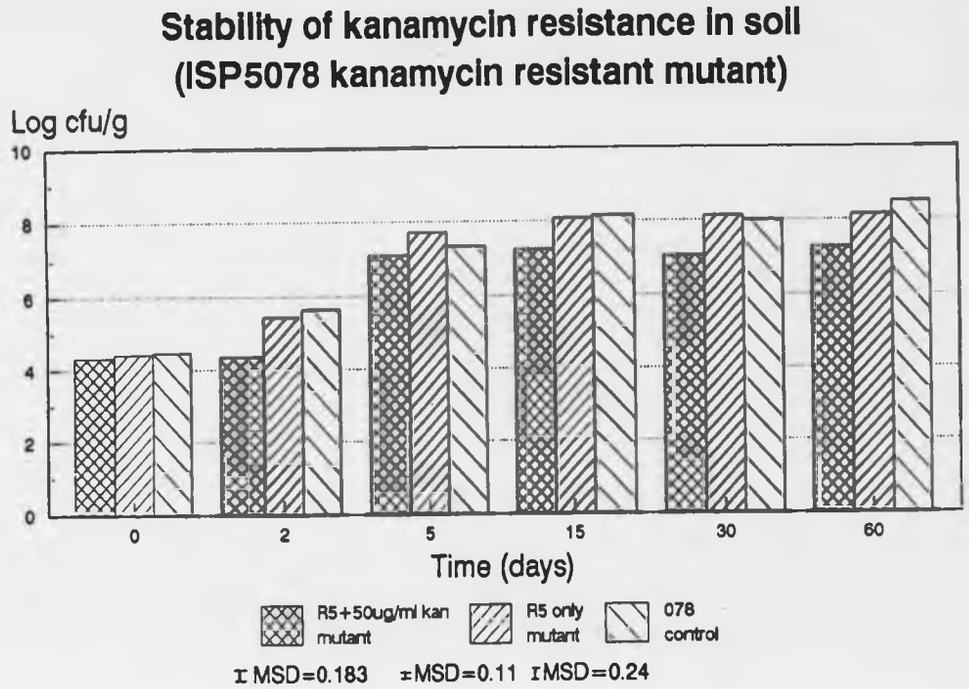


Figure 48a

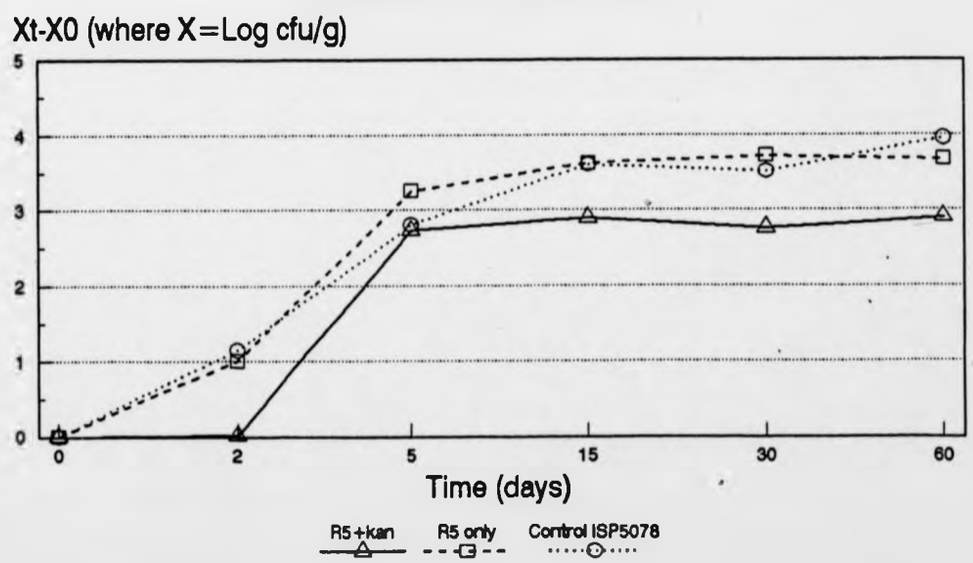


Figure 48b

Fig.49 Stability of plasmid pIJ673 in ISP5078 in sterile soil.

Fig.49a Histogram of log10 c.f.u. g^{-1} soil. The stability of the plasmid was monitored by plating ISP5078 harbouring the plasmid on R5 with and without neomycin selection. Comparisons in the population count were made for the inoculant with the plasmid and the control, ISP5078 parent strain. The population of ISP5078 + pIJ673 recovered on neomycin was significantly lower than the population recovered without selection on day 2. The difference between the count in the presence and absence of selection varied throughout the course of the experiment. It was however, maintained. Counts in the absence of selection for the plasmid containing strain were comparable to those obtained from the control parent strain. Loss of the plasmid at day 2 was coincident with the initial burst of germination, growth and cell divison.

Fig.49b The scaled graph clearly depicts the difference between the plasmid containing population recovered on selection and that recovered in the absence of selection. The population differences were indicative of plasmid loss and instability in this host background.

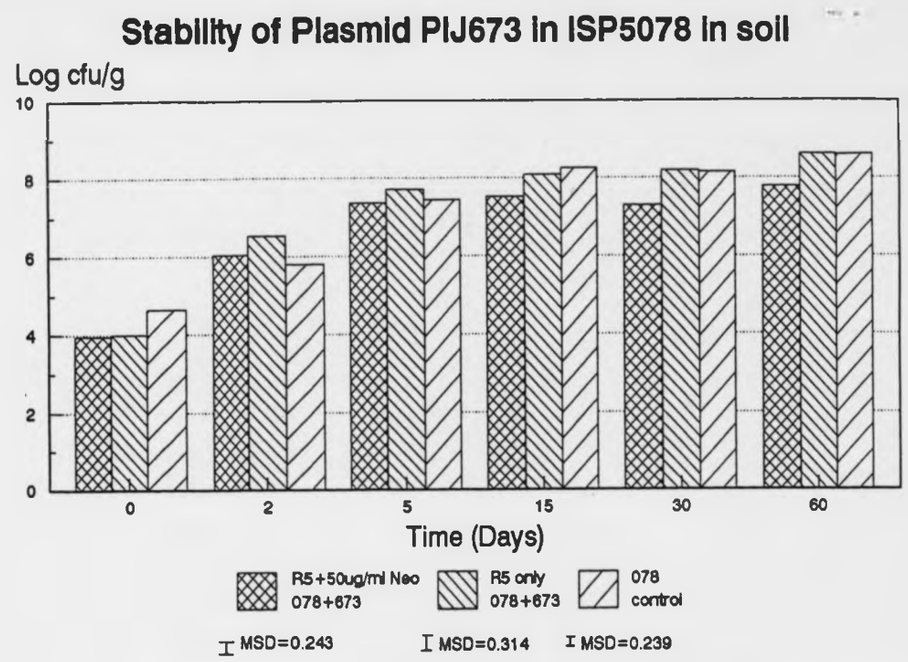


Figure 49a

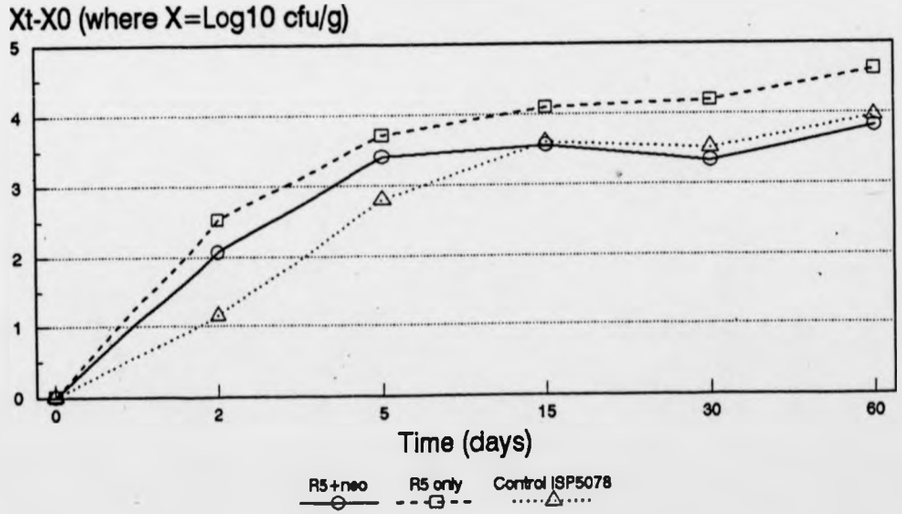


Figure 49b

ISP5078 Km^R population was resistant to kanamycin. However, by day 2 the proportion of the Km^R strain recoverable on kanamycin had decreased significantly from the total population recovered on non-selective media (counts were 1 log lower). The log difference in the proportion of the population recoverable on kanamycin was maintained throughout the course of the experiment. This initial reduction was probably associated with the initial burst of germination, growth and sporulation that occurred following inoculation. The strain having exhausted the available nutrients, sporulated and remained in the soil as spores. To obtain a broader picture on the reversion of the mutation in soil it would be necessary to expose the strain to further rounds of germination, growth and sporulation. This could be achieved through the addition of nutrients and moisture or with a transfer to uncolonised soil to stimulate growth and activity (Cresswell *et al.*, 1992). The growth and survival of the ISP5078 control appeared comparable to the total Km^R population recoverable on R5 only. The Km^R population differed morphologically from the original strain, the difference was most marked in the absence of selection.

6.3.5.3.2 ISP5078 harbouring pIJ673

Similar to the findings of the proceeding experiment a proportion of the ISP5078 (harbouring pIJ673) population lost the plasmid at day 2. This co-incided with germination and mycelial development and would be associated with segregation at cell division. The histogram illustrates the discrepancy between the total and plasmid containing population, which becomes greater between days 15 and 30 and was maintained throughout the course of the experiment. The scaled graph suggests that the transformed population have a higher growth rate than the original ISP5078 strain and obtain higher populations relative to day 0 at day 60. However, the control strain was

introduced at a higher population density than the mutant, so that direct comparisons of growth rate are difficult. Both strains reached similar final populations in soil. Again, it is possible that the plasmid loss observed resulted of one round of growth and sporulation of the strain in soil and that the strain remained as spores in the soil for the duration of the experiment. Exposing the strain to several rounds of germination and sporulation and monitoring plasmid loss after each round might indicate a similar proportion of loss with each cycle.

6.3.5.4 Conclusions

The latter two experiments, do signify that both the kanamycin mutation and plasmid are unsuitable candidates as markers in ISP5078. The kanamycin mutation appeared to have a high reversion rate and the plasmid to be unstable in soil. Rates of reversion and stability were not investigated *in vitro*, which would be an important preliminary step in the evaluation of a promising mutation or plasmid. Both experiments resulted in reduced resistant populations after one round of germination and sporulation with populations an order of magnitude smaller than the total population. Thus it would be possible to lose the strain in certain dynamic environments after several rounds of germination and sporulation. However, since pIJ673 appeared quite stable in the MP2 host background, it would be interesting to compare the growth of ISP5078 + pIJ673 and MP2 + pIJ673 under the same growth conditions. This may allow further insights into the behaviour of the plasmid in different host backgrounds and under different environmental conditions.

General Conclusions

The microcosm experiments have allowed initial studies of specific scab-causing streptomycetes in association with potato plants and qualitative and

quantitative measurement of the rhizosphere effect. They have also permitted the evaluation of several approaches to monitoring in sterile soil that might be applied to studies *in situ*. Studies under non-sterile conditions assisted in demonstrating the short-comings of viable plate count procedures for monitoring despite the culturability of the inoculant. They indicated the need for alternative approaches such as the direct extraction and characterisation of nucleic acids from environmental samples under these conditions. Approaches for marking genetically uncharacterised strains by transformation with plasmids and induced mutations for antibiotic resistance highlighted the problems that either of these strategies may result in unstable markers and that all strains that are generated should be rigourously tested for loss of markers and for comparable ecological fitness to the parent strain. The direct observation approaches of RNA *in situ* hybridization and scanning electron microscopy though not quantitative yielded most information about the growth and activity of inoculants in association with plants and soil.

Chapter 7

Discussion

Chapter 7 Discussion

Confusion over the taxonomic status of *Streptomyces scabies*, the causal agent of common scab of potatoes developed when the type strain put forward by Waksman & Henrici (1948) did not match the original description of the strain (Thaxter, 1891). Confusion was compounded with the deposition of many taxonomically distinct strains found to be common scab pathogens, in culture collections under the name of *Streptomyces scabies*.

Attempts were made to clarify the taxonomic position of scab-causing strains using phenotypic variation (Lambert & Loria, 1989) and more recently on the basis of DNA homology (Healy & Lambert, 1991) [Chapter 3]. Common scab strains have also been included in larger comprehensive studies on the organisation and taxonomy of the *Streptomyces* genus (Williams *et al.*, 1983a, Kamper *et al.*, 1991). Phenotypic characterizations by Lambert & Loria (1989) indicated that scab-causing streptomycetes formed a taxonomically defined group consistent with Thaxter's original description of *Streptomyces scabies*. In contrast the DNA homology studies indicated the genetic distinctness of *Streptomyces scabies* strains conforming to the revived description of *S.scabies* Lambert & Loria (1989) from two other groups of plant pathogenic streptomycetes.

These current studies were initiated in response to the need for an integrated approach to clarify further, the taxonomic position of scab-causing streptomycetes. The aim was to take a diverse collection of plant pathogenic streptomycetes isolated and obtained from agricultural centres throughout the world, and to compare them on the basis of phenotype, pathogenicity in the glass house and hybridization with 16S rRNA targeted probes complimentary to sequences of *Streptomyces scabies* ISP5078 (Witt *et al.*, 1989).

These findings support the views of others that plant pathogenic streptomycetes form a taxonomically heterogeneous grouping (Wollenweber, 1920; Millard & Burr, 1926; Corbaz, 1964; Labruyere, 1971; Archuleta & Easton, 1981) rather than a taxonomically defined group (Lambert & Loria, 1989). The strains were clustered on the basis of phenotypic characters, rRNA hybridization signals and pathogenicity. Dendrograms were produced that were consistent with the phenotypic identification of isolates using the computer assisted probability matrix (Williams *et al.*, 1983b). The pathogenic streptomycetes appeared to form three centres of variation, with the *S.albidoflavus* and *S.atroolivaceus* (cluster 1 and 3) strains clustering together, the *S.rochei* (cluster 12) strains clustering together and the *S.cyaneus* and *S.diastatochromogenes* (cluster 18 and 19) strains grouping together (Fig.s 8,9,10; Section 3.6). The well characterized strain that has been used throughout this study, ISP5078 clusters with the *S.albidoflavus* strains. Each group contained pathogenic isolates although a number of highly virulent isolates clustered with the strains identifying to *S.cyaneus* and *S.diastatochromogenes*. The recently designated type strain for *S.scabies* ATCC 49173 (Lambert & Loria, 1989) fell within this cluster.

These studies also highlighted the relative merits of 16S rRNA targeted probes in the characterization of streptomycetes. Hybridization signals obtained with 16S rRNA probes complimentary to the α (position 982 to 998, *S.ambofaciens* nomenclature) and β (position 1102 to 1122) variable regions (Stackebrandt *et al.*, 1991) gave results consistent with the phenotypic identifications. These probes appeared useful in the identification of strains (pathogenic and non-pathogenic) taxonomically related to ISP5078. Hybridization results obtained with the probes were consistent with the findings Stackebrandt *et al.* (1991) on

the distribution of these sequences among specific *Streptomyces* species groups. Stackebrandt *et al.* (1991) established that the presence of specific 16S sequences correlated well with the taxonomic identity of strains (derived on the basis of phenotypic criteria). Phenotypic characterizations did however provide more information on the identity and relatedness of the isolates to each other and other streptomycetes in the genus, than probing with 16S rRNA targeted probes.

The variable regions of the 16S rRNA molecule contain only limited sequence variation, with 19 differences identifiable in the α region and 12 in the β region, from an analysis of 77 strains from 55 *Streptomyces* species (Stackebrandt *et al.*, 1991). These differences were not sufficient to allow each described species to be distinguished from each other, although some sequence variations do appear unique for particular species (Witt *et al.*, 1989; Stackebrandt *et al.*, 1991). A third variable region has also been described and designated the γ region (positions 158 to 203). This region contains the greatest sequence variation on the 16S rRNA molecule and is considered to provide more reliable targets for oligonucleotide probes and primers than the α and β regions (Stackebrandt *et al.*, 1991).

While the approach may assist in the elucidation of taxonomic relatedness, sequence variation on the 16S rRNA molecule alone is insufficient for the identification of unknown isolates. Sequence data is currently being collected for the larger 23S rRNA molecule, which contains greater amounts of variation and promises to provide more species and strain-specific sequences.

This study also illustrated that optimal conditions for the use of these probes should be ascertained. Problems of poor binding were encountered in this

study with the β probe and poor functioning of the β *S.lividans* probe were also found in another study (D.Hahn pers. comm.). It is possible that these difficulties could have been overcome through rigorous experimenting with the hybridization methodology. Points to consider include strategies for denaturing the target RNA, the empirical determination of the Tm of probes and the optimisation of hybridization conditions (Hames & Higgins, 1987).

The approaches considered may be divided into those that rely on the isolation, cultivation and then enumeration of the inoculant from soil or plant material on specific isolation media. Such approaches require a selectable phenotype. Antibiotic resistance has been frequently used as it confers the advantage of being able to deselect the indigenous background contaminants. Alternative approaches involve the isolation of specific and diagnostic molecules, such as nucleic acids. Enumeration of inoculants in environmental materials is thus calibrated in terms of the relative strengths of hybridization signals from specific, diagnostic nucleic acid target sequences.

An initial investigation into the patterns of naturally occurring resistance and susceptibility to antibiotics was made for ISP5078. Particular resistances were evaluated alone and in combination for the selective recovery of strain ISP5078 from the indigenous streptomycetes in soil. The advantage of this approach was that the strain would not have been manipulated to obtain a characteristic phenotype. Strains marked via mutation or the introduction of plasmids and reporter genes may exhibit an altered physiology which may in turn affect their ecology. This approach proved unsuccessful as it appeared difficult to discriminate strain ISP5078 from phenotypically similar soil isolates harbouring similar patterns of antibiotic resistance and susceptibility. In addition, when several selective agents were combined in a minimal media

synergistic, toxic effects occurred suppressing the growth of the inoculant and the indigenous streptomycetes in the soil. These findings emphasised the difficulties associated with the taxonomy and pathogenicity of scab-causing strains, as avirulent isolates appeared morphologically and physiologically undistingishable from virulent isolates.

Attempts were also made to mark the chromosome of ISP5078 with a construct containing the *nptII* and *xylE* reporter genes. The combination of the *nptII* gene conferring resistance to high levels of kanamycin and the *xylE* gene encoding the production of catachol 2,3 dioxygenase, an enzyme which catalyses the conversion of catechol into a distinctive yellow compound (2-hydroxy muconic acid), allows instant recognition of the inoculant. This system confers the advantage of selective isolation of the inoculant from environmental samples using antibiotic resistance and the catechol 2,3 dioxygenase activity, which being restricted to pseudomonads harbouring *Tol* plasmids is absent from indigenous streptomycete populations. In addition the *xylE* system has been shown to work very efficiently in streptomycetes (Clayton & Bibb, 1989; Ingram *et al.*, 1989). Despite obtaining the desired construct in *E.coli* DH5α. Problems were encountered in delivering the construct to the chromosome of ISP5078, a genetically uncharacterized strain with no specific genetic vectors. The procedures that were used in this study utilised PEG mediated protoplast transformation (Hopwood *et al.*, 1985). The protoplasting step induced widespread spontaneous kanamycin resistance in regenerants making the recognition of a low frequency chromosomal recombination event impossible. Induction of spontaneous kanamycin resistance via protoplasting has also been reported by Hotta *et al.* (1988a) in *S.griseus*, ie. strains with a similar identity to ISP5078. Hotta and colleagues have characterised this phenomenon and identified the production of a unique

actyltransferase enzyme (AAC 3) in *S.griseus* protoplast regenerants. Strategies that obviate the need for transformation methods which incorporate a protoplast step, may overcome these difficulties. The induction of kanamycin resistance in protoplasted cells does prompt questions concerning the affects that protoplasting has on the physiology, morphology and fitness of strains that undergo this manipulation and consequently their value as marked strains in ecological investigations. Alternative strategies might focus on the application of phage delivery vectors to genetically uncharacterized strains such as ISP5078. These studies also drew attention to the fact that ISP5078 and some other scab-causing streptomycetes are characterized by a distinctive yellow substrate mycelium, similar in colour to the *xylE* and catechol product, 2-hydroxy muconic acid. This similarity in colour could cause confusion in the discrimination of *xylE* marked strains. Thus future attempts to mark these strains with reporter genes might consider alternative combinations of strains and markers.

Initial characterization studies with 16S rRNA targeted probes indicated that the β1 probe might be specific for common scab strain ISP5078. Although further investigations revealed this not to be the case. Studies were initiated to evaluate the application of a 16S rRNA targeted oligonucleotide probe for the detection and monitoring of scab inoculants *in situ*. Non-sterile microcosm work had revealed the difficulties associated with the study of these inoculants in the presence of a prolific background soil microflora using traditional plate count methods. An approach for detection utilising rRNA targeted probes was attractive as it obviated the need to deselect the indigenous background soil microflora. Furthermore the multiple copies of ribosomes estimated per cell (10^4 to 10^5) meant that an RNA approach would be more sensitive than probing genomic DNA (6 rDNA operons have been estimated per

streptomycete cell).

Obtaining nucleic acids free of humic acid contamination was the greatest challenge in this type of approach. Humic acids are small negatively charged molecules that behave in a biochemically similar way to nucleic acids and hence will co-purify with them. Humic acid contamination of nucleic acids may affect the binding of the nucleic acids to hybridization membranes and the efficiency of hybridization. Some progress was made in the development of this approach as ribosomal RNA could be isolated fairly reproducibly from soil samples (as visualised by gel electrophoresis). However obtaining efficient hybridizations using a dot-blot approach where the nucleic acids and humic acids were concentrated together was more difficult. The concentration of nucleic acids into a small spot on a manifold was desirable in order to enhance sensitivity. Hybridization signals were obtained from RNA that had been recovered from inoculants spiked into soils using the methodology of Hughes & Galau (1988) and an additional extraction with chloroform saturated in TE buffer. However, hybridization signals were only recovered from samples that appeared to contain significant amounts of rRNA when examined visually using gel electrophoresis. It should be possible to increase sensitivity by one or two orders of magnitude. This indicated that samples were still considerably contaminated with humic acids and or hybridization procedures were very inefficient. Estimates of detection limits for streptomycete inoculants in soil, indicated that ribosomal RNA from 10^7 spores could be recovered from soil and visualised on an agarose gel. Detection limits may then be reduced 1 to 2 orders of magnitude with the application of a reliable and efficient hybridization system. However, if detection limits were brought down to 10^4 to 10^5 spores g^{-1} soil, then the rRNA approach does not actually offer enhanced sensitivity over traditional plate count approaches. Plate

counts produce quantitative data that is amenable to statistical analysis and hence assuming the inoculant being investigated is amenable to cultural approaches, the plate count will provide more information. If the culturability assumption does not hold, methods involving the direct extraction of target nucleic acids may offer more ecological information on the strain under study.

With the advent of PCR technology, the combination of DNA extraction and PCR will undoubtedly offer enhanced sensitivity over the natural amplification of the ribosomal sequences. Furthermore PCR technology may also be applied to ribosomal RNA, with the incorporation of a reverse transcriptase step to produce cDNA which may then be amplified by PCR (Medlin *et al.*, 1989). The success of the reverse transcriptase step will be determined by the secondary structure of the molecule surrounding the target sequence and the length of the target sequence. Since this approach utilises a short target sequence i.e. the length of an oligonucleotide probe this approach may be worth considering where the need for the most sensitive means of detection out weighs the need for rapid routine testing and minimum manipulations.

Perhaps the fact that approaches based on RNA have not been adopted widely by environmental scientists, who have instead focused on the direct probing or amplification of the DNA, emphasises the preference to work with DNA. DNA being more stable requires less vigilance in its preparation and handling and will consequently be more amenable to use in the routine detection and monitoring analyses required in environmental studies.

Strains (MP2 + pIJ673 and TK24) marked with a plasmid and a mutation were studied initially under near-environmental conditions in non-sterile soil

(Section 6.3.1). These studies illustrated that *S.lividans* TK24 harbouring a streptomycin resistance mutation was less able to compete in this environment than the scab isolate MP2 harbouring plasmid pIJ673. Hence by day 120, TK24 was not detectable, while the scab strain MP2, harbouring pIJ673, was. Differences in survival and fitness were attributed to the presence of the debilitating streptomycin resistance mutation in TK24. However, there may also have been a selective advantage in the presence of the plasmid and therefore the antibiotic resistance determinants as in the rhizosphere environment which is characterised by intense microbial competition and antagonism (Fravel, 1988). While there may be some loss of the plasmid from the MP2 strain (which could not be assessed), a significant proportion of the population did maintain it and remain within detectable limits. Interestingly, the plasmid did not appear to represent a significant metabolic burden on the strain or its ability to secure a niche in an environment with an apparent prolific soil microflora (Fig. 30 & 31).

This study in non-sterile soil emphasised the difficulties of working under environmental conditions, as estimates of error based on minimum significant difference values were so high, that the analysis of trends in the data was compromised. These difficulties were due to the abundant indigenous soil microflora, a problem that appeared particularly exaggerated in the presence of a developing potato plant and decomposing mother tuber. It was concluded that in order to sensitively evaluate the growth and survival of scab inoculants under non-sterile conditions that methods for detection and monitoring had to be able to overcome the problems of the intense background soil microflora, that contributed to insensitive detection using the plate count even with very selective media. Under optimal conditions the sensitivity of the plate count is such that if 100 microbes are present in a soil sample only one may be detected

once the soil has been diluted 1 : 10 and a 1/10 fraction of that plated out. The problems of microbial contamination make this count 2 to 3 orders of magnitude more insensitive. Difficulties like this prompted an enquiry into other methods of detection that are not dependent on cultivation such as nucleic acid based methodology. This is in addition to the difficulties associated with large error margins, which could be compensated for to some degree by increasing the amount of replication in the experiment. However, working with 1000 g soil pots, with and without plants was very time consuming and unfortunately imposed constraints on the amount of replication that could be included in the experiment.

For the reasons described above and in order to evaluate alternative methodology for monitoring and detection of inoculants it was considered necessary to focus on studies of the plant microbe interaction in sterile soil, under reproducible conditions. Once the system has been characterised and methodology evaluated in sterile soil, increasing complexity can be introduced until ultimately studies may be conducted *in situ*. By characterising the system in sterile soil and then introducing further complexity, such as competition etc. it is possible to assess the affect of these factors on the plant microbe interaction and hence gain more insight into the dynamics of microbial communities.

Studies therefore continued by focusing on the interaction between *Streptomyces scabies* ISP5078 and an axenic potato plant in 20 g sterile soil microcosms. These studies build upon the findings of Wellington *et al.* (1989) and other SEM studies of the streptomycete lifecycle in soil (Mayfield *et al.*, 1972). The same patterns of the germination of streptomycete spores, mycelial development followed by exhaustion of nutrients and moisture and sporulation

were observed using scanning electron microscopy and *in situ* hybridization.

It was apparent that the potato rhizosphere did indeed provide a significant source of nutrients and amendment for inoculants, as scanning electron micrographs show the roots covered in mycelial filaments and streptomycete spore chains. This effect was localised and not detectable when the bulk soil including root material was sampled and enumerated using viable plating procedures. Enhanced population levels of inoculants in association with the roots were detectable by plate count methods when the roots and associated soil were sampled and enumerated. Furthermore the potato plant added an additional dynamic element to the microcosms as inoculants could be visualised in both the mycelial and spore growth forms at day 30. In contrast, our studies in amended soil indicated that after day 15 mycelial development was no longer visible, illustrating that the developing plant is supplying fresh sources of nutrients to the inoculants after those present in an amended soil would have been exhausted. SEM and *in situ* hybridization studies in the sterile unamended soil reveal little growth of inoculants (Fig.s 43 &44), while in sterile soils amended with 1% starch and chitin the prolific development of streptomycete mycelium may be observed (Fig. 37a)

Under sterile conditions scanning electron microscopy and *in situ* hybridization studies were able to provide similar information about the activity and growth form of the inoculant in soil. Scanning electron microscopy has the advantage in sterile soil that higher magnification gives better resolution, where as in non sterile environments or mixed cultures, *in situ* hybridization when used in combination with specific probes offers the potential to visualise specific inoculants in the context of the background soil microflora. Its application to actinomycetes may be limited to nutrient rich

environments like the rhizosphere where microbes may be observed in the mycelial state. Since streptomycete spores cannot be easily identified from the background autofluorescence of soil minerals. The technique may therefore be limited to the study of unicellular bacteria in bulk soil, as the cells are larger than spores and may appear distinctive from the autoflourescence of soil minerals.

The importance of the viable plate count to the monitoring of scab-causing streptomycetes in soil (it provides a base line for the comparison of alternative strategies and has been used for detection and monitoring in earlier studies of *Streptomyces* ecology) prompted the evaluation of a plasmid pIJ673 conferring resistance to thiostrepton, neomycin and viomycin and a kanamycin resistant mutation as selectable markers in strain ISP5078, in soil. Both the plasmid bearing and mutant strains exhibited significant rates of instability and reversion in sterile soil conditions. The kanamycin mutants also differed morphologically, suggesting the potential for other alterations to the fitness and ecology of the strain. These preliminary studies indicated that both markers would offer a problematic means of monitoring inoculants *in situ*, where it is not possible to assess the frequency of reversion or instability.

These findings place further pressure on the need to introduce reporter genes into microbes in order to use plate count methods of enumeration in non-sterile soils. Hence they enforce the need to find appropriate reporter genes and technology to deliver the constructs onto the chromosome of genetically uncharacterized strains. They also add further weight to the need to develop molecular methods of detection that are not dependent on the manipulation of the strain under study or the need to deselect it from the indigenous soil microflora.

Probe target sequences for use in detection and monitoring studies may be derived from the catalogues of ribosomal RNA sequences that are currently being compiled. Significant data exists for the 16S rRNA molecule however, limited sequence variation is available for the separation of closely related taxa. Current 23S rRNA sequencing work may highlight more taxon, species and strain specific target sequences since the molecule is larger and contains longer stretches of variable sequences (Stackebrandt *et al.*, 1991). The value of these approaches will be dependent on obtaining specific or well defined probes. Introduced reporter genes may also serve as unique target sequences for probes to a particular strain.

Perhaps the principal objective of microbial ecologists is to investigate and hence to acquire insight into the composition and dynamics of particular microbial communities. Those objectives more often than not are translated into the desire to follow the fate of introduced inoculants in specific environments. However, the information obtained on monitoring one individual strain, that has probably been subject to some type of manipulations in order to mark it distinctively may be all but irrelevant to the quest for answers about the character of specific ecosystems. Molecular approaches that include the cloning and sequencing of nucleic acids direct from environmental samples have begun to provoke questions about the validity of this type of study as these new approaches reveal previously undescribed centres of microbial diversity (Ward *et al.*, 1990) .

Of interest to this study and arising from the identification of the different pathogenic and taxonomic scab pathogens are the questions surrounding their relative contributions to common scab disease and prevalence in agricultural

soils. Which strains are ubiquitous, most competitive and indeed most important to control or suppress in prevention of the disease? Until we have a handle on pathogenicity or some other means of discerning the virulent from the avirulent strains these questions will continue to elude us as we are unable to perform the most basic of analyses on community composition. In the mean time the best we can do is study marked strains with the hope that their ecology is representative of the group.

General Conclusions

Characterisation of a group of common scab strains revealed that isolates were phenotypically distinct. Isolates were found to identify to three centres of variation, identifying with the *S.albidoflavus/ S.atroolivaceus* strains (cluster 1 and 3), the *S.rochei* (cluster 12) and the *S.cyaneus/ S.diastatochromogenes* (clusters 18 and 19) species groups (Williams *et al.*, 1983a). Hybridizations with16S rRNA targeted probes complimentary to the variable α and β sequences of strain ISP5078 (Witt *et al.*, 1989) indicated that phenotypic differences were supported by genetic differences in the 16S rRNA sequences. These strains appeared phenotypically and genetically indistinguishable from avirulent strains of a similar taxonomic identity. The latter point was reaffirmed in attempts to selectively isolate strain ISP5078 on the basis of naturally occurring phenotypic traits from non-sterile soil. The suitability of a kanamycin resistant mutation induced by protoplasting and a multicopy plasmid conferring multiple resistances to antibiotics as suitable markers for following the fate of specific scab-causing strains were investigated in sterile soil. Both markers appeared unsuitable and demonstrated significant rates of reversion and instability. Attempts to develop methodology for the detection and monitoring of scab causing inoculants using the 16S rRNA targeted probes were made. Ribosomal RNA was recovered reproducibly from soil samples

as monitored by agarose gel electrophoresis, however problems of contaminating humic acids prevented reproducible and efficient hybridization. Detection limits for the method were tentatively estimated (assuming efficient hybridisation can be achieved) to be of the order of 10^5 to 10^6 spores per gram soil. The two phase lifecycle of streptomycetes, the active mycelial form and dormant or resting spore growth form allow studies of the activity of the strain via microscopy. RNA *in situ* hybridization and scanning electron microscopy were applied to studies of the growth of ISP5078 in sterile soil with and without potato plants and also with and without chitin and starch amendments. Both approaches illustrated the abundance of the microorganism in the mycelial state in the presence of nutrients i.e. either with amendments or at the potato root surface. In contrast little mycelial development could be observed in sterile unamended soil.

Future Work

Of great relevance to studies of the epidemiology of the common scab strains are the findings of Lawrence and his colleagues on the production of a vivotoxin by pathogenic common scab strains. Since the present investigations highlight that common scab strains form a taxonomically heterogeneous grouping, with individual strains being indistinguishable (phenotypically and genetically) from non-pathogenic strains of a similar taxonomic identity. It would appear that that the only way of discriminating between strains would be in terms of a pathogenicity determinant. Lawrence's group have characterised the toxin (King *et al.*, 1989) and hopefully it will not be long before the genes are cloned and sequenced. Probes and PCR primers may then be designed to complement specific regions of these gene sequences. The suitability of this approach would be dependent on identifying sequences that are unique to plant pathogenic streptomycetes harbouring genes conferring

production of the toxin. Hence preliminary studies would have to compare scab toxin sequences against other available toxin sequences and also check for specificity by screening other streptomycetes and plant pathogenic streptomycetes for the presence of these sequences. In addition to the genetic methods of monitoring that may be used, it may also be possible to use an assay for the toxin in the same way that Morgan *et al.* (1989) use the product of the *xylE* gene for phenotypic detection of the strain. It may also be possible to raise antisera to the toxin and look for its production and hence to extrapolate to the activity of pathogenic strains in the potato-soil environment.

Experiments that develop upon the present studies might consider the introduction of further complexity into the basic axenic potato soil microcosm, in order to obtain further information about some of the interactions occurring in this plant microbe interaction. For instance it would be interesting to do various co-inoculation experiments with scab isolates that are representative of the three areas of variation identified in the dendrograms. Since the isolates come from three phenotypically distinct backgrounds it would be possible to distinguish between them on the basis of morphology, melanin pigment production, antibiotic resistances etc. Such studies might reveal any differences in the competitive ability and fitness of these strains in sterilised soils. Various moisture and amendment regimes might also be experimented with in order to assess how representatives from the three phenotypic groups respond to sudden flushes of moisture and nutrients. Studies that investigate the ability of inoculants to gain a niche in plant soil microcosm that have been preinoculated with a strain from one of the phenotypic groups and challenged 15 to 30 days later (the period associated with optimal mycelial development in soil) with another inoculant.

The lifecycle of the *Streptomyces* being characterised by an active mycelial component and dormant spores offers a valuable means of monitoring the activity of the inoculant *in situ* via microscopy. In co-inoculation studies *in situ* hybridizations in conjunction with probes that differentiate between the different taxonomic groups may be used to study colonisation of the soil, potato roots and developing tubers. Such an approach may offer information on the relative abilities of inoculants to compete for and colonise these niches. Although the β probe would not hybridize to fixed cells, for reasons that were not determined, the α probe was not actually tested and therefore may offer a means to visually differentiate *S.albidoflavus* strains from *S.cyaneus*/ *S.diastatochromogenes* strains in soil.

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Appendix 1

Raw data from streptomycete identifications

	ISP5078	ATCC 3352	ATCC 15485	ATCC 10246	ASS8112	ISS	S46	S47
Adonitol	-	-	-	-	-	-	-	-
Cellobiose	+	+	+	+	+	+	+	+
L-Rhamnose	+	+	+	-	+	+	+	+
D-Raffinose	-	-	-	-	+	-	-	-
Meso-inositol	-	-	-	-	+	+	-	-
NaAzide 0.01%	+	-	+	+	-	+	+	+
D-Mannitol	+	+	-	+	+	+	+	+
D-xylose	+	+	+	+	+	+	+	+
NaCl 7%	+	-	-	-	-	+	+	-
Phenol 0.1%	+	+	+	-	-	+	+	+
Growth at 45C	-	-	-	-	+	+	+	+
Rifampicin	-	-	-	+	+	-	+	-
Neomycin	-	+	-	-	+	-	-	+
Arbutin	+	+	+	+	+	+	+	+
Xanthine	+	+	-	+	-	+	+	+
Allantoin	+	+	+	+	+	+	+	-
Inhib. of A.niger	-	-	-	-	-	+	-	-
Inhib. of S.murinus	-	-	-	-	-	-	-	+
Nitrate reduction	-	-	-	-	-	-	+	-
H2S production	+	+	+	-	+	+	-	-
Pectin hyrolysis	+	-	-	-	+	-	+	+

Table 1a

Raw data from streptomycete identifications

	ISP5078	ATCC 3352	ATCC 15485	ATCC 10246	ASS8112	ISS	S46	S47
Lecthinase activity	-	-	-	-	-	-	-	-
L-hydroxyproline	-	-	+	-	-	-	+	-
L-histidine	+	+	+	+	-	+	+	-
a-aminobutyric acid	-	+	-	-	-	-	-	-
Fragmented mycelium	-	-	-	-	-	-	-	-
Melanin pigmentation	-	-	-	-	+	-	-	+
Substrate pig. RO	-	-	-	-	-	-	-	-
Inhib. B.subtilis	-	-	-	-	-	-	-	+
Spore mass grey	+	+	+	-	+	+	+	+
Spore mass red	-	-	-	-	-	-	-	-
Spore surface rugose	-	-	-	-	-	-	-	-
Spore surface smooth	+	+	+	+	+	+	+	-
Spore chain vert (V)	-	-	-	-	-	-	-	-
Spore chain spiral	-	-	-	-	-	-	-	-
Spore chain RA	-	-	-	-	-	+	-	+
Spore chain RF	+	+	+	-	+	-	+	-
Inulin	-	-	-	-	+	-	-	-
D-fructose	+	+	+	-	+	-	+	+
Substrate pig. YB	+	-	+	-	+	+	+	-
Spore mass green	-	-	-	-	-	-	-	-

Table 1b

Raw data from streptomycete identifications

	8.2	8.6	8.7	8.8	8.16	8.17	R1	R2
Adonitol	-	-	-	-	-	-	-	-
Cellobiose	+	+	+	+	+	+	+	+
L-rhamnose	+	+	+	-	+	+	+	+
D-raffinose	-	-	+	+	+	+	-	-
Meso-inositol	+	-	+	-	-	+	-	+
NaAzide 0.01%	+	+	-	-	-	-	+	+
D-Mannitol	+	+	+	+	+	+	+	+
D-xylose	+	+	+	+	+	+	+	+
NaCl 7%	+	+	-	-	-	-	+	+
Phenol 0.1%	+	+	-	-	+	+	+	+
Growth at 45C	+	+	-	-	-	-	+	-
Rifampicin	-	+	-	-	-	-	+	-
Neomycin	+	-	+	+	+	+	+	+
Arbutin	+	+	+	+	+	+	+	+
Xanthine	+	+	-	-	-	-	+	+
Allantoin	+	+	+	+	-	+		+
Inhib A.niger	-	-	-	-	-	-	-	-
Inhib S.murinus	-	-	-	-	-	-	+	-
Nitrate reduction	+	+	-	+	+	+	-	+
H2S production	+	-	+	-	+	+	-	-
Pectin hydrolysis	+	+	+	+	+	+	+	-

Table 2a

Raw data from streptomycete identifications

	8.2	8.6	8.7	8.8	8.16	8.17	R1	R2
Lecthinase activity	-	-	-	-	-	-	+	-
L-hydroxyproline	-	-		-	-	-	+	-
L-histidine	+	+	-		+	-		-
a-aminobutyric acid	-	-	-	-	-	-	-	-
Fragmented mycelium	-	-	-	-	-	-	-	-
Melanin pigmentation	-	+	+	+	+	+	+	-
Substrate pig. RO	-	-	-	-	-	-	-	-
Inhib. B.subtilis	-	-	-	-	-	-	+	-
Spore mass grey	+	+	+	+	+	+	+	+
Spore mass red	-	-	-	-	-	-	-	-
Spore surface rugose	-	-	-	-	-	-	-	-
Spore surface smooth	-	+	+	+	+	-	+	+
Spore chain vert.	-	-	-	-	-	-	-	-
Spore chain spiral	-	-	+	-	+	+	+	-
Spore chain RA	+	+	-	-	-	-	-	-
Spore chain RF	-	-	-	+	-	-	-	+
Inulin	-	+	+	+	+	+	-	-
D-fructose	+	+	+	+	+	+	+	+
Substrate pig. YB	+	+	-	+	+	+	-	-
Spore mass green	-	-	-	-	-	-	-	-

Table 2b

Raw data from streptomycete identifications

	ATCC 49173	MP2	MP9	PD259	PD260	1028	1033	1034
Adonitol	+	-	-	-	+	+	-	+
Cellobiose	+	+	+	-	+	+	+	+
L-rhamnose	+	+	+	-	+	+	+	+
D-raffinose	+	-	-	+	+	+	+	+
Meso-inositol	+	+	-	-	+	+	+	+
NaAzide 0.01%	+	+	+	+	+	+	-	-
D-Mannitol	+	+	+	+	+	+	+	+
D-xylose	+	+	+	-	+	+	+	+
NaCl 7%	-	+	-	-	-	-	-	-
Phenol 0.1%	+	+	+	-	-	-	+	-
Growth at 45C	+	+	+	-	-	+	-	-
Rifampicin	+	-	+	-	-	+	-	-
Neomycin	-	+	+	+	-	-	-	-
Arbutin	+	-	+	+	+	+	+	+
Xanthine	-	-	-	-	-	-	-	-
Allantoin	+	+	-	-	-	-	-	-
Inhib A.niger	+	-	-	-	-	+	+	-
Inhib S.murinus	+	-		-	-	-	-	-
Nitrate reduction	+	+	-	-	-	-	-	-
H2S production	-	-	-	+	+	-	-	+
Pectin hydrolysis	-	-	+	-	-	+	-	-

Table 3a

Raw data from streptomycete identifications

	ATCC 49173	MP2	MP9	PD259	PD260	1028	1033	1034
Lecthinase activity	-	-	-	-	-	-	-	-
L-hydroxyproline	-	-	-	+	-	-	-	-
L-histidine	-		-	+	+	+	+	-
a-aminobutyric acid	-	-	-	-	-	-	-	-
Fragmented mycelium	-	-	-	-	-	-	-	-
Melanin pigmentation	+	-	+	+	+	+	+	+
Substrate pig. RO	-	-	-	-	-	-	-	-
Inhib. B.subtilis	+	-		-	-	-	-	-
Spore mass grey	+	+	+	+	+	+	+	+
Spore mass red	-	-	-	-	-	-	-	-
Spore surface rugose	-	-	-	-	-	-	-	-
Spore surface smooth	+	+	-	+	+	+	+	+
Spore chain vert.	-	-	-	-	-	-	-	-
Spore chain spiral	+	-	-	+	+	+	-	+
Spore chain RA	-	+	+	-	-	-	+	-
Spore chain RF	-	-	-	-	-	-	-	-
Inulin	+	-	-	-	+	+	-	+
D-fructose	+	+	+	+	+	+	+	+
Substrate pig. YB	+	+	-	+	+	+	+	+
Spore mass green	-	-	-	-	-	-	-	-

Table 3b