INTERACTIONS BETWEEN ACTINOPHAGE AND STREPTOMYCETES IN SOIL

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CONTENTS

SUMMARY

A method was developed based upon soil dispersion using an ion exchange resin and differential centrifugation that allowed the selective isolation of *Streptomyces* spores rather than mycelia; this allowed the characterisation of a germination/sporulation cycle *in situ*. The method was able to detect relatively low numbers of streptomycetes in soil, through its concentrating action. The ecology of a temperate actinophage derived from ΦC31, containing the thiostrepton-resistance gene, was studied in conjunction with strains of *Streptomyces lividans*. In sterile amended soil, phage numbers showed initial increases due to a corresponding germination of the host spore inoculum; subsequently phage numbers declined when the host streptomycete was no longer in the mycelial state and hence receptive to phage infection. Lysogens were readily obtained in sterile amended soil; use of the spore extraction method and another method that isolated both spores and mycelia enabled lysogenic mycelia to be first detected after 2 days and lysogenic spores after 5 days. Phage-mediated gene transfer of the thiostrepton-resistance gene from a lysogenic donor to a non-lysogenic recipient was also demonstrated in sterile amended soil. In nonsterile soil, host growth was considerably retarded with respect to sterile amended soil, although phage numbers showed a similar pattern with an initial burst of activity followed by subsequent decline. Lysogens were only rarely found in nonsterile soil and were also found to be less fit *in situ* than the parent strain. No difference was found in sterile amended soil.

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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 and all sources of information have been specifically acknowledged by source of reference.

P.R. Herron

ABBREVIATIONS

Ab^r	Antibiotic resistance
AGS	Arginine glycerol salts agar
APB	Phage buffer
aph	Neomycin phosphotransferase gene
c.f.u.	Colony forming units
Cont.	Continued
c.p.s.	Counts per second
DAPI	4,6-diamino-2-phenylindole
DMSO	Dimethyl sulphoxide
EDTA	diaminoethanetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
GEM	Genetically-engineered microorganism
H.S.D.	Honestly significant difference
Hg^r	Mercury resistance
Km^r	Kanamycin resistance
M.S.D.	Minimum significant difference
Min.	Minute(s)
MM	Minimal medium
NB	Nutrient broth
NCG	Nutrient agar
NS	Nonsterile
PCR	Polymerase chain reaction
p.f.u.	Plaque forming units
PEG	Poly ethylene glycol
PVPP	Polyvinyl pyrrolidone
RASS	Reduced arginine salts agar
rif^r	Rifampicin resistance

SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
SNA	Soft nutrient agar
St. Am.	Sterile amended
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TES	*N*-Tris (hydroxymethyl) methyl-2 aminoethane sulphonic acid
TNE	Tris-NaCl-EDTA buffer
T.D.	Doubling time
Tn	Transposon
Tris	Tris(hydroxymethyl)aminoethane
TSB	Tryptone soya broth
tsr	Thiostrepton-resistance ribosomal methylase gene
Vol.	Volume(s)
vph	Viomycin phosphotransferase gene

GLOSSARY

Lysogeny:

This is the state whereby a temperate phage has infected a host which results in a semistable association with the bacterium. The prophage is then replicated with the bacterial genome, and is inherited by each of the daughter cells (Lenski, 1988).

Pseudolysogeny

This can be defined as a state whereby a virulent phage is maintained within a population of host cells over a number of generations, but not by the action of a phage-encoded repressor protein (Barksdale & Arden, 1974; Lenski, 1988).

Phage conversion:

A change in a phenotype of the host, brought about by a gene within the genome of a temperate bacteriophage .

Generalised transduction

A change in a phenotype of the recipient host brought about by infection of a defective phage particle containing only donor bacterial DNA, followed by subsequent recombination.

Specialised transduction

A change in the phenotype in the recipient host brought about by lysogenisation by a bacteriophage; the host gene encoding that phenotype being attached to the phage genome by means of incorrect excision of the prophage from the donor's chromosome.

CHAPTER 1

GENERAL INTRODUCTION

1.1 The terrestrial environment

Soil is essentially a nutrient poor environment subject to great variations in, moisture, temperature, pH, ionic concentration, atmospheric conditions and nutrient availability. A consequence of the abundance of solids is that movement of water, gases, substrates and organisms is limited. This leads to the development of environmental heterogeneity, which, in turn, may lead to microbial diversity. The soil matrix is a particulate environment with periodic disturbance by burrowing animals; this causes gradients to appear between the atmosphere and the soil's parent material. These gradients are caused by differences in the relative rates of production and consumption of the organic matter and gases, and the rates of physical/chemical interactions between organisms, inorganic solids and the percolating water (Nedwell & Gray, 1987). Thus, over a period of time these gradients result in the formation of many different microenvironments for microbial growth.

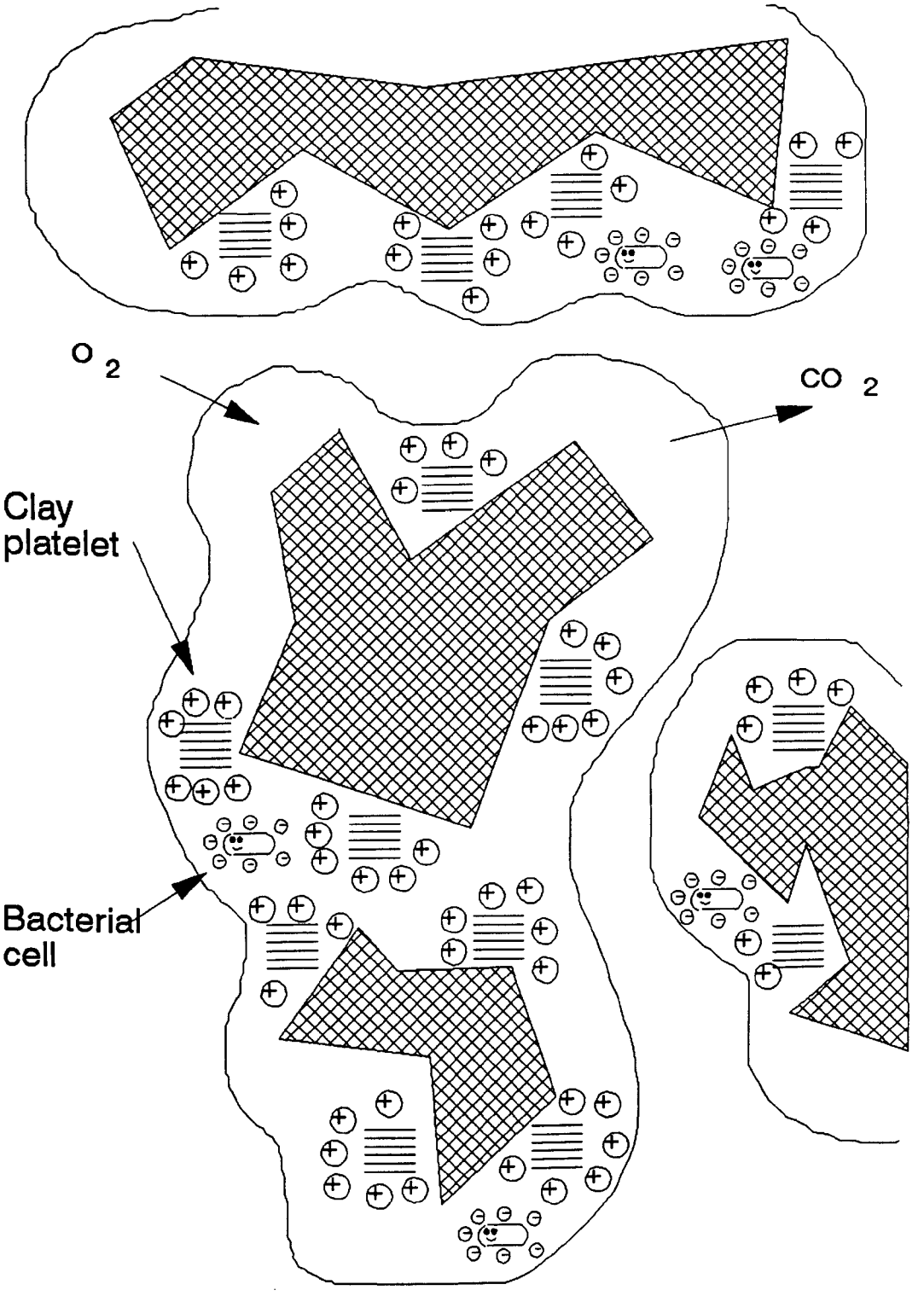
1.1.1 Soil structure

Soil is comprised of five major components: mineral matter, water, air, organic matter and living organisms (reviewed by Gray & Williams, 1971; Alexander, 1977; Lynch, 1979; Nedwell & Gray, 1987; Stotzky, 1989). The air and water fraction comprises *ca.* 30-50% of the soil volume and represents the soil's pore space, the proportion of air to water varying according to climatic conditions. The amount of pore space is dependent on the texture, structure and organic matter content. The air space controls the level of aeration of the soil, whilst the liquid phase is a dilute solution of salts and nutrients necessary for growth and survival of microorganisms. These two fractions are related as any pores not filled with water will be filled with gas. The soil atmosphere contains

CO_2 at levels 10-100 fold higher than that in the above ground atmosphere, whilst O_2 is less plentiful; this effect is magnified with increasing depth. The unusual composition of the soil atmosphere stems from the respiration of soil animals, plants and microorganisms. The mineral fraction arises (comprising slightly less than 50% of the soil volume) through the weathering of the underlying parental rock. The inorganic components of the soil can be divided into several categories, the distribution of which, determines its type. The larger soil particles (sands and silts) are of little influence on the physical, chemical and biological activities of soil. They are not responsible for the retention of water in unison, but rely on the formation of soil aggregates. Clays are composed of silicon, oxygen and aluminium, also iron, magnesium, potassium, calcium, sodium and other elements may be found to varying degrees. Clay particles have a crystalline structure (see Fig. 1) composed of several plate-like layers and exist as a coating on, or lie between, larger sand and silt particles, thus binding the aggregate together through electrostatic interactions. The entire structure is stabilised by organic matter, insoluble inorganic material, polysaccharide gums produced by microorganisms, and filamentous microorganisms themselves. These microaggregates are temporary structures and vary between 0.5 and 5 mm in diameter. The chemical reactivity and high surface area of clays means that this is the most influential fraction in terms of microbiological activity. Little microbial activity takes place on the surface of sand and silt particles due to the lack of suitable sites for the attachment of microorganisms to these minerals. If the particle is coated in clay the microbe is able to attach through electrostatic interactions. Clay particles are coated in metal oxides which have a net negative charge that causes the attraction of a cloud of cations giving an overall positive charge (Sykes & Williams, 1978). Thus it is possible for microorganisms to bind to clay via electrostatic interactions. Charged organic matter (and inorganic cations) can also facilitate this attachment by acting as a bridge between the clay particle and bacterium.

Fig. 1. Soil microhabitat.

The diagram represents a stylized soil microsite. Hatched areas represent silt or sand particles bathed in water, surrounded in a water coat. Stacks of lines represent packets of clay mineral, surrounded by positively charged ions, the diffuse double layer, that are able to bind microorganisms through electrostatic interactions or through entrapment by organic polymers.


O 2
CO 2
Clay platelet
Bacterial cell

The organic fraction (see Section 1.1.3) of soil (termed humus) is a product of the synthetic and decomposing activities of the microflora and is one of the principal microbial food supplies as well as being critical in the formation and maintenance of soil structure.

1.1.2 Features of the soil environment

There are many features that are likely to affect the microbial activity of a soil (e.g. temperature, pH, available water), some of which are mentioned in Section 1.1.1 and in the reviews by Gray & Williams (1971); Alexander (1977); Lynch (1979); Nedwell & Gray (1987); Stotzky (1989). The relative influence of these individual ecological factors differs from soil to soil and is usually greater on introduced rather than indigenous microorganisms (Stotzky & Krasovsky, 1981). Furthermore none of these factors operate individually but in concert with numerous other factors. The most important factor influencing soil temperature is sunlight, the degree of which striking a soil is dependent on a number of other soil characteristics (e.g. direction of slope, soil type, depth, water content, density of vegetation). Temperature varies with latitude, climatic conditions and altitude and is cyclic both annually and diurnally. The diurnal fluctuation can be as much as 35°C with minima below freezing and maxima about 60°C. Soil temperature has an obvious affect on microbial metabolic activity. As soils are heterogeneous environments, there will be microenvironments where the temperature is considerably different to the air temperature as soil is a poor conductor of heat.

Soil pH tends to be acidic rather than alkaline under normal conditions due to: leaching of base metals, CO_2 production by microorganisms and the formation of organic acids in the degradation of dead plant material. External factors such as climate, temperature and land-use determine pH, the overall trend

is towards acidic soils, although some processes in soil (e.g. NO_3^-/SO_4^{2-} reduction) may reverse this drift towards acidity. Again, microenvironments exist where the pH is widely different from the macroenvironment e.g. pH is relatively low around clay particles (Sykes & Williams, 1978)

Soil type, whilst not influencing a microorganism directly, determines the levels at which some of the above characteristics are set. As the soil type is dependent on the prevailing climatic conditions and the parental material of the soil, these factors indirectly control all the others. For example, brown earth soils develop in conditions of moderate to high rainfall over a basal material with low permeability, such as clay or loam. Hence the soil is subjected to moderate leaching with the resulting formation of an acidic A horizon (Gray & Williams, 1971).

1.1.3 Substrate availability and microbial response to low nutrient conditions

With the exception of photosynthetic microorganisms at the soil surface and other autotrophs within the soil bulk, all of the soil microflora are heterotrophic. In soil the growth limiting substrate is generally nitrogen. The sources, of which are: leaf litter; root exudates; or dead microorganisms, either through parasitism (Zeph & Casida, 1986) or cryptic growth (Chapman & Gray, 1986). Dead plant material is incorporated into soil and a variety of products formed. As the initial products and original material are degraded they are converted to black or brown organic complexes, whilst the less resistant compounds are sequentially degraded. The arrival of plant litter at the soil surface, therefore, provides microbes with a variety of energy sources, some of which are readily available and others that require extensive degradation (Williams, 1985). The second source of nutrients for soil microorganisms is

from the death of plant roots, the sloughing off of root cells as the root moves through the soil and root exudation of organic molecules through the root cell wall. Initially, nutrients are decomposed at the soil or root surface before being transferred into the soil body by leaching, diffusion or by soil animals (Anderson, 1987). Thus, these substrates are present in discrete packages surrounded by gradients of soluble nutrients for a limited period of time prior to their utilisation by the soil microflora (Williams, 1985, Nedwell & Gray, 1987). This introduces temporal as well as spatial heterogeneity into the system. Whilst some microorganisms feed on soluble substrates in the bathing water layer, others make use of the discrete bundles of organic matter.

The survival of a bacterium in its natural habitat depends on its ability to grow at a rate sufficient to balance that of death from starvation and other natural causes (Dawes, 1989). There are essentially two methods of achieving this. The first is that used by oligotrophic microbes (also called `autochthonous' microbes or `*k* strategists') and the second by copiotrophic microorganisms (also called `zymogenous' microbes or `*r* strategists'). The first group refer to microorganisms that grow continuously, but at low levels of activity; and the second group exist primarily in a resting phase with brief periods of activity upon the supply of nutrients (Poindexter, 1981). At first glance, soils would seem to be oligotrophic environments, in that the overall level of nutrients is low (Williams, 1985). However, the situation in soil is complicated by the spatial and temporal variation in nutrient levels. Organic materials entering the soil are either diluted in the nutrient mass or remain at sites of nutrient concentration; the former being the site of autochthonous activity, and the latter, zymogenous (Williams, 1985). This distinction, however, is somewhat blurred in soil (unlike aquatic systems [Poindexter, 1981]) as there is no unequivocal evidence for the existence of obligate oligotrophs in the terrestrial environment (Williams, 1985); and even the resting stage of a so-called copiotrophic organism (e.g. *Streptomyces* spores) shows metabolic activity (Ensign, 1978).

1.2 Soil microorganisms

The soil is a vast store of a large number and variety of microorganisms engaged in a myriad of activities. Whilst many different species and genera appear in this environment , some are obviously more common than others. Problems exist in actually classifying this population for the reasons described in Section 1.2.4. It is essentially meaningless, therefore, to present a long list of the percentage of the population from different genera supported in a given natural soil, suffice to say that most groups of microorganisms would be represented in different soils. The presence of a given genus/species/subspecies is dependent on that particular environment. Soil microbes should not be thought of as simply a group of organisms growing in mixed culture, but more a diverse assembly existing in a heterogeneous environment; these assemblies consisting of varying numbers of microorganisms, varying diversity and varying levels of interactions.

1.2.1 *Streptomyces* in soil

The genus *Streptomyces* contains aerobic, Gram-positive actinomycetes that are highly oxidative and form extensive branching substrate mycelia of 0.5-1.0 μm in diameter containing LL-diaminopimelic acid and glycine, but no characteristic sugar in the cell wall (Williams & Wellington, 1982; Williams *et al.*, 1983). Aerial mycelia are produced from the substrate mycelia that obtain their nutrients by means of parasitism on the substrate mycelia. The aerial mycelia in turn differentiates to form spores. Streptomycetes have a mol% (G+C) of between 69 & 73 (Williams, 1989) and are best known for a great variety of antibiotics and secondary metabolites produced late in growth (Wellington & Cross, 1983). Actinomycetes are found in many habitats (e.g.

freshwater, cold and warm-blooded animals, and composts [Williams & Wellington, 1982]). Their most important habitats, however, are soils, where they form a significant part of the microbial population. Viable counts of several million per gram are common for over 20 genera. Even in waterlogged, anaerobic or acidic soils numbers of up to 10^3 c.f.u./g can be found (Williams & Wellington, 1982). The vast majority of actinomycetes isolated are from the genus *Streptomyces* (Williams, 1978). Streptomycete numbers in soil range from 10^5 to 10^6 propagules/g (Lloyd, 1969) and are estimated to comprise 95.34% of the actinomycete population in most nonanaerobic soils (Williams & Wellington, 1982).

Early studies on the classification of *Streptomyces* were based on morphology (reviewed by Williams & Wellington, 1980) and ability to produce bioactive compounds (for patenting purposes), a much clearer picture emerged with the use of numerical taxonomic approaches. In the most comprehensive study to date (Williams *et al.*, 1983), the type strains of just under 300 recognised species of *Streptomyces* were assigned to 21 major clusters or subclusters (6-38 strains), 37 minor clusters (<5 strains) and 13 single member clusters. The data obtained from such taxonomic studies can then be used to develop selective procedures for the isolation of specific streptomycetes from the environment (Vickers *et al.*, 1984; Wellington *et al.*, 1987).

In order to isolate and enumerate streptomycetes from natural habitats it is necessary to eliminate or reduce fungal or bacterial growth on isolation plates; the numerous strategies evolved for this have been reviewed by Williams & Wellington (1982) and involve such procedures as the use of antibiotics, soil drying and inclusion of large organic molecules as a carbon source in the isolation medium (e.g. starch, chitin). The high numbers of streptomycetes commonly found in soil must be interpreted with care as direct observation and homogenisation experiments have shown that the vast majority of colonies on an isolation plate originate from spores (Goodfellow & Simpson, 1985). Since a

colony on a plate may originate from either a spore or a mycelial fragment, these numbers give no indication of the actual activity of the organism *in situ* (Skinner, 1951). Soil is the natural habitat for most streptomycetes; where they find the environment suitable for their growth and proliferation. These conditions may not be optimal *per se*, but allow suitable competition with other members of the soil microflora, i.e., they can degrade organic matter not easily attacked by other microorganisms; they are non-fastidious and are satisfied with inorganic nitrogen; they are able to utilise the soil matrix to support their mycelial growth and, although their spores are not as resistant toward unfavourable conditions as bacterial endospores , they do contribute to survival over conditions of environmental hardship (Kutzner, 1981).

Streptomyces exist in soil mainly as spores (Skinner, 1951; Lloyd, 1969, Mayfield *et al.*, 1972), whilst active growth is discontinuous in space and time (Mayfield *et al.*, 1972); i.e. is limited to sites of nutrient availability (Kutzner, 1981; Goodfellow & Simpson, 1985). Direct observation studies show that particulate organic substrates, such as root fragments and fungal hyphae are rapidly colonised by hyphal growth. The growth sites of streptomycetes are restricted, ranging from 20-200μm in diameter (Goodfellow & Simpson, 1985). Hyphal growth disappears and is replaced by spores after nutrient depletion (Lloyd, 1969). However the evidence that sporulation is linked to nutrient limitation is by no means conclusive. The appearance of spores is also discontinuous in space (Mayfield *et al.*, 1972). Thus, the main means for the survival of the species seems to be the partial resistance of spores, coupled with intermittent replenishment of the original spore population by mass germination at sites of nutrient concentration (Lloyd, 1969), with spores being dispersed by water movement and arthropods (Goodfellow & Simpson, 1985).

Streptomycete spores, and even mycelia, are able to withstand considerable desiccation (Williams *et al.*, 1972); some being able to grow (albeit in a severely limited manner) at a water potential of -100 bars. Soil acidity

appears to be an important limiting factor in the occurrence of streptomycetes (Davies & Williams, 1970). Acidophilic streptomycetes are readily found in acid soils, but it is also possible to isolate neutrophilic strains from the same soils (Williams *et al.*, 1971). Streptomycetes are able to survive on an assortment of substrates *in situ*. In addition to simple carbohydrates *Streptomyces* also plays a major role in the degradation of organic matter (Williams & Wellington, 1982) such as lignocellulose (Crawford, 1978; Crawford & Sutherland, 1979) and chitin (Lloyd *et al.*, 1965), which is one of the most characteristic features of soil streptomycetes (Williams & Robinson, 1981).

1.2.2 Bacteriophage ecology in soil

The ecology of bacteriophage in soil is less well understood than that of their potential hosts, with most information being based on numbers of p.f.u. (Williams *et al.*, 1986). If phage exist at 0.1% of the titres obtained in the laboratory, they would be the most numerous genetic objects in soil (Reanney & Marsh, 1974). Phage may be involved in either lysogenic or virulent associations with their host in soil, however, current detection procedures provide no indication of the absolute counts in the environment as these methods rely on the virulent reaction of the virus with its host. The importance of this point was illustrated by Reanney (1968); free phage of *Bacillus stearothermophilus* in soil were estimated to comprise only 20% of the total population; the remaining 80% being present as lysogens.

The abundance of any species is determined by the resources available to it. For bacteriophage, this resource is primarily the presence of susceptible hosts (Williams *et al.*, 1986); thus in order for a phage to survive it must not eliminate its host species and a balance must exist between them (Alexander, 1981). The fact that bacteriophage exist is *a priori* evidence that mechanisms have evolved

to enable coexistence with their hosts (Alexander, 1981). In order to survive, a phage must encounter, adsorb to and infect a metabolically active host. Bacteriophage with copiotrophic hosts must, therefore, mimic their hosts by undergoing rapid replication during short periods of bacterial growth (Williams *et al.*, 1986), whilst bacteriophage with oligitrophic hosts must continuously replicate at levels sufficient to cause no significant detriment to the slow growing host. It must be remembered that evolution favours those predators/parasites that are not excessively exploitive of their prey/host (Alexander, 1981) and thus the bacteriophage must complement the life style of its host so as not to eliminate it and itself.

The concentration of host within a system affects the level of phage replication; phage in an ecosystem with a high host titre will take less time to contact (and hence infect) a host than one with a lower host titre. In aquatic systems there is no threshold level of host numbers below which phage replication cannot take place (Kokjohn *et al.*, 1991), although there was thought to be an effective one of *ca.* 10^4 c.f.u./ml (Wiggins & Alexander, 1985) due to the time necessary for a phage to contact low numbers of host and initiate replication. It was thought that low numbers of free phage found in aquatic environments meant that bacteriophage would have little effect on bacterial numbers, however, recent advances in concentration techniques have allowed greater sensitivity of measurement of phage populations *in situ*. This has shown that bacteriophage populations are commonly several orders of magnitude higher than their host populations (Miller & Sayler, 1991), and as such, can have a significant effect in controlling host, resulting in a stabilisation of phage/host numbers (Ogunseitan *et al.*, 1990).

In soil the situation is different due to the heterogeneity and the paucity of mixing of the system; thus if phage and their host are to interact it must be in individual microsites where their effective concentrations are not equal to the total number of phage in a given mass of soil divided by the number of available

microniches. This threshold level was determined to be between 100 and 1000 c.f.u./g for phage of *Azospirillum brasilense* (Germida, 1986). The level of phage replication must be sufficient to supply enough active virions to survive the next period of environmental adversity. Temperate phage, obviously, have a mechanism to facilitate their survival during periods of environmental hardship, and their endurance is dependent on that of their host. However, virulent phage must survive for considerable periods as free virions and be subject to environmental fluctuations (Williams *et al.*, 1986) and thus must be as resistant to these conditions as their host (Williams & Lanning, 1984). Some virulent phage may exist in a pseudolysogenic state (e.g. *Pseudomonas aeruginosa* phage, UT1 [Ogunseitan *et al.*, 1990]); where the prophage exists alongside the bacterial genome and is thus able to hide from environmental adversity (the differences between lysogeny and pseudolysogeny are reviewed by Barksdale & Arden, 1974).

The survival of a free phage is dependent on a number of different effects. Williams & Lanning (1984) showed that the titre of free actinophage generally decreased faster in nonsterile than in sterile soil, whilst increased temperature and moisture content of soil also decreased survival. Cheo (1980), found that whilst increased moisture content of soil reduced the survival of virions, desiccated soil quickly destroyed virus particles. Interestingly, this study also reported that little inactivation occurred in flooded soils, indicating an importance of aerobic microbial activity in this process. The occurrence of actinophage is dependent on soil pH. Sykes *et al.* (1981) were unable to detect actinophage in soils below pH 6.1, phage survival was also found to be negligible in acidic soils. However, phage can adsorb to clay particles (Bystricky *et al.*, 1975) and when in this state were found to have enhanced resistance to soil acidity (Sykes & Williams, 1978). At neutral pH, phage carry a net negative charge (Sykes & Williams, 1978; Schiffenbauer & Stotzky, 1982), thus phage adsorption to clay is dependent on the presence of cations (for bridging) or the

occurrence of positively charged regions on the clay particle (Schiffenbauer & Stotzky, 1982). Whilst, adsorbed phage may be protected from the environment (Babich & Stotzky, 1980), the net negative charge of clays is likely to cause the attraction of a cloud of cations (including H^+ ions) surrounding the clay particle. This leads to localised areas of acidity and might result in phage inactivation in acidic soils (Sykes & Williams, 1978).

1.2.3 Detection and monitoring of microorganisms in the environment

Many methods are available for the detection and enumeration of microorganisms in the environment, additional techniques have been developed following the recent advances in molecular biology (Trevors & Van Elsas, 1989). Most studies in microbial ecology make use of techniques that involve isolation and culturing of the microorganism as they have the advantage of being simple. All such methods rely on the ability of the target organism to grow following its extraction from the environment. The problems associated with such an approach are that only culturable cells can be cultivated rendering those microbes that are viable but non-culturable undetectable (Rozack & Colwell, 1987). In soil, plate counts can be up to 2-3 orders of magnitude below direct counts (Bakken, 1985; Fægri, 1977). There are also problems associated with the relation of c.f.u. to actual numbers of cells through clumping of cells, this difficulty is especially evident when using viable counts for the monitoring of mycelial microorganisms in the environment. Direct counting allows the enumeration of microorganisms without the need for culturing of the sample. The sample is visualised by staining with fluorescent dyes that bind to various cellular constituents (e.g. DNA, which is bound by DAPI [Coleman, 1980] and acridine orange [Porter & Feig, 1980]). Antibodies raised against strains of microorganisms may be used to determine direct counts for a specific

subpopulation (Howgrave-Graham & Steyn, 1988; Brooker & Stokes, 1990), for their detection by means of an ELISA (Kemp *et al.*, 1988; Morgan *et al.*, 1989; Bashan *et al.*, 1991) or for their enumeration using flow cytometry (Saunders, *et al.*, 1990). All such methods require a high degree of specificity for the target microorganism, so as to eliminate non-specific binding of the stain to nontarget organisms or inanimate objects in the sample. The use of antibodies, whilst being relatively specific, still requires the initial isolation of the target microorganisms from the environment and thus is also incapable of detecting nonculturable organisms.

Progress in the sequencing of 16S rRNA genes (Lane *et al.*, 1985) has allowed the development of taxonomically significant oligonucleotide probes (Giovannoni *et al.*, 1988, Amann *et al.*, 1990a) that can be used for the detection of microbes by microautoradiography and epifluorescence microscopy respectively *in situ*. The advantage of such a strategy is that the target organism does not need to be cultured during the preparation of the probe, as a probe can be selected that is specific for a particular species/genus/Kingdom (Woese, 1987). 16S rRNA directed probes can also be used to differentiate between species by the use of flow cytometry (Amann *et al.*, 1990b).

Nucleic acids extracted from the environment can be used to monitor microbial populations; use of such methods is discussed in Chapter 3. Once the nucleic acids have been extracted from the environmental sample it is possible to use them to gain information about their nature and diversity. Apart from the cases discussed in Chapter 3, one such approach is the use of DNA reassociation kinetics. Once the DNA has been purified it is possible to melt it to its single stranded form (Torsvik *et al.*, 1990b), the rate of reassociation is then proportional to the concentration of homologous DNA. $C_0t_{1/2}$ curves can then be used to estimate the number of clones from which the DNA extract is derived from (Torsvik *et al.*, 1990b). Such an approach enables an estimation of the diversity of a given environment to be attempted without the need for culturing

of the inhabitants.

1.2.4 Problems in studying microbial ecology in soil

Perhaps the greatest problem in studying the microbial ecology of soil is actually isolating the entire population. Viable counts of soil bacteria can be 2% of the values obtained by direct counting (Bakken, 1985). Torsvik *et al.* (1990a) attempted to recover the entire population of a soil sample on isolation plates, following $C_0t_{1/2}$ curve analysis it was shown that the entire 200 strains isolated (by phenotypic analysis) were from *ca.* 20 different clones. However, when total DNA was extracted from the soil itself and subjected to DNA reassociation analysis, the DNA extract was derived from *ca.* 4000 different clones (Torsvik, 1990b). Thus, it seems that only a small fraction of the microbial community can be isolated using viability based methods. As many soil microorganisms are oligotrophic, the use of rich (in terms of soil nutrient levels) media is actually inhibitory to some microbes (Williams, 1985). This concept is not universally true as many copiotrophs (e.g. *Escherichia coli*) do have the ability to operate in oligotrophic conditions (Williams, 1985); whilst Horowitz *et al.* (1983) showed that subarctic bacteria isolated in low nutrient conditions exhibited greater nutritional diversity than those isolated on rich media. The other problem in culturing soil microorganisms is due to the concept of the viable, but non-culturable organism (Colwell *et al.*, 1985; Roszak & Colwell, 1987). Such a microorganism can be detected by direct methods and shown to be viable (using a direct viable count [Kogure *et al.*, 1979]); but the organism will not grow on isolation media. This presents special problems in the case of water hygiene (Colwell *et al.*, 1985), as the detection of pathogenic organisms is based on the viable most probable number test.

1.3 Gene transfer in the environment

Recent concern over the release of genetically-engineered microorganisms into the environment (Sussman *et al.*, 1988; Fry & Day, 1990) has prompted an interest in the study of micobial gene transfer *in situ*. The conditions to allow such processes as conjugation to take place in the environment exist in terms of temperature and pH (Singleton & Anson, 1981 & 1983). However, most of the evidence, prior to the last five years, for environmental gene exchange, was retrospective, in that the phenotypic diversity of a species was interpreted to be a result of horizontal gene transfer. However, since the development of molecular techniques the ability to produce markers that are not native to the indigenous population has been obtained. Thus, it is now possible to use marked strains to assess transfer mechanisms *in situ*.

1.3.1 Natural gene transfer

The tracking of natural gene transfer between microorganisms presents difficulties as it must be done in a retrospective manner (e.g. glutamine synthetase II (GSII) of the family Rhizobiaceae is distinct from all other prokaryotic glutamine synthetases, but its amino acid sequence is highly homologous to plant GSII, indicating that the GSII gene is of eukaryotic origin [Carlson & Chelm, 1986]). This gene is also homologous to one of the glutamine synthetases of *Streptomyces* and *Frankia*. Similar work, carried out by Brisson-Noël *et al.* (1988), pointed to the common origin of antibiotic resistance genes of *E. coli*, *Streptococcus* and *Enterococcus*. There is evidence *in vitro*, for genetic exchange between a large number of different microorganisms. The Ti plasmid of Agrobacterium has been well documented as a vehicle for gene transfer between bacteria and plants (Zambryski *et al.*, 1989). The wide host

range plasmid RSF1010 can also facilitate exchange by this route, in addition to intergeneric transfer between bacteria (Buchanan-Wollaston *et al.*, 1987). Another example of transkingdom genetic exchange has been reported by Heinemann & Sprague (1989), who demonstrated the mobilisation of DNA between *E. coli* and *Saccharomyces cerevisiae*. Many intergeneric matings have also been reported making use of either bacteriophage (e.g. *E. coli* to *Myxococcus* [Kaiser & Dworkin, 1975]) or shuttle vectors (e.g. *E. coli* to *Bacteroides* [Guiney *et al.*, 1984], *E. coli* to *Bacillus* [Trieu-Cuot *et al.*, 1985], *E. coli* to *Clostridium* [Ross-Williams *et al.*, 1990] and *E. coli* to *Mycobacterium* [Lazraq *et al.*, 1990]). Thus, the occurrence of these events, as well as the apparent promiscuity of naturally occurring plasmids such as RSF1010, indicates that widely differing DNA is able to survive and replicate in a different organism.

Many bacteria that have been isolated from the environment were found to contain genetic elements that are capable of mediating gene transfer *in vitro*. Nodulating ability of *Rhizobium* is often carried on Sym plasmids and can be transferred to a non-symbiotic strain (Johnston *et al.*, 1978; Broughton *et al.*, 1987); indeed similar Sym plasmids were found in widely differing *Rhizobium* isolates, whilst identical strains carried different Sym plasmids (Schofield *et al.*, 1987). McPherson & Gealt (1986) isolated several strains (*E. coli* & *Klebsiella*) that contained both antibiotic resistance markers and high molecular weight plasmids that were capable of transferring the resistance markers to a recipient *in vitro*. Many other bacterial characteristics are coded on transmissible plasmids e.g. copper resistance in *Pseudomonas syringae* (Bender & Cooksey, 1986), mercury resistance in many organisms (Olson *et al.*, 1979; Radford *et al.*, 1981 [transposon encoded]; Kelly & Reanney, 1984). Thus, the ability to exchange genetic information exists in the environment, measurement of *in situ* transfer itself is discussed in Sections 1.3.4 to 1.3.6.

If portions of the DNA of two microorganisms are identical, whilst most

is dissimilar, this can be taken as evidence for genetic exchange, although genetic diversity by itself is not real proof for gene transfer. In the soil system there is heterogeneity in the level of diversity, in that some micosites will contain a monoculture, while others a multitude of species. Even then, the measurement of species diversity in an ecosystem is essentially meaningless as it is impossible to actually culture all of the organisms in the soil, although techniques such as DNA reassociation are beginning to address this problem (see Section 1.2.6). Even if it were possible to culture everything, measuring the diversity of a community does not give any indication as to its stability because microbial populations respond to environmental variation by changing; unlike animal/plant populations which resist that change (Brock, 1987). Some useful information can be gained from the measurement of species diversity. For example, enzyme polymorphism studies on clinical isolates of *E. coli* (Selander & Levin, 1980) suggest that *in situ* gene transfer between populations is rare due to the low degree of enzyme polymorphism exhibited by these isolates. If gene transfer were common, combinations of genes would be broken up to a large extent resulting in many different kinds of protein electrophoresis patterns, although Caugant *et al.* (1981) suggested that some diversity in *E. coli* isolates was due to recombination. Similar work on isolates of *Rhizobium* (Young, 1985; Young *et al.*, 1987; Harrison *et al.*, 1989) also found substantial enzyme polymorphism existed between strains (for three loci, 63 different permutations were possible, but only 15 detected [Young *et al.*, 1987]). Thus indicating that populations exist in a non-random fashion, either as distinct genotypes with little recombination between them or if recombination is frequent, then selection against the hybrid genotypes must be strong (i.e. hybrids are less fit than their parents).

It would seem, therefore, that the mechanisms for genetic exchange exist within microbial communities. But, that this potential is rarely utilised resulting in a lower degree of diversity in the population than would exist if genetic

exchange were common.

1.3.2 Monitoring of gene transfer using marked inoculants

Over the last five years many studies have attempted to measure environmental gene transfer using either indigenous markers and plasmids (e.g. Bale *et al.*, 1987) or genetically-engineered constructs (Richaume *et al.*, 1989). In order to measure these events, new techniques have become available to detect and quantify microorganisms *in situ* (see Section 1.2.3). The environment is not a monoculture, thus, of equal importance to the extraction of an organism or gene from a sample, is the use of markers that allow a target organism to be differentiated from the background microflora. As most markers were originally obtained from organisms present in the environment the problems of false positives can be reduced and sensitivity optimised by the use of multiple markers e.g. selective (antibiotic resistance) marker, elective marker and a silent marker for hybridisation.

Elective markers make use of a metabolic characteristic that allows their host to appear extraordinary compared to the indigenous population upon the addition (or incorporation in the growth media) of a particular substrate or can be used as hybridisation markers (silent genes). These genes can also be probed in environmental DNA extractions. Difficulties are found through the masking of these effects by indigenous bacteria when hosts carrying these markers are isolated from fertile environments.

The *lux* gene cassette is responsible for producing luciferase (Shaw & Kado, 1986). In the presence of *n*-decyl aldehyde, bacteria containing the *luxAB* cassette are able to produce light. Bacteria containing the *luxAB* genes can be used for the *in situ* monitoring and detection of an organism in soil (Rattray *et al.*, 1990). Light production was detected *in situ* as well as in liquid culture, in both cases there was correlation with specific biomass. The perceived problem in

this system is the need for ATP within the cell to allow reduction of the aldehyde. Although constitutive *lux* plasmid-bearing strains of *E. coli* did not show any significant difference in growth rate when compared with wild type strains, this has yet to be investigated in soil (Rattray *et al.*, 1990). However, Shaw & Kado (1986) found no difference in infectivity or survival of such a plasmid in plant pathogenic bacteria. Another marker that has been used in this fashion is catechol 2,3 dioxygenase (Morgan *et al.*, 1989; Pickup *et al.*, 1990), which is coded for by the gene *xylE* which is derived from the pseudomonad Tol plasmid. Colonies carrying this marker are visualised through yellow pigment production by spraying isolation plates with catechol (Morgan *et al.* 1989). Tol is a naturally occurring *Pseudomonas* plasmid and thus its presence within the natural population should also be assessed. The *E. coli lacZ* gene product (ß-galactosidase) can also be used as an elective marker (Drahos *et al.*, 1986; O'Gara *et al.*, 1988). It catalyses the breakdown of the chromogenic X-gal (5-chloro-4-bromo-3-indoyl-ß-*D*-galactopyranoside) to a blue coloured derivative. Thus bacteria expressing *lacZ* can easily be visualised on plates. Fluorescent pseudomonads are universally free of the ß-galactosidase phenotype. Drahos *et al.* (1986) cloned *lacZ* and *lacY* (lactose membrane transport protein) into a rifampicin resistant wild isolate of *Pseudomonas fluorescens*, which, coupled with the organisms inherent ability to fluoresce under ultra-violet light, created a series of tests that were highly diagnostic for the recombinant pseudomonad amongst soil/rhizosphere bacteria.

Markers involving selection pressures rely on the ability of a selective agent to either kill or prevent the growth of competitive microorganisms on isolation plates. As the target population is no longer masked by the indigenous microflora relatively low levels of detection can be achieved. Perhaps the most common of such markers are antibiotic resistance markers, which can either be cloned into a host from a foreign organism or obtained by mutation of the host. Regulatory difficulties exist in the release of cloned antibiotic resistance

determinants through their possible transfer *in situ* to pathogenic microorganisms and/or the indigenous microbial populations. However, naturally occurring antibiotic resistance genes are found extensively in clinical (Talbot *et al.*, 1980), wastewater (Alcaide & Garay, 1984), aquatic (Goyal *et al.*, 1979; Jones *et al.*, 1986) and terrestrial systems (Van Elsas & Pereira, 1986), thus their use in foreign organisms for ecological studies should not present a problem with regard to transfer to pathogenic strains. Heavy metal resistance markers are similar to antibiotic resistances, in that nonresistant organisms are killed by the presence of that metal, (Bale, *et al.*, 1987; Top *et al.*, 1990), although potential risks concerning transfer to pathogens are not as great. Theoretically heavy metal resistances could be used in polluted environments provided that the natural resistance of the indigenous population does not cause problems with the level of background activity. The ability to grow on a rare substrate (such as a pollutant) can be used both to study the degradative capacity of the host organism in the environment as well as using this capability to deselect for indigenous microflora on isolation plates (Dwyer *et al.*, 1988a & 1988b; Fulthorpe & Wyndham, 1989; Ramos *et al.*, 1991).

1.3.3 Survival of genetically engineered microorganisms in the environment

There is a certain amount of contradictory evidence concerning the survival of microorganisms carrying cloned DNA. Chemostat studies have shown that the possession of nonchromosomal genetic elements (e.g. plasmids, transposons and temperate bacteriophage) reduced (Jones *et al.*, 1980; Helling *et al.*, 1981; Whan Lee & Edlin, 1985), enhanced (Edlin *et al.*, 1975; Helling *et al.*, 1981; Hartl *et al.*, 1983; Edlin *et al.*, 1984) and had no effect on the fitness of a host bacterium (Jones *et al.*, 1980). It thus appears that the effect of extra

DNA in such systems is dependent on the nature and products of the DNA rather than the DNA burden itself. Some species of microorganisms (genetically-engineered or non-genetically-engineered) persist following their introduction into the environment. On the other hand, other species introduced into the same environment in large numbers die out readily. Many observations have been made on the decline or persistence of different bacterial species (see Table 1), but rarely is it known why microorganisms do or do not survive in natural ecosystems (Alexander, 1981). The competitiveness of non-genetically engineered inoculants in the environment has been best studied in *Rhizobium* with respect to environmental conditions, although many other microorganisms have also been considered. As would be expected, some environmental conditions exert a negative effect, some a positive and some have no effect at all on the survival of a microorganism in the environment. Similarly, the possession of cloned DNA or a chromosomal mutation conferring antibiotic resistance affects the behaviour of a bacterium *in situ* in different ways (see Table 2), with the same antibiotic resistance exerting neutral or negative effects in the same organism (Compeau *et al.*, 1988) or the possession of a seemingly unimportant plasmid exerting a positive effect on the survival of a bacterium (Levy *et al.*, 1980).

Table 1. Survival of bacteria in the environment: Effect of environmental conditions

Organism	Environment	Condition	Effect on survival	Reference
Bacillus	soil	nutrient availability, moisture	+ +	West *et al.* (1985)
	pH (increase)		+	
Escherichia /Salmonella	faeces buried in soil	time	- -	Temple *et al.* (1980)
Escherichia	river-water	competition	-	Flint (1987)
Escherichia	water	(sea-water) (freshwater) (+humic acids)	- -- +	Davies & Evison (1991)
Escherichia	soil	nutrient availability, competition	+ -	Klein & Casida (1967)

Table 1. Survival of bacteria in the environment: Effect of environmental conditions (cont.)

Organism	Environment	Condition	Effect on survival	Reference
Faecal indicator bacteria	estuary	time	-	Pettibone *et al.* (1987)
Flavobacterium /*Arthrobacter*	soil -"-	sterility -"-	+ +	Thompson *et al.* (1990)
Fusarium	soil	inoculum source	0	Couteudier & Alabouvette (1990)
Gram-negatives	lake-water	time	-	Scheurman *et al.* (1988)
Klebsiella /Gram negatives	lake-water	time	- -	Sjogren & Gibson (1981)

Table 1. Survival of bacteria in the environment: Effect of environmental conditions (cont.)

Organism	Environment	Condition	Effect on survival	Reference
Klebsiella/ *Escherichia*	Tropical marine	time	0/-	Lopez-Torres *et al.*(1988)
Pseudomonas/	lake	amendment	+	Henis & Alexander (1990)
Escherichia/	water		+	
Klebsiella/			+	
Micrococcus/			0	
Arthrobacter/			0	
Rhizobium/			+	
Bacillus (asporogenous)			-	
Pseudomonas/	sediment	time	-	Burton *et al.* (1987)
Salmonella/			-	
Escherichia/			-	
Klebsiella			-	
Pseudomonas/ *Actinomyces*	soil	location	S	Miller *et al.* (1989)

Table 1. Survival of bacteria in the environment: Effect of environmental conditions (cont.)

Organism	Environment	Condition	Effect on survival	Reference
Pseudomonas (Ice nucle-ation ability)	soil/ water/ snow	time	- - -	Goodnow *et al.* (1990)
Pseudomonas/ Arthrobacter	soil	nutrient availability, moisture, competition	+/0 -/0 0/0	Labeda *et al.* (1976)
Rhizobium	Bean rhizos-phere	protozoan inhibition	+	Ramirez & Alexander (1980)
Rhizobium	nodule	acidity	S	Dughri & Bottomley (1983 & 1984)
Rhizobium	soil	competition	S	Meade *et al.* (1985)

Table 1. Survival of bacteria in the environment: Effect of environmental conditions (cont.)

Organism	Environment	Condition	Effect on survival	Reference
Rhizobium	nodule	competition	S	Hynes & O'Connell (1990)
Rhizobium	soil	predation	-	Postma *et al.* (1990)
Rhizobium	soil	clay content, raised water content	+ -	Heijnen & Van Veen (1991)
Rhizobium	soil	protection from predation by clay	+	Heijnen *et al.* (1988)
Rhizobium	liquid culture	protection from predation by clay	+	Heijnen *et al.* (1991)

Table 1. **Survival of bacteria in the environment: Effect of environmental conditions** (cont.)

Organism	Environment	Condition	Effect on survival	Reference
Rhizobium	soil	raised water content	-	Postma & Van Veen (1990)
Rhizobium	soil	raised water content	-	Postma *et al.*, 1989
Rhizobium	soil	temperature	+	Boonkerd & Weaver (1982)
		moisture	-	
Rhizobium	soil	time	-	Crozat *et al.* (1982)
Rhizobium	soil/ nodule	inoculum size	+	Mårtensson (1989)
Rhizobium	soil/ peat	soil type	S	McLoughlin & Dunican (1981)

Table 1. **Survival of bacteria in the environment: Effect of environmental conditions** (cont.)

Organism	Environment	Condition	Effect on survival	Reference
Ten strains	soil	transport	0	Gannon *et al.* (1991)
(i.e. the species of bacteria had no effect on the extent of tranfer through soil)				
Total soil bacteria	soil	climate	0	Lundgren & Söderström (1983)

Key. +, enhanced survival; -, reduced survival; 0, no effect; S, strain specific survival.

Table 2. Survival of bacteria in the environment: Effect of cloned or mutated DNA

Organism	Environment	Cloned or mutated DNA	Effect on survival	Reference
Agrobacterium/ Xanthomonas/ Erwinia	phyto-patho-genicity	pRD1 (plasmid)	+ +/- +/-	Kozyrovskaya *et al.* (1984)
Alcaligenes	lake-water	pBR60 (chloro-benzoate degrading plasmid)	+/-	Fulthorpe & Wyndham (1989)
Azospirillum	soil	Tn*5*	S	Bentjen *et al.* (1989)
Enterobacter	rhizo-sphere	pRD1 (plasmid)	S	Kleeberger & Kling-müller (1980)
Erwinia	pond-water	km^r	0	Scanferlato *et al.* (1989 & 1990)

Table 2. **Survival of bacteria in the environment: Effect of cloned or mutated DNA** (cont.)

Organism	Environment	Cloned or mutated DNA	Effect on survival	Reference
Erwinia	soil	km^r	0/+	Orvos *et al.* (1990)
Escherichia	soil	plasmids	+	Devanas & Stotzky (1986a)
Escherichia	soil	pBR322 (plasmid containing *Drosophila* gene)	0	Devanas *et al.* (1986b)
Escherichia	air	ColE1::Tn*5* (plasmid)	0/+	Marshall *et al.* (1988)
Escherichia	intestine	Ab^r (mut^n)	-/0/+	Onderdonk *et al.* (1981)
Escherichia	intestine	pBR322 (plasmid)	+	Levy *et al.* (1980)

Table 2. Survival of bacteria in the environment: Effect of cloned or mutated DNA (cont.)

Organism	Environment	Cloned or mutated DNA	Effect on survival	Reference
Escherichia/ Pseudomonas	lake-water	plasmid	- 0	Awong *et al.* (1990)
Escherichia	river-water, soil	plasmids	0 +	Chao & Feng (1990)
Escherichia	sea-water	pBR322, pUC8 (plamids)	0 0	Byrd *et al.* (1990)
Escherichia *Pseudomonas*	well-water	R388, pRO101 R388, pRO101 (plasmids)	- - - 0	Caldwell *et al.* (1989)

Table 2. **Survival of bacteria in the environment: Effect of cloned or mutated DNA** (cont.)

Organism	Environment	Cloned or mutated DNA	Effect on survival	Reference
Pseudomonas	activated sludge	plasmid (pollutant degrading)	S	Dwyer *et al.* (1988 a & b)
Pseudomonas	activated sludge	pD10 (plasmid)	0	McClure *et al.* (1990)
Pseudomonas/ Klebsiella	drainage water	plasmids	-	Van Overbeek *et al.* (1990)
Pseudomonas	ground-water	pWWO RK2 (plasmids)	0 0	Jain *et al.* (1987)
Pseudomonas	Phyllo-plane	ice minus	+	Lindow (1987)
Pseudomonas	soil	pBS3 (Kelthane degrading plasmid)	+	Golovleva *et al.* (1988)

Table 2. **Survival of bacteria in the environment: Effect of cloned or mutated DNA** (cont.)

Organism	Environment	Cloned or mutated DNA	Effect on survival	Reference
Pseudomonas	soil	pLAFR3,	0	Yeung *et*
		pJE8	0	*al.* (1989)
Pseudomonas	soil	pRO101, pRO103 (herbicide degrading plasmids)	0	Short *et al.* (1990)
Pseudomonas	soil	plasmid (herbicide degrading plasmid)	0	Ramos *et al.* (1991)
Pseudomonas	soil	rif^r (mut^n)	0/-	Compeau *et al.* (1988)
Pseudomonas	soil	Tn*5*	0	Van Elsas
Bacillus		pFT30 (plasmid)	0	*et al.* (1986)

Table 2. Survival of bacteria in the environment: Effect of cloned or mutated DNA (cont.)

Organism	Environment	Cloned or mutated DNA	Effect on survival	Reference
Pseudomonas	soil	Tn*5*/ TN*5*::*tox*	- -	Van Elsas *et al.* (1991)
Pseudomonas	tropical marine	mega-plasmid	0	Cruz-Cruz *et al.* (1980)
Rhizobium	desert soil	Tn*5*	0	Pillai & Pepper (1990)
Rhizobium	soil	Tn*5*	-	Hirsch & Spokes (1988)
Rhizobium	sand/ soil	Tn*5*	0	Catlow *et al.* (1990)
Rhizobium	soil	ab^r (mut^n)	0	Kuykendall & Weber (1978)

Table 2. Survival of bacteria in the environment: Effect of cloned or mutated DNA (cont.)

Organism	Environment	Cloned or mutated DNA	Effect on survival	Reference
Rhizobium	root nodule	ab^r (mut^n)		Turco *et*
		(Nod^n)	-	*al.* (1986)
		(N_2 fix^n)	0/-	
		(T.D.)	+	
Rhizobium	root nodule (nodulation)	rif^r (mut^n), pTA2 (plasmid)	- +	Bromfield *et al.* (1985)
Rhizobium	root nodule (nodulation)	rif^r (mut^n)	-	Lewis *et al.* (1987)
Streptomyces	soil	plasmids,	0	Wang *et al.*
		mut^n	0	(1989)

Key. +, enhanced survival; -, reduced survival; 0, no effect on survival; S, organism survived; rif^r, rifampicin resistance; km^r, kanamycin resistance; ab^r, antibiotic resistances; mut^n, chromosomal mutation (i.e. not carried on vector); TD, doubling time; nod^n, nodulation; N_2 fix^n, nitrogen fixation.

1.3.4 Gene transfer *in vivo*

Much of the concern about the release of GEMs into the environment stems from the possibility of resistance/pathogenicity genes entering populations of clinically important bacteria, as well as dangers of ecosystem destabilisation. *E. coli* is the major host for research in molecular biology. As well as being an inhabitant of the intestine it is also sexually promiscuous and can transfer genetic information to over 40 gram-negative (Stotzky & Babich, 1986), and one gram-positive bacterium (E. Gormley & J. Davies, unpublished data). Thus there is a perceived risk of such genes transferring to potential pathogens.

The intestinal tract should certainly provide an ideal site for bacterial matings to occur, if only, because of the suitable temperature for mating pair formation (Wamsley, 1976) and the consistent high cell density (Reanney, 1976). For gene transfer to occur efficiently in the intestinal tract, the gut must be colonised by large numbers of donor and recipient organisms (Stotzky & Babich, 1986). However, it is difficult for bacteria to colonise the intestine unless the indigenous microflora is in some way suppressed (e.g. use of germ free animals or antibiotic suppression has enabled gene transfer to be visualised [Walton, 1966; Jarolman & Kemp, 1969; Guinée, 1970; Marshall *et al.*, 1981]). Detection of these events is not well documented in systems containing a gut flora, although Freter *et al.* (1983) did find plasmid transmission in gnotobiotic mice carrying a synthetic microflora. Plasmid transfer was detected between *E. coli* strains in the undisturbed gut of calves (Timoney & Linton, 1982), however in normal intestines containing an undisturbed microflora this seems to be a rare occurrence (Freter *et al.*, 1983; Stotzky & Babich, 1986). Clinically important bacteria also harbour bacteriophage that might facilitate transfer of genetic information, e.g. Hyder & Streitfield (1978) isolated strains of *Streptococcus* which carried temperate phage that could transfer erythromycin resistance *in vitro*, although whether this was due to transduction or phage conversion is not

clear.

1.3.5 Gene transfer in aquatic systems

Aquatic ecosystems encompass a wide range of habitats with many different characteristics. The potential for gene transfer varies depending on the nature of that system (see review by Saye & Miller, 1989). The input of sewage into these environments stresses the importance of genetic exchange with a view to the survival of antibiotic resistant strains (Grabow *et al.*, 1976) and antibiotic resistance transfer to medically important bacteria (Trevors *et al.*, 1987). Mechanisms of gene transfer can be divided into three mechanisms, based on their method of transmission: conjugation, transformation and transduction.

In the environment conjugation is, perhaps, the best studied mechanism because of the potential for transfer of R-factors from faecal contaminants (Mach & Grimes, 1982; Alcaide & Garay, 1984). Matings between pairs of bacteria on some sort of support (e.g. dialysis sacs, nylon filters) have been readily shown in aquatic systems (Grabow *et al.*, 1975; Gowland & Slater, 1984; Trevors & Oddie, 1986; O'Morchoe *et al.*, 1988). Genthner *et al.* (1988) assessed the capacity of freshwater isolates to act as recipients for conjugation, and found that *ca.* 40% of these isolates were capable of receiving plasmid DNA (wide host range, self-transmissible) *in vitro*. This approach was reversed by Gauthier *et al.* (1985), who examined the transfer of naturally occurring plasmids (Hg^r) to *E. coli in vitro*. Also using metal resistance as a marker, Pickup (1989) found that 60% of copper tolerant bacteria isolated from lake water carried at least one plasmid. Genther *et al.* (1988) reported that transfer frequencies between a *Pseudomonas* donor and natural isolate recipients were higher on plates than in liquid culture. This suggests that conjugation in aquatic systems may occur more readily when the mating pairs are attached to suspended solids or mechanical

supports, although Singleton (1983) showed that colloidal clay reduced conjugation of *E. coli* through clay adhering to the cell surface and producing a barrier that inhibited mating. In waste-water systems, which contain higher levels of suspended solids than potable water, conjugation can also be readily demonstrated (Grabow, *et al.*, 1976; Mach & Grimes, 1982; Gealt *et al.*, 1985; Mancini *et al.*, 1987), although Altherr & Kasweck (1982) could only detect gene transfer in raw sewage, and not in waste-water. An unusual observation was made by McClure *et al.* (1990), who, after inoculating a nonconjugative plasmid-bearing strain of *Pseudomonas* into an activated sludge unit, subsequently re-isolated the inoculum to find that it had acquired indigenous plasmids that had the ability to mobilise the original plasmid to recipient strains *in vitro*.

Conjugation also occurs extensively in the epilithon (the slime layer covering stones in aquatic habitats). Naturally isolated, mercury resistance plasmids that were able to transfer over a wide range of environmentally significant conditions (Rochelle *et al.*, 1989a) were used to demonstrate gene transfer between *Pseudomonas* strains in the epilithon (Bale *et al.*, 1987; Bale *et al.*, 1988; Day *et al.*, 1988; Fry & Day, 1990; Rochelle *et al.*, 1989b). *Agrobacterium* can conjugate *in vivo* (tomato plants), where Kerr *et al.* (1977) demonstrated the transfer of virulence. Gene transfer can also take place *in planta* (e.g. from *E.coli* to *Pseudomonas* [Lacy & Leary, 1975; between *Klebsiella* strains on radish plants [Talbot *et al.*, 1980]) and has been reviewed by Farrand (1989).

Extracellular DNA is produced and excreted by aquatic bacteria (Paul & David, 1989, Paul *et al.*, 1990); thus, along with DNA released from lysed bacteria, there is a potential for transformation of aquatic microorganisms in the aquatic ecosystem (reviewed by Stewart, 1989). If this DNA can adsorb to sand or sediment particles it then becomes much more resistant to the action of exonucleases (Lorenz *et al.*, 1981; Aardema *et al.*, 1983; Lorenz &

Wackernagel, 1987). Natural transformation of *Pseudomonas stutzeri* occurs *in vitro* (Carlson *et al.*, 1983; Stewart & Sinigalliano, 1989) and also when attached to solids *in situ* (Lorenz & Wackernagel, 1990; Stewart & Sinigalliano, 1990); although Stewart *et al.* (1991) found that exogenous DNA is only biologically active when the solids (autoclaved sediment) were first saturated with nonhomologous DNA. This process would seem to be ecologically important to the *Pseudomonas* donor strain involved as Stewart *et al.* (1983) showed the process to be active and plasmid independent. In addition to *Pseudomonas*, many other genera undergo natural transformation e.g. *Bacillus* (Lorenz *et al.*, 1988; Lorenz & Wackernagel, 1988), epilithic *Acinetobacter* (Rochelle *et al.*, 1988) and *Vibrio* (Frischer *et al.*, 1990; Jeffrey *et al.*, 1990).

The first report of transduction in the environment was by Baross *et al.* (1974), who showed the transduction of agarase genes between strains of *Vibrio parahaemolyticus* in the guts of oysters in sterile sea water. The possible mechanisms of this process in the environment have been reviewed by Kokjohn (1989). Transduction and co-transduction have been shown between strains of *Pseudomonas* in lakewater (Morrison *et al.*, 1978; Saye *et al.*, 1987; Saye *et al.*, 1990). Morrison *et al.* (1978) found that, as well as transduction of a recipient by an inoculated free phage lysate, lysogenic donors could be induced *in situ* and subsequently that transducing phage particles, carrying chromosomal marker genes, could reinfect a non-lysogenic recipient. Later work by Saye *et al.* (1987) only detected transduction (of plasmids) in a system which used a lysogenic recipient strain and a non-lysogenic plasmid donor. Saye *et al.* (1990) found no significant difference in the transduction of chromosomal DNA and plasmid DNA *in vitro*, although higher frequencies of plasmid transduction were found *in situ*. This study also showed that chromosomal markers could be cotransduced *in situ* and that transductants of lysogenised strains were obtained 10 to 100 fold more frequently than from non-lysogenic parents.

1.3.6 Gene transfer in terrestrial systems

Gene transfer in soil differs in one important respect from other environments, in that, through the nature of the environment, it is likely to be more discontinuous in both time and space due to the greater heterogeneity of soil compared to other environments. Soil microorganisms can either be oligotropic or copiotrophic, thus one would expect the gene transfer strategies of these kinds of organisms to reflect their overall lifestyle; i.e. oligotrophic microorganisms exchanging DNA at a continuous low level and copiotrophic organisms in brief periods of high activity.

All bacteria, in order to exchange DNA, must physically move the genetic information from donor to recipient. In as much as all soil microorganisms are aquatic (ie exist in a covering of water), their movement (and hence that of genetic information) is restricted by the absence of soil water, apart from periods of water saturation (Stotzky, 1989).

Much work has used sterile soil or non-indigenous strains (e.g. *E. coli*) to investigate genetic interactions in soil (e.g. Trevors & Oddie, 1986). Whilst, sterile soil is not truly reflective of field conditions, it can certainly give an indication of the likelihood of gene transfer in that system (Trevors & Oddie, 1986). Soil is a heterogeneous system, therefore it is likely that microenvironments exist that do containing only one bacterial species. Several methods can be used to sterilise soil (e.g. autoclaving, gamma irradiation or methyl bromide treatment). Autoclaving is probably the most destructive of these in terms of loss of soil structure and effect on metabolism, respiration and nitrogen status (Powlson & Jenkinson, 1976; Ramsay & Bawden, 1983). Acridine-orange direct counts indicated that 55% of the microbial population was lysed by autoclaving, and storage at 5°C for 7 days, whilst in gamma irradiated soil the soil microflora, measured by direct counting, was still intact after 21 days (Ramsay & Bawden, 1983). Work by Powlson & Jenkinson (1976) demonstrated that autoclaving caused the greatest increase in the amount of

exctractable organic carbon when compared to fumigation, air drying and gamma irradiation. Sterilised soil is therefore devoid of indigenous microorganisms, but is also nutrient amended through the release of previously unavailable nutrients.

E. coli is not a natural inhabitant of soil, however experiments investigating its gene transfer capability have relevance to the safety and potential escape of genes from laboratory strains. For example *E. coli* can survive for at least three weeks in soil (Krazovsky & Stotzky, 1986) and mobilise a plasmid to indigenous bacteria (Henschke & Schmidt, 1990). Conjugation takes place in soil within many genera. The first report was by Weinberg & Stotzky (1972), who demonstrated conjugation between strains of *E. coli* in sterile soil (both plasmids and chromosomal DNA were transferred). It was found that the addition of montmorillonite clay to the system enhanced the frequency of conjugation, presumably through enhanced adhesive properties of the soil and stabilisation of the mating pair. Since then *E. coli* conjugation has been demonstrated in sterile amended soil (Trevors & Oddie, 1986; Trevors, 1987) and in nonsterile soil (Krasovsky & Stotzky, 1986; Trevors & Starodub, 1987), where the process was shown to be pH dependent.

Intrageneric gene transfer has also been observed between bacilli in sterile amended soil and nonsterile soil amended with bentonite (Van Elsas *et al.*, 1987), but was undetectable in wheat rhizosphere (Van Elsas *et al.*, 1988a). *In situ* conjugation has been detected between pseudomonads in sterile soil (Trevors & Berg, 1989) and in a sterile peat-vermiculite mixture, rhizosphere and phyllosphere (Knudsen, *et al.* 1988). It has also been demonstrated in nonsterile bulk soil and wheat rhizosphere, but was enhanced in the rhizosphere or when bulk soil was amended with bentonite or nutrients (Van Elsas, *et al.*, 1988a; 1988b; 1989 & 1990). Another genus that has been shown to conjugate in a soil based system is *Rhizobium*, which can conjugate in sterile alfalfa nodules (Pretoriüs-Guth *et al.*, 1990).

Less information on intergeneric conjugation in soil is available. It has been demonstrated in sterile soil from *E. coli* to *Rhizobium*, where transfer frequency was shown to be dependent on a number of abiotic factors (Richaume *et al.*, 1989). Conjugation was also demonstrated in sterile soil (Henschke & Schmidt 1989) between *E. coli* and *Enterobacter* and between *Enterobacter* and *Pseudomonas*. Meanwhile, Top *et al.* (1990) were able to show conjugation in sterile & nonsterile amended soil between *E. coli* and *Alcaligenes*. Transfer of plasmid pFL67-2 (based on the promiscuous plasmid RSF1010) from *E. coli* to indigenous *Pseudomonas fluorescens* strains was shown in natural soil by Henschke & Schmidt (1990).

Whilst transformation has been studied extensively in aquatic systems (see Section 1.3.5) it is a poorly examined area in the soil ecosystem. The only reports of the transfer of auxotrophic and resistance markers between *Bacillus subtilis* strains used in sterile amended soil are those of Graham & Istock (1978 & 1979). As both donor and recipient were plasmid and transducing phage free this transfer was attributed to transformation. The addition of DNase did not prevent the process, as would be expected if the transfer was due to transformation, however the inactivity of the DNase was assumed to be due to either inactivation of the enzyme or DNA protection by clay particles. Many bacterial species in soil are naturally transformable (Lorenz & Wackernagel, 1988), and some more recent work has demonstrated that a number of genera can be transformed e.g. *Bacillus* and *Pseudomonas* (Lorenz & Wackernagel, 1988 & 1990; Lorenz *et al.* 1988), albeit in highly artificial conditions.

As with transformation, transduction in soil is also a poorly studied process. Germida & Khachatourians (1988) raised a transducing lysate from a Tn10-containing strain of *E. coli* and used this to transduce an auxotrophic and tetracycline sensitive strain *in situ*. *E. coli* was also used to demonstrate phage-mediated gene transfer in soil by Zeph *et al.* (1988), although this was not transduction but a phage conversion (Barksdale & Arden, 1974). A derivative of

phage P1 (containing Tn*501*) was used to create lysogens of a marked *E. coli* strain, this was then inoculated into soil with a marked non-lysogenic recipient. The appearance of the resistance markers of Tn*501* in the recipient showed the phage had been induced from the donor and reinfected the recipient. The transfer was confirmed by the use of nucleic acid probing (Zeph & Stotzky, 1989).

1.4 Mechanisms of gene transfer in *Streptomyces*

There are four methods of genetic exchange in *Streptomyces*; conjugation, transduction, transformation/transfection, and protoplast fusion-recombination (reviewed by Hopwood, 1967; Hopwood, *et al.*, 1973; Chater & Hopwood, 1984; Rafii & Crawford, 1989a). The first two processes are essentially natural, in that they can take place without substantial *in vitro* manipulation. The latter two processes, however, require severe treatment of the participant cells in order to achieve gene transfer. Thus, it is likely that conjugation and transduction are the two methods that facilitate genetic exchange between streptomycetes in the environment.

Most genetic studies have been carried out on two closely related strains of species cluster 21 (Williams *et al.*, 1983); *Streptomyces coelicolor* A3(2) and *Streptomyces lividans* 66 (also called 1326 [John Innes strain number]). Many derivatives have been constructed from them because of their amenability to genetic analysis (Hopwood *et al.*, 1985).

1.4.1 *Streptomyces* gene transfer *in vitro*

Streptomyces are able to undergo chromosomal recombination (reviewed by Hopwood, 1967; Hopwood, *et al.*, 1973; Chater & Hopwood, 1984; Rhodes, 1986; Rafii & Crawford, 1989a). With the exception of situations where the parental strains have been subjected to protoplast fusion, the exchange of chromosomal genes is governed by the presence of a plasmid in one of the parental strains. A tentative model for plasmid transfer and spread in streptomycetes was proposed by Hopwood *et al.* (1986). The model proposes that transfer between hyphae occurs by means of hyphal fusion and may require host genes. Many plasmids have been isolated from *Streptomyces* (reviewed by Hopwood *et al.*, 1986). Wild type S.*coelicolor* A3(2) contains four plasmids (Hopwood, 1983): SCP1 (Vivian, 1971); SCP2 (Bibb *et al.*, 1977, Bibb & Hopwood, 1978); SLP1 (this plasmid exists as a chromosomal integrate in S.*coelicolor* A3(2), but can also exist as an autonomous plasmid in *S.lividans* 66 [Bibb *et al.*, 1981]); and SLP4 (Hopwood *et al.*, 1983). Meanwhile *S.lividans* 66 contains two plasmids (SLP2 and SLP3 [Hopwood *et al.*, 1983]), when these plasmids are cured no conjugation or chromosomal transfer is detectable in this species. pIJ101, a plasmid isolated from *S.lividans* ISP 5434, was found to be much smaller (8.9 kb) than other plasmids so far isolated from *S.lividans* or S.*coelicolor* A3(2) (Kieser *et al.*, 1982). This plasmid is self-transmissible and can promote conjugation and chromosomal transfer; derivatives of pIJ101 (Hopwood *et al.*, 1985) are probably the most common cloning vectors used in *Streptomyces*. The transfer functions of this plasmid have been characterised (Kendall *et al.*, 1988) which have enabled it to be used to construct a shuttle vector that is capable of mediating conjugation between *Streptomyces* and *E. coli* (Mazodier *et al.*, 1988).

Few transducing phage of streptomycetes have been characterised to date. SV1 and related phage (Stuttard, 1979, Stuttard, 1989), infecting the

chloramphenicol-producing strains *Streptomyces venezuelae* and *Streptomyces phaeochromogenes* (Stuttard, 1982), were the first examples of generalised transducing phage in *Streptomyces*. The prophage of ΦSF1 exists as two plasmids (Chung, 1982; Chung & Thompson, 1985) and is capable of transducing *Streptomyces fradiae*. A transducing phage of *Streptomyces olivaceus* was reported previously (Alikhanian *et al.*, 1960), but few genetic markers were available and no subsequent data was published, so the significance of this report remains uncertain (Hopwood *et al.*, 1973). SH10 (see Section 1.4.3) was also thought to be a generalised transducing phage (Süss & Klaus, 1981), however, the transduction frequencies were approximately the same as the reversion frequencies of the markers used. No transducing phage have yet been isolated for S.*coelicolor* A3(2) or *S.lividans* 66 (Chater, 1986), although attempts were made to isolate phage that package by the headful using chelating agent resistance (Stuttard, 1989). Cosmids can also be used to mediate gene transfer between streptomycetes; Morino *et al.* (1985) constructed such a vector by inserting the *cos* sequence of R4 into pIJ365 (Kieser *et al.*, 1982). This cosmid was efficiently packaged and transduced as a head to tail concatamer. Further studies used this molecule as a vector for transfer of melanin synthesis genes (Morino *et al.*, 1988). The phage FP43 (see Section 1.4.3) has also been used to construct a cosmid by cloning a segment of the phage genome into a pIJ101 derivative, pIJ702 (Kieser *et al.*, 1982).

S.*coelicolor* A3(2) and *Streptomyces parvulus* protoplasts were first transformed by SCP2* in the presence of PEG 1000, the transformants were then confirmed as such by plating onto a lawn of an SCP2^{-} strain, followed by scoring for pock formation (Bibb *et al.*, 1978). Subsequent work demonstrated that *S.lividans* 66 and strains of S.*coelicolor* could be transfected with a number of different actinophage (including ΦC31), using a similar protocol (Suárez & Chater, 1980a). This process was further developed by the encapsulation of transforming chromosomal DNA in liposomes (Makins & Holt, 1981) and the

use of a liposome-assisted method of transfection (Rodicio & Chater, 1982). Since then it has been possible to transform/transfect many other *Streptomyces* species e.g. *Streptomyces peucetius* (Lambel & Strohl, 1986), *Streptomyces tendae* (Engel, 1987), *Streptomyces griseus* (Daza *et al.*, 1990) and *Streptomyces venezuelae* (Aidoo *et al.*, 1990; Anné *et al.*, 1990a). There is no evidence for natural transformation of *Streptomyces* (Chater & Hopwood, 1983), although *Thermoactinomyces vulgaris* is able to undego transformation without such treatment as protoplasting (Hopwood & Wright, 1972). However, this strain has now been reclassified in the *Bacillus/Clostridium* branch of the Gram positives.

Protoplast fusion was first demonstrated in *Streptomyces* by Hopwood *et al.* (1977), using the PEG treatment of a protoplast mixture of two marked streptomycetes. Recombination occurred in the absence of both known sex factors (at the time), indicating that this recombination was independent of sex factor activity. This process results in multiple cross-over events between chromosomes and thus a higher proportion of recombinants in the progeny (Hopwood & Wright, 1978; Baltz, 1980).

1.4.2 *Streptomyces* gene transfer in the environment

Streptomyces are capable of exchanging genetic information *in situ* by means of conjugation (reviewed by Rafii & Crawford, 1989a). Transformation and transduction have not yet been demonstrated (Rafii & Crawford, 1989a). It is thought, however, that gene transfer by these mechanisms would be rare, due to the lack of transducing phage of *Streptomyces* (Chater, 1986), the high level of restriction in this genus (Cox & Baltz, 1984) and the fact that *Streptomyces* are not naturally competent for transformation. The first reports of conjugation in sterile soil were by Rafii & Crawford (1988) and Wellington *et al.* (1988), who showed that derivatives of pIJ101 could be transferred and mobilised

between strains of *S.lividans* and other streptomycetes. Bleakley & Crawford (1989) reported that the number of transconjugants in sterile soil was increased by amendment of the soil and decreasing water content, although Rafii & Crawford (1989b) detected conjugation in liquid culture and suggested that *Streptomyces* may be capable of mating in aqueous systems. Also using a system based on *S.lividans* and a pIJ101 derivative it was found that these organisms could conjugate intra- and inter-specifically in nonsterile soil (Wellington *et al.*, 1990a; Wellington *et al.*, 1990b; Wellington *et al.*, 1991, in press). This system was used for the development of a mathematical model based on the Pearl-Verhulst logistic equation, which was capable of accurately predicting populations and response to nutrient and moisture limitation (Clewlow *et al.*, 1990).

1.4.3 Actinophage

Many *Streptomyces* phage have been isolated, some of which are specific for individual species and others polyvalent (Korn *et al.*, 1978; Wellington & Williams, 1981). The ability of a bacteriophage to infect a particular host, in addition to adsorption requirements, is affected by host restriction endonuclease production (Chater & Wilde, 1976; Chater & Carter, 1978; Cox & Baltz, 1984). Whilst many bacteriophage have been isolated, relatively few have been studied in depth. The best characterised actinophage is ΦC31 (see Section 1.4.4), although molecular studies are now being carried out on other actinophage. VWB (Anné *et al.*, 1984 & 1990c), isolated on *Streptomyces venezuelae* is resistant to chelating agents and also has cohesive ends (Anné *et al.*, 1985), suggesting a capacity for carrying extra DNA (Anné *et al.*, 1990b) and thus have potential use as a cloning vector. A temperate wide host range actinophage, R4 (Chater & Carter, 1979), was isolated using a restriction deficient mutant of

Streptomyces albus G. A naturally lysogenic culture of *Streptomyces lavendulae* yielded a phage, Bα (Nakano *et al.*, 1981), which is now considered analogous to R4 (Chater, 1986). This phage and FP43 (McHenney & Baltz, 1988) have been extensively manipulated *in vitro* to create cosmid vectors for use in cloning experiments (see Section 1.4.1). FP22 is, in genome terms, the largest (131 kb) streptomycete bacteriophage so far described (McHenney & Baltz, 1988; Hahn *et al.*, 1990a). It was isolated from soil by enrichment with *Streptomyces fradiae* and *Streptomyces griseofuscus*. This phage is unusual as it has a very wide host range (lysing 70% of strains tested [Hahn *et al.*, 1990a]), has few restriction sites for endonucleases produced by *Streptomyces* strains and has a Mol% (G+C) of 46% (Hahn *et al.*, 1990a). As FP22 has at least 23 kb of dispensable DNA, coupled with its headfull packaging mechanism, this makes it a potentially powerful vehicle for cloning in *Streptomyces*. The final group of well-characterised *Streptomyces* phage are those infecting *Streptomyces hygroscopicus* (SH3, SH10, SH11 & SH12). These actinophage were isolated from soil and lysogenic cultures of *S. hygroscopicus* (Klaus *et al.*, 1979). The temperate phage SH10 was characterised by Klaus *et al.* (1981) and was found to have a wide host range and be capable of transfecting *S.lividans* 66 (Krügel *et al.*, 1980). This phage was also thought to mediate transduction (see Section 1.4.1), although frequencies of transduction were similar to the spontaneous mutation rates of the markers used.

1.4.4 Biology of ΦC31

The actinophage most extensively studied is ΦC31 (Lomovskaya *et al.*, 1980; Chater, 1986), which was first isolated from a culture of S.*coelicolor* A3(2) (Lomovskaya *et al.*, 1970). Early work on ΦC31 showed it to be a true temperate phage. When lysogenic and non-lysogenic strains were crossed, the

prophage segregated like any other chromosomal marker of S.*coelicolor* A3(2) (Lomovskaya *et al.*, 1971). The frequency of lysogenisation of ΦC31 in S.*coelicolor* A3(2) is 20-40% and 1% of germinated lysogenic spores spontaneously release phage (Lomovskaya *et al.*, 1972). ΦC31 has a wide host range and infects about half of the 137 strains it has been tested against (Voeykova *et al.*, 1979; Chater, 1986). The phage is naked, showing a polyhedral head (53 nm wide), a long non-contractile tail (100 x 5 nm), a basal plate (15 nm in diameter) with at least one pin and a prominent knot between the head and tail (Suárez *et al.*, 1984). Up to 17 polypeptides have been found in the virion, four of them (51, 38.5, 29.5 and 28 kda) make up *ca.*84% of the total protein of the particle (Suárez *et al.*, 1984). The DNA of ΦC31 is 63 Mol% (G+C), has a molecular weight of 2.67 x 10^7 (39.02 kb [Lomovskaya *et al.*, 1980] or 41.2 kb [Chater *et al.*, 1981]) and contains cohesive ends (Chater, 1980). The left half of the molecule encodes essential late functions, whilst the central 3 kb contains the *c* (repressor) gene (Chater *et al.*, 1985). To the right of the *c* region is about 10 kb of essential DNA encoding early functions, and then 8 kb of inessential DNA, including the *attP* site required for prophage integration and at least two functions that interact with the host restriction-modification system (Chater *et al.*, 1985). ΦC31 is not able to transduce host DNA (Lomovskaya *et al.*, 1980) and adsorbs by the tail to the surface of susceptible cells (Lomovskaya, 1972). Newly-germinated mycelia are especially susceptible to ΦC31 (Lomovskaya, 1972). However, Rosner & Gutstein (1980) suggested that older mycelia might be more efficient at adsorbing actinophage. The DNA enters the host by an unknown mechanism in a non-permuted linear form. The molecule then circularises at the cohesive ends, which are then ligated to give a covalently closed circular molecule (Lomovskaya *et al.*, 1980). In the case of a lysogenic response, this molecule is inserted into the S.*coelicolor* A3(2) chromosome between *uraA1* and *pheA1* (Lomovskaya *et al.*, 1973). The establishment and maintenance of lysogeny involve the action of a single

repressor protein (72 kDa, with a predicted DNA binding domain [Sinclair & Bibb, 1988]) the reduction in concentration of which (e.g. due to hyphal fusion) can result in zygotic induction (Emeljanova *et al.*, 1973; Lomovskaya *et al.*, 1980). Induced lysogens show a latent period of 45 minutes, followed by a rise period of 20-30 minutes giving a burst size of 100-200 (Rodríguez *et al.*, 1986). Induction of lysogens also causes a reduction in host RNA synthesis (Rodríguez *et al.*, 1986; Clayton & Bibb, 1990). DNA replication involves a "rolling circle" mechanism generating concatameric molecules; the packaging of these into mature phage involves the site specific cleavage of the concatamers into monomers (Chater, 1980). Many cloning vectors have been constructed from ΦC31 (see Chapter 4 and Chater *et al.*, 1985)

1.5 General and specific aims of project

The aims of this project were to study the of a marked actinophage in soil and assess its potential to act as a vector for the introduction of genes to soil streptomycetes (phage conversion). Other aims were to develop new techniques to study the ecology of marked streptomycetes *in situ* and gain more information about this group of microorganisms and their phage in the environment.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial strains

Streptomyces lividans 1326, *Streptomyces lividans* TK23 and TK24 were used as hosts for phage propagation and for soil microcosm studies, their characteristics are listed in Table 3.

2.2 Phage

The actinophage used in this study were ΦC31 c1, KC301 and SV1. Their characteristics are given in Table 4.

2.3 Chemicals

The source and type of chemicals used in this study are listed in Table 5.

2.4 Media

All media were made up with double distilled water and sterilised by autoclaving at 121°C for 15 minutes unless otherwise stated. A list of media and their constituents is given in Table 6, whilst a list of antibiotics is given in Table 7.

Table 3. Bacterial strains

Strain	[1]Characteristics	Source	Reference
S.lividans 1326	SLP2$^+$ SLP3$^+$ (plasmids)	T.Kieser	Hopwood *et al.* (1985)
S.lividans TK23	[2]*spc-1*	T.Kieser	Hopwood *et al.* (1985)
S.lividans TK24	[3]*str-6*	T.Kieser	Hopwood *et al.* (1985)

[1]Known plasmids, Chromosomal resistance markers.

[2]Spectinomycin resistance.

[3]Streptomycin resistance.

Table 4. Phage

Phage	Characteristics	Source	Reference
ΦC31 c1	Clear plaque mutant	K. Chater	Lomovskaya *et al.* (1970)
KC301	Temperate, carries [1]*tsr* fragment	K. Chater	Chater *et al.* (1982)
SV1	Generalised transducing phage	C. Stuttard	Stuttard (1979)

[1]Thiostrepton-resistance. (Thompson *et al.*, 1980).

Table 5. Chemicals

Chemical	Supplier
Agar, Bacto	Difco
Agarose	Sigma
L-arginine	Sigma
L-asparagine	Sigma
Bovine serum albumin (fraction V)	Sigma
Bromophenol blue	Sigma
Cesium chloride	BDH
Casaminoacids	Difco
Chelex-100	Biorad
Chitin (crabshell)	Sigma
Chloroform	BDH
Cycloheximide	Sigma
DMSO	FSA
DNA (Herring sperm)	Sigma
Egg albumin	Sigma
Ethanol	Hayman Ltd.
Ficoll 400	Sigma
Gelatin	Sigma
Glucose	BDH
Glycerol	BDH
8-Hydroxyquinoline	BDH
Isopropanol	BDH
Lysozyme (egg white)	Sigma

Table 5. Chemicals

Chemical	Supplier
Methanol	FSA
Neomycin sulphate	Sigma
Nutrient agar	Oxoid
Nutrient broth	Difco
Nystatin	Sigma
Phenol (chromotography grade)	BDH
Phenol (analar)	BDH
PEG 6000	BDH
PVPP (MW 360 000)	Sigma
Potassium acetate	BDH
L-proline	Sigma
Restriction enzymes	BRL
Restriction buffers	BRL
Rifampicin	Sigma
RNase I	Sigma
Sodium acetate	BDH
Sodium citrate	BDH
Sodium-EDTA	BDH
SDS	BDH
Spermine tetrahydrochloride	Sigma
Starch (soluble)	BDH
Streptomycin sulphate	Sigma
Sucrose	BDH

Table 5. Chemicals

Chemical	Supplier
TES buffer	BDH
Thiostrepton	Sigma
Tris-HCl	BDH
Tryptone soya broth	Oxoid
Yeast extract	Oxoid

Table 6. Media

Medium	Constituents per litre[1] (unless otherwise indicated)	
R2YE	Sucrose	103 g
	K_2SO_4	0.25 g
	$MgCl_2.6H_2O$	10.1 g
	Glucose	10 g
	Casamino acids	0.1 g
	Distilled water	800 ml
	Bacto agar	25 g
	(10 ml 0.5% (w/v) KH_2PO_4)	
	(80 ml 3.68% (w/v) $CaCl_2.6H_2O$)	
	(15 ml 20% (w/v) L-proline)	
	(10 ml TES buffer, pH 7.2)	
	(2 ml Trace elements solution)	
	(5 ml 1 N NaOH)	
	(50 ml 10% (w/v) Yeast extract)	
R5 (R2YE alternative method)	Sucrose	103 g
	K_2SO_4	0.25 g
	$MgCl_2.6H_2O$	10.1 g
	Glucose	10 g
	Casamino acids	0.1 g
	Yeast extract	5 g
	Bacto agar	25 g

Table 6. Media

Medium	Constituents per litre[1] (unless otherwise indicated)	
(R5 [cont.])	TES buffer	5.73 g
	Trace elements solution	2 ml
	(10 ml 0.5% (w/v) KH_2PO_4)	
	(4 ml 5 M $CaCl_2.2H_2O$)	
	(15 ml 20% (w/v) L-proline)	
	(7 ml 1 N NaOH)	
Trace elements 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
	$(NH_4)_6Mo_7O_{24}.4H_2O$	10 mg
Oatmeal agar	[2]Oatmeal	20 g
	Yeast extract	5 g
	Bacto agar	15 g
Arginine glycerol salts agar (AGS)	L-Arginine	1 g
	Glycerol	12.5 g
	Bacto agar	15 g
	K_2HPO_4	1 g

Table 6. Media

Medium	Constituents per litre[1] (unless otherwise indicated)	
(AGS [cont.])	NaCl	1 g
	$MgSO_4.7H_2O$	0.5 g
	$Fe(SO_4)_3.6H_2O$ (10 g/l)	1 ml
	$CuSO_4.5H_2O$ (1 g/l)	1 ml
	$ZnSO_4.7H_2O$ (1 g/l)	1 ml
	MnSO4.4H2O (1 g/l)	1 ml
	[pH 8.0]	
Reduced arginine salts agar (RASS)	L-Arginine	0.1 g
	Starch (soluble)	12.5 g
	Bacto agar	15 g
	K_2HPO_4	1 g
	NaCl	1 g
	$MgSO_4.7H_2O$	0.5 g
	$Fe(SO_4)_3.6H_2O$ (10 g/l)	1 ml
	$CuSO_4.5H_2O$ (1 g/l)	1 ml
	$ZnSO_4.7H_2O$ (1 g/l)	1 ml
	$MnSO_4.4H_2O$ (1 g/l)	1 ml
	[pH 8.0]	
Minimal medium (MM)	Bacto agar	1 g
	L-asparagine	0.5 g

Table 6. Media

Medium	Constituents per litre[1] (unless otherwise indicated)	
(MM [cont.])	K_2HPO_4	0.5 g
	$MgSO_4.7H_2O$	0.2 g
	$FeSO_4.7H_2O$	0.01 g
	[pH 7.0-7.2]	
	(20 ml 50%(w/v) [3]glucose)	
Nutrient agar (NCG)	Nutrient agar	23 g
	Glucose	5 g
	(10 ml 1 M $MgSO_4.7H_2O$)	
	(10 ml 0.8 M $Ca(NO_3)_2.6H_2O$)	
Soft nutrient agar	Nutrient broth	8 g
(SNA)	Glucose	5 g
	Bacto agar	3 g
	(10 ml 1 M $MgSO_4.7H_2O$)	
	(10 ml 0.8 M $Ca(NO_3)_2.6H_2O$)	
Tryptone soya broth	Tryptone soya broth	30 g
+ 10% (w/v) sucrose (TSB)	Sucrose	100 g
Nutrient Broth	Nutrient broth	8 g

Table 6. Media

Medium	Constituents per litre[1] (unless otherwise indicated)	
Phage buffer (APB)	10 mM Tris HCl, pH 7.5	1000 ml
	0.8 M $Ca(NO_3)_2.6H_2O$	5 ml
	5 % (w/v) Gelatin	1 ml

[1]Items in parentheses were added after autoclaving from sterile stocks.

[2]The oatmeal was boiled for one hour before autoclaving..

[3]Filter sterilised through 0.22 μm nitrocellulose filter.

Table 7. Antibiotic stocks

Antibiotic	Stock solution (mg/ ml)	Final concentration in media (μg/ ml)		
		Defined[6]	Complex	Liquid
[1]cycloheximide	25	50	-	-
[2]neomycin (sulphate)	10	1	10	1
[3]nystatin	50	50	-	-
[4]rifampicin	10	10	10	-
[2]spectinomycin	50	50	100	-
[2]streptomycin (sulphate)	50	10	50	50
[5]thiostrepton	50	50	50	5

[1]Dissolved in distilled water and sterilised by autoclaving.

[2]Dissolved in distilled water and sterilised through a 0.22 μm nitrocellulose filter.

[3]Dissolved in small volume sterile 0.1 N NaOH and made up to volume with SDW.

[4]Dissolved in methanol.

[5]Dissolved in chloroform or dimethylsulphoxide (DMSO).

[6]Defined media refers to RASS, AGS and MM. Complex to R2YE, R5 and Oatmeal. Liquid refers to TSB.

2.5 Buffers, reagents and solutions

Buffers, reagents and solutions were made up with double distilled water and sterilised by autoclaving at 121°C for 15 minutes when appropriate. Buffers, reagents and solutions along with their constituents are listed in Table 8.

2.6 Growth and maintenance of *Streptomyces* cultures

S.lividans strains were grown on R2YE, R5 (Hopwood *et al.*, 1985) or oatmeal plates containing appropriate antibiotics at 30°C. A well-sporulating plate was flooded with 10 ml SDW and the spores scraped off the mycelia with an inoculating loop. The spore suspension was transferred to a sterile universal bottle and vortexed (*ca.* 1 minute) and filtered through non-absorbant cotton wool. Following this, the suspension was centrifuged for (3000 r.p.m., 10 minutes) in a MSE benchtop centrifuge and the pellet vortexed briefly before being mixed with 20% (v/v) glycerol and stored at -20°C. *Streptomyces* cultures were also stored at 4°C as slope cultures on the agars described above.

Liquid cultures of *S.lividans* were grown in 25 ml TSB with appropriate antibiotics in 250 ml baffled flasks (fitted with springs) using a Gallenkamp Orbital Shaker (180-200 r.p.m.) at 30°C.

Table 8. Buffers, reagents and solutions

Reagent	Constituents[1]	
Acid phenol-chloroform	Phenol (analar)	5 g
	Chloroform	5 ml
	SDW	1 ml
	8-Hydroxyquinoline	5 mg
Alkaline SDS	SDS	2 g
	0.3 M NaOH	100 ml
Denaturing solution	0.5 M NaOH in 1 M NaCl	
Denhardt's solution, 100x	Bovine serum albumin	2 g
	Ficoll	2 g
	PVPP	2 g
	Distilled water to	100 ml
Loading buffer, x5	Sucrose	6 g
	Sodium-EDTA	0.37 g
	SDW to	10 ml
	[pH 8]	
	25% (w/v) Bromophenol blue	0.1 ml

Table 8. Buffers, reagents and solutions

Reagent	Constituents[1]	
Lysozyme solution	Lysozyme	100 mg
	0.3 M sucrose	50 ml
MT buffer	1 M Tris-HCl, pH 7.5	1 ml
	1 M $MgCl_2$	0.1 ml
	SDW	100 ml
Neutral phenol chloroform	Acid Phenol/ chloroform Equilibrated by shaking, first with 0.5 volumes 1 M Tris-HCl (pH 8.8), then with 0.5 volumes 0.1 M Tris-HCl (pH 8)	1 vol.
Neutralising solution	3 M NaCl in 0.5 M Tris-HCl, pH 7.5	
Non-homologous DNA[2]	DNA (herring sperm)	10 mg
	SDW	1 ml
Nutrient broth + egg albumin	Nutrient broth	0.4 g
	SDW to [pH 8]	50 ml

Table 8. Buffers, reagents and solutions

Reagent	Constituents[1]	
(Nutrient broth + egg albumin [cont.])	(5% (w/v) Egg albumin)	1 ml
Phenol/chloroform mix	Phenol (chromotography grade)	100 g
	Chloroform	100 ml
	Saturated (1 volume) with TE buffer (pH 8)	
Phenol solution	Phenol (chromotography grade)	500 g
	8-Hydroxyquinoline	0.5 g
	TE buffer (+ 0.1 M NaCl)	65 ml
Phosphate buffer	1 M Na_2HPO_4	473.5 ml
	1 M NaH_2PO_4	26.5 ml
	[pH8]	
	SDW to	1000 ml
Prehybridisation solution	100x Denhardt's solution	4 ml
	20x SSC	15 ml
	SDW	50 ml
	Non-homologous DNA[3]	1 ml

Table 8. Buffers, reagents and solutions

Reagent	Constituents[1]	
RNase solution	RNase (10 mg/ ml [TE buffer])	50 µl
	MT buffer	950 µl
SDS mix	10% (w/v) SDS	1 vol.
	2 M Tris-HCl, pH 9.6	1 vol.
	0.5 M sodium EDTA, pH 7.4	2 vol.
SM buffer	1 M Tris-HCl, pH 7.5	20 ml
	1 M $MgSO_4$	1 ml
	5 M Nacl	20 ml
	Gelatin	1g
	SDW to	1000 ml
20x SSC	NaCl	175.3 g
	Sodium citrate	88.23g
	SDW to	1000 ml
¼ Strength Ringer's solution	NaCl	2.25 g
	KCl	0.105 g
	$CaCl_2$	0.12 g
	$NaHCO_3$	0.05 g

Table 8. Buffers, reagents and solutions

Reagent	Constituents[1]	
(¼ str. Ringer's [cont.])	SDW to	1000 ml
TE buffer	2 M Tris-HCl, pH 8	5 ml
	0.25 M sodium-EDTA, pH 8	4 ml
	SDW to	1000 ml
TNE buffer	2 M Tris-HCl, pH 8	5 ml
	5 M NaCl	20 ml
	0.25 M sodium-EDTA, pH 8	4 ml
	SDW to	1000 ml
TBE buffer (10x TBE)	Tris-HCl	108 g
	Boric acid	55 g
	0.5 M sodium-EDTA, pH 8	40 ml
	SDW to	1000 ml

[1]Items in parentheses were added after autoclaving.

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

[3]Boiled 10 minutes, chilled rapidly on ice before use.

2.7 Growth and maintenance of actinophage

The method of Hopwood *et al.* (1985) was followed. Phage were either stored in APB or nutrient broth at 4°C. The lysate was serially diluted in nutrient broth or APB and 100 μl spotted onto 5 cm NCG plates. SNA (0.8 ml, cooled to 45°C) containing spores of an indicator strain (*ca.* 10^7 c.f.u./ ml) was then mixed with the lysate. The plates were incubated overnight at 30°C.

2.7.1 Preparation of phage lysates

Lysates were prepared by the method of Hopwood *et al.* (1985). A well-isolated single plaque was transferred using a sterile pasteur pipette to *ca.* 1.5 ml NB and soaked out for 2 hours. This was then assayed as described above. Next morning two almost confluent plates were flooded with NB (2.5 ml) and stood at room temperature for 2 hours without shaking. The resulting lysate was pipetted from the two plates and filtered (0.45 μm nitrocellulose filter). The filtrate was then assayed as described in section 2.7 and stored in a sterile 5 ml bijou bottle. This method typically yielded *ca.* 10^8 p.f.u./ ml.

2.7.2 Preparation of high titre phage lysates

High titre phage lysates were prepared by the method of Stuttard (1979). *Ca.* 10^4 p.f.u. were spotted onto each of four 9 cm NCG plates, which were inoculated with SNA (2.5 ml, seeded with *ca.* 10^7 c.f.u./ ml of host spores) and incubated overnight at 30°C. Next morning each plate was flooded with 13 ml APB and left at room temperature for 2 hours. The agar and APB were scraped into a polycarbonate Oakridge tube and centrifuged (2750 r.p.m., 10 minutes at

4°C in a JA-17 rotor [Beckman J2-21 centrifuge]) to remove the SNA. The supernatant was filtered (0.45 μm nitrocellulose filter) and centrifuged (20000 r.p.m., 90 minutes in a Beckman SW28 rotor [Beckman L8 ultracentrifuge]). The pellet was resuspended overnight at 4°C in 2 ml of APB. The lysate was further clarified by centrifugation (3000 r.p.m., 10 minutes) and assayed as described in section 2.7. This method typically yielded *ca.* 10^{10}-10^{11} p.f.u./ ml.

2.8 Preparation of lysogens

Generally, the method outlined in 2.8.1 was adequate for producing a lysogenic culture of KC301 in *S.lividans*. In certain soil experiments (see Chapter 9) a lysogenic spore suspension was required that contained no contaminating free KC301 particles. In this case, further treatment of the spore suspension was necessary to ensure this (2.8.2).

2.8.1 Routine preparation of lysogens

The method of Hopwood *et al* (1985) was followed during this procedure. R5 (or R2YE) plates supplemented with streptomycin were spread with 100 μl of concentrated spore suspension of *S.lividans* TK24. After being allowed to dry thoroughly, *ca.* 20 μl of KC301 lysate was spotted onto the plate and incubated at 30°C until the area within the spot was sporulating. The plate was then replicated to MM supplemented with thiostrepton and streptomycin, and incubated until sporulation occurred in the area corresponding to the original spot. Lysogens could then be purified by single colony isolation, again on MM.

2.8.2 Preparation of phage-free lysogenic spore suspensions

A spore suspension of a *S.lividans* TK24::KC301 lysogen was prepared as described above (see sections 2.8.1 and 2.6). The spores were washed (x6) in 10 ml APB to remove any free phage and pelleted by centrifugation (3000 r.p.m., 10 minutes). Any contaminating mycelia (that might spontaneously release KC301) were killed by treatment with 2% SDS for 1 hour at room temperature, with occasional shaking. The spores were then further washed with 10 ml 0.1 M sodium citrate (x6) to inactivate any remaining free phage. The final supernatant was filtered (0.45 μm nitrocellulose filter) and assayed for KC301, whilst the pellet was resuspended in 20% glycerol and stored at -20°C.

2.9 Isolation of phage DNA

2.9.1 Large scale isolation of phage DNA

The method of Hopwood *et al.* (1985) was followed during this procedure, $6x10^4$ p.f.u. in 1 ml of NB were added to each of six bioassay dishes containing NCG. The plates were then overlaid with SNA (35 ml, seeded with host) and incubated overnight. Next morning the top layers were scraped into a Beckman 500 ml centrifuge pot (containing 180 ml of NB) and left for 2 hours at room temperature. After centrifugation (10000 r.p.m., 10 minutes in a JA-10 rotor [Beckman J2-21 centrifuge]), the pellet was re-extracted for 1 hour (90 ml NB) and the supernatant stored at 4°C. The two supernatants were pooled and phage particles pelleted by centrifugation (25000 r.p.m., 75 minutes in a SW28 rotor [Beckman L8 ultracentrifuge]). The pellet was resuspended in 1 ml of SM buffer (4°C, 3 hours), the suspensions pooled and made up to 5 ml with SM buffer and sufficient CsCl added to give 0.85 g/ ml CsCl. This was then transferred to a Beckman Polyallomer tube and the remaining space filled with

liquid paraffin. The tube was centrifuged (30500 r.p.m., 18 hours, 20°C in a Vti 65 rotor [Beckman L8 ultracentrifuge]) and the white/blue phage band was removed using a size 25 needle. After dialysis (4 hours against 4 x 100 ml SM buffer, 4°C), the lysate was transferred to a conical glass test tube along with 1/20 volume 20x SSC and heated (60°C, 10 minutes). To this extract 5M NaCl, to give a final concentration of 0.25M, and an equal volume of phenol solution were added. The mixture was then inverted 50 times and centrifuged (3000 r.p.m., 10 minutes). The upper aqueous layer was removed and re-extracted (x2) with phenol solution (see section 2.11), whilst the phenol phases were re-extacted with an equal volume of TE buffer (containing 0.25M NaCl). The aqueous layers were pooled and transferred to a glass universal bottle and mixed with an equal volume of ether to remove any contaminating phenol. This was then centrifuged (3000 r.p.m., 2 minutes) and the aqueous phase removed. This was repeated twice and the final upper layer ethanol precipitated (see section 2.12). KC301 DNA was confirmed as such by digestion with *Bam*H1, and agarose gel electrophoresis (see Fig. 2); this yielded three bands including the 1.75 kb *tsr* fragment (Hopwood *et al.*, 1985).

2.9.2 Small scale isolation of phage DNA

The method of Hopwood *et al.* (1985) was followed during this procedure. $2x10^4$ p.f.u. were inoculated onto each of three 9 cm NCG plates and overlaid with 2.5 ml SNA (seeded with host). After growth overnight at 30°C, the SNA was scraped into a polycarbonate Oakridge tube (containing 12 ml NB). The mixture was sucked through a 10 ml pipette twice and stood at room temperature (2 hours). After this the tube was centrifuged (10000 r.p.m., 10 minutes) to remove the SNA. Phage were then pelleted by centrifugation (25000 r.p.m., 75 minutes in a SW28 rotor [Beckman L8 ultracentrifuge]). The

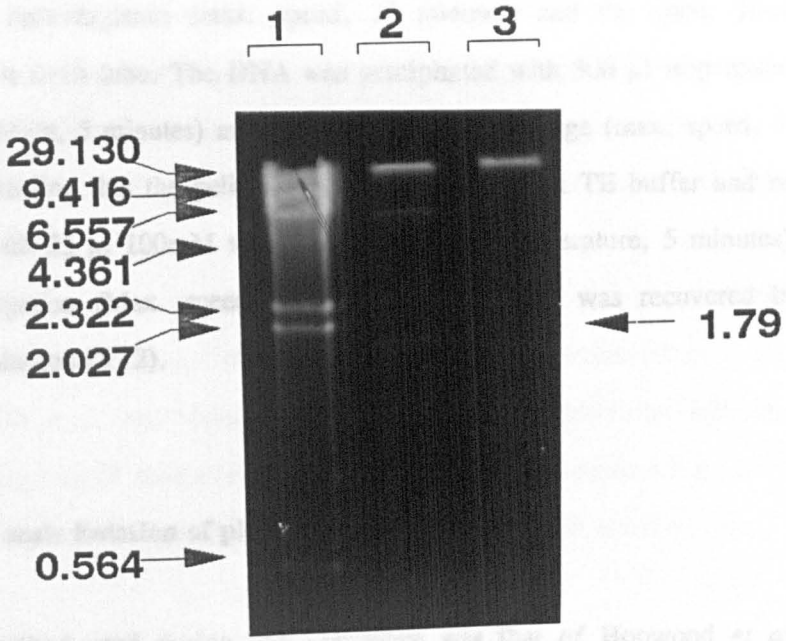
Fig. 2. Digestion of KC301 DNA with *Bam*H1

KC301 DNA was prepared in the manner described in sections 2.9.1 or 2.9.2. The isolated DNA was restricted as described in Section 2.14 with *Bam*H1 after first heating for 10 minutes at 70°C to break the cohesive ends of the phage genome. Restricting KC301 DNA in this manner results in three bands, as the *tsr* gene was originally cloned into ΦC31 via a single *Bam*H1 site on pBR322 (see Chapter 4). Thus the 1.79 kb *tsr* gene is excised by this treatment.

Track 1. Lambda *Hin*dIII

Track 2. KC301 *Bam*H1

Track 3. KC301 undigested


1
2
3
29.130
9.416
6.557
4.361
2.322
2.027
1.79
0.564

pellet was resuspended and kept in 400 μl RNase solution in an orbital shaker (37°C, 20 minutes) and transferred to an Eppendorf tube along with 80 μl SDS mix (70°C, 30 mins.). Immediately afterwards, 100 μl 8M potassium acetate were added and the extract placed on ice for 15 minutes. After this the mixture was sedimented in a MSE Microcentaur microfuge (Max speed, 10 minutes) and the supernatant transferred to a fresh Eppendorf tube. Phenol/chloroform mix was then added to this and shaken by hand (5 minutes). The phases were separated by centrifugation (max. speed, 10 minutes) and the upper phase transferred to a fresh tube. The DNA was precipitated with 500 μl isopropanol (room temperature, 5 minutes) and sedimented in a microfuge (max. speed, 10 minutes). Following this the pellet was dissolved in 500 μl TE buffer and re-precipitated with 25 μl 100mM spermine-HCL (room temperature, 5 minutes). After centrifugation (Max. speed, 10 minutes) the DNA was recovered by ethanol precipitation (2.12).

2.10 Large scale isolation of plasmid DNA

The method used during this procedure was that of Hopwood *et al.* (1985). 500 ml of *S.lividans* TK24 containing pIJ673 (*aph*, *tsr* and *vph* , T.Kieser *et al.*, unpublished data) was grown for 2-3 days in shake flasks of TSB supplemented with appropriate antibiotics. The culture was harvested by centrifugation (10000 r.p.m., 10 minutes) in Beckman 500 ml centrifuge pots and washed (x2) with 500 ml 10% (w/v) sucrose. The final pellet was resuspended in a total volume of 50 ml lysozyme solution and incubated at 37°C for 30 minutes. 25 ml alkaline SDS were then added to this and immediately vortexed before incubating at 70°C (15 minutes). After transferring to a 300 ml MSE polypropylene centrifuge pot acid-phenol/chloroform (8 ml) was added, the mixture vortexed and cell debris pelleted by centrifugation (10000 r.p.m., 10

minutes). The supernatant was removed with an inverted 25 ml pipette and 0.1 volume 3 M sodium acetate and equal volume isopropanol added (room temperature, 5 minutes). DNA was pelleted by centrifugation (10000 r.p.m., 10 minutes) and dissolved in 5 ml TE buffer. The DNA solution was transferred to a polypropylene Oakridge tube and reprecipitated with ethanol at -20°C (2 hours), it was then dissolved in 5 ml TE buffer containing RNase (50 μg/ ml) and incubated at 37°C for 1 hour. The mixture was next removed to a glass McCartney bottle and phenol extracted (see section 2.11) with neutral phenol/chloroform. After this the aqueous phase was mixed with 1 volume of ether, heated (68°C) to evaporate the ether and ethanol precipitated (2.12). The DNA was dissolved in 1 ml TE buffer and 5.25g CsCl added, followed by sufficient TE buffer to make a final volume of 5 ml (1.05 g/ ml CsCl). The remaining space was filled with liquid paraffin and the tube centrifuged (50000 r.p.m., 18 hours, 20°C in a Vti 65 rotor [Beckman L8 ultracentrifuge]). The lower (plasmid) band was visualised under long wave ultra-violet light and removed using a size 25 needle. After dialysis (4 hours, 4°C) against 4 x 500 ml TE buffer the DNA was recovered by ethanol precipitation (see section 2.12).

2.11 Extraction of DNA with phenol

Phenol reagents were prepared as described previously (2.5). In general, an equal volume of the phenol reagent was mixed with the DNA sample by shaking and separated by centrifugation (3000 r.p.m., 10 minutes in a MSE benchtop centrifuge or Max. speed 2 minutes. in a MSE microcentaur microfuge). The upper aqueous phase was recovered (taking care not to disturb the interface) and the DNA reclaimed by ethanol precipitation (2.12).

2.12 Ethanol precipitation

To a DNA solution, 0.1 volume of 3 M sodium acetate and 2 volumes of ice-cold ethanol were added, mixed and chilled at -20°C for 2 hours. The DNA was recovered by centrifugation (max. speed, 10 minutes in a MSE microcentaur microfuge or 3000 r.p.m., 10 minutes in a MSE benchtop centrifuge) and washed in 1 ml ice cold 70% ethanol. After centrifugation the DNA was dried under vacuum and resuspended in TE buffer.

2.13 Agarose gel electrophoresis

Horizontal agarose gels were prepared by boiling agarose with 1 x TBE electrophoresis buffer. 1% (w/v) gels were used routinely. Ethidium bromide (0.5 μg/ ml final concentration) was added and the agarose cooled before pouring. DNA samples were prepared by adding 0.2 volumes of loading buffer. The sample was then loaded into the gel slots. Electrophoresis was carried out with the gel completely submerged in buffer at 75 volts for *ca.* 3 hours or 30 volts overnight. Lambda bacteriophage DNA digested with restriction enzyme *Hin*dIII was used as DNA molecular weight markers. This generated sizes of: 23.13, 9.416, 6.557, 4.361, 2.322, 2.027, 0.564 and 0.125 kb (Hopwood *et al.*, 1985).

2.14 Restriction endonuclease digestion of DNA

DNA restriction digests were carried out by mixing 2 μl 10x enzyme buffer, 2 μl DNA solution (in TE buffer), 14 μl SDW, and 2 μl restriction endonuclease and incubating at 37°C for *ca.* 2 hours.

2.15 Isolation of DNA fragments from agarose gels

DNA fragments were electrophoresed until acceptable band separation had been achieved. The gel was removed from the gel rig and the bands visualised under long-wave ultra-violet light. A cut was made just ahead of the band to be removed and a piece of filter paper (Whatman, 3MM) inserted. A section of dialysis tubing was also inserted into the slit, but on the side of the filter paper facing away from the band. The gel was then returned to the gel rig. After most of the electrophoresis buffer had been removed (sufficient was left so that the buffer surface was above the gel base plate, but below the top of the gel), the current was switched on and the gel electrophoresed for 10-15 minutes. The 3MM paper was then removed from the gel and checked for fluorescence under ultraviolet light. The paper was placed in a small (0.5 ml) Eppendorf tube and a hole punctured in the base with a size 25 needle. This Eppendorf was in turn placed in a large (1.5 ml) Eppendorf and centrifuged (max. speed, 10 minutes) in a MSE microcentaur. The filtrate in the large Eppendorf was stored on ice, whilst the paper was washed with 200 μl TNE buffer. After centrifugation, the two filtrates were pooled, phenol extracted with phenol solution (see section 2.11) and the DNA finally recovered by ethanol precipitation (see section 2.12). This method typically gave *ca.* 70% recovery of the DNA present in a given band (J. Ridley, personal communication).

2.16 Southern blotting

The method of Hopwood *et al.* (1985) was used during this procedure. After electrophoresis the gel was trimmed to the minimum size required and one corner cut off to assist orientation. The gel was then transferred to a plastic

sandwich box and washed with gentle rocking in 0.25 M HCL (2 x 10 minutes) to partially depurinate the DNA. After rinsing in SDW the gel was washed in denaturing solution (2 x 15 minutes), before being rinsed (x3) in SDW. After this the gel was washed in 200 ml neutralising solution. Two pieces of filter paper (Whatman, 3MM) and one piece of Nylon (Hybond) filter paper (or Nitrocellulose [Hybond]) were cut to the exact size of the gel, the latter being soaked in distilled water and the former in 2x SSC. An inverted gel rig base plate was placed in a plastic tray and covered in 3MM filter paper so the paper touched the floor of the tray on two sides. The gel was then placed (well side down) on the gel rig base plate and the nylon filter paper plus the two sheets of 3MM paper positioned on its surface respectively. Bubbles were expelled from each layer in turn by rolling with a 25 ml pipette and the tray filled with 20x SSC. After this, a stack of paper towels were placed on the gel and a heavy weight (*ca.* 1 Kg) put on top. The gel was then surrounded by plastic sheeting to prevent contact between the paper towels and the 3MM paper soaked in 20x SSC. After overnight blotting, the towels and 3MM paper were removed and the DNA fixed to the nylon filter by short-wave ultra-violet irradiation (3 minutes DNA side, 1 minute reverse side). DNA was fixed to nitrocellulose filters by baking in a vacuum oven (105°C, 2 hours). Finally the filter was sealed in a plastic bag and stored until ready for use.

2.17 Preparation of filters for colony hybridisation

Filters for colony hybridisation were prepared by the method of Hopwood *et al.* (1985). A nylon (or Nitrocellulose) filter (cut to size and autoclaved) was placed on a R5 plate supplemented with appropriate antibiotics, whilst bubbles were removed with a glass spreader. *Ca.* 50 colonies were then picked onto this in a grid like pattern and incubated for 20-30 hours (until young

mycelia could be seen). The filter was then removed, marked with a pencil and placed (colony side up) on three sheets of 3MM paper soaked in lysozyme solution (4 mg/ ml in TE buffer). After incubation at room temperature (30-45 minutes) the filter was transferred to a fresh set of three sheets of 3MM paper soaked in SDS solution (1% [w/v] SDS in 1 M NaOH) and left for 20 minutes. Following this, the filter was dried (one sheet of 3MM paper to remove excess liquid) and transferred to a further three sheets soaked in neutralizing solution (7 volumes 1 M tris-HCl, pH 7.5, 3 volumes 5 M NaCl) for 5 minutes. This was repeated three times. The filter was briefly placed on a sheet of 3MM soaked with 90% ethanol and then dried at room temperature. The DNA was fixed to the filter as described in section 2.16.

2.18 Preparation of filters for plaque hybridisation

The method of Hopwood *et al.* (1985) was followed during this procedure. Plaques were transferred from NCG plates using sterile toothpicks to a 9 cm NCG plate previously seeded with SNA and a host streptomycete (see section 2.7.2). After overnight incubation (30°C) the plate was kept at 4°C for 2 hours to harden the SNA. A nylon (or nitrocellulose) filter, cut to size, was placed on the surface of the plate (bubbles removed with glass spreader) and left for 10 minutes. The filter was then transferred to three sheets of 3MM paper soaked in 0.5 M NaOH for 10 minutes. After this it was moved to three sheets of 3MM soaked in 1 M tris-HCl, pH 7.5 for 2 minutes (x2), before being moved to a final three sheets soaked in 1 M tris-HCl, pH 7.5 and 1.5 M NaCl. Lastly the filters were dried at room temperature (30 minutes) and fixed as described in section 2.16.

2.19 Hybridisation with ^{32}P-labelled DNA probes

Method B of Hopwood *et al.* (1985) was followed. This method is as described in Section 2.19, except, a random primed DNA labelling kit (Boehringer Manheim) was used instead of nick translation (2.19.2) and incorporated label was separated from unincorporated by the method described in Section 2.19.3.

2.19.1 Prehybridisation

A corner was cut from the plastic bag containing the nylon filter and 100 μl/ cm^2 (of filter area) prehybridisation solution were added to the bag. Bubbles were removed using a 25 ml pipette. The bag was then sealed and incubated at 70°C for 3 hours in a shaking water bath.

2.19.2 Labelling of probe

DNA to be used as a template was ethanol precipitated and resuspended in 10 μl SDW. This was then boiled (10 minutes) and immediately placed on ice. A kit containing all of the necessary requirements (except dGT^{32}P) for random primed DNA labelling was used (Boehringer-Mannheim) to label the probe . The following reaction was set up; 2 μl 10x reaction mix (containing hexanucleotide primers), 1 μl dATP, 1 μl dTTP, 1 μl dCTP, 5 μl DNA, 8 μl SDW, 1 μl dGT^{32}P (10 μCi, Amersham) and 1 μl Klenow enzyme. This mix was then incubated at 37°C for 30 minutes. After this time the reaction was stopped by the addition of 2 μl 5x loading buffer.

2.19.3 Separation of incorporated from unincorporated label (S. McGowan, personal communication)

A hole was pierced in the bottom of a small Eppendorf tube with a size 25 needle. A 1 ml pipette tip with the top and bottom removed was inserted into the tube. *Ca.* 20 μl of 0.17 mm diameter glass beads in TE buffer were placed at the bottom of the Eppendorf tube and the tube filled to the top of the blue tip with Sephadex G25 in TE buffer. The Eppendorf tube was placed in a larger tube with the bottom removed, which in turn was placed in a narrow walled 14 mm diameter test tube. This was centrifuged (2000 r.p.m., 4 minutes [Gallenkamp bench top centrifuge]) to compact the Sephadex G25. After centrifugation the large Eppendorf tube was replaced with a fresh whole tube and the blue tip removed.

The stopped reaction mixture was then placed inside the small Eppendorf (on the surface of the Sephadex G25) and centrifuged as described previously. *Ca.* 20 μl of incorporated label was deposited in the large Eppendorf, whilst unincorporated label remained at the surface of the G25 along with the blue coloured loading buffer. The incorporated label was monitored with a Geiger counter, if the count was higher than 1000 c.p.s. the reaction was deemed to have been successful.

2.19.4 Hybridisation

After 3 hours incubation at 70°C, the prehybridisation reaction was removed from the water bath, a corner removed from the bag and most of the prehybridisation solution removed. Hybridisation was carried out at 70°C because this temperature equivalent to 15°C below the melting temperature of streptomycete DNA. The incorporated label was boiled for 10 minutes (to obtain

single stranded DNA), before immediately being placed on ice. 1 ml of ice cold fresh prehybridisation solution was added to the probe, mixed and transferred to the plastic bag containing the filter. This was then sealed and incubated overnight at 70°C in a shaking water bath.

2.19.5 Stringency washes

After incubation overnight at 70°C the hybridisation mixture was removed from the water bath and the hybridisation solution drained from the bag. The filter was then transferred to a polypropylene box, with sealable lid, and washed (70°C, 30 minutes) with 500 ml 2x SSC, 0.1% SDS (x2) and 0.2x SSC, 0.1% SDS (x2). After the stringency washes the filter was again sealed inside a plastic bag.

2.20 Autoradiography

The filter was placed in a Harmer film cassette along with Fuji X-ray film and two intensifying screens (both screens facing the film). The cassette was then sealed in aluminium foil and left at -70°C for a period of time varying between 4 hours and 1 week. The exposed X-ray film was developed using Kodak LX-24 developer (5 minutes), followed by a 15 second wash in water and finally fixed for 2 minutes in Kodak FX-40 X-ray fixer.

2.21 Soil microcosm conditions

Soil was taken from a local wheat field site (Cryfield Hall, University of Warwick). This soil is a brown earth and can be described as a coarse loam. Analysis of particle size gave a composition of (percent, dry weight); 63.6% sand, 18.4% silt, 11.7% clay and 6.2% loss on ignition. The pH of fresh soil was 6.5-6.8 (Wellington *et al.*, 1990). For microcosm experiments soil was stored at room temperature, before being sieved through a 2 mm sieve at the time of use. The storage of soil typically caused a reduction in the moisture content to *ca.* 2% (w/w). Soil was sterilised by autoclaving the soil in the containers the experiment was to be carried out in at 121°C on two separate days. Soil was amended (when required) with 1% (w/w) chitin and 1% (w/w) soluble starch. The soil was routinely wetted to 15% moisture (40% moisture holding capacity, -3 bar [-300 kPa]) with SDW. Inoculations of phage and host streptomycete were carried via this wetting stage. Microcosms of various sizes were used in this study. 200g soil in 500 ml beakers (sealed with aluminium foil), 100g soil in 300 ml glass bottles, 20g soil in 50 ml glass bottles and 10 g soil in glass MacCartney bottles. After inoculation soil was incubated at 22°C.

2.22 Isolation of soil bacteria

Section 2.22.1 describes a method traditionally used for the isolation of micro-organisms from soil, whereas section 2.22.2 describes a method developed during the course of this study, that specifically isolates streptomycete spores from soil (see Chapter 3).

2.22.1 Isolation of total soil *Streptomyces*

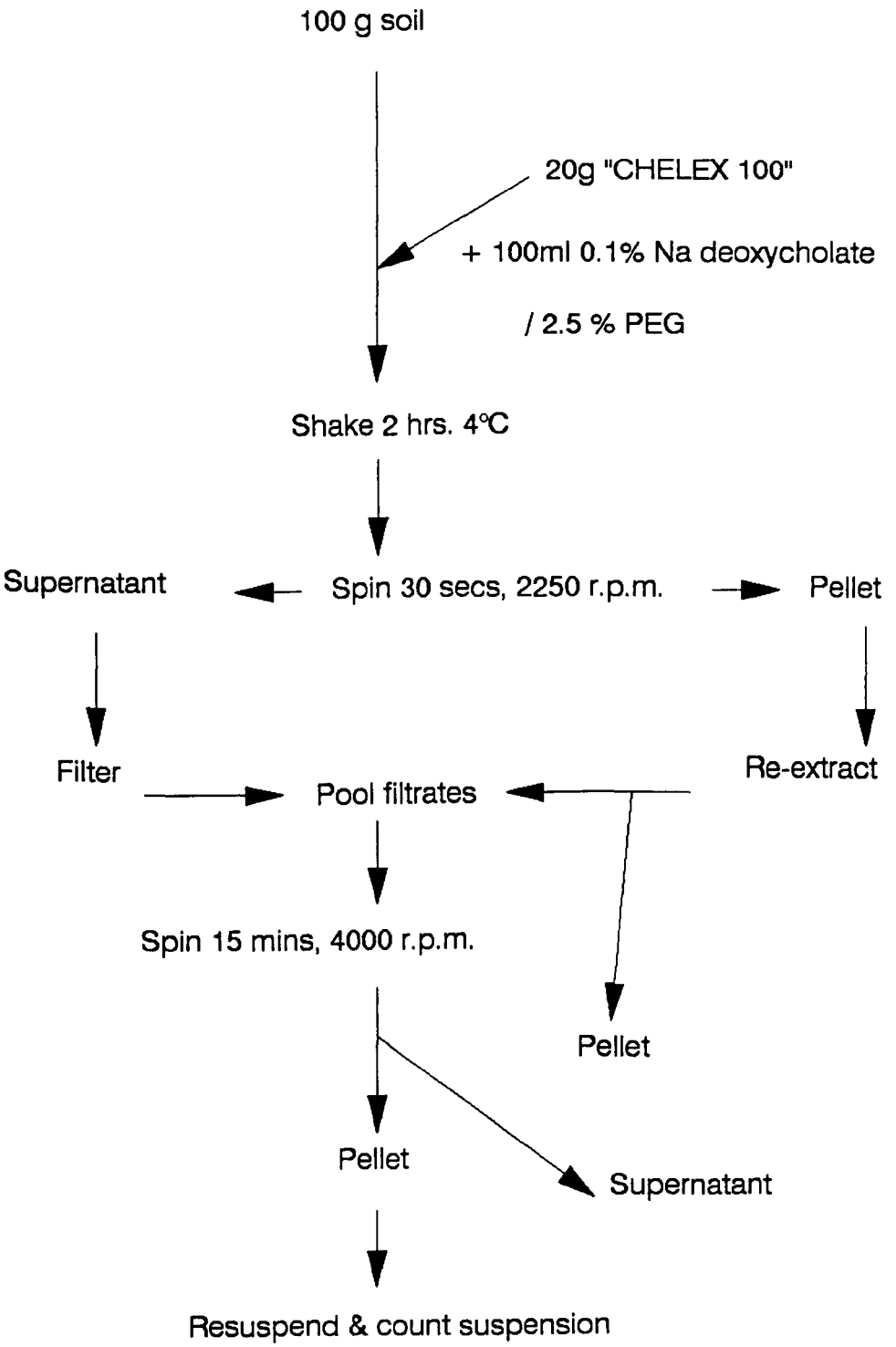
1g of soil (wet weight) was transferred to 9 ml of ¼ strength Ringers solution and shaken (max. speed, 10 minutes) on a Griffin wrist action shaker. The supernatant was serially diluted and spread onto RASS or AGS plates containing cycloheximide, nystatin and rifampicin. In addition antibiotics were included that were neccessary for the selection of a certain genotype. A number of soil streptomycetes were resistant to rifampicin, however the distinctive colony morphology of *S.lividans* allowed the indigenous microflora to be easily distinguished from inoculant strains.

2.22.2 Isolation of *Streptomyces* spores from soil (see Fig. 3)

Soil (100 g) was transferred to a Beckman 500 ml centrifuge pot, to which were added 20g Chelex-100 and 100 ml 0.1% (w/v) sodium deoxycholate/2.5% (w/v) PEG. This was shaken for 2 hours on a Griffin wrist action shaker (4°C, setting 7), before being centrifuged to remove the larger soil particles (2250 r.p.m., 30 seconds, JA-10 rotor). The supernatant was filtered through a 5 cm diameter metal filter holder (Millipore) and stored at 4°C, whilst the pellet was re-extracted with fresh sodium deoxycholate/PEG (4°C, 1 hour). After filtration the two supernatants were pooled and *Streptomyces* spores pelleted by centrifugation (4000 r.p.m., 15 minutes, JA-10 rotor). The pellet was resuspended in 9 ml of ¼ strength Ringers solution, the volume of the suspension determined and serially diluted and plated out as described in section 2.21.1.

Fig. 3. Spore extraction method.

The diagram represents a flow diagram of the spore extraction method. A full description is given in Section 2.22.2.


100 g soil
20g "CHELEX 100"
+ 100ml 0.1% Na deoxycholate
/ 2.5 % PEG
Shake 2 hrs. 4°C
Supernatant
Spin 30 secs, 2250 r.p.m.
Pellet
Filter
Re-extract
Pool filtrates
Spin 15 mins, 4000 r.p.m.
Pellet
Pellet
Supernatant
Resuspend & count suspension

2.23 Isolation of soil actinophage

2.23.1 Isolation of chloroform-sensitive actinophage

This method is method C of Lanning and Williams (1982) for the isolation of chloroform-sensitive actinophage. 50 ml NB+0.1% (w/v) egg albumin, pH 8 were added to 20g soil in a 50 ml glass bottle. This was shaken on a wrist action shaker (maximum speed, 30 minutes, 4°C). After this the soil was left for 16 hours at 4°C, before being transferred to an Oakridge tube and centrifuged (3000 r.p.m., 30 minutes) to pellet soil particles. The supernatant was filtered (0.45 μm nitrocellulose filter) and assayed as described in section 2.7.

2.23.2 Isolation of chloroform-tolerant actinophage

This method is method D of Lanning & Williams (1982) and was carried out as above except that after centrifugation 5 ml of the supernatant was transferred to a sterile vial and 0.1 ml chloroform added. Vials were shaken on a reciprocal shaker for 5 minutes and allowed to stand (4°C, 2 minutes). The aqueous layer was then assayed as described in section 2.7.

2.24 Isolation of soil DNA

Both methods described here (Cresswell *et al.* 1991) are developed from the *in situ* lysis procedure of Ogram et al. (1987)

2.24.1 *In situ* lysis of soil microflora using SDS

To 10g of soil in an Oakridge tube were added 0.3g SDS and 20 ml phosphate buffer. This was then heated (70°C, 1 hour, mixed occasionally by inversion) and shaken on a Griffin wrist action shaker (max. speed, 10 minutes). Larger soil particles were pelleted by centrifugation (6500 r.p.m., 15 minutes). The pellet was re-extracted (shaking only) with a further 20 ml phosphate buffer, after which the supernatants were pooled and mixed with 0.2 volumes of 8 M potassium acetate (placed immediately on ice for 15 minutes). The mixture was centrifuged (10500 r.p.m., 30 minutes) and the supernatant transferred to a 300 ml MSE polypropylene centrifuge pot. The extract was then made up to 0.5 M with 5 M NaCl, 20 ml 50% (w/v) PEG were added and the DNA precipitated overnight at 4°C. Next morning the DNA was pelleted by centrifugation (8000 r.p.m., 10 minutes) and dissolved in 500 μl TE buffer. The soil DNA extract was then transferred to a polypropylene Oakridge tube and phenol extracted (x2) with phenol solution (see section 2.11) and the DNA recovered by ethanol precipitation (see section 2.12).

2.24.2 *In situ* lysis of soil microflora using bead-beating

10g of soil was washed into a bead-beating bottle using phosphate buffer, *ca.* 10 ml of 0.10-0.11 mm diameter glass beads added and the bottle filled to the top with phosphate buffer (*ca.* 40 ml total volume). The bottle was then placed inside a bead beater (B. Braun, Melsungen A.G., bead beater) and shaken for 5 x 1 minute bursts with CO_2 cooling. After transferring to an Oakridge tube larger soil particles and glass beads were pelleted by centrifugation (6500 r.p.m., 15 minutes). The pellet was re-extracted (1 minute vortex) with a further 20 ml phosphate buffer, after which the supernatants were pooled and mixed with 0.2

volumes of 8 M potassium acetate (placed immediately on ice for 15 minutes). The mixture was centrifuged (10500 r.p.m., 30 minutes) and the supernatant transferred to a 300 ml MSE polypropylene centrifuge pot. The extract was then made up to 0.5 M with 5 M NaCl, 20 ml 50% (w/v) PEG were added and the DNA precipitated overnight at 4°C. Next morning the DNA was pelleted by centrifugation (8000 r.p.m., 10 minutes) and dissolved in 500 μl TE buffer. The soil DNA extract was then transferred to a polypropylene Oakridge tube and phenol extracted (x2) with phenol solution. The DNA extract was transferred to a large eppendorf tube and precipitated with 1 volume isopropanol (room temperature, 5 minutes). After pelleting by centrifugation (max speed, 10 minutes) the DNA was resuspended in 500 μl TE buffer and reprecipitated with 25 μl 100 mM spermine-HCL. Finally the DNA was washed in 1 ml 70% ethanol and resuspended in 50 μl TE buffer.

2.25 Statistical analysis

All points on graphs are means of three replicate samples counted in triplicate unless stated. Statistical analysis was by the MINITAB software package (Minitab Statistical software, State College, Pa., USA). Minimum significant differences were calculated from analysis of variance using the method of Petersen (1985). When the number of replicates for each sample point was the same, Tukey's H.S.D. was used; however when the number of replicates between sample days were different, the M.S.D calculation was used (e.g. when one set of three from nine phage assay plates were contaminated and thus the number of plaques was unreadable), the average value at that day was calculated from six plates, however as averages from other sample days had been calculated from nine values it was necessary to use the M.S.D. calculation ($n_i=6$, $n_j=9$).

Tukey's honestly significant difference

$$H.S.D. = Q_a\sqrt{(m.s.e./r)}$$

Minimum significant difference

$$M.S.D. = Q_a\sqrt{[(m.s.e.[1/n_i + 1/n_j])/2]}$$

Where:

Q_a:	value from Studentised range (Q) table (95%) confidence limits. Depending on μ (error degrees of freedom [from ANOVA]) and n (number of sample points [days]).
m.s.e.:	mean square error (from ANOVA).
r:	sample size (number of replicates)
n_i:	lowest number of replicates
n_j:	highest number of replicates

Chapter 3

Isolation and detection of soil inoculants

3.1 Development of spore extraction procedure

Traditional methods for the isolation and enumeration of microorganisms from soil have relied upon the mechanical desorption of bacteria from soil particles by shaking the soil with an eluent such as ¼ strength Ringer's solution or SDW (Wellington *et al.*, 1990; Bleakley & Crawford, 1989 respectively). The drawback of such approaches is that it is difficult to detect less than 10^2 c.f.u./g by viable counting. Whilst such approaches are quick and cheap to operate, it is unlikely, by their simple nature, that these methods are capable of extracting a representative sample of the soil microflora. Thus, there is a perceived need for more sensitive detection methods in soil microbial ecology (reviewed by Trevors & Van Elsas, 1989).

3.1.1 Extraction of microorganisms from soil

The extraction of microorganisms from soil can be divided into four areas (Hopkins & O'Donnell, 1991): dispersion of soil aggregates, dissociation of microorganisms from soil particles, separation of soil material from microorganisms and purification of bacteria from the extract. Many methods are published that take an extraction procedure through some or all of these steps. The simplest methods simply involve shaking a given mass of soil with an eluent such as distilled water, whilst others involve complex regimes of soil dispersion and biomass concentration. To obtain a realistic and sensitive method of isolating microorganisms from the environment it is necessary to examine closely the physical and chemical characteristics of the soil matrix before deciding on a protocol that is able to carry out the steps outlined above.

(i) Mechanical desorption

A simple development of traditional methods was that of Ramsay (1984) who compared shaking, blending and ultrasonication along with different extractants as methods of removing bacteria from soil. It was found that ultrasonication removed the highest proportion of indigenous bacteria from sand grains. When a shaken suspension was further treated with ultrasonication the bacterial titre was further increased, indicating that bacteria were still bound to clay particles, thus many bacteria were only appearing as one colony. The effect of the ultrasonication, therefore, was to release microbes from this bound state (Ozawa & Yamaguchi, 1986), but, whilst not seeming to affect indigenous organisms, ultrasonication did cause lysis of inoculated *Bacillus cereus*. Ramsay (1984) also found that 50 mM Tris buffer, pH 7.5 proved to be the best soil extractant, thus agreeing with Griffiths & Ritz (1988) who found this to be the most efficient extractant in the recovery of protozoa from soil.

Jay & Margitic (1979) compared the extraction of microorganisms and endotoxins from meat samples using various homogenisers and found that a stomacher gave higher recoveries of bacteria than a Waring blender due to the greater sensitivity of Gram-negative bacteria to blending. However, Martin & Macdonald (1981) demonstrated that blending was a more efficient method for recovery of non-filamentous microorganisms from soil than the use of a stomacher, presumably because of the more adsorbant nature of soil coupled with the more intensive disruption regime of the blender. Baecker & Ryan (1987) further examined the use of homogenisation techniques in the efficient isolation of actinomycetes from soil. The soil was pretreated with hammer mill comminution before extraction using an identical homogeniser to one employed by Bakken (1985) to reduce soil damage to the equipment. This pretreatment gave a greater recovery of actinomycetes than had previously beem available.

Fægri *et al.* (1977) developed a technique to separately determine

bacterial and fungal activities in soil. This method used rapid fractionated centrifugation of a soil homogenate (Waring blending); the soil was subjected to successive rounds of homogenisation and centrifugation, the resulting pellets being pooled and subjected to high speed centrifugation. Virtually all fungal activity was found in the final pellet after first series of spins, whilst the bacterial activity was predominantly found in the pellet of the high speed centrifugation. This method was further developed by Martin & Macdonald (1981), who subjected the final pellet (high speed centrifugation) to additional purification by Percoll gradient centrifugation. Bakken (1985) also used the basic method of Fægri *et al.* (1977) whilst attempting to obtain a representative and essentially pure fraction of bacterial cells by the use of Percoll gradient centrifugation. The suitability of different soil homogenisers (Waring blending proved the best method) was investigated and the soil homogenate was subjected to additional rounds of fractionated centrifugation. It was found that cells larger than 1.9μm did not appear in the final pellet (0.4% of soil fungi), thus agreeing with Fægri *et al.* (1977) that fungi (spores and hyphae) were pelleted in the low speed series of spins (90% of soil fungi).

(ii) Chemical desorption

The mechanical extraction of microorganisms from soil involves the use of vigorous physical techniques such as homogenisation. The problem with this is that this introduces a bias in the sampling conditions by damaging fragile cells (e.g. protozoa) during the isolation procedure (Macdonald, 1986). If microbial cells can be desorbed by chemical means from soil particles this bias can be reduced by using a gentle shaking regime. The three strongest factors involved in this adsorption are: physical entrapment of cells in soil aggregates, ionic bridging between cells and clay particles and the adhesion of microbes to

inanimate soil particles by means of polymeric gums (Macdonald, 1986). Dispersion of soil and thus the negation of the first two of these interactions can be effected by the use of iminodiacetic acid ion exchange resins, e.g. Dowex A1 (Sigma) or Chelex-100 (Biorad). This operation is facilitated by the resin exchanging Na^+ ions for Ca^{2+} ions of clay particles in the soil matrix, thus bringing about the disruption of the electrostatic forces that bind soil aggregates together (Edwards & Bremner, 1965) and microorganisms to these aggregates. Maximal dispersion of mineral particles without the destruction or irreversible alteration of soil particles was obtained by shaking (low speed) soil with Dowex A1 for 2 hours (4°C) (Edwards & Bremner, 1965).

Tris buffer is a fairly common extractant used for the collection of soil microflora (Niepold et al., 1979; Ramsay, 1984; Griffiths & Ritz, 1988 and Hopkins and O'Donnell, 1991), when compared with other eluents Tris buffer gave the best microbial recovery (Ramsay, 1984; Griffiths & Ritz, 1988). Many other extractants have been used to recover microorganisms from soil e.g. ¼ strength Ringer's solution (Wellington *et al.*, 1990) or SDW (Bakken, 1985). However, in extractions where soil dispersion is carried out by means of an ion exchange resin the sole remaining source of attachment for microorganisms to soil particles is by means of polymeric gums thus making inorganic extractants (e.g. Tris buffer) redundant. These extracellular polymers which bind cells to soil surfaces and to one another are a complex mixture of polysaccharides of plant and microbial origin. The action of detergents should, therefore, bring about the destruction of these gums. Macdonald (1986) reported that the use of several different detergents in conjunction with Dowex A1 increased the yield of microorganisms from soil. It was found that 0.1% sodium deoxycholate gave the highest yield (84%). Hopkins & O'Donnell (1991) found that shaking with anionic exchange resin and a chelating ion exchange resin gave consistently greater dispersion of soil aggregates than shaking with Tris buffer or distilled water or the use of ultrasonication.

A further development of dispersion techniques (Hopkins *et al.*, 1991a & 1991b), using ion exchange resins, was to disperse soil during the initial stages of an extraction, followed by progressively more rigourous treatments (e.g. homogenisation, ultrasonication) in order to maximise the recovery of the more fragile members of the microbial community. Thus, the advantage of using chemical desorption techniques over physical techniques is that, whilst they may be longer and more labourious, a gentler sampling regime means that less damage is inflicted on microbial cells during the extraction procedure and thus a more representative sample is obtained.

An extraction procedure was developed to disperse soil particles using an ion exchange resin, followed by removal of bulk soil by centrifugation and subsequent concentration of soil biomass by further concentration (see Section 2.22.2 for description). The method developed was based on the soil dispersion conditions of Edwards & Bremner (1965) and Macdonald (1986). Maximal concentration of streptomycete propagules was then achieved by subsequent optimisation of centrifugation conditions.

3.1.3 Optimisation of centrifugation conditions

Following the design of a procedure for soil dispersion (see Fig. 3 and Section 2.22.2), the next stage was to optimise centrifugation conditions so that the bulk of the soil mass could be removed whilst still leaving a significant proportion of the streptomycete population in the supernatant. Fægri *et al.* (1977), used a 1000 x g spin for 15 minutes in order to remove the majority of the soil mass. The way in which the optimum centrifugation conditions for this system were developed are detailed below. With most the larger soil particles removed (*ca.* 90% of the soil bulk) the resulting supernatant could be filtered to remove any remaining resin, and then centrifuged at high speed to pellet spores.

A known titre ($6.2x10^4$ c.f.u.) of *S.lividans* TK24 spores were inoculated into 40 ml of 0.1% (w/v) sodium deoxycholate and centrifuged at 2750 r.p.m. (1000 x g) in a JA17 rotor (Beckman J2-21 centrifuge) for various lengths of time. The titre remaining in the supernatant was then used to determine the proportion of the initial streptomycete population remaining in the supernatant.

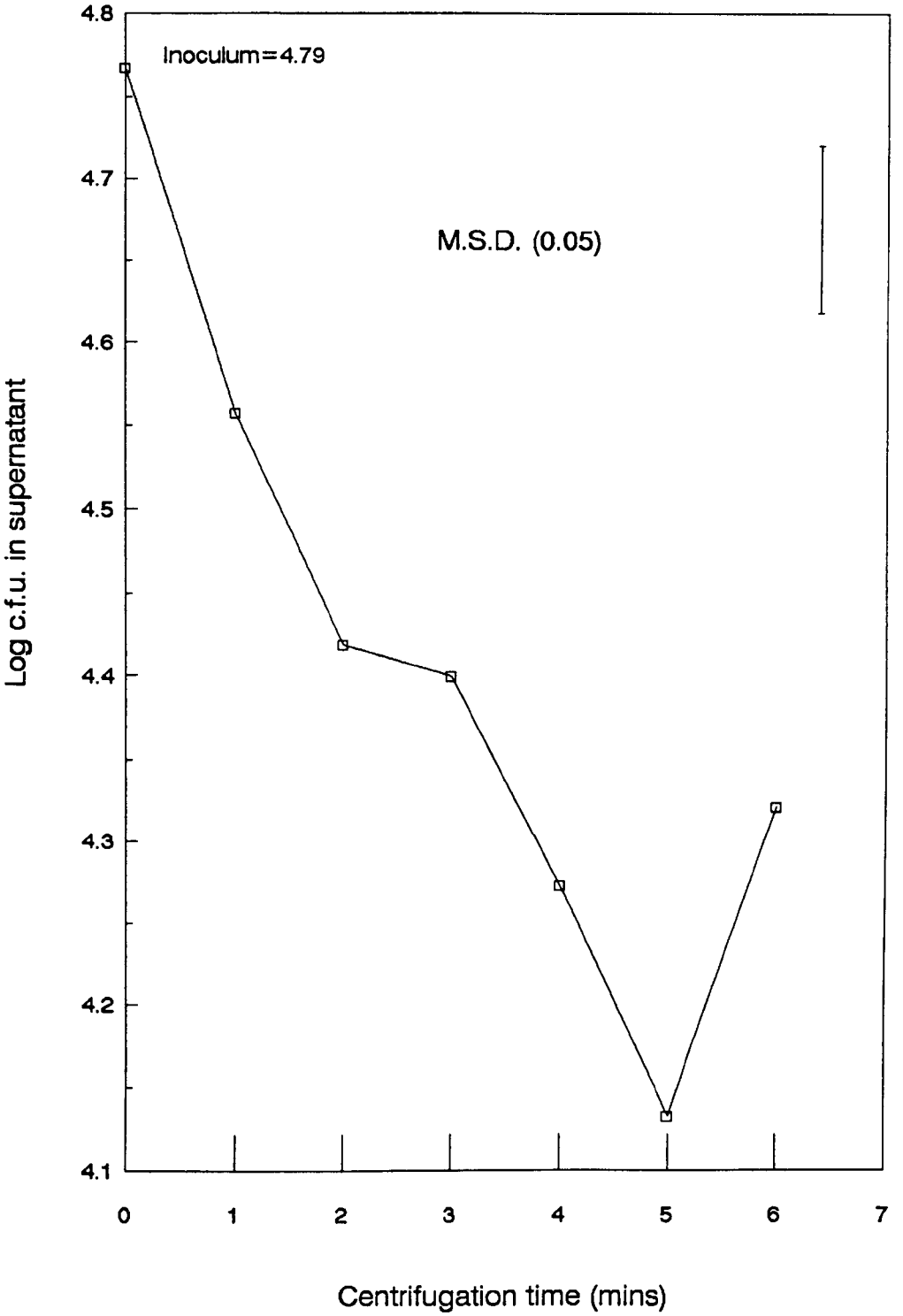
A graph of log c.f.u. remaining in the supernatant after centrifugation is shown in Fig. 4. With no centrifugation, ca. 95% of the inoculum was recovered (calculated from arithmetic values). However, even after 1 minute of centrifugation a significant drop in recovery to ca. 59% was found. Thus, a time of 30 seconds was chosen for the initial centrifugation so as to minimise the pelleting of spores during this centrifugation. The centrifugation step to facilitate the concentration of biomass was carried out under slightly (see Section 2.6 and 2.22.2) more severe conditions of that used by Hopwood *et al.* (1985) to pellet *Streptomyces* spores.

3.1.4 Optimisation of soil dispersion conditions

After the development of centrifugation conditions for the isolation method, it was necessary to optimise the conditions of the soil dispersion step. This was done by chosing the most efficient eluent and testing the effect of shaking speed on the extraction efficiency.

Fig. 4. Optimisation of centrifugationconditions for the spore extraction method.

A known titre of *S.lividans* TK24 spores were inoculated into 0.1% sodium deoxycholate and centrifuged at 2750 r.p.m. (1000 x g) for various lengths of time. The titre remaining in the supernatant was then assayed by viable counting and plotted against time.


Inoculum=4.79
M.S.D. (0.05)
Log c.f.u. in supernatant
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
0
1
2
3
4
5
6
7
Centrifugation time (mins)

(i) Comparison of eluents in spore extraction method

MacDonald (1986) found that optimal recovery of microorganisms during soil dispersion was achieved using 0.1% sodium deoxycholate as an eluent. It was decided to examine the recovery of streptomycete spores from soil using this eluent and 2.5% (w/v) PEG. $6.2x10^4$ c.f.u. of *S.lividans* TK24 spores were inoculated into each of six 100g samples of sterile soil. Sterile soil was used so as to avoid any masking of recovered inoculants by indigenous streptomycetes. Three samples were extracted using 0.1% sodium deoxycholate alone and three with 0.1% (w/v) sodium deoxycholate/2.5% (w/v) PEG as an eluent. 0.1% (w/v) sodium deoxycholate recovered 34.8% (2.49%) of the inoculum, whilst 0.1% (w/v) sodium deoxycholate/2.5% PEG yielded 125.8% (6.68%). Figures in parentheses represent standard errors of the mean. Thus, the latter eluent mixture gave the best recovery. Subsequent extractions using this method (see Chapters 5 to 9), more commonly gave extraction efficiencies of *ca.* 30%. This demonstrates the variability of recovering microorganisms from an environmental sample. In way of comparison, extraction using the ¼ strength Ringer's solution method could give recoveries varying from *ca.* 15% to almost 1000%, although it was more commonly between 100 and 200% of the added inoculum. Of course all such figures are subject to the obvious difficulties in basing these percentages on c.f.u. for a mycelial microorganism, however they can certainly give an indication as to the efficiency of a given method.

(ii) Comparison of different speeds of shaking during soil dispersion

The extraction method using the ¼ strength Ringer's solution regularly indicated that more colonies were being counted on plates than spores initially inoculated into the soil sample. This implied that spore chains in the inoculum,

counted as one c.f.u. during the inoculum assay, were being broken up during the (relatively) high speed shaking regime of this method. This could be determined by the use of direct counting procedures, e.g. acridine orange. This principle was tested using the spore extraction method by increasing the speed at which the sample was shaken during the dispersion process. $9.8x10^5$ c.f.u. of *S.lividans* TK24 spores were inoculated into six samples of 100g sterile soil. Three were then extracted as usual (see Section 2.22), with a shaking speed of 7 on a Griffin flask shaker. The other three samples were extracted as usual, but shaken at speed 10 on the flask shaker. 102.1% (10.0%) of the inoculum was recovered when the soil was shaken at speed 7, and 371.9% (90.3%) at speed 10. Figures in parentheses represent standard errors of the mean. It seems, therefore, that the use of a higher shaking speed caused fragmentation of spore chains and resulted in more streptomycete colonies being recovered. Thus, many more colonies were recovered than should be produced from the inoculum. It was felt that, although a higher shaking speed gave more efficient recovery of spores, the use of the lower speed would give a more meaningful representation of the bacterium in soil. This, again, is subject to the drawbacks of using c.f.u. to measure a mycelial organism *in situ*.

(iii) Comparison of Chelex-100 and Sodium Hexametaphosphate as soil dispersants

A common soil dispersant is sodium hexametaphosphate, its mode of action being to desorb clay minerals from each other by blocking cation exchange sites, thus breaking up soil crumbs and facilitating the release of microorganisms from these structures (Stotzky, 1986). It was decided to compare the action of sodium hexametaphosphate and Chelex-100/0.1% (w/v) sodium deoxycholate/2.5% (w/v) PEG. $1.06x10^4$ c.f.u. of *S.lividans* TK24 were inoculated into six 100g samples of sterile soil. Three were extracted with 100ml

5% sodium hexametaphosphate/0.7% (w/v) $CaCO_3$ (Stotzky, 1986; Hopkins *et al.*, 1991) and three with Chelex-100/0.1% (w/v) sodium deoxycholate/2.5% (w/v) PEG. The extraction procedure was the same in both cases (see Section 2.22.2) with Chelex-100/0.1% (w/v) sodium deoxycholate/2.5% (w/v) PEG being replaced with 5% (w/v) sodium hexametaphosphate/0.7% (w/v) $CaCO_3$). The former gave a recovery of 91.35% (3.03%) and the latter 1.71% (0.27%). Figures in parentheses represent standard errors of the mean. The reason for the poor performance of sodium hexametaphosphate is not known, but is probably due to an unexpected toxicity effect

3.1.5 Detection limit of spore extraction method in sterile and nonsterile soil

The perceived advantage of biomass concentration techniques is that they reduce the final volume of the extract that the microbial population is suspended in. In theory it should be possible to detect one bacteria in whatever the starting mass of soil is, assuming that the centrifugation conditions are sufficiently stringent. This means that the detection limit of the method is relatively low; i.e. fewer (than traditional methods) organisms per unit soil can be detected. To determine the detection limit of the spore extraction method 10, 100, 1000 and 10000 c.f.u. of *S.lividans* TK24 spores were inoculated into 100g samples of sterile and nonsterile soil (i.e. 0.1, 1, 10 and 100 c.f.u./g). Colonies were detected on isolation plates from all samples from sterile soil. Apart from those inoculated with 0.1 c.f.u./g, *S.lividans* TK24 colonies were detectable on all plates derived from nonsterile soil extracts. Thus, this method is capable of detecting 0.1 c.f.u./g in sterile soil and 1 c.f.u./g in nonsterile soil. It should be stressed, however, that only one or two colonies were detected over a series of 10 plates at the lowest dilutions and thus enumeration is impossible at these

inoculum levels. Fig. 5 shows a graph of coefficient of variation ([standard deviation/mean] x 100). This graph shows that as the inoculum size is increased the accuracy of the mean increases (i.e. the variation around the mean is a smaller percentage of the mean). It can be seen that for a given inoculum size the coefficient of variation is larger in nonsterile soil than in sterile soil. This demonstrates that it is possible to be more confident at enumerating low numbers of bacteria in sterile soil than in nonsterile.

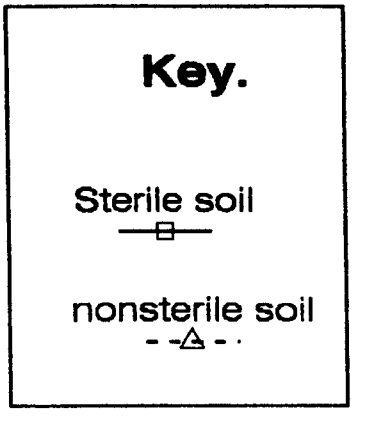
3.1.6 Comparative recovery of spores and mycelia from soil using spore extraction method

In order to ascertain the selectivity of the spore extraction method with respect to the state of the organism, each fraction of the procedure was examined for the appearance of both spores and mycelia. This was done by inoculating 1.03×10^6 c.f.u. of *S.lividans* TK24 mycelia into 3 samples of 100 g of sterile soil and 2.63×10^7 c.f.u. of *S.lividans* TK24 spores into a separate set of 3 sterile 100g portions.

Fig. 6 shows the percentage of the inoculum recovered from each fraction of the spore extraction method. A simplified flow diagram is shown next to it (see Fig. 3 and Section 2.22.2 for complete procedure). More mycelia were recovered than was originally inoculated. This is presumably due to fragmentation of the hyphae during the extraction procedure. Although only 21.95% of spores were retrieved in the final slurry, less than 1% of mycelia also appeared in this fraction, thus demonstrating the selectivity of the method for spores. These conclusions are again subject to the difficulties in using c.f.u. as a measure of a mycelial microorganism, however data given in Chapter 5 further confirms the selectivity of this method for streptomycete spores rather than mycelia.

Fig. 5. Variation of counted c.f.u./g against inoculum size after isolation from soil by the spore extraction method.

Known titres of *S.lividans* TK24 spores were inoculated into different 100 g samples of sterile and nonsterile soil. After isolation by the spore extraction method, the final soil slurry was assayed for *S.lividans* TK24 by viable counting, and the coefficient of variation of each sample (calculated from 10 replicates) plotted against the corresponding inoculum size.



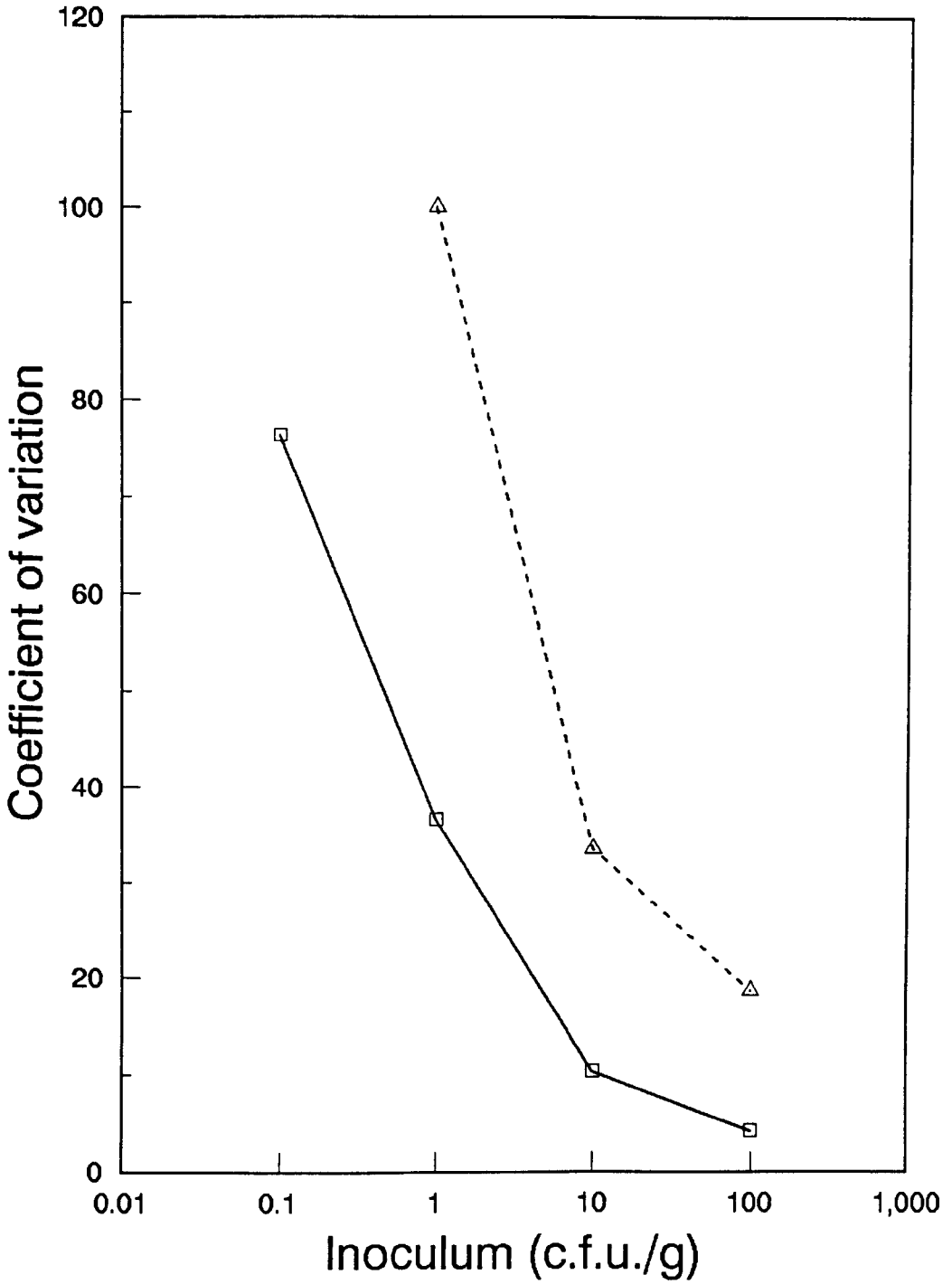
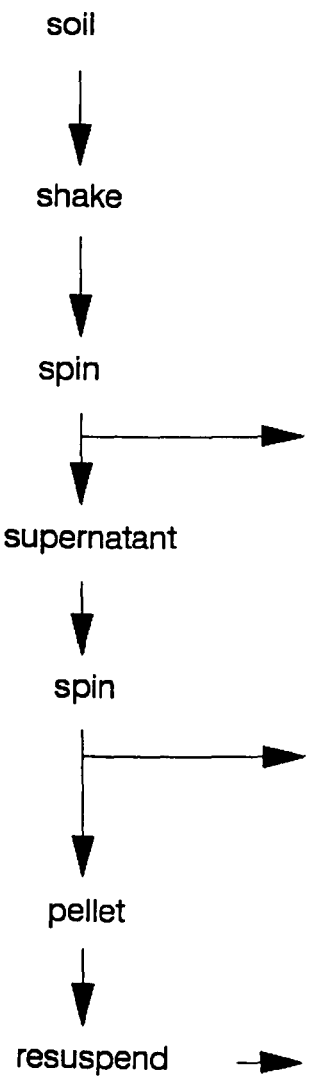

120
100
80
60
40
20
0
Coefficient of variation
0.01
0.1
1
10
100
1,000
Inoculum (c.f.u./g)

Fig 6. Separation of spores from mycelia using the spore extraction method.

Known titres of *S.lividans* TK24 spores and mycelia were inoculated into different 100 g soil samples. The percentage recovered at each stage of the extraction procedure is displayed in the table. Figures in parentheses represent standard errors of the mean.



Fraction	% of inoculum recovered: Spores	Mycelia
pellet	7.32 (0.479)	61.31 (9.04)
super-natant	31.96 (7.58)	85.0 (10.9)
slurry	21.95 (1.55)	0.225 (0.058)
total	59.31 (7.91)	163.3 (14.6)

3.2 Extraction of actinophage from soil

Methods for the isolation and enumeration of actinophage from soil are described by Lanning & Williams (1982). Their most efficient methods relied on the desorption of phage from clay particles by the action of nutrient broth (pH 8.0) containing 0.1% (w/v) egg albumin. This method works on the principle that protein suspended in a basic solute desorbs negatively charged phage particles from cation exchange sites in soil (Lanning & Williams, 1982).

3.2.1 Optimisation of phage extraction procedure for KC301

Lanning and Williams (1982) found that the highest levels of recovery of an introduced actinophage (108.7% from an arable soil) were found when chloroform was used to sterilise the soil extract (i.e. kill indigenous bacteria). However, not all actinophage are chloroform-tolerant, thus an alternative method of extract sterilisation (extract filtered through 0.45 μm nitrocellulose filter) was also developed which gave reduced recovery (74%) from the same arable soil. Thus, prior to deciding which method to use in soil microcosm studies it was necessary to determine whether KC301 was chloroform-tolerant. This was done by extracting KC301 and a known chloroform-resistant phage, SV1 (Stuttard, 1979) from sterile sand. This material was used to minimise any problems of phage desorption from soil, thus any differences in recovery could be attributed to the effects of chloroform. 1.21×10^5 p.f.u. of KC301 and 2.5×10^5 of SV1 were inoculated separately into two sets of four bottles. One bottle contained no sand and the other three, 20g of sterile sand. All samples were extracted according to methods C and D of Lanning & Williams (1982) prior to the sterilisation step (see Sections 2.23.1 and 2.23.2 respectively). The empty bottle and one of the sand-containing bottles was assayed directly (from 50ml DNB

added [see Section 2.23]), the remaining two sand containing bottles were treated by filtration (see Section 2.23.1) and chloroform (2.23.2). The results of this experiment are summarised in Table 9.

Highest recoveries with both phage were found in the absence of any treatment. Obviously this would be impractical when using such a method in nonsterile soil. Whilst, filtration caused a loss of phage, presumably through adsorption to the filter, only KC301 showed a significant drop in titre when treated with chloroform. Thus, it was decided that filtration would be used as a method for soil sterilisation as it appeared that KC301 is a chloroform sensitive actinophage.

3.3 Extraction of KC301 DNA from soil

An alternative route for the tracking of a microbial population in soil, rather than viable counting, is provided by using nucleic acid techniques. Whilst many workers (see section 3.3.1) have looked at the extraction of bacterial DNA from the environment, the recovery of bacteriophage DNA (either as free phage or lysogen DNA) has not yet been studied. The purpose of this section of work was to assess the ability of currently available DNA extraction techniques (Ogram *et al.*, 1987, Cresswell *et al.*, 1991) to monitor and detect KC301 in soil.

Table 9. Recovery of KC301 and SV1 from sterile sand.

Phage	% Phage recovered			
	Bottle	Sand		
		no treatment	filtration (0.45 μm)	chloroform (2% [v/v])
KC301	100.55 (2.59)	82.19 (2.03)	68.64 (2.78)	35.58 (2.18)
SV1	88.08 (3.63)	76.35 (2.80)	64.28 (2.75)	68.76 (3.45)

Figures in parentheses represent standard errors of the mean.

3.3.1 Detection of nucleic acids in the environment

It is possible to extract both RNA (Hahn *et al.*, 1990b) and DNA (reviewed by Trevors & Van Elsas, 1989) from an environmental sample. There are a number of advantages in using RNA extracts for monitoring a soil population, however as KC301 is a DNA bacteriophage (Lomovskaya *et al.*, 1970) it was necessary to use DNA extraction.

Initial attempts at studying environmental DNA were based on fluorometric analysis (Torsvik & Goksøyr, 1978; Deflaun *et al.*, 1986) which quantified the total amount of DNA within an extract and related it to bacterial numbers. Torsvik, (1980) further developed the purification process, using ion exchange and hydroxyapatite chromatography, to investigate soil DNA denaturation characteristics (T_m=90.8°C, %G+C≈50%). More recently the development of molecular techniques (Holben & Tiedje, 1988) has allowed the ecology of individual species or genes to be investigated (Holben *et al.*, 1988). DNA extraction from soil (or sediment) may be carried out in two ways, both of which subsequently involve hybridisation of the DNA to a labelled probe (Holben & Tiedje, 1988). The first procedure (Ogram *et al.*, 1987; Cresswell *et al.*, 1991) involves the lysis of microbial cells *in situ* by a variety of methods, the DNA is then extracted from the environmental sample and further purified before being subjected to further analysis (restriction, hybridisation, PCR). The second procedure is one of indirect lysis (Torsvik, 1980; Holben *et al.*, 1988; Trevors & Berg, 1989) where the microbial biomass is first extracted from the sample (using the method of Fægri *et al.*, 1977), subsequently lysed and the DNA purified. Direct lysis gives a relatively high yield of DNA, but contains larger amounts of impurities (e.g. humic acids) than DNA indirectly isolated (Holben & Tiedje, 1988; Trevors & Van Elsas, 1989). Either of the two extraction methods listed above can be used to provide DNA for PCR (Steffan *et al.*, 1988; Bej *et al.*, 1990) if the DNA is of sufficient purity for Taq polymerase

to work. Quantification of a microbial population can be carried out by first extracting DNA from the environmental sample, enumeration could then be attempted by using a probable number system based on hybridisation with a labelled probe (Fredrickson *et al.*, 1988), or possibly by correlating the amount of hybridised DNA with c.f.u. by means of a densitometer or betascope.

The drawback of these methods are that they are unable to distinguish between viable and nonviable microorganisms. Theoretically single-copy sequences may be detected at levels down to 10^2 c.f.u./g (Cresswell *et al.*, 1991), however, using direct lysis methods, it is possible to detect *ca.* 10^5-10^6 c.f.u./g of a single copy gene in a unicellular microorganism (Holben *et al.*, 1988), although the use of multicopy plasmids can lower the detection limit of this method. Using PCR as few as 10^4 c.f.u./g can be detected in soil or even 1 c.f.u./g in sediment (Steffan *et al.*, 1988) although as better methods for DNA purification become available this detection limit is certain to improve (Holben & Tiedje, 1988).

3.3.2 Extraction of KC301 DNA from soil

Soil DNA extractions were carried out by the SDS lysis procedure of Cresswell *et al.* (1991), which is a derivative of the direct lysis procedure of Ogram *et al.* (1987). Later work (see Chapter 8) used the direct lysis by bead-beating method of Cresswell *et al.* (1991). The SDS lysis procedure (see Section 2.24.1) lyses bacterial cells *in situ*, followed by subsequent purification of the DNA. KC301 was added to nine 10 g samples of nonsterile soil to give a 10 fold dilution series containing the phage at titres ranging from 9.57×10^9 p.f.u./g to 95.7 p.f.u./g. A further 10 g soil sample was prepared as a negative control. These were then extracted as in Section 2.24.1, except that a 700 μl aliquot of PEG precipitate, dissolved in TE buffer, (*ca.* 5ml total volume) was transferred

to an Eppendorf tube and subjected to the remainder of the procedure. This dilution series of extracts was then run on an agarose gel, Southern blotted and probed with ^{32}P-labelled KC301 DNA. The gel photograph and autoradiograph are shown in Fig. 7 (a). Although soil DNA can be seen in all wells, hybridisation is only visible with DNA extracts containing 9.57×10^8 or more p.f.u./g. This experiment was repeated using the whole of the PEG precipitate for subsequent DNA purification (as in Section 2.24.1). Again, six 10g nonsterile soil samples were prepared, five of which were inoculated with a 10 fold dilution series of KC301, ranging from 4.93×10^8 p.f.u./g to 4.93×10^4 p.f.u./g. The remaining sample was inoculated with SDW as a negative control. Following DNA purification the extracts were run on an agarose gel, southern blotted and probed with ^{32}P-labelled KC301 DNA. The gel and autoradiograph of these extractions are shown in Fig. 7 (b). Again, DNA can be seen in all tracks, but hybridisation can only be detected in wells containing DNA derived from 4.93×10^6 p.f.u./g or more. As would be predicted, the use of the entire PEG precipitate resulted in a significantly lower detection limit for this approach to monitoring the phage *in situ*. Such a method might provide a means for following a phage population in soil, together with traditional assay procedures.

Fig 7 (a) i and ii. Extraction of KC301 DNA from soil using a portion of TE dissolved PEG precipitate.

KC301 was inoculated into 10 g nonsterile soil samples in sufficient SDW to give a percentage moisture of 15%. DNA was extracted as in Section 2.24.1; except that after PEG precipitation and addition of TE buffer, 700 μl were transferred to an Eppendorf tube and phenol extracted (x2) with phenol solution (see Section 2.11). A dilution series of KC301 DNA was prepared and also subjected to electrophoresis on the same gel (see facing page). The gel was southern blotted and probed with ^{32}P-labelled KC301 DNA as described in Chapter 2. The resulting autoradiograph is displayed overleaf.

Left hand wells.

Track 1. 9.57×10^9 p.f.u./g KC301

Track 2. 9.57×10^8 p.f.u./g KC301

Track 3. 9.57×10^7 p.f.u./g KC301

Track 4. 9.57×10^6 p.f.u./g KC301

Track 5. 9.57×10^5 p.f.u./g KC301

Track 6. 9.57×10^4 p.f.u./g KC301

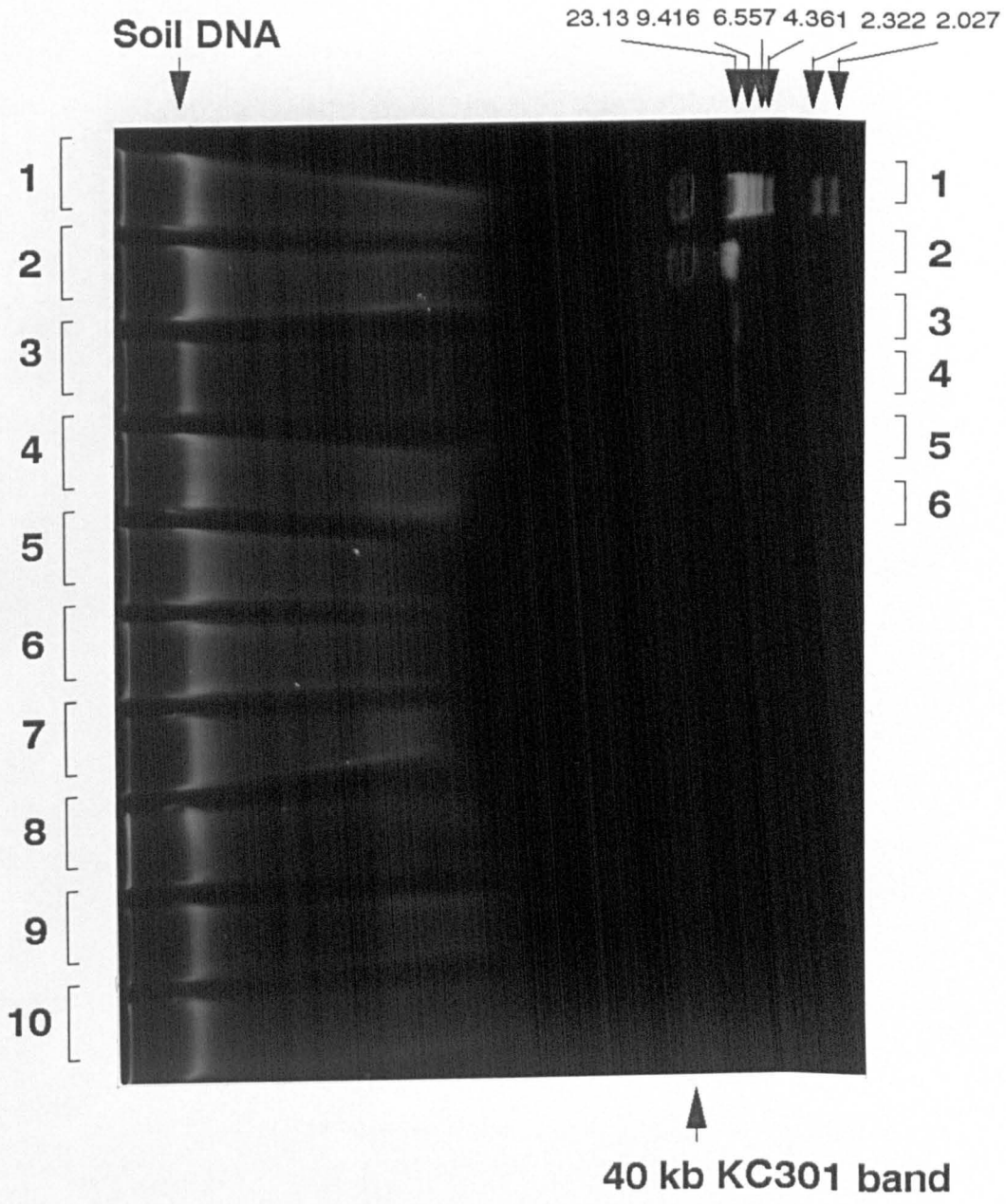
Track 7. 9.57×10^3 p.f.u./g KC301

Track 8. 957 p.f.u./g KC301

Track 9. 95.7 p.f.u./g KC301

Track 10. SDW.

(cont. overleaf)


Soil DNA
23.13 9.416 6.557 4.361 2.322 2.027
1
2
3
4
5
6
7
8
9
10
1
2
3
4
5
6
40 kb KC301 band

Right hand wells.

Track 1.	Lambda *Hin*DII
Track 2.	345 ng KC301 DNA
Track 3.	79 ng KC301 DNA
Track 4.	34.5 ng KC301 DNA
Track 5.	7.9 ng KC301 DNA
Track 6.	3.45 ng KC301 DNA

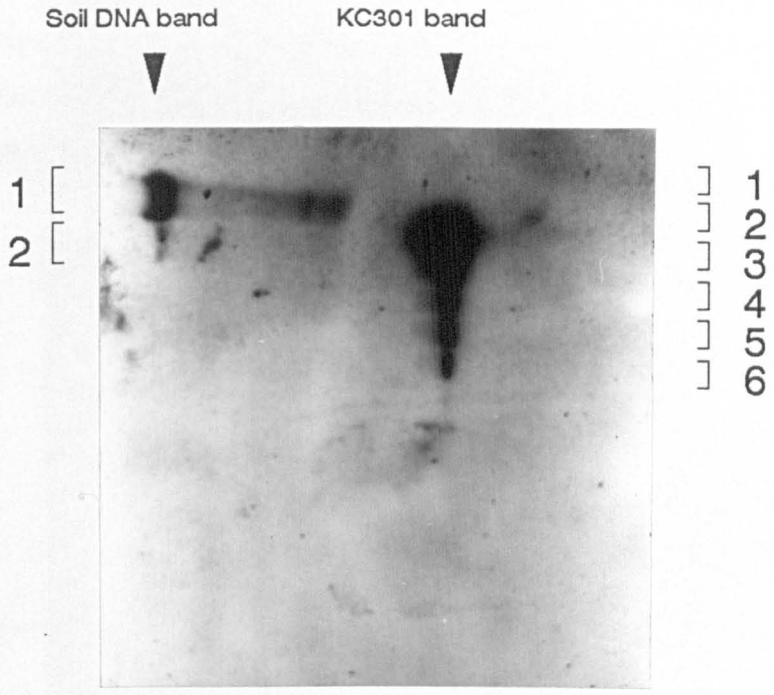

Soil DNA band
KC301 band
1
2
1
2
3
4
5
6

Fig 7 (b) i and ii. Extraction of KC301 DNA from soil using a complete PEG precipitate.

KC301 was inoculated into 10 g nonsterile soil samples in sufficient SDW to give a percentage moisture of 15%. DNA was extracted as in Section 2.24.1. A dilution series of KC301 DNA was prepared and also subjected to electrophoresis on the same gel (see facing page). The gel was southern blotted and probed with 32P-labelled KC301 DNA as described in Chapter 2. The resulting autoradiograph is displayed overleaf.

Upper wells.

Track 1. 4.93×10^8 p.f.u./g KC301

Track 2. 4.93×10^7 p.f.u./g KC301

Track 3. 4.93×10^6 p.f.u./g KC301

Track 4. 4.93×10^5 p.f.u./g KC301

Track 5. 4.93×10^4 p.f.u./g KC301 (not shown)

Track 6. SDW (not shown)

Lower wells.

Track 1. Lambda *Hin*DIII

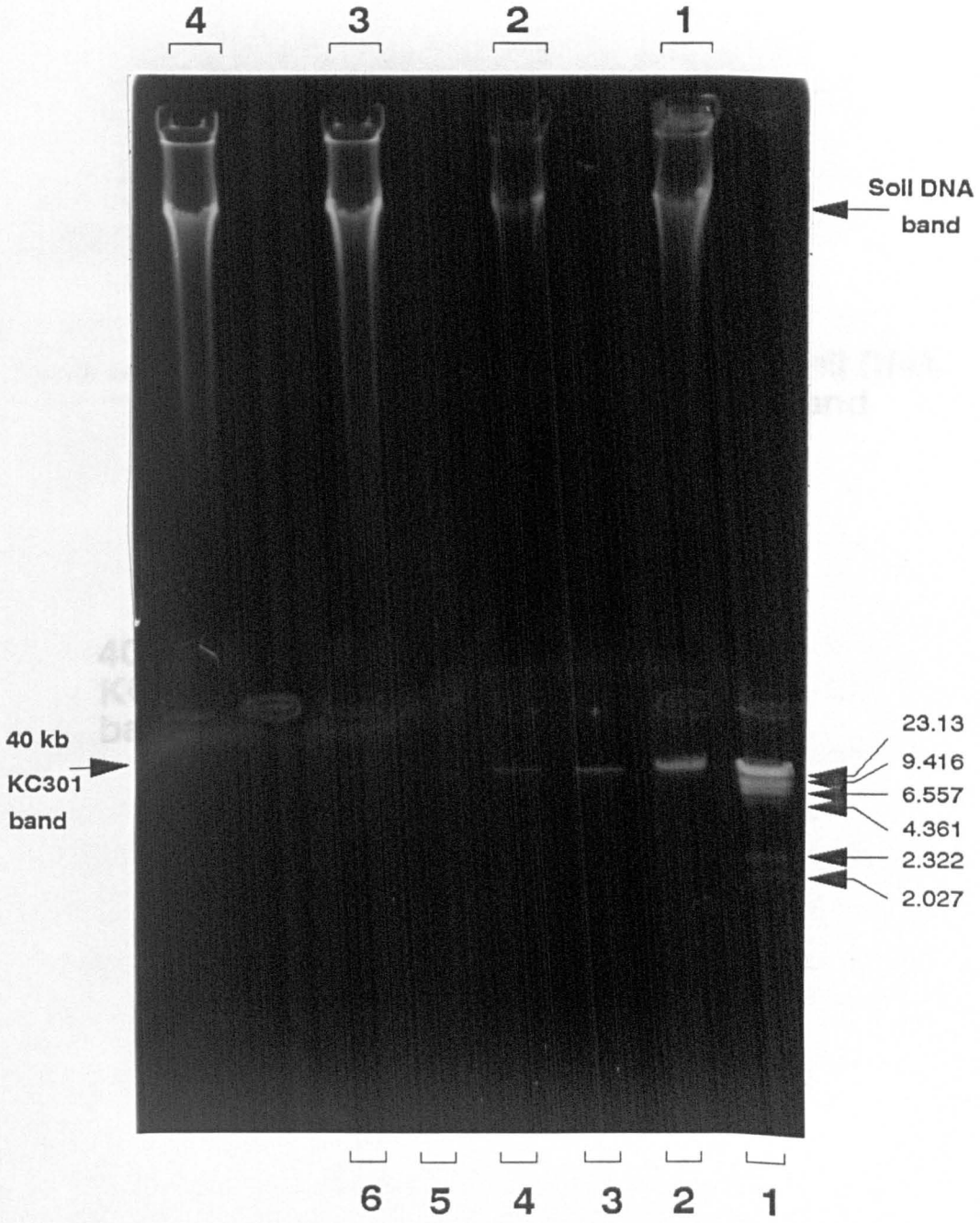
Track 2. 345 ng KC301 DNA

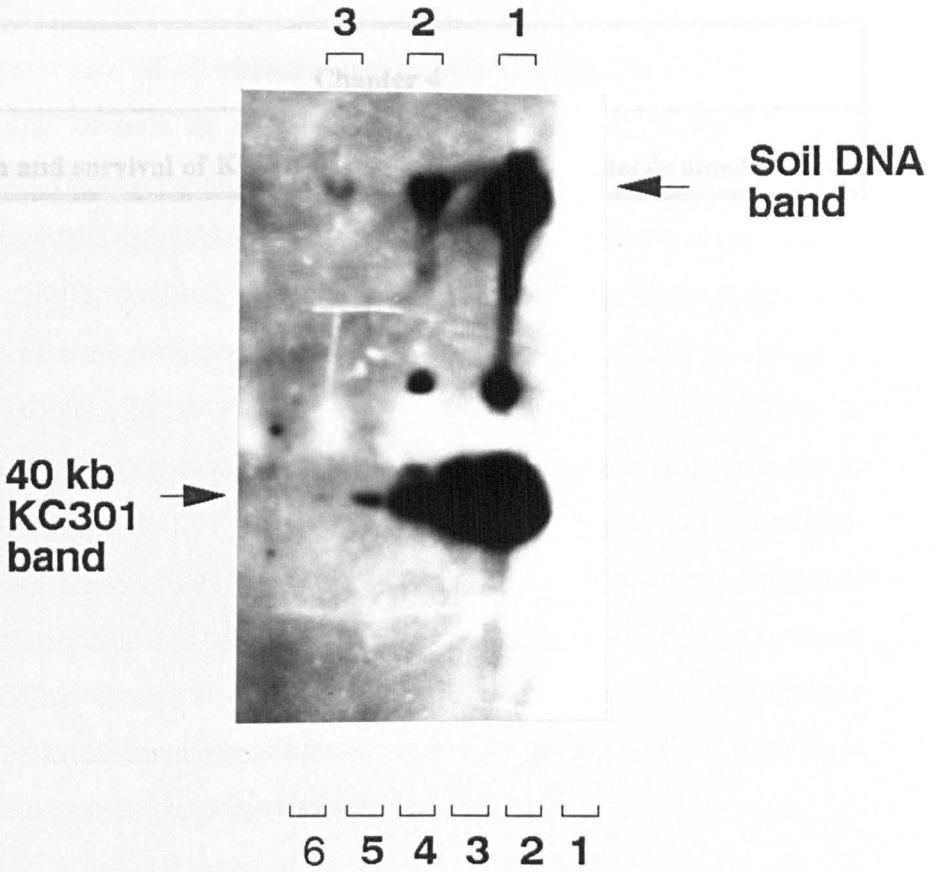
Track 3. 79 ng KC301 DNA

Track 4. 34.5 ng KC301 DNA

Track 5. 7.9 ng KC301 DNA

Track 6. 3.45 ng KC301 DNA


4
3
2
1
Soil DNA
band
40 kb
KC301
band
23.13
9.416
6.557
4.361
2.322
2.027
6
5
4
3
2
1


3
2
1
Soil DNA band
40 kb KC301 band
6
5
4
3
2
1

Chapter 4

Growth and survival of KC301 and *S.lividans* 1326 in sterile amended soil

4.1 Development of KC301 from ΦC31

Many derivatives of ΦC31 have been constructed by spontaneous deletions and subsequent subcloning (Chater *et al.*, 1985; Hopwood *et al.*, 1985; Chater, 1986). Viable deletion mutants that are still able to produce turbid plaques, i.e are capable of lysogeny, can be obtained by treatment with chelating agents (Chater, 1980). The *E. coli* plasmid, pBR322 was cloned into an *EcoR1* site (Chater *et al.*, 1981a) of such a mutant, ΦC31△M*cts*△23 (1.43kb deletion) at a site 6.35 kb from the right hand of the phage genome (Suárez & Chater, 1980b). Further deletion mutants were obtained from ΦC31△M*cts*△23::pBR322 (Chater *et al.*, 1981b). One of these deletion mutants, △W12, lacking part of the ΦC31 genome and part of pBR322, was used to insert the 1.9 kb *tsr* resistance gene from *Streptomyces azureus* (Chater *et al.*, 1982). This 40 kb derivative ΦC31△M*cts*△23::pBR322△W12::*tsr* is known as KC301 (Hopwood *et al.*, 1985) and is apparently unimpaired in any of its replication functions *in vitro*. The *tsr* gene (23S rRNA pentose methylase gene) codes for thiostrepton-resistance (Cundliffe, 1978) and was originally shotgun cloned from *S. azureus* into SLP1.2 (Thompson *et al.*, 1980; Thompson *et al.*, 1982a; Thompson *et al.*, 1982b). Thus, lysogens of KC301 can be detected by the incorporation of thiostrepton into the growth medium, since *tsr* is expressed in its host (phage conversion).

4.2 Survival of KC301 and *S.lividans* 1326 in sterile amended soil: aims

The aims of this experiment were to examine the fate of KC301 to lysogenise *in situ* in the presence of a host, *S.lividans* 1326, and to determine the capacity of KC301 to lysogenise *S.lividans* 1326 *in situ*. Sterile amended soil was chosen, rather than natural soil, so as to give the best conditions for these

interactions to occur.

4.3 Sterile amended soil conditions

Five 200g samples of sterile amended soil were prepared as described in Section 2.21. The inocula added are described in Table 10. Phage and hosts were added to sufficient SDW to give a final percentage moisture of 15% (about 26 ml), which was then mixed with the soil. In this experiment and all subsequent experiments the time during which phage and host were stored together in SDW prior to inoculation was kept to a minimum. This was to reduce the chances of phage-host aggregation as much as possible. It should be remembered that KC301 requires divalent cations in order for phage adsorption to a host to occur. Phage were extracted as in Section 2.23.1 and host as in 2.22.1. Two 1 g samples were taken from each soil pot per sample day, extracted using the ¼ strength Ringer's solution method, serially diluted and plated out in triplicate for enumeration of hosts and lysogens. One 20 g sample was taken per sample day from each soil pot. An aliquot of the centrifugation supernatant (see Section 2.23.1) was filtered, serially diluted and assayed for KC301 in triplicate as described in Section 2.7. Hosts were enumerated on AGS, supplemented with cycloheximide (see Section 2.4) and lysogens were enumerated by incorporating thiostrepton in the medium (see Table 7). Cycloheximde was included as precautionary measure should the soil be contaminated with fungi, as the 200g microcosms used in this experiment proved very difficult to keep sterile.

Table 10. Inocula

Soil sample	KC301 inoculum (p.f.u./g)	*S.lividans* 1326 inoculum (c.f.u./g)
A	-	$5x10^6$
B	$8.5x10^4$	-
C	$8.5x10^4$	$5x10^6$
D	$8.5x10^4$	$5x10^6$
E	$8.5x10^4$	$5x10^6$

4.4 Survival of inoculants in sterile amended soil

Fig. 8 shows the growth and survival of *S.lividans* 1326 in this experiment. Graphs are displayed as logged counts at each day and as scaled counts; the latter allows changes in populations to be compared between different microcosms, without any masking by inoculum size. Over the course of the experiment total host counts rose from *ca.* 10^5 c.f.u./g, to a peak of *ca.* 10^8 before falling back to *ca.* 10^7 c.f.u./g. The reason for this die-back is thought to be due to drying of the soil. The pots in this experiment were covered with aluminium foil, thus, the percentage moisture was able to drop to <5% by day 39. It is interesting to note that the *S.lividans* 1326 titre in soil co-inoculated with KC301 (see Fig. 8) increased to a lesser extent than soil containing the *S.lividans* 1326 alone. This effect could be due to KC301 lysing a proportion of newly-germinated mycelia during the first few days of the experiment, thus reducing the total level of sporulation. However, it should be stressed that the differences in populations are not significant. Lysogens were first detected at day 2 (as the ¼ strength Ringer's method extracts both spores and mycelia, these lysogens must have been mycelial) and rose to a peak of *ca.* 10^4 c.f.u./g by day 39. Obviously, most of the lysogen counts in the latter part of the experiment would be due to sporulation of lysogenic mycelia, however, there must still have been a significant number of infections resulting in lysogeny during the first few days after inoculation. Lysogens were confirmed as such by colony hybridisation with ^{32}P-labelled KC301 DNA (See Fig. 10). No lysogens were detected in soils inoculated without both phage and host, no colonies were detected in soils uninoculated with *S.lividans* 1326.

The survival of KC301 in this system is shown in Fig. 9; it can be seen that phage numbers only increase from that of the inoculum in soils co-inoculated with a host, thus KC301 does infect and propagate in sterile amended soil containing a viable host. After this initial burst, phage numbers decrease

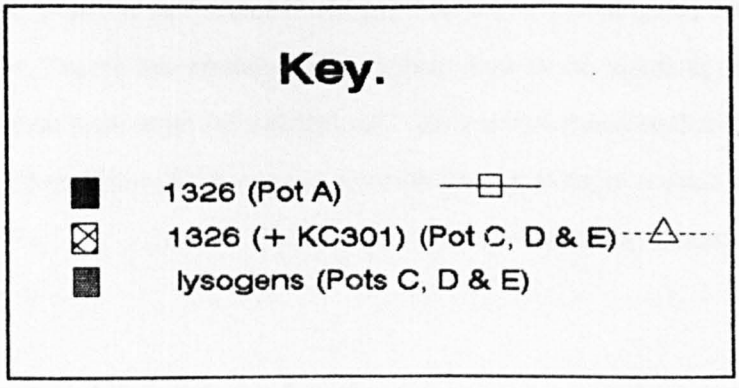
during the course of the experiment, until at day 39 their titre has dropped below the detection limit of the phage extraction method (25 p.f.u./ g). It is thought that KC301 was undetectable at day 39 due to phage inactivation through drying of the soil. Williams & Lanning (1984) found that increased moisture had a negative effect on actinophage survival, but did not investigate the effects of low moisture contents. Thus, it is thought that the drop in moisture content of the soil caused a concentration of phage in cracks and crevices of soil particles, this resulted in increased binding of the virions. It is not possible to determine whether the failure to detect plaques at day 39 was due to phage inactivation, or simply that the extraction procedure was not able to remove them from their bound state. Plaques were not detected from soils uninoculated with KC301.

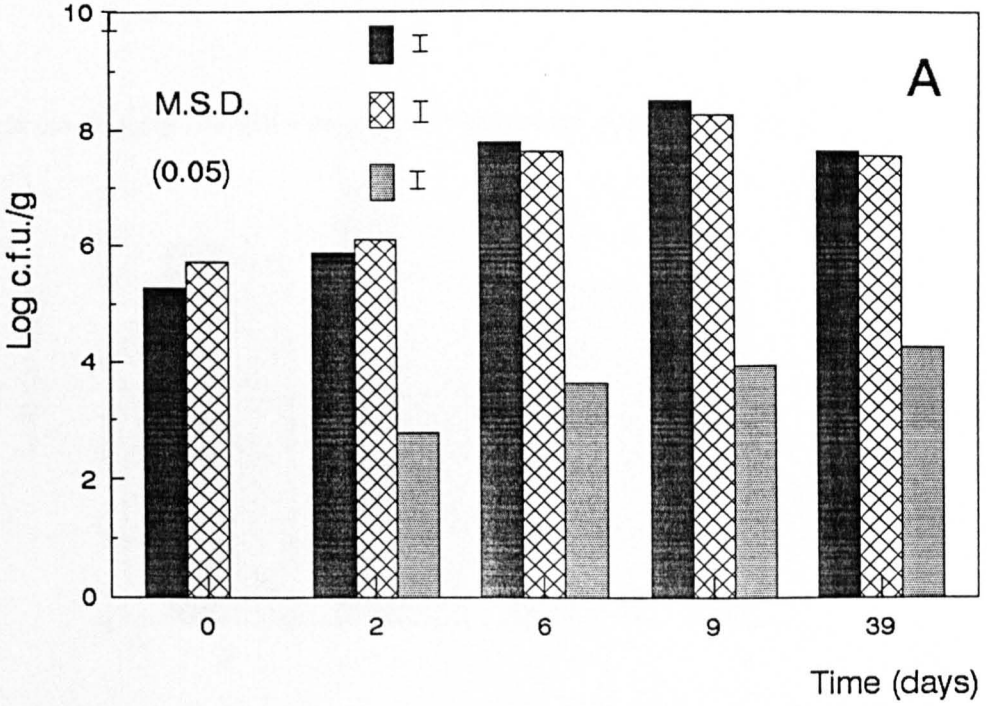
4.5 Phage and host survival in sterile amended soil: conclusions

S.lividans 1326 grew in sterile amended soil, increasing its titre between two and three logs. It was receptive to phage infection, which could result in both lysis and lysogeny. Free KC301 could survive in soil for a limited period, the survival being linked to the soil moisture content i.e. as the soil dried out phage were probably drawn into crevices in soil crumbs and became adsorbed to clay particles (and hence inactivated). The fact that lysogens were detectable and free phage were not, gives an indication as to how ΦC31 might survive periods of environmental adversity.

Fig. 8 Growth and survival of *S.lividans* 1326 in sterile amended soil.

Graph A represents the logged counts at each day, whilst graph B shows the scaled counts (calculated by dividing the logged count at a given day by the count for that sample set at day 0 [counted c.f.u./g at day 0, not theoretical titre estimated from the inoculum count]).




A
M.S.D.
(0.05)
I
I
I
Log c.f.u./g
10
8
6
4
2
0
0
2
6
9
39
Time (days)

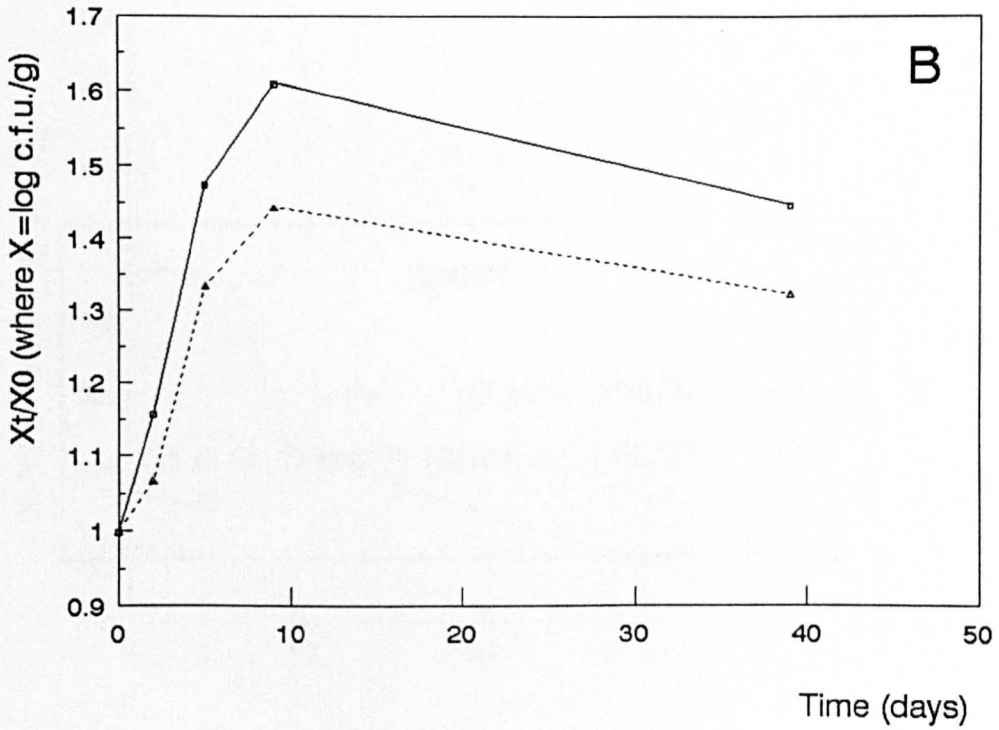
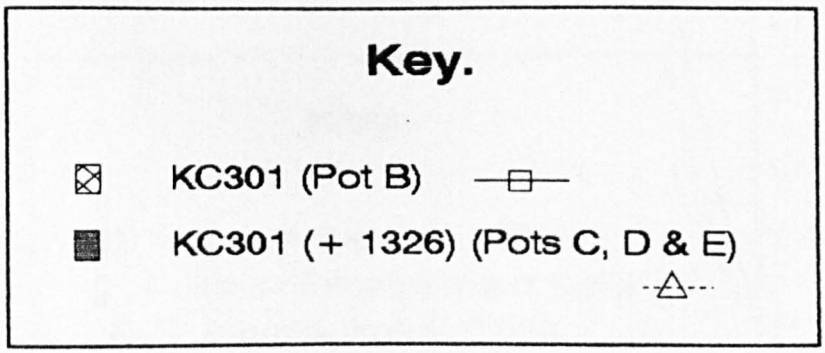
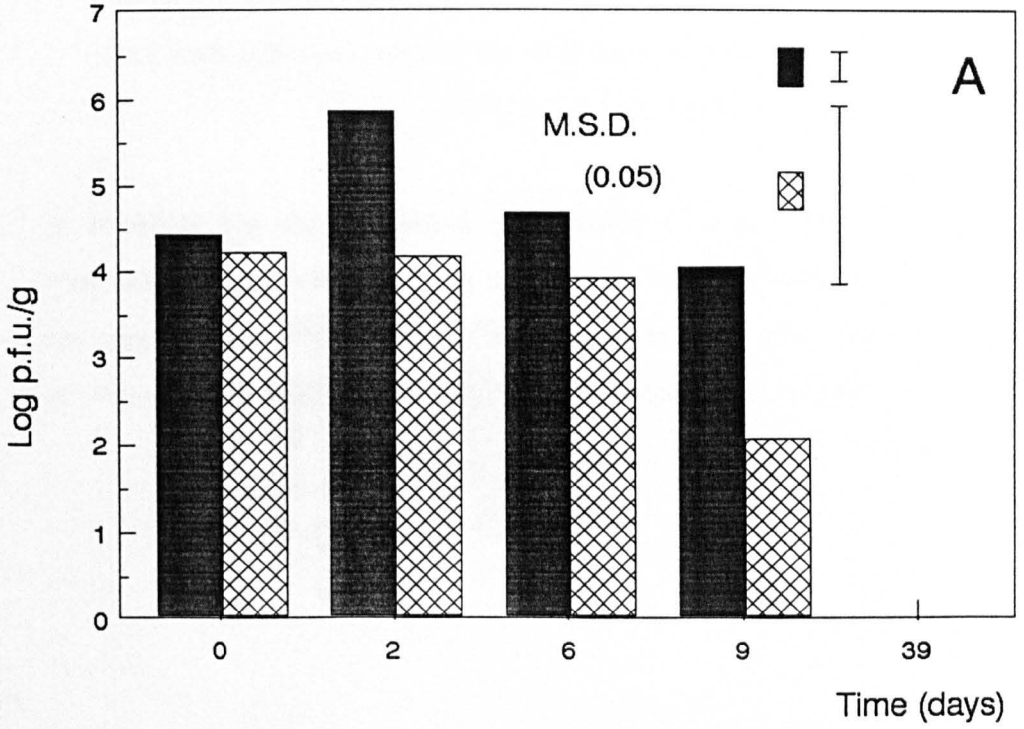

B
Xt/X0 (where X=log c.f.u./g)
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0
10
20
30
40
50
Time (days)

Fig. 9. Survival of KC301 in sterile amended soil

Graph A represents the logged counts at each day and graph B the scaled counts.




A
M.S.D.
(0.05)
Log p.f.u./g
7
6
5
4
3
2
1
0
0
2
6
9
39
Time (days)

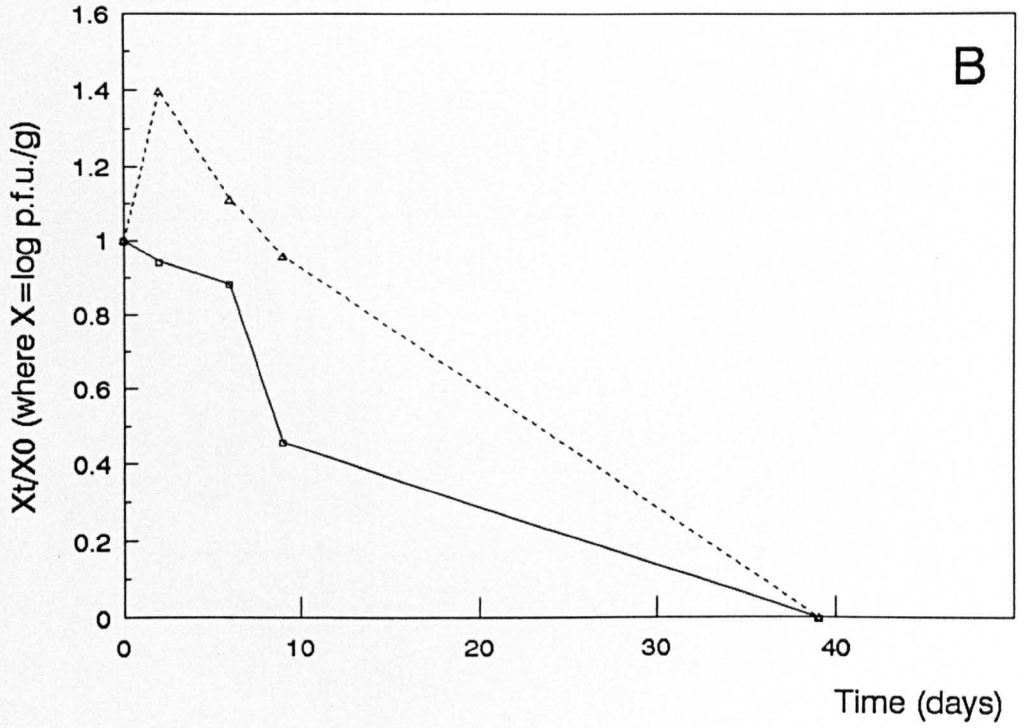

B
Xt/X0 (where X=log p.f.u./g)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
Time (days)

Fig 10. **Autoradiograph of thiostrepton-resistant *S.lividans* 1326 isolated from sterile amended soil probed with ^{32}P-labelled KC301 DNA.**

Colonies were picked from isolation plates and subjected to colony hybridisation as described in Chapter 2. Hybridisation was only detected with thiostrepton-resistant colonies (see facing page), no hybridisation was detected with colonies isolated from plates containing no thiostrepton.

Chapter 5.

Survival of *S.lividans* TK24 and KC301 in sterile amended and nonsterile soil.

5.1 Monitoring of phage and host using the spore extraction method: aims

Following the development of the spore extraction method, it was decided to apply this procedure to tracking *S.lividans* TK24 in both sterile amended and nonsterile soil. Chapter 4 describes the interactions of the phage and host in sterile amended soil, it was therefore hoped that similar events could be tracked in nonsterile soil. Previous work by Wellington *et al.* (1990) had indicated that conjugation between strains of *S.lividans* could be found in nonsterile soil; it was thus anticipated that the low detection limit of this method would allow the recovery of quantifiable numbers of lysogens from nonsterile soil.

5.2 Conditions for monitoring inoculants in nonsterile and nonsterile amended soil

Previous experiments using 200g sterile amended soil microcosm (see Section 2.21) were difficult to keep sterile (data not shown), due to the need to open and sample frequently. It was decided to carry out this experiment using sealed glass bottles that could be sampled destructively, thus eliminating contamination problems after inoculation had taken place (see Section 2.21). Sufficient air space remained in the bottles to prevent the onset of anaerobic conditions. A series of seven microcosms were prepared for each sample set (see Table 11). 100g soil samples were used for spore extractions from sets A, C, D, F and G; whilst 20g samples were prepared for phage extractions from sets B, C, E, F, G. In addition a further set of seven 100g samples were prepared for use in percent moisture calculations. Phage and host were added to aliquots of SDW, that when added to the soil, gave a final percentage moisture of 15%. In

this experiment, the samples were not mixed, and water was allowed to penetrate the soil through diffusion (uniform wetting of the soil was seen within 24 hours of inoculation). In soil experiments carried out in such sealed microcosms, percentage moisture would fall from *ca.* 15% to *ca.* 13% during the first fifteen days of the experiment before levelling off and remaining at this point for up to sixty days. Phage were extracted as in Section 2.23.1 and host as in 2.22.2. One 100g microcosm was extracted using the Chelex-100 method per sample day, the final extract being serially diluted and plated out in triplicate for enumeration of hosts and lysogens. One 20g microcosm was extracted per sample day for the enumeration of KC301. Three aliquots of the centrifugation supernatant (see Section 2.23.1) were filtered, serially diluted and assayed for KC301 in triplicate as described in Section 27. Hosts were enumerated on RASS (see Section 2.4), containing rifampicin, streptomycin, cycloheximide and nystatin (see Table 7). Lysogens were visualised by incorporating thiostrepton in the medium (see Table 7).

Table 11. Inocula

Sample set	[1]conditions	*S.lividans TK24* (c.f.u./g)	KC301 (p.f.u./g)
A	St. Am.	$1.42x10^4$	-
B	St. Am.	-	$9.15x10^3$
C	St. Am.	$1.42x10^4$	$9.15x10^3$
D	NS.	$1.42x10^4$	-
E	NS.	-	$9.15x10^3$
F	NS.	$1.42x10^4$	$9.15x10^3$
G	NS.	-	-

[1]St. Am., sterile amended soil; NS., nonsterile soil.

5.3 Survival of *S.lividans* TK24 and KC301 in nonsterile and sterile amended soil

The growth and survival of *S.lividans* TK24 during this experiment is shown in Fig. 11; in sterile amended soil the titre of *S.lividans* TK24 dropped to almost undetectable levels during the first few days of the experiment, before recovering with a large increase. As the Chelex method is spore specific it is assumed that this drop was due to germination of the spore inoculum, which then became undetectable by this extraction procedure. Following this mycelial development, the depletion of nutrients resulted in massive sporulation of the hyphae causing the dramatic increase in titre after day 2. It seems that this germination/sporulation cycle was complete by day 15 (i.e. there were no more great changes in population numbers), and the spores were able to survive for up to 60 days. In nonsterile soil there was a similar drop in numbers during the initial part of the experiment, although this effect was not as extensive as in sterile amended soil. Following this, numbers of *S.lividans* TK24 remained broadly constant during subsequent sample days; presumably because the bulk of the spore inoculum did not germinate and was able to survive in this relatively resistant form. Streptomycin- and rifampicin-resistant indigenous streptomycetes were recovered from both nonsterile control and experimental soils. Their numbers were *ca.* 10^2 c.f.u./g; however all such colonies had a uniformly white spore mass, in contrast to the grey colouration of *S.lividans* TK24, and were thus easily distinguishable. Lysogens were first isolated from sterile amended soil at day 5. When, this experiment was carried out using the ¼ strength Ringer's solution extraction method, lysogens were first found at day 2 (see Fig. 8). As the Ringer's method isolates both spores and hyphae, it is thought that lysogenic mycelia appeared at day 2 and lysogenic spores at day 5 (see Chapter 9). If this is the case, the lysogen count at day 5, in this experiment is not reflective of the initial number of lysogenic infections, rather it represents the number of spores

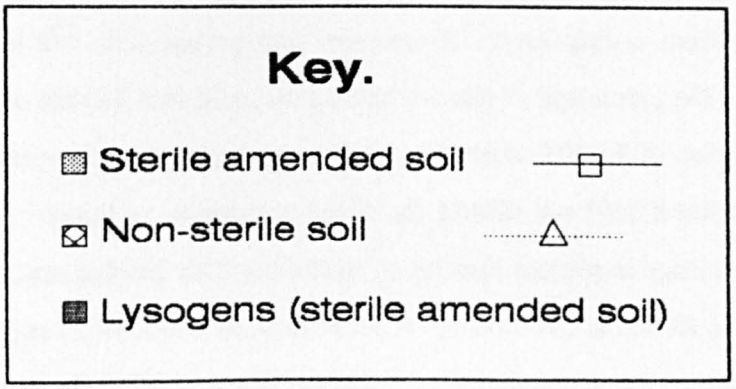
produced by mycelia harbouring KC301 in a temperate state. No thiostrepton-resistant colonies were isolated from soils uninoculated with phage and host. No lysogens were isolated from nonsterile soil. It is thought that this was due to insufficient germination of the spore inoculum, resulting in too few host cells being receptive to phage infection. Putative lysogens were confirmed as such by colony hybridisation with ^{32}P-labelled KC301 DNA and ^{32}P-labelled *tsr* removed from pIJ673 with *BclI* (see Fig. 13 [b]); as described in Chapter 2 and Fig. 14. Thiostrepton-resistant colonies were also picked onto SNA overlays seeded with *S.lividans* TK24 to determine whether they were capable of plaque formation by spontaneous phage release (see Fig. 13 [a]).

The survival of KC301 during this experiment is shown in Fig. 12; in all cases, except sample set B (KC301 in sterile amended soil), phage numbers showed an increase between days 0 and 1. This shows that KC301 was able to infect and propagate in a host; even in nonsterile soil uninoculated with *S.lividans* TK24, KC301 was able to infect indigenous streptomycetes that were able to support its propagation (sample set E). However, indigenous streptomycetes were not monitored so it is not possible to confirm this. Set B showed no increase as there was no host available (sterile amended soil). It is interesting that raised phage titres were only found when the host was in the mycelial state indicating that a receptive host is critical to the development of a high phage titre; when *S.lividans* TK24 began to sporulate phage numbers decreased. The soil used in this experiment, although termed nonsterile, was not truly fresh soil; in that it had been dug several months previously and stored during this period. The result of this was % moisture had dropped to *ca.* 2%. It is inconceivable that no death of the soil microflora had taken place during this period of drying. Thus, upon inoculation (and remoistening) there is likely to have been a nutrient flush, which may have caused germination of indigenous streptomycetes (see Section 1.3.6), resulting in their becoming receptive to phage infection. It is not known, therefore, whether this increase in phage

numbers during the initial stages of the experiment would be found if truly fresh soil were used. Surprisingly, KC301 showed better survival in nonsterile soil than in sterile amended soil, contradicting the work of Williams & Lanning (1984). The reason for this is thought to be the presence of a greater number of potential hosts in nonsterile soil, coupled with a wide range of streptomycetes that have germination/sporulation cycles of greater duration than *S.lividans* TK24 i.e. hosts were present in the mycelial state for longer periods (e.g. *Streptomyces halstadeii* does not sporulate in soil until 25-30 days after inoculation (J. Eccleston, personal communication). No indigenous phage were isolated at day 0 from nonsterile control soils, however at day 1 their titre was *ca.* 10^3 c.f.u./g. Both turbid and clear plaques were seen. It is assumed that these temperate phage were present within the indigenous *Streptomyces* population as lysogens and induced by the action of germination. Virulent phage were either present beneath the detection limit of free phage (25 p.f.u./g) or possibly existing as pseudolysogens at day 0. Obviously, KC301 counts in nonsterile soil after day 0 represent total actinophage numbers. By probing plaques from nonsterile soil isolation plates (see Fig. 15) it was possible to determine the proportion of KC301 within the entire actinophage population that infected *S.lividans* TK24. Forty nine plaques per sample day were picked from isolation plates from sample set F. These were then probed with ^{32}P-labelled KC301 DNA. The percentage of plaques lighting up could then be used to determine the proportion of KC301 within the total actinophage population (see Fig. 16). It seems that KC301 was diluted out of the population, as shown by the reduction in percentage in plaques lighting up with time. This dilution could be due to the fact that KC301 is considerably modified from wild type ΦC31 and is, somehow, less fit in the environment, the parent phage, ΦC31, is less fit than its competitors or that the introduced phage is unable to establish itself in the indigenous community.

Fig. 11. **Growth and survival of *S.lividans* TK24 in sterile amended and nonsterile soil.**

Graphs A and B represent the logged counts at each day, whilst graphs C and D represent scaled counts. A and C show counts from sample sets A and D (*S.lividans* TK24 in sterile amended and non sterile soil), whilst graphs B and D give counts from sample sets C and F (*S.lividans* TK24 x KC301 in sterile amended and nonsterile soil).



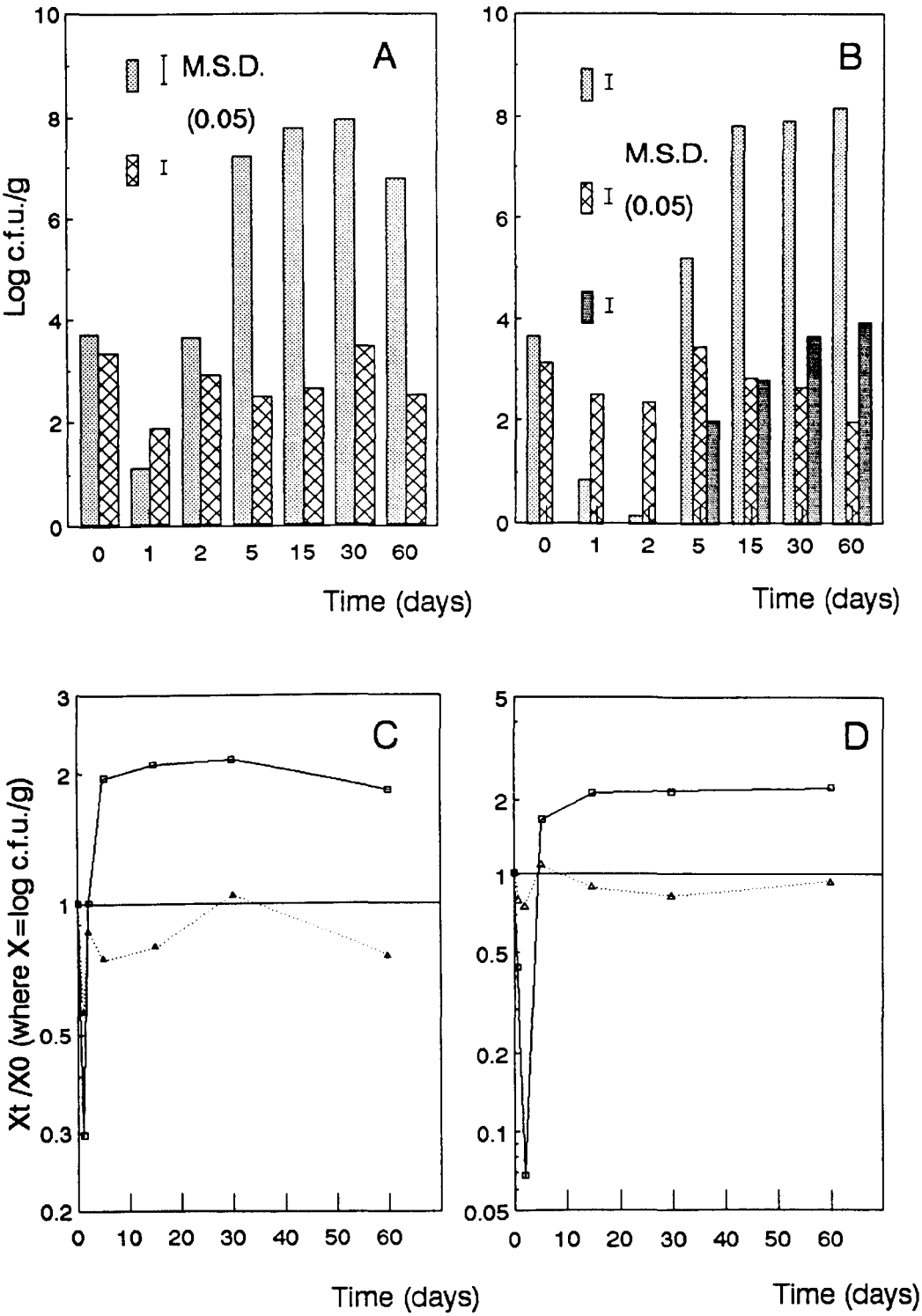
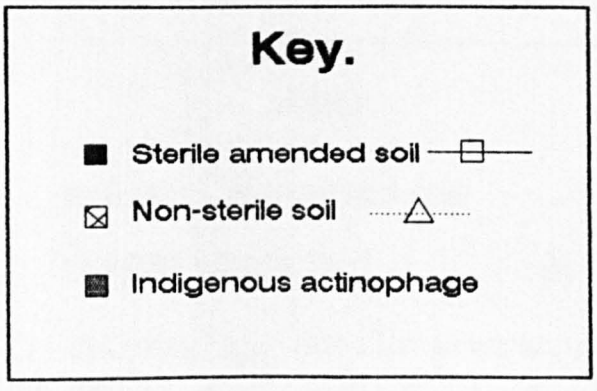
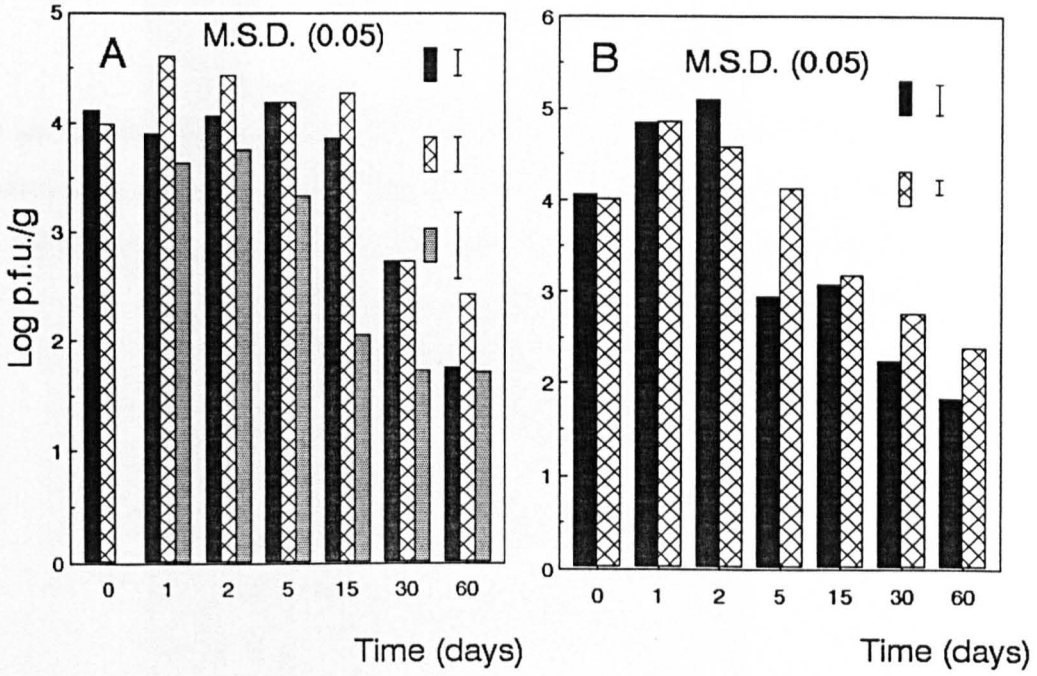

A
M.S.D.
(0.05)
Log c.f.u./g
Time (days)
B
M.S.D.
(0.05)
Time (days)
C
Xt /X0 (where X=log c.f.u./g)
Time (days)
D
Time (days)

Fig. 12. Survival of KC301 in sterile amended and nonsterile soil.

Graphs A and B show the logged counts at each day, whilst graphs C and D give the scaled counts. Graphs A and C show counts from sample sets B, E and G (KC301 in sterile amended soil, nonsterile soil and indigenous actinophage [that infect *S.lividans* TK24] isolated from sample set G), whilst graphs B and D show counts from sample sets C and F (KC301 x *S.lividans* TK24 in sterile amended and non sterile soil).




A
M.S.D. (0.05)
Log p.f.u./g
0
1
2
3
4
5
0 1 2 5 15 30 60
Time (days)
B
M.S.D. (0.05)
0
1
2
3
4
5
6
0 1 2 5 15 30 60
Time (days)

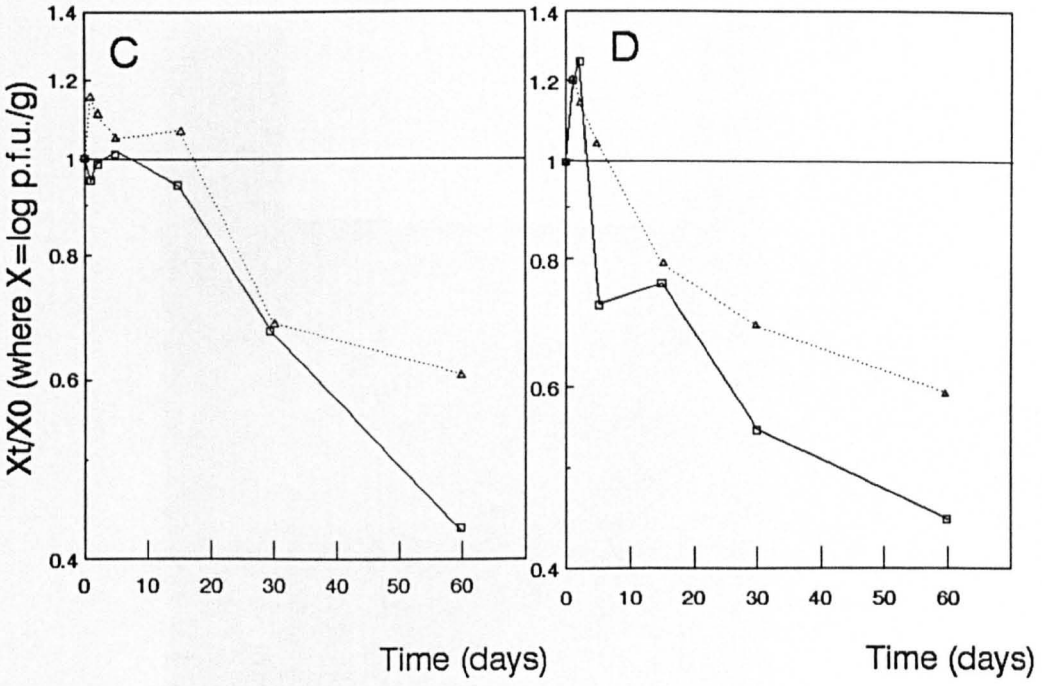
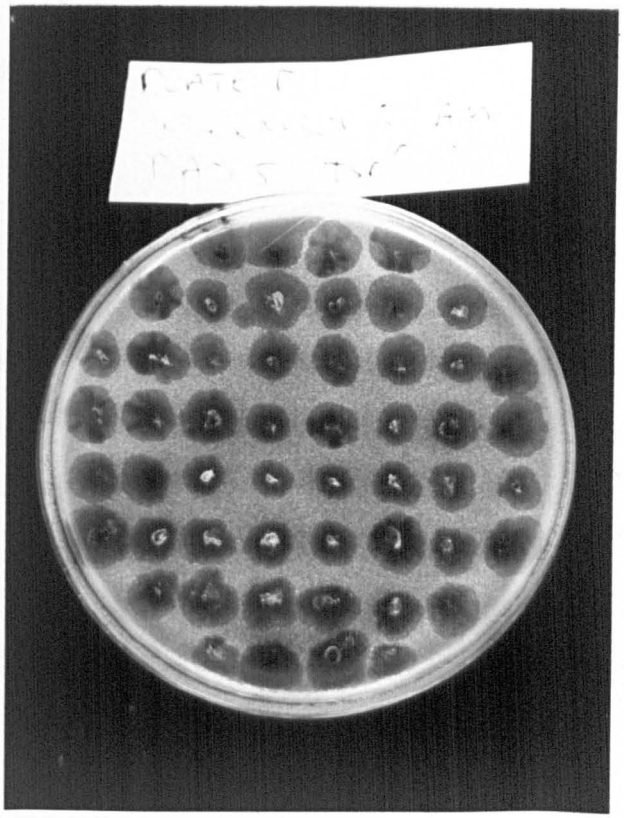
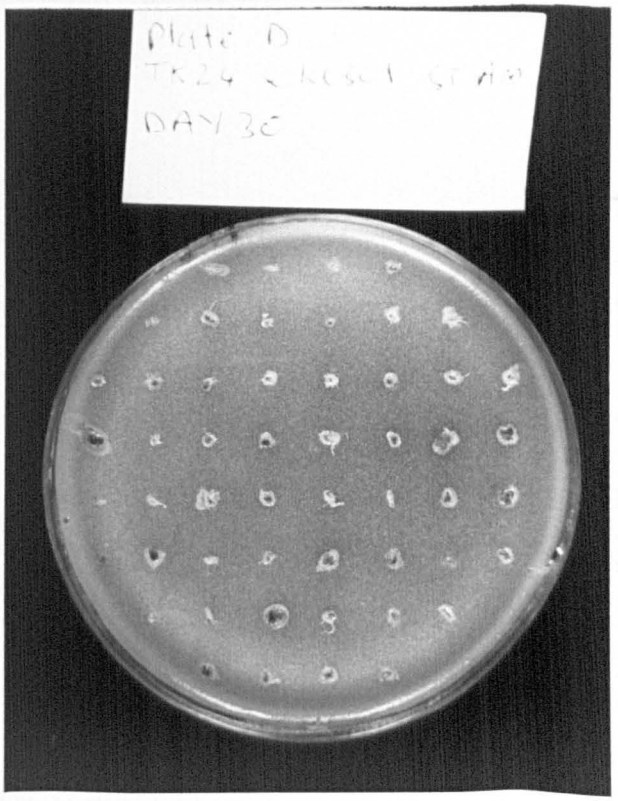

C
D
Xt/X0 (where X=log p.f.u./g)
0.4
0.6
0.8
1
1.2
1.4
0 10 20 30 40 50 60
Time (days)
Time (days)

Fig 13 (a). Confirmation of thiostrepton-resistant colonies isolated from sterile amended soil as lysogens.

Colonies isolated from isolation plates containing streptomycin (B) and thiostrepton and streptomycin (A) were picked onto 9 cm NCG plates overlaid with SNA seeded with *S.lividans* TK24. Those colonies lysogenic for KC301 can be visualised by the surrounding plaque.



A

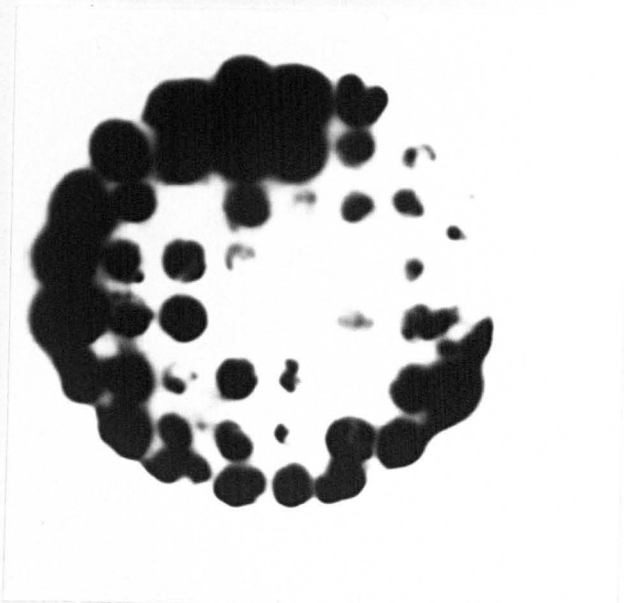


B

Fig 13 (b). Probing of thiostrepton-resistant colonies with ^{32}P-labelled KC301 DNA and *tsr gene*.

Colonies were picked from isolation plates and subjected to colony hybridisation as described in Chapter 2. The filters were probed with ^{32}P-labelled KC301 DNA (A) and with ^{32}P-labelled *tsr* gene (B) taken from pIJ673 (see Fig. 14) Hybridisation was only detected with thiostrepton-resistant colonies (see facing page), hybridisation was not detected with colonies isolated from plates containing no thiostrepton.

A



B

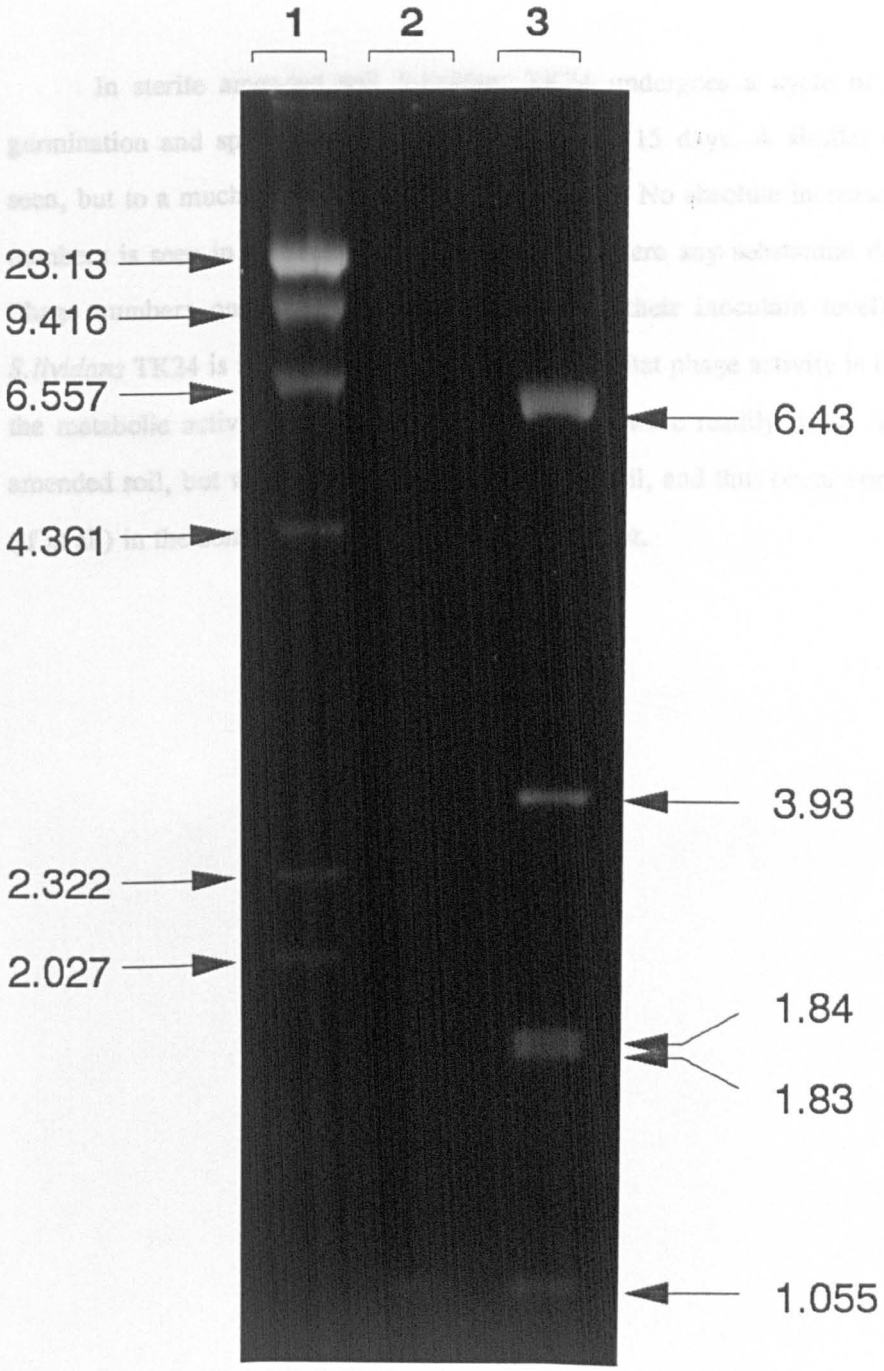
Fig. 14. Excision of *tsr* gene from pIJ673 using *Bcl*1.

pIJ673 is a multicopy plasmid derived from pIJ101 (T. Kieser, unpublished data), containing the genes *vph, aph* and *tsr*. It contains 5 *Bcl*1 sites, two of which fall inside *tsr* at 302 bp and 1357 bp into the gene (Hopwood *et al.*, 1985). Thus digestion with this enzyme yields a 1.055 kb fragment containing the bulk of the gene. This fragments was removed from the gel as described in Chapter 2 and used to probe for the *tsr* gene in thiostrepton-resistant colonies of *S.lividans* TK24 isolated from soil (see Fig. 13 [b]).

Track 1. Lambda *Hind*III

Track 2. Isolated *tsr* fragment

Track 3. pIJ673 *Bcl*1


1
2
3
23.13
9.416
6.557
4.361
2.322
2.027
6.43
3.93
1.84
1.83
1.055

5.4 Survival of *S.lividans* TK24 and KC301 in sterile amended and nonsterile soil: conclusions

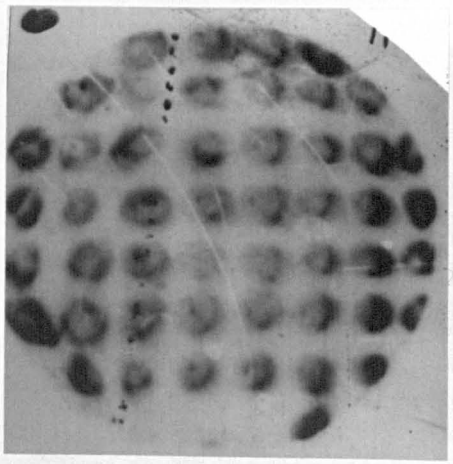
In sterile amended soil *S.lividans* TK24 undergoes a cycle of profuse germination and sporulation lasting approximately 15 days. A similar cycle is seen, but to a much lesser extent, in nonsterile soil. No absolute increase in host numbers is seen in nonsterile soil, but neither is there any substantial decrease. Phage numbers only show a raised titre (above their inoculum level), when *S.lividans* TK24 is in the mycelial state, indicating that phage activity is linked to the metabolic activity of its host. Again lysogens were readily found in sterile amended soil, but were not detected in nonsterile soil, and thus occur very rarely (if at all) in the conditions created by this experiment.

Fig. 15. Probing of plaques isolated from nonsterile soil with ^{32}P-labelled KC301 DNA.

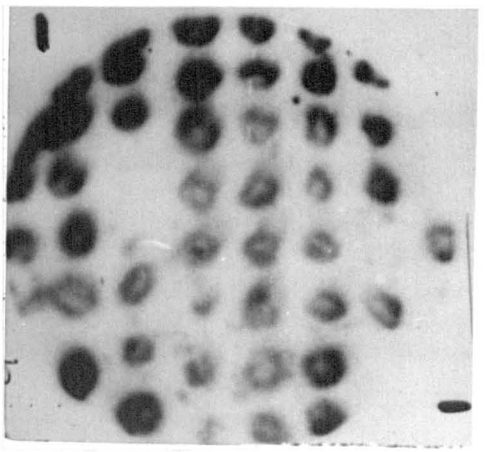
Forty nine plaques were picked from phage isolation plates from sample set F at each sample day and subjected to plaque hybridisation (see Section 2.18). The number of plaques lighting up could then be used to determine the proportion of KC301 within the free entire phage population infecting *S.lividans* TK24 (See Fig. 16).

Sample day	no. of plaques showing hybridisation
0	49
1	44
2	31
5	35
15	39
30	24
60	8

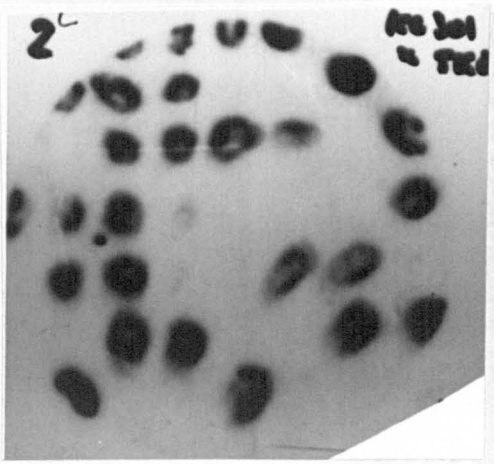
Day 0



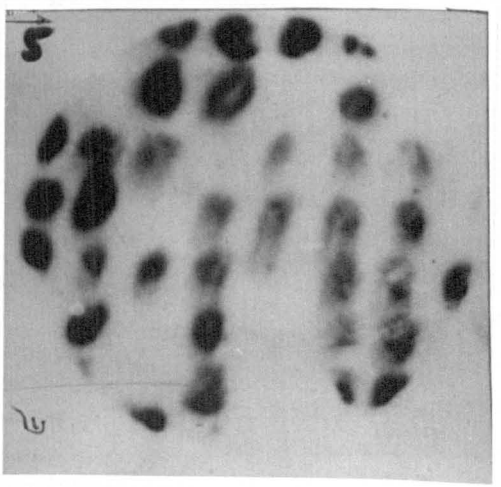
Day 1



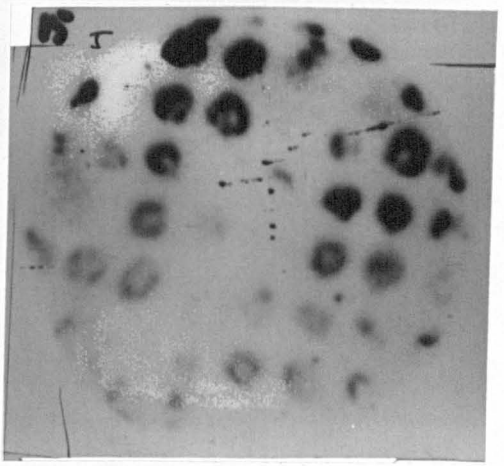
Day 2



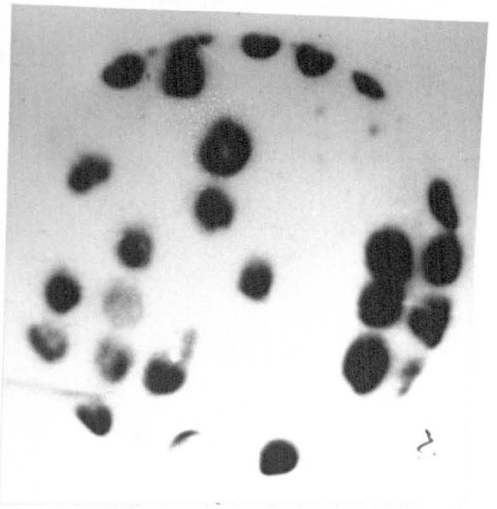
Day 5



Day 15



Day 30



Day 60

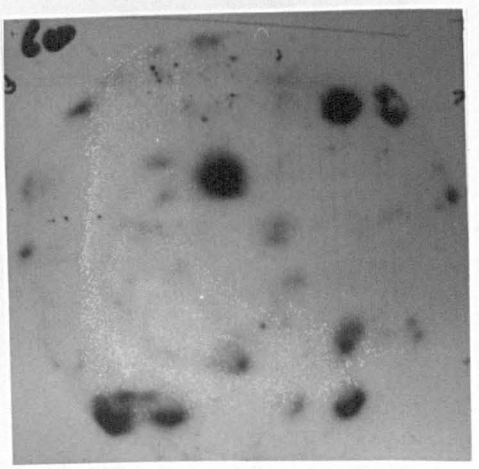
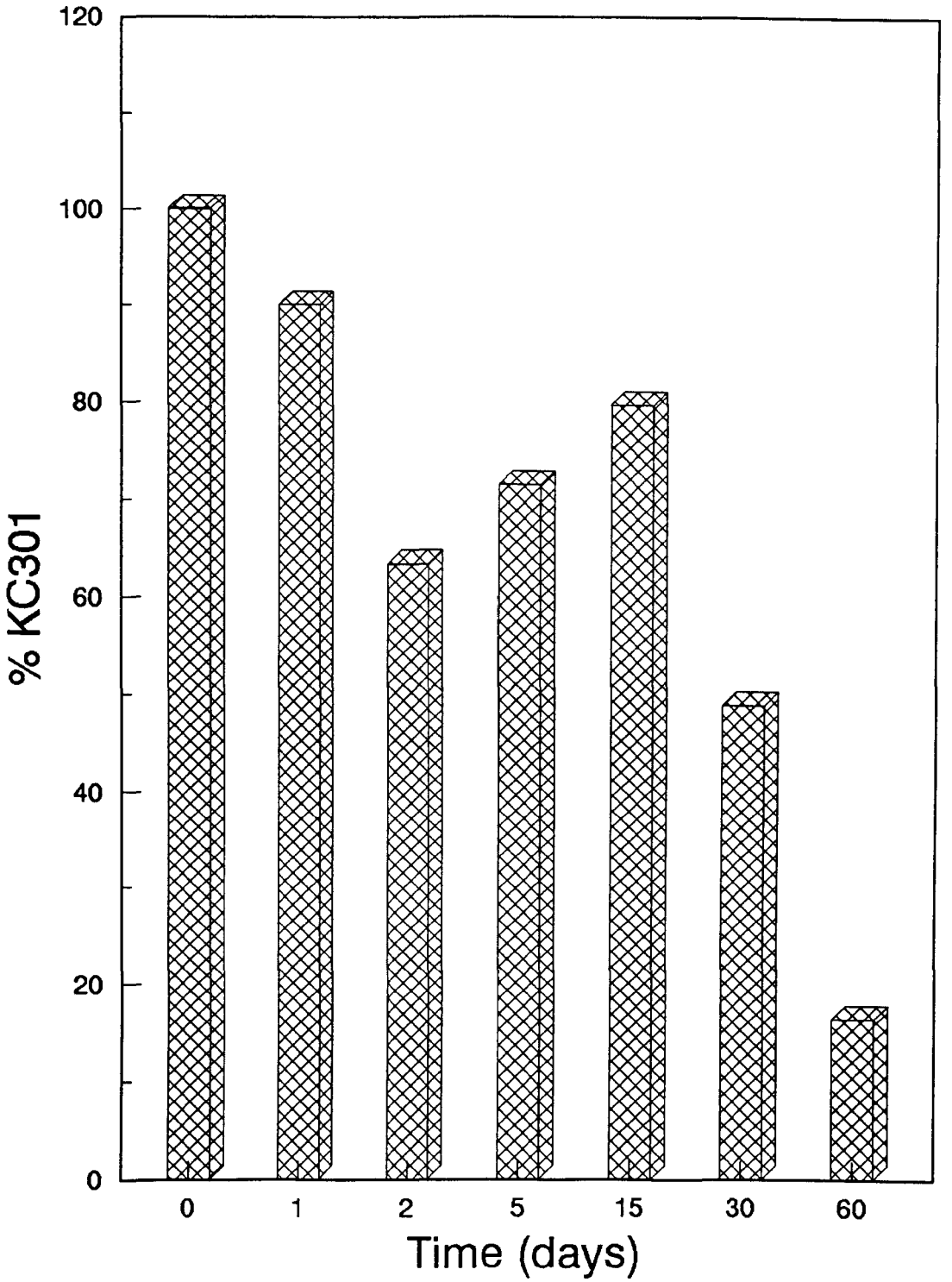


Fig. 16. **Proportion of plaques on *S.lividans* TK24 isolated from nonsterile soil caused by KC301.**

This graph represents the percentage of plaques showing hybridisation with KC301, as described in Fig. 15.


120
100
80
60
40
20
0
% KC301
0
1
2
5
15
30
60
Time (days)

Chapter 6

Growth and survival of *S.lividans* TK24 and KC301 in nonsterile amended soil

6.1 Survival of inoculants in nonsterile amended soil: aims

Previous work investigated the appearance of lysogens of KC301 in sterile amended and nonsterile soil (see Chapters 4 and 5). Lysogens of KC301 in *S.lividans* TK24 were readily detected in sterile amended soil, but could not be found in nonsterile soil. Plasmid transfer between streptomycetes is enhanced in nonsterile soil by the addition of nutrients (Bleakley & Crawford, 1989; Wellington *et al.*, 1990). It is likely that this is brought about by germination and increased growth of the organism and thus a greater likelihood of hyphal contact taking place. If this is true, it follows that phage infection should be encouraged for the same reasons. Wiggins and Alexander (1985) found there was a minimum bacterial density necessary for bacteriophage replication to take place. It was therefore hoped that amendment of the soil might bring about this minimum host density. In addition if the titre of the host in soil can affect the number of lysogens produced, it is likely that raising the number of phage in the inoculum would achieve the same result. To investigate this, two soil crosses in amended soils were set up, one at a multiplicity of infection of 10, and the other at 1000 (the host inoculum was kept constant).

6.2 Conditions for monitoring *S.lividans* TK24 and KC301 in nonsterile amended soil

Nonsterile soil was amended and weighed out into 20 g and 100 g microcosms as described in chapter 5 and Section 2.21, for destructive sampling, except that two sets of 10 g microcosms were also prepared (see Section 2.21). Phage and hosts were added to aliquots of SDW, that when added to the microcosms gave a final percentage moisture of 15% (see Table 12). In this experiment, the samples were not mixed, and water was allowed to penetrate the

soil through diffusion. From one set of 10 g microcosms, three 1 g aliquots were removed and extracted with ¼ strength Ringer's solution per sample set per sample day (see Section 2.22.1). The remaining soil was then used to calculate the percent moisture of the soil at each sample day. These 1 g samples were serially diluted and plated out in triplicate. One 100 g microcosm was extracted using the Chelex-100 method per sample set per sample day, serially diluted and plated out in triplicate for enumeration of hosts and lysogens (see Section 2.22.2). Phage were extracted as in Section 2.23.1. One 20 g microcosm was extracted per sample set per sample day for the enumeration of KC301. Three aliquots of the centrifugation supernatant (see Section 2.23.1) were filtered, serially diluted and assayed for KC301 in triplicate as described in Section 2.7. Hosts were enumerated on RASS (see Table 6), containing rifampicin, streptomycin, cycloheximide and nystatin (see Table 7). Lysogens were visualised by incorporating thiostrepton in the medium (see Table 7). The final set of 10 g microcosms were used for the extraction of soil DNA by the SDS lysis procedure (see Section 2.24.1). These extracts were run on an agarose gel, southern blotted and probed with ^{32}P-labelled KC301 DNA (see Chapter 2).

Table 12. Inocula

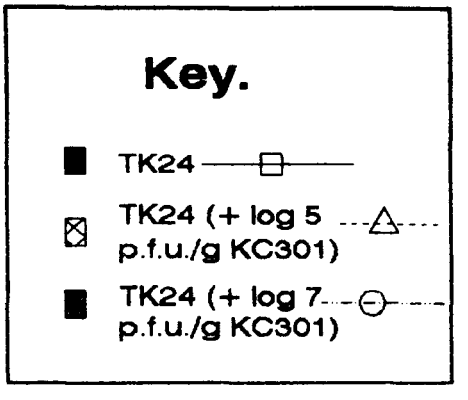
Sample set	*S.lividans* TK24 (c.f.u./g)	KC301 (p.f.u./g)
A	1.06×10^4	-
B	-	1.02×10^5
C	-	1.02×10^7
D	1.06×10^4	1.02×10^5
E	1.06×10^4	1.02×10^7
F	-	-

6.3 Survival of phage and host in nonsterile amended soil

Graphs of the survival and recovery of *S.lividans* TK24 are given in Fig. 17. Both sets of extractions indicated an initial drop in the titre of *S.lividans* TK24 in the soil, although not to the same extent as was found using the spore extraction method with sterile amended soil (see Chapter 5). The drop in recoverable propagules using ¼ strength Ringer's solution was probably due to mycelial death through the action of the fast shaking used during this procedure (Skinner, 1951). Later in the experiment (days 5-15) numbers increased (graphs A and B), possibly because of growth and the greater predominance of spores in the population. Again, there was a drop in recovery using the Chelex method (graphs C and D). This was due to germination of the spores during days 1-2 (the drop in titre was greater with this method than during the corresponding period with the Ringer's extraction method), numbers then rose due to sporulation of the bacteria. For the spore extraction, counts were consistently lower than those using Ringers; this is best illustrated at day 0 where the percentage recovery for the spore extraction method was around 20% of the inoculum, whereas that for ¼ strength Ringers was greater than 100%. The percentage recovery for the former is generally in the order of 20-30% , however as the final extract was taken from 100g soil, a much larger total number of streptomycete propagules was actually isolated than using the ¼ strength Ringers method (1 g sample size). The high percentage recovery achieved in the Ringer's extraction was probably due to spore chains and mycelial fragments in the inoculum (counted as one c.f.u.) being broken up by the vigorous shaking used in this method. This highlights the problems associated with the use of c.f.u. as a measure of growth for a mycelial microorganism.

Fig. 17. **Growth and survival of *S.lividans* TK24 in nonsterile amended soil.**

Graphs A and C represent logged counts at each day, whilst graphs B and D show scaled counts. A and B are derived from ¼ strength Ringers solution extractions and graphs C and D from spore extractions.



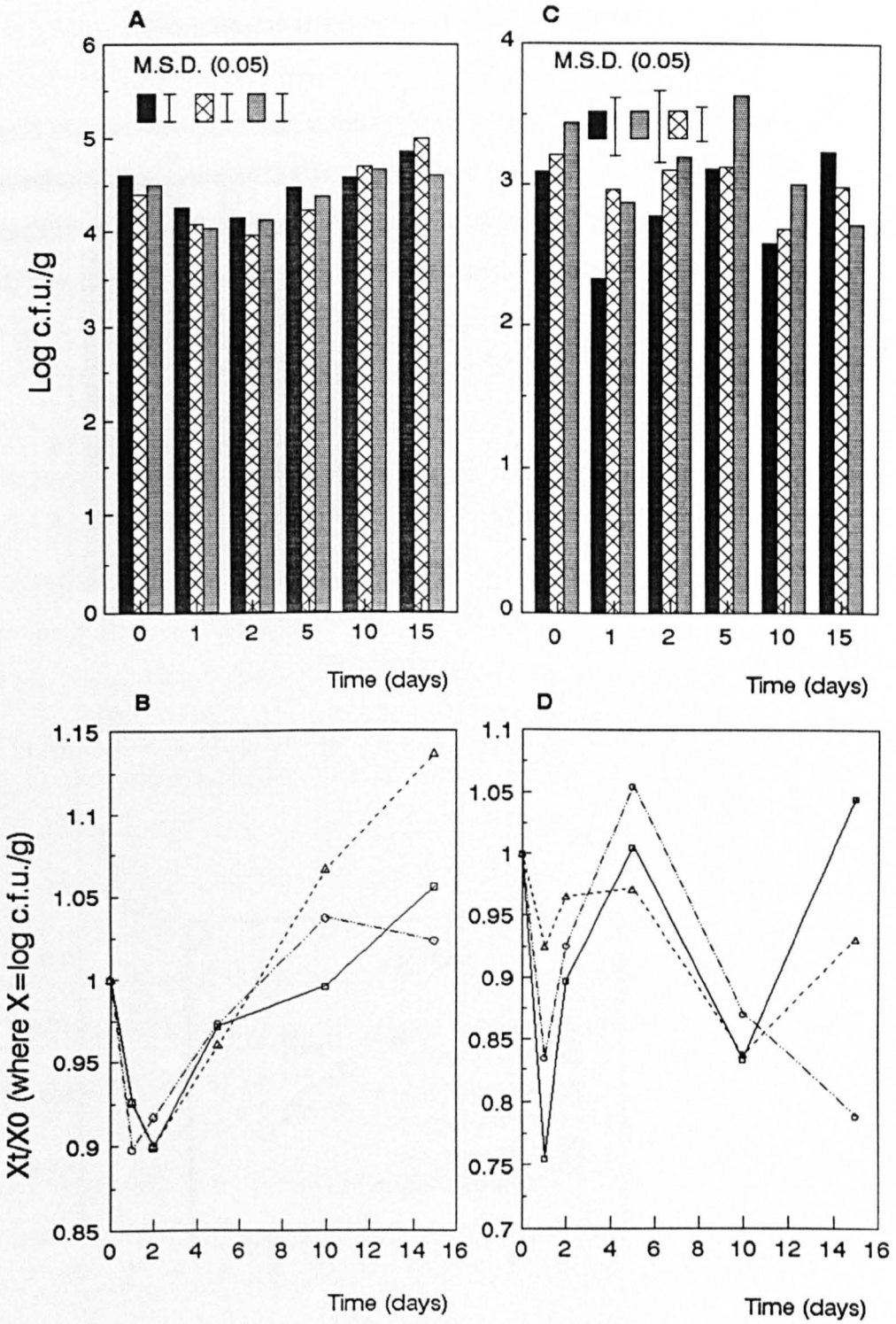
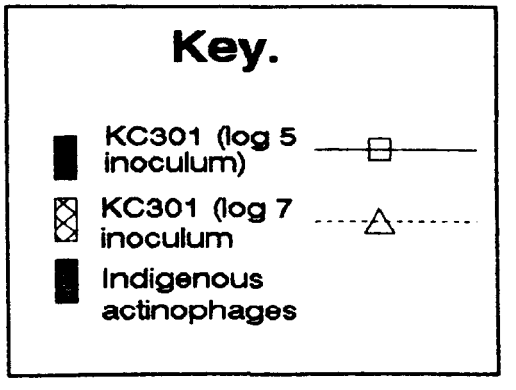
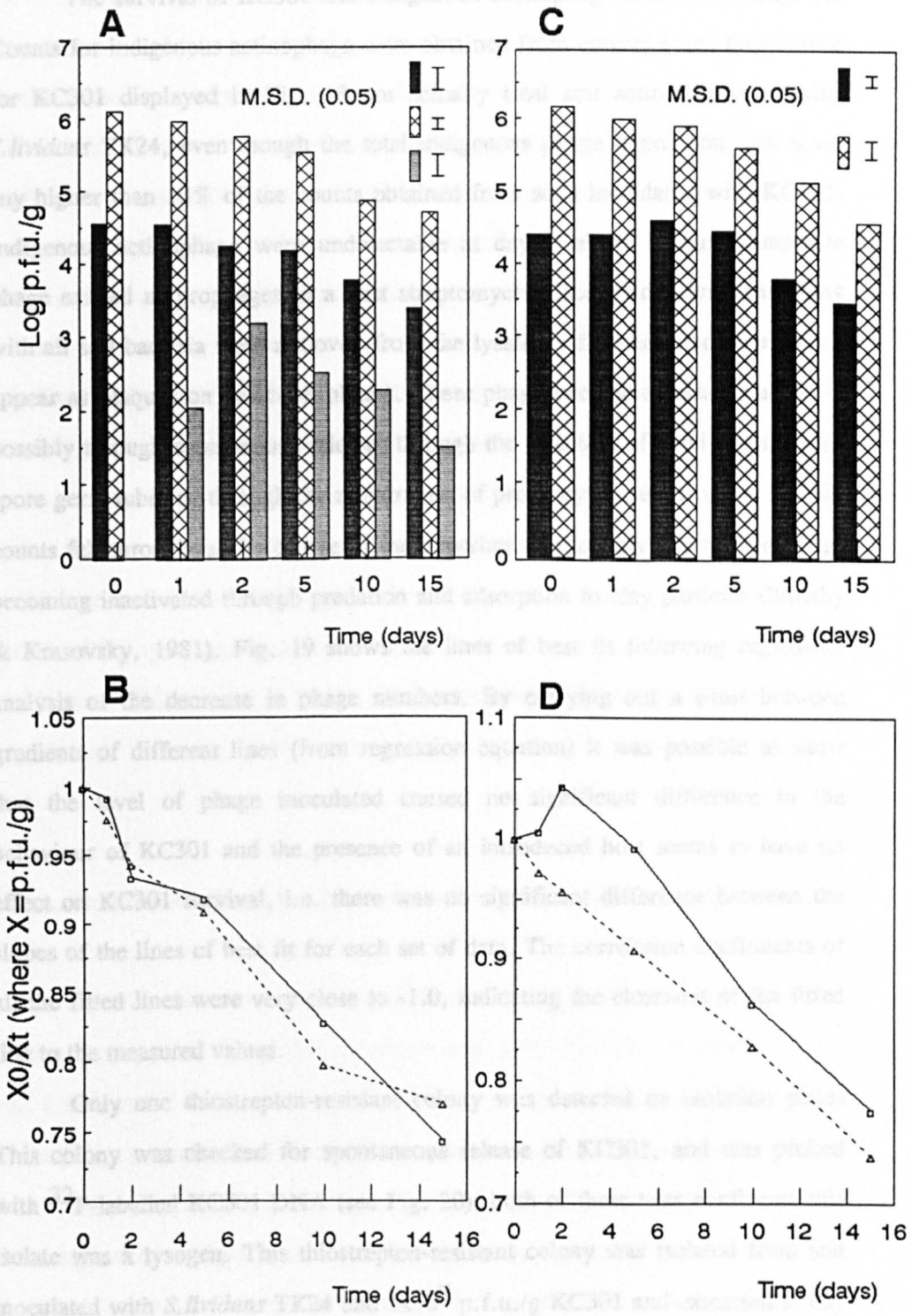

A
M.S.D. (0.05)
Log c.f.u./g
Time (days)
C
M.S.D. (0.05)
Time (days)
B
Xt/X0 (where X=log c.f.u./g)
Time (days)
D
Time (days)

Fig. 18. **Survival of KC301 in nonsterile amended soil.**

Graphs A and C show logged counts at each day, whilst graphs B and D show scaled counts. Graphs A and B represent KC301 survival in the absence of an inoculated host (Sample sets B, C and F), whilst KC301 counts in C and D were taken from soils co-inoculated with *S.lividans* TK24 (Sample sets D and E).




A
C
M.S.D. (0.05)
M.S.D. (0.05)
Log p.f.u./g
Time (days)
Time (days)
B
D
X0/Xt (where X=p.f.u./g)
Time (days)
Time (days)

The survival of KC301 and indigenous actinophage is shown in Fig. 18. Counts for indigenous actinophage were obtained from control soils, thus counts for KC301 displayed in Fig. 18 are actually total soil actinophage infecting *S.lividans* TK24, even though the total indigenous phage population was never any higher than 10% of the counts obtained from soils inoculated with KC301. Indigenous actinophage were undetectable at day 0 either because temperate phage existed as prophages in a host streptomycete (indigenous lysogens along with all soil bacteria were removed from the lysate by filtration and thus do not appear as plaques on detection plates). These phage became detectable at day 1 possibly through spontaneous release, through the infection of newly germinated spore germ tubes or through the rehydration of pseudolysogenic mycelia. KC301 counts fell throughout the course of the experiment, probably due to free phage becoming inactivated through predation and adsorption to clay particles (Stotzky & Krasovsky, 1981). Fig. 19 shows the lines of best fit following regression analysis of the decrease in phage numbers. By carrying out a t-test between gradients of different lines (from regression equation) it was possible to show that the level of phage inoculated caused no significant difference in the behaviour of KC301 and the presence of an introduced host seems to have no effect on KC301 survival, i.e. there was no significant difference between the slopes of the lines of best fit for each set of data. The correlation coefficients of all the fitted lines were very close to -1.0, indicating the closeness of the fitted line to the measured values.

Only one thiostrepton-resistant colony was detected on isolation plates This colony was checked for spontaneous release of KC301, and was probed with ^{32}P-labelled KC301 DNA (see Fig. 20); both of these tests confirmed this isolate was a lysogen. This thiostrepton-resistant colony was isolated from soil inoculated with *S.lividans* TK24 and $1x10^7$ p.f.u./g KC301 and extracted at day 15 using the spore extraction method, when host counts were at their highest. No thiostrepton-resistant colonies were isolated using the ¼ strength Ringers

method. Obviously it is impossible to draw too many conclusions concerning the detection of a single lysogen from soil, but it does illustrate the need for a certain minimum phage density to produce detectable lysogeny. The colony was found using the spore extraction method. This emphasises the enhanced detection limit for rare genotypes by use of this method. A problem that became apparent upon amendment of the soil was that the fungal population proliferated greatly, and fungal spores were then concentrated in the final extract of the Chelex-100 method. When this concentrate was plated out, the high fungal population interfered with the identification of streptomycetes at low dilutions, even with the incorporation of cycloheximide and nystatin in the agar.

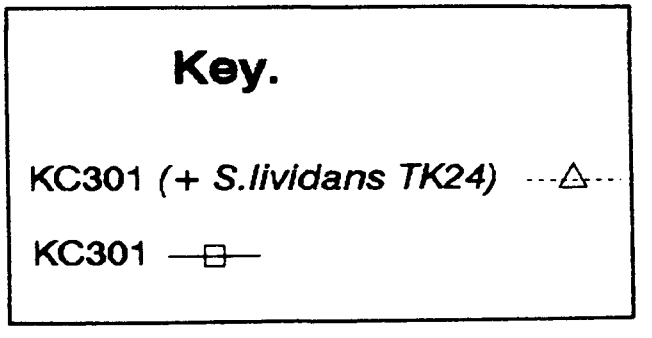
No hybridisation was detected between soil DNA extracts and a KC301 probe after Southern blotting, although hybridisation was detected with the positive control (data not shown). This was not surprising as the detection limit for the SDS lysis procedure with Southern blotting is *ca.* 10^7 p.f.u./g, and numbers of KC301 did not rise above this level during the course of the experiment.

6.4 Survival of phage and host in nonsterile amended soil: conclusions

It seems that streptomycete growth does occur in this nonsterile amended system, to a greater extent than in unamended nonsterile soil (i.e. in this system soil *S.lividans* TK24 titre increased), however the germination sporulation cycle is much less extensive than in sterile amended soil. Phage survival was similar to that in other experiments (see Chapters 4 and 5), in that numbers declined with time; regression analysis demonstrated that the presence of an inoculated host or the phage inoculum titre had no effect on the rate of decline, on a log scale, of KC301 numbers.

Fig. 19. **Regression analysis of KC301 survival in nonsterile amended soil.**

Linear regression analysis on log p.f.u./g KC301 counts for each relevant sample set was carried out using the MINITAB statistical software package, generating an equation that produced the lines displayed in the Figure. From the regression equations, a Student's t-test was carried out to determine if there was any significant differences between the slopes of any two lines (hence the rate of die off of KC301 *in situ*).



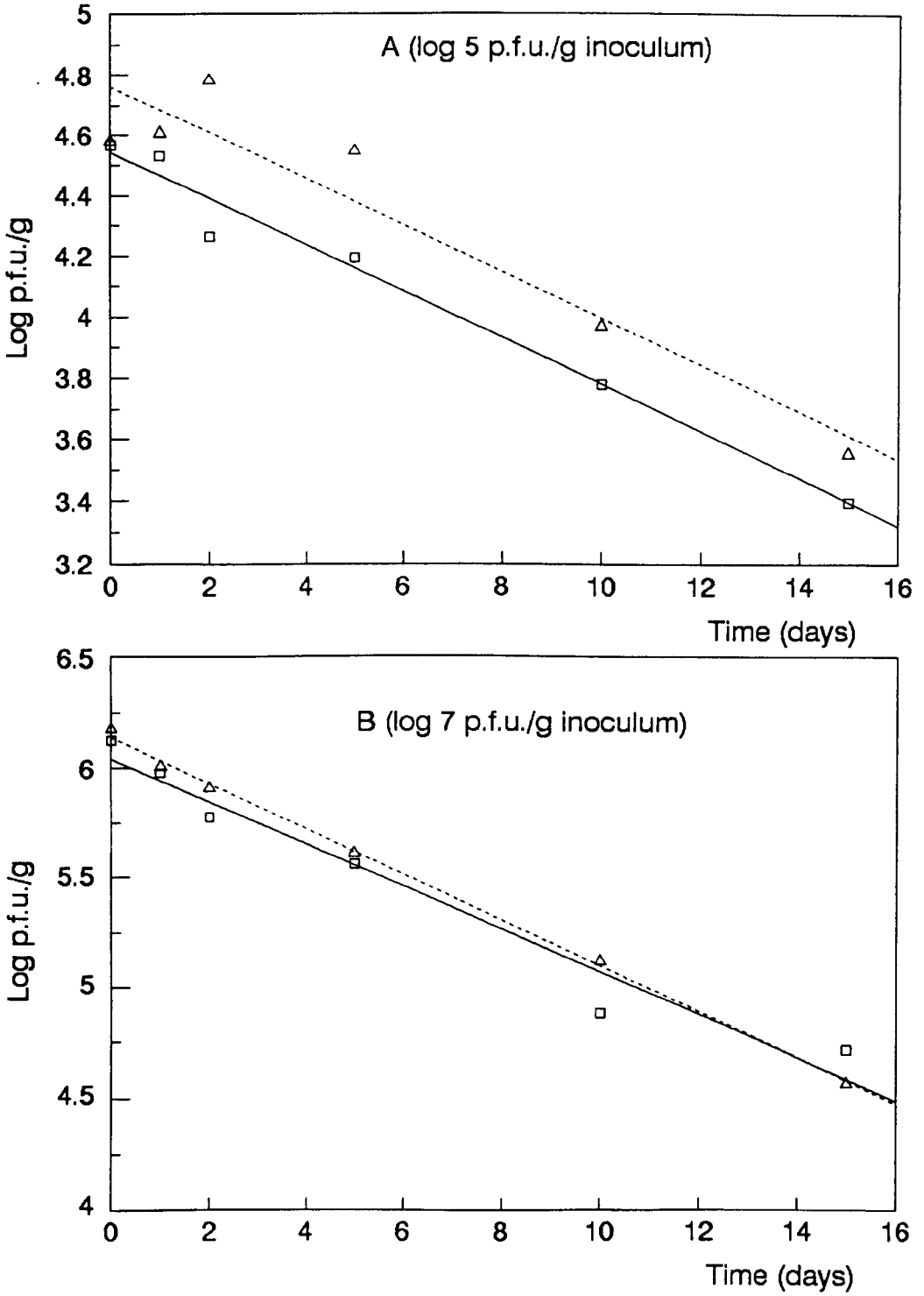
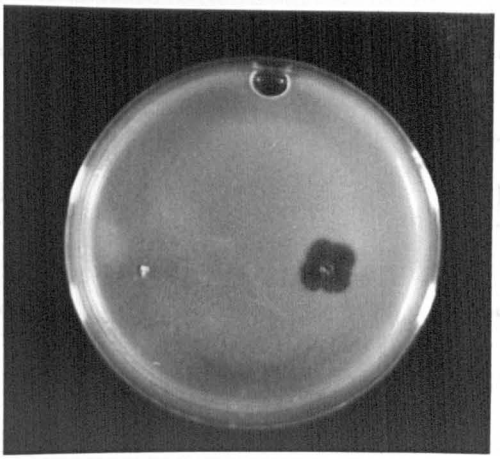

A (log 5 p.f.u./g inoculum)
Log p.f.u./g
5
4.8
4.6
4.4
4.2
4
3.8
3.6
3.4
3.2
0
2
4
6
8
10
12
14
16
Time (days)
B (log 7 p.f.u./g inoculum)
Log p.f.u./g
6.5
6
5.5
5
4.5
4
0
2
4
6
8
10
12
14
16
Time (days)

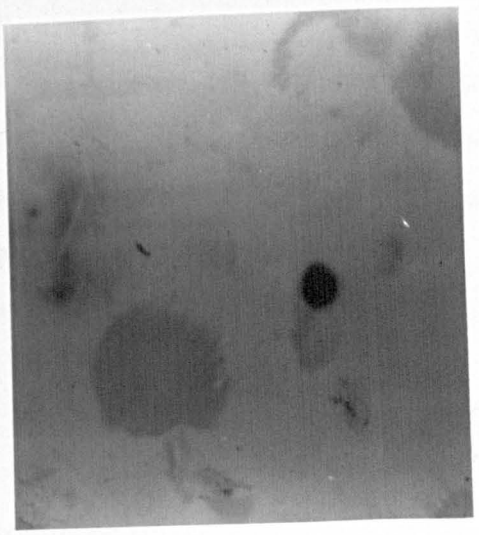
Fig. 20. **Confirmation of a thiostrepton-resistant colony isolated from nonsterile amended soil as a lysogen.**

The one thiostrepton-resistant colony isolated during the course of this experiment was picked onto SNA seeded with *S.lividans* TK24 (A, right) along with a *S.lividans* TK24 negative control (A, left). This colony was also probed with KC301 DNA (B, right), as was the negative control (B, left).

A



B



Chapter 7.

Survival of *S.lividans* TK24 and KC301 in a fed-batch nonsterile amended soil system

7.1 Fed-batch soil system: aims

Previous work (see Chapters 5 and 6) had shown that streptomycete growth in soil followed a cyclical pattern of germination and sporulation after inoculation into sterile amended, and to a lesser extent in nonsterile soil. In a natural soil, microbial growth would not take place in such a simple manner; nutrients, moisture, inorganic compounds or any growth limiting substance would constantly be added to the environment. Any of these events could initiate a new round of growth. It was decided to construct an experiment to attempt to model such a system, i.e. to determine whether further cycles of germination could be induced after inoculation, and whether such germination periods were accompanied by a corresponding period of phage activity. In sterile amended soil in previous experiments, it seemed that germination reached a peak two days after inoculation (as did phage numbers) and sporulation after fifteen days. Thus, by adding fresh soil and nutrients at fifteen days it was hoped that another round of germination could be stimulated, coupled with another round of phage propagation. In effect the soil would be treated as a fed-batch system, rather than a less realistic batch culture as had been done previously. It was intended to sample the microcosms two days and fifteen days after inoculation. Fifteen days later, and every subsequent fifteen days, half of the soil would be replaced with fresh nonsterile amended soil, and the microcosms resampled. Microcosms were also sampled two days after feeding with fresh soil, in an attempt to detect maximal germination.

7.2 Monitoring of inoculants in a fed-batch system *in situ*

Nonsterile soil was amended and weighed out into 200g microcosms as described in Section 2.21. Four sets of nine microcosms were prepared (see

Table 13). Phage and host were added to aliquots of SDW that when added to soil gave a final moisture content of 15% (see Table 13). All microcosms were inoculated at the same time. At days 0, 2, 15, 17, 30, 32, 45, 47 and 60 one microcosm (from each sample set) was destructively sampled. However, after fifteen days (and every subsequent fifteen days) the remaining microcosms were mixed and 50% of their mass replaced with fresh nonsterile amended soil. This newly constituted soil was then rewetted to 15% and thoroughly mixed. Over a fifteen day period the percentage moisture would drop from *ca.* 15% to *ca.* 13%. At each sample day, three 1g aliquots were removed and extracted with ¼ strength Ringer's solution per sample set (see Section 2.22.1). Also 100g of soil was extracted using the Chelex-100 method per sample set per sample day, serially diluted and plated out in triplicate for enumeration of hosts and lysogens (see Section 2.22.2). Phage were extracted as in Section 2.23.1. 20g were extracted per sample set per sample day for the enumeration of KC301. Three aliquots of the centrifugation supernatant (see Section 2.23.1) were filtered, serially diluted and assayed for KC301 in triplicate as described in Section 2.7. The remaining soil was then used to calculate the % moisture of the soil at each sample day. Hosts were enumerated on RASS (see Table 6), containing rifampicin, streptomycin, cycloheximide and nystatin (see Table 7). Lysogens were visualised by incorporating thiostrepton in the medium (see Table 7). Indigenous streptomycetes were isolated on RASS containing only antifungal agents and identified by their ditinctive colony morphology.

Table 13. Inocula

Sample set	*S.lividans* TK24 (c.f.u./g)	KC301 (p.f.u./g)
A	1.05×10^5	-
B	-	1.04×10^5
C	1.05×10^5	1.04×10^5
D	-	-

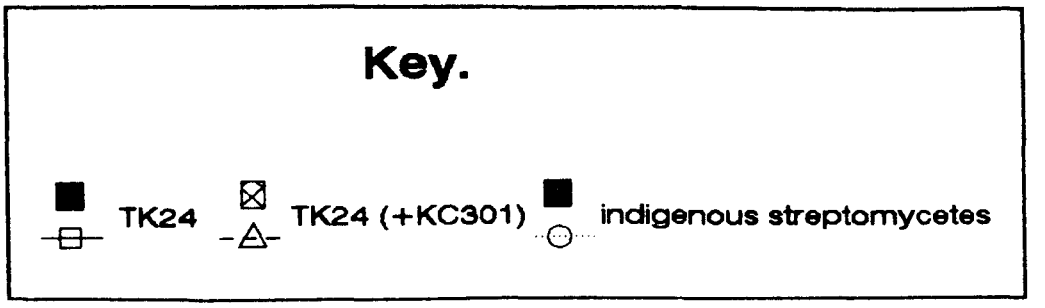
7.3 Survival of *S.lividans* TK24 in a fed-batch soil system

The survival of *S.lividans* TK24 in this fed-batch system is shown in Fig. 21. It can be seen that growth only occurred during the first fifteen day period of the experiment. *S.lividans* TK24 numbers reached a peak at day 15, before declining in a stepwise manner for the remainder of the experiment. This indicates that *S.lividans* TK24 was diluted out of the system, at each feeding of the soil. Thus, it would seem that the growth of the organism between feeding dates was not sufficient to make up for the loss when half of the soil was removed. Indigenous streptomycetes were not diluted out, this could be due to their re-inoculation every time the microcosms were fed with fresh soil. Although growth was not detected after day 15 in *S.lividans* TK24, it is possible that indigenous streptomycetes germinated and sporulated during the course of the experiment. Unfortunately, indigenous streptomycetes were only counted by means of the ¼ strength Ringer's solution method. *S.lividans* TK24 was not detectable using the ¼ strength Ringer's solution at day 60. Counts using the spore extraction method at day 60 indicated that this strain's numbers had reached the detection limit of this method (*ca.* 10^2 c.f.u./g). This indicates the usefulness of the spore extraction method in detecting low numbers of inoculants in soil. One thiostrepton-resistant *S.lividans* TK24 colony was detected at day 15, it was confirmed as a lysogen by screening for spontaneous phage release and by probing with P^{32} labelled KC301 DNA (see Fig. 23). Again, problems occurred with fungal contamination at low dilutions of the final suspension of the spore extraction method, hindering the screening of these plates for lysogens. However, it is important to point out that this colony was detected at the peak of host numbers. No streptomycetes were isolated from control soils that were resistant to streptomycin, rifampicin and thiostrepton.

The survival of KC301 in this system is shown in Fig. 22. Phage numbers declined throughout the course of the experiment, until at day 30 counts

Fig 21. **Growth and survival of *S.lividans* TK24 in fed-batch nonsterile amended soil.**

Graphs A and B give the logged counts at each day, whilst graphs C and D give the scaled counts. Graphs A and C represent total numbers of *S.lividans* TK24 and indigenous streptomycetes, whilst graphs B and D represent spore counts.



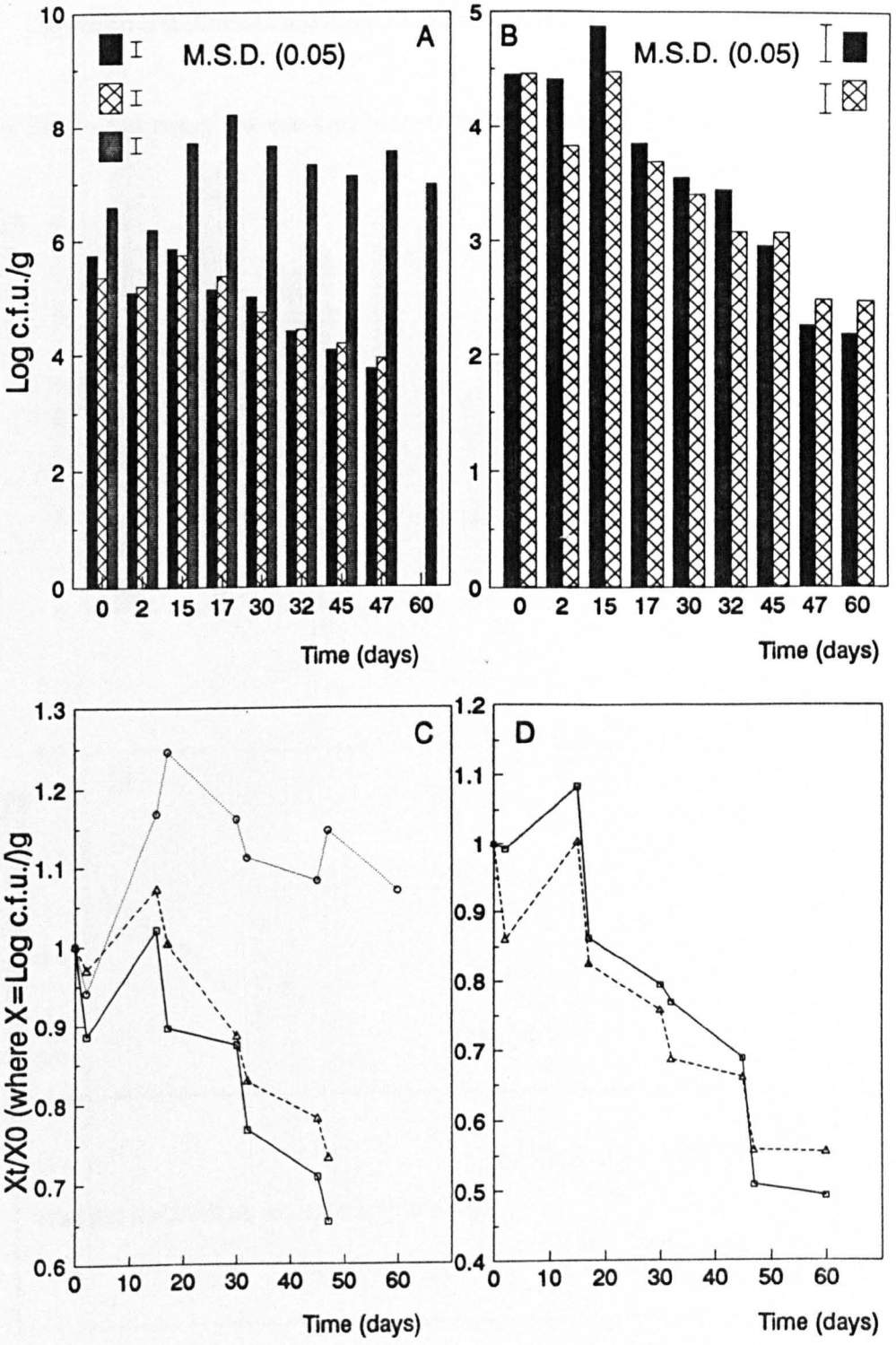
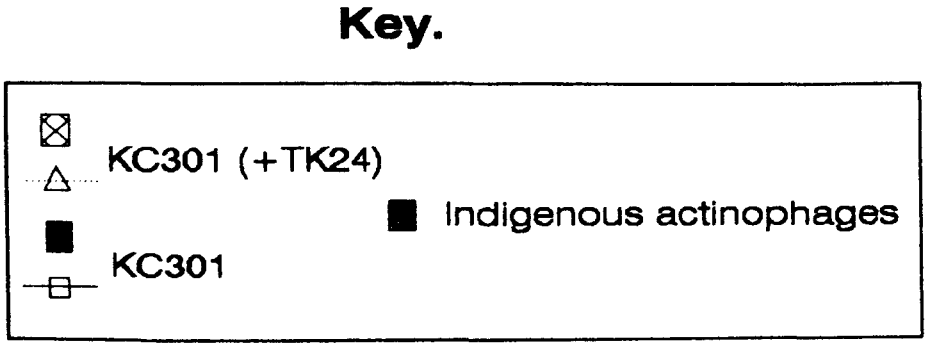
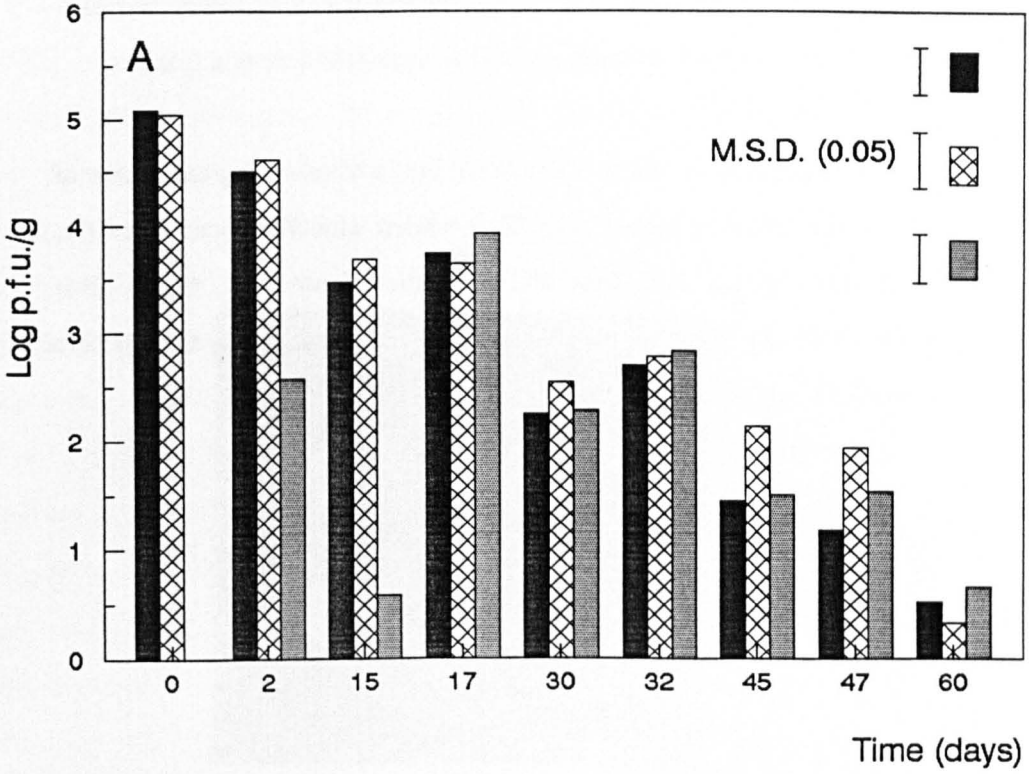

A
M.S.D. (0.05)
Log c.f.u./g
Time (days)
B
M.S.D. (0.05)
Time (days)
C
Xt/X0 (where X=Log c.f.u./)g
Time (days)
D
Time (days)

Fig. 22. **Survival of KC301 in fed-batch nonsterile amended soil.**

Graph A gives the logged count at each day and graph B gives the scaled count at each day.




A
M.S.D. (0.05)
Log p.f.u./g
0
1
2
3
4
5
6
0
2
15
17
30
32
45
47
60
Time (days)

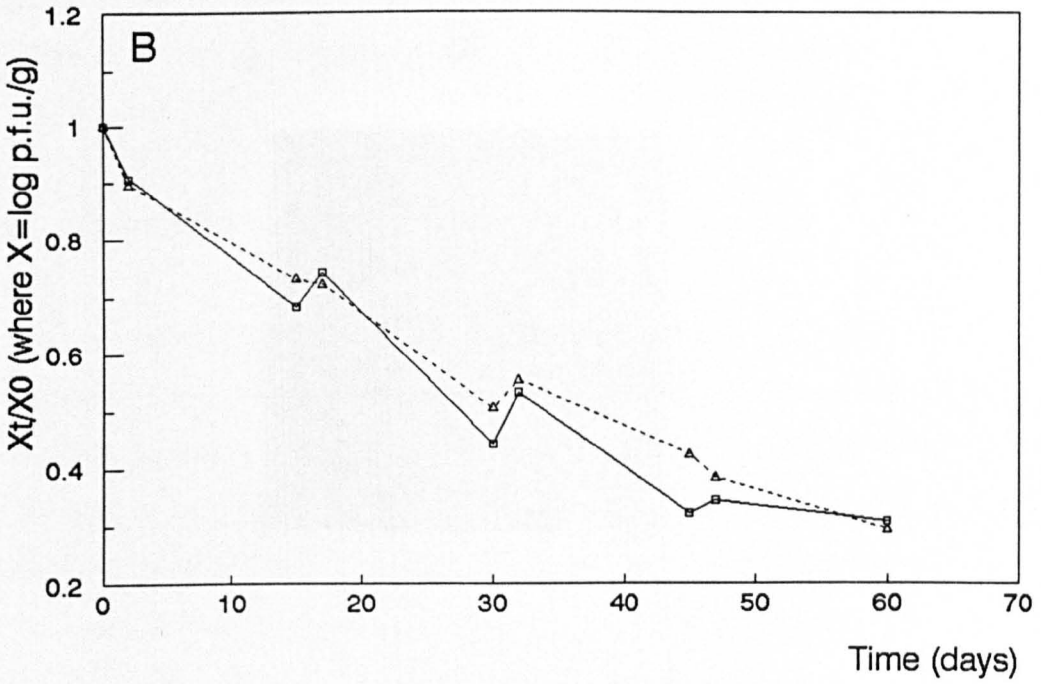
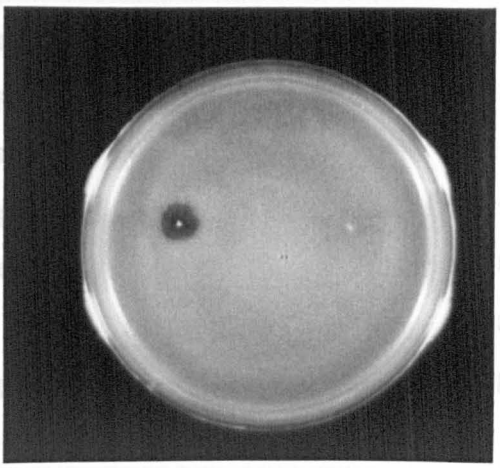

B
Xt/X0 (where X=log p.f.u./g)
0.2
0.4
0.6
0.8
1
1.2
0
10
20
30
40
50
60
70
Time (days)

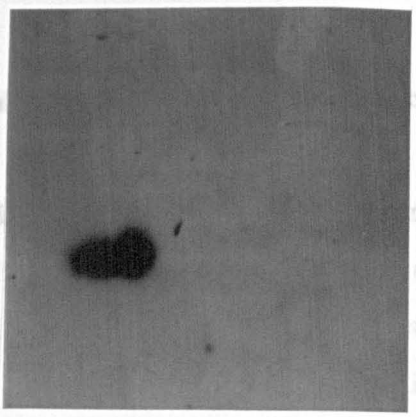
Fig. 23. **Confirmation of a thiostrepton-resistant colony isolated from fed-batch nonsterile amended soil as a lysogen.**

The one thiostrepton-resistant colony isolated during the course of this experiment was picked onto SNA seeded with *S.lividans* TK24 (A, left) along with a *S.lividans* TK24 negative control (A, right). This colony was also probed with KC301 DNA (B, left), as was the negative control (B, right).

A



B



from soils containing KC301 were indistinguishable from those of indigenous phage in control soils; again indigenous actinophage appeared at day 1, before declining during the course of the experiment. It is interesting that each time the soil was fed, phage counts increased as if the virus was propagating in newly germinated mycelia. However, the increases at these points were not significant by the statistical test used in this study, and thus it is impossible to draw too many conclusions about the state of the host during these periods. It is intriguing however, that phage numbers showed periodic increases in titre; this is, after all how a predator-parasite should behave *in situ*; indeed a mathematical model developed for streptomycete-phage interactions (Manchester, 1986; Williams *et al.*, 1986) predicted that such a pattern would occur in natural soil. Although, the establishment of a theoretical dynamic predator-prey relationship was dependent on the parameter values chosen for the modelling equation, infection efficiency had to be reduced two orders of magnitude from that actually measured in soil, in order for a prolonged period of phage-host coexistence to be established, if measured values were used both phage and host died out.

7.4 Behaviour of phage and host in fed-batch soil: conclusions

Growth of the inoculated host only occurred during the first fifteen days of the experiment; *S.lividans* TK24 was then diluted out of the system, although indigenous streptomycete numbers remained high, presumably through continued re-inoculation in the fresh soil. Increases in phage titres immediately after amendment gave some indication that limited germination was taking place. It is not clear whether this increase in phage titre was due to the action of nutrients or moisture. These cycles were perhaps analogous to the cyclical predator-prey relationship predicted by Manchester (1986), however, increases in phage titre during such periods were found not to be significant by the statistical test used in

this study.

Chapter 8.

Growth and survival of *S.lividans* TK24::KC301 lysogens in sterile amended and nonsterile soil.

8.1 Comparison of host strain and lysogen survival *in situ*: aims

Previous work had examined the fate of free phage in combination with a streptomycete host in soil. There is a great deal of information in the literature about the relative fitness of genetically-engineered, plasmid-bearing and phage bearing organisms with respect to their parent strains in the environment (see Section 1.3.3). It was therefore decided to investigate the fitness of a lysogenic strain of *S.lividans* TK24 in soil, and compare its survival to that of the parent organism. This was done by examining the fate of *S.lividans* TK24 and *S.lividans* TK24::KC301 in sterile amended and nonsterile soil. Lysogens of ΦC31 are capable of spontaneously releasing phage particles *in vitro* (Lomovskaya *et al.*, 1972). This ability would provide a means whereby the phage could replenish a depleted population after a period of environmental adversity; thus the capacity of *S.lividans* TK24::KC301 to spontaneously release phage *in situ* was also assessed.

8.2 Monitoring of lysogenic and non-lysogenic streptomycete populations in soil

Soil was amended and weighed out into 20g and 100g microcosms as described in Chapter 5 and Section 2.21; except that two sets of 10g microcosms were also used (see Section 2.21). Lysogens were prepared as described in Section 2.8.1. Host was added to aliquots of SDW that, when added to the soil, gave a final percentage moisture of 15% (see Table 14). In this experiment, the samples were not mixed, and water was allowed to penetrate the soil through diffusion. From one set of 10g microcosms, three 1 g aliquots were removed and extracted with ¼ strength Ringer's solution per sample set per sample day (see Section 2.22.1). The remaining soil was then used to calculate the % moisture of

the soil at each sample day. These 1 g samples were serially diluted and plated out in triplicate. One 100 g microcosm was extracted using the Chelex-100 method per sample set per sample day, serially diluted and plated out in triplicate for enumeration of hosts and lysogens (see Section 2.22.2). Phage were extracted as in Section 2.23.1. One 20 g microcosm was extracted per sample set per sample day for the enumeration of KC301. Three aliquots of the centrifugation supernatant (see Section 2.23.1) were filtered, serially diluted and assayed for KC301 in triplicate as described in Section 2.7. The final set of 10g microcosms were used for the extraction of total soil DNA using the bead-beating method of Cresswell *et al.* (1991) (see Section 2.24.2) rather than the SDS lysis procedure (see Section 2.24.1) used in Chapter 3. *S.lividans* TK24 were enumerated on RASS (see Table 6), containing rifampicin, streptomycin, cycloheximide and nystatin (see Table 7). *S.lividans* TK24::KC301 inoculants were counted by incorporating thiostrepton in the medium (see Table 7).

Table 14. **Inocula**

Sample set	[1]Conditions	*S.lividans* TK24 (c.f.u./g)	*S.lividans* TK24::KC301 (p.f.u./g)
A	St. Am.	$2.2x10^4$	-
B	NS.	$2.2x10^4$	-
C	St. Am..	-	$2.2x10^4$
D	NS.	-	$2.2x10^4$
E	NS.	-	-

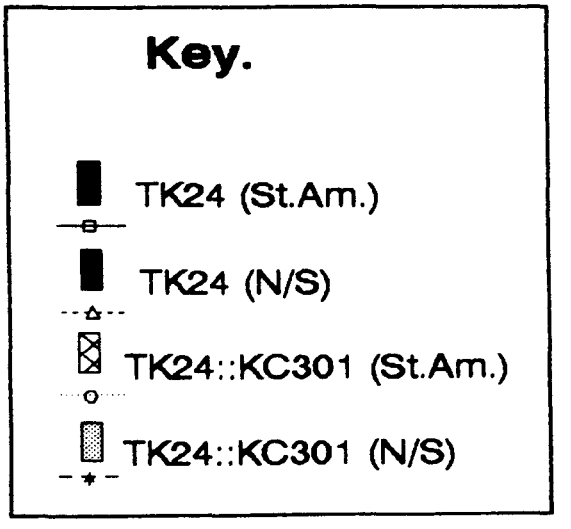
[1]St. Am., Sterile amended soil; NS., nonsterile soil.

8.3 Survival of *S.lividans* TK24::KC301 and *S.lividans* TK24 *in situ*

The growth and survival of *S.lividans* TK24 and *S.lividans* TK24::KC301 in sterile amended and nonsterile soil is shown in Fig. 24. In general terms the host performed as predicted, i.e. there was a pronounced germination/sporulation cycle of both *S.lividans* TK24 and *S.lividans* TK24::KC301 in sterile amended soil (see graphs C and D), whilst numbers of *S.lividans* TK24 spores did not increase or decrease significantly during the course of the experiment in nonsterile soil (see Chapters 5 and 6). Interestingly, lysogens grew and sporulated just as well as the parent organism in sterile amended soil. *S.lividans* TK24::KC301 numbers (both of total propagules and spores) did decrease rapidly in nonsterile soil, the rate of decline becoming less after day 5. In nonsterile soil *S.lividans* TK24::KC301 numbers declined, indicating that the carriage of the phage in a temperate state was somehow detrimental to the host's survival. This is presumably due to phage induction and lysis during a brief period of germination of the organism. Numbers of *S.lividans* TK24::KC301 dropped by more than two logs during the initial period of the experiment. This is considerably more than would be expected if one considers that ΦC31 is spontaneously released from *ca.* 1% of germinating spores (Lomovskaya *et al.*, 1972). No thiostrepton-resistant streptomycete cells were detected in soils uninoculated with *S.lividans* TK24::KC301. A proportion of thiostrepton-resistant colonies from sample sets C and D were checked to ascertain if they still carried the KC301 genome by screening for spontaneous phage release and by probing with ^{32}P-labelled KC301 DNA (see Fig. 26). It is possible that in the conditions of nutrient limitation found in nonsterile soil that the phage might have preferred to be in the free state i.e. KC301 preferred not to lysogenise a host whose death might be imminent.

Fig. 24. **Growth and survival of *S.lividans* TK24::KC301 in sterile amended and nonsterile soil.**

Graphs A and C give the logged counts at each day, whilst graphs B and D give the scaled counts. Graphs A and B represent total *S.lividans* TK24 counts, whilst graphs C and D give spore counts.



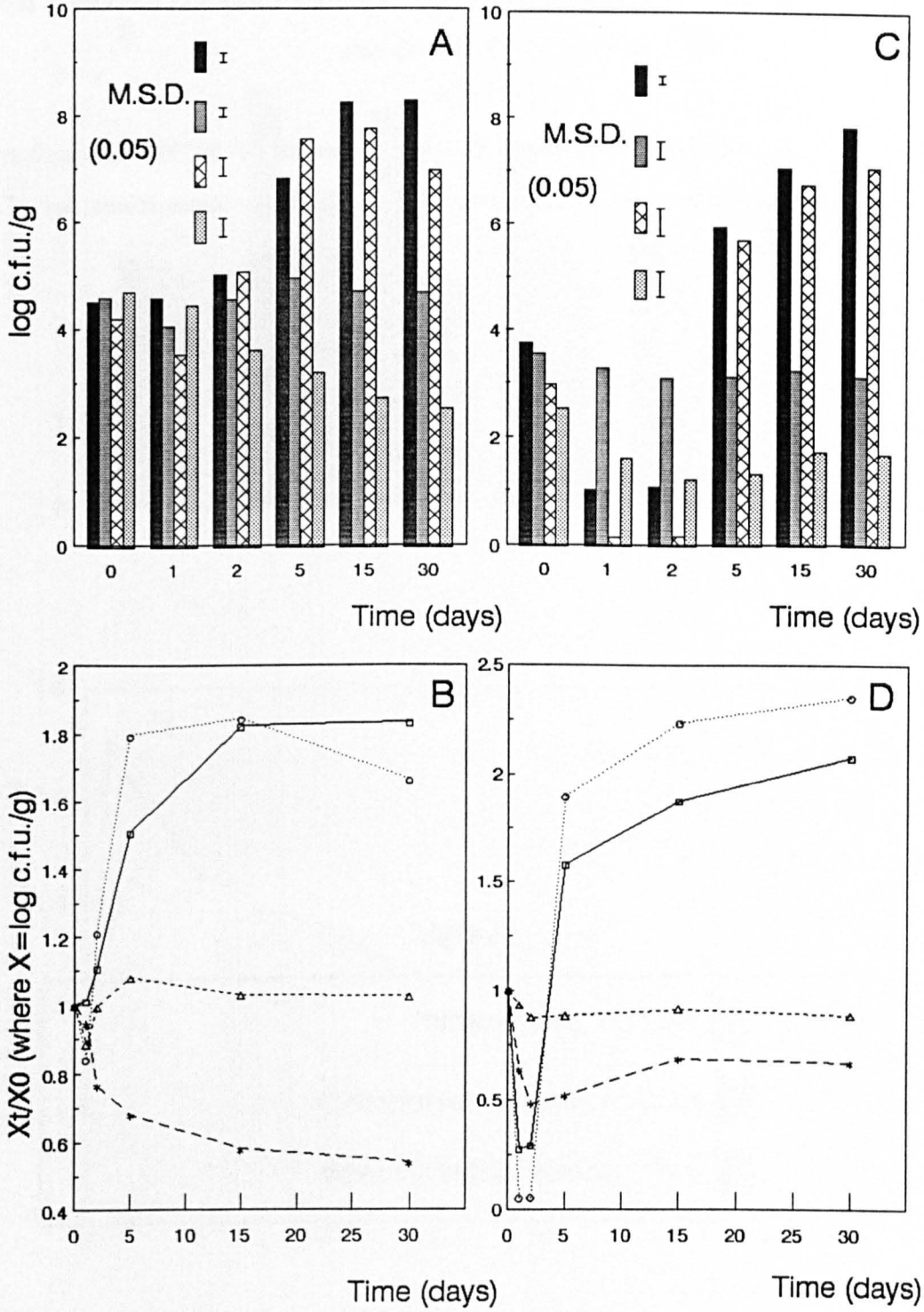
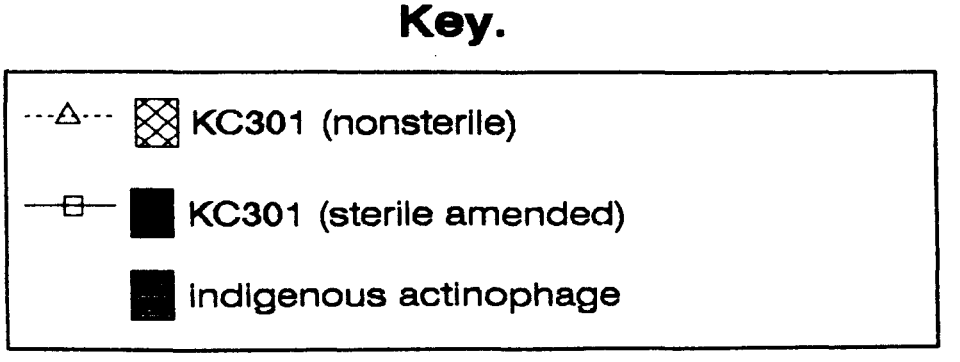
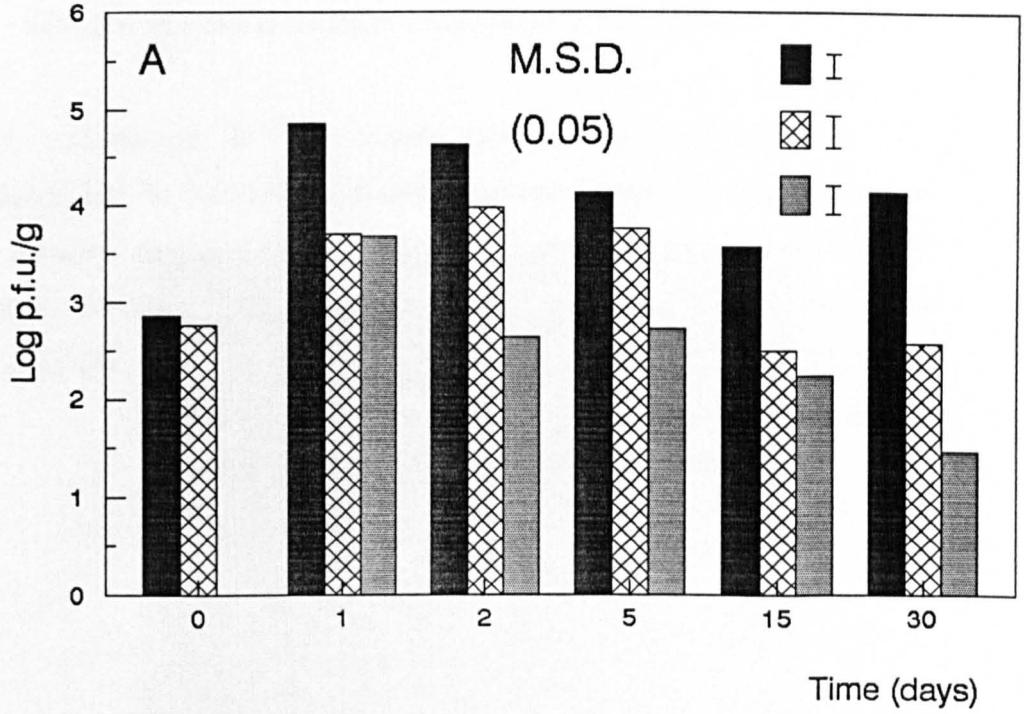

A
M.S.D.
(0.05)
log c.f.u./g
Time (days)
C
M.S.D.
(0.05)
Time (days)
B
Xt/X0 (where X=log c.f.u./g)
Time (days)
D
Time (days)

Fig. 25. **Release of KC301 from a lysogenic host and survival in sterile amended and nonsterile soil.**

Graph A represents the logged counts of KC301 and indigenous actinophage numbers, whilst graph B shows the scaled counts at each day.




A
M.S.D.
(0.05)
Log p.f.u./g
0
1
2
3
4
5
6
0
1
2
5
15
30
Time (days)

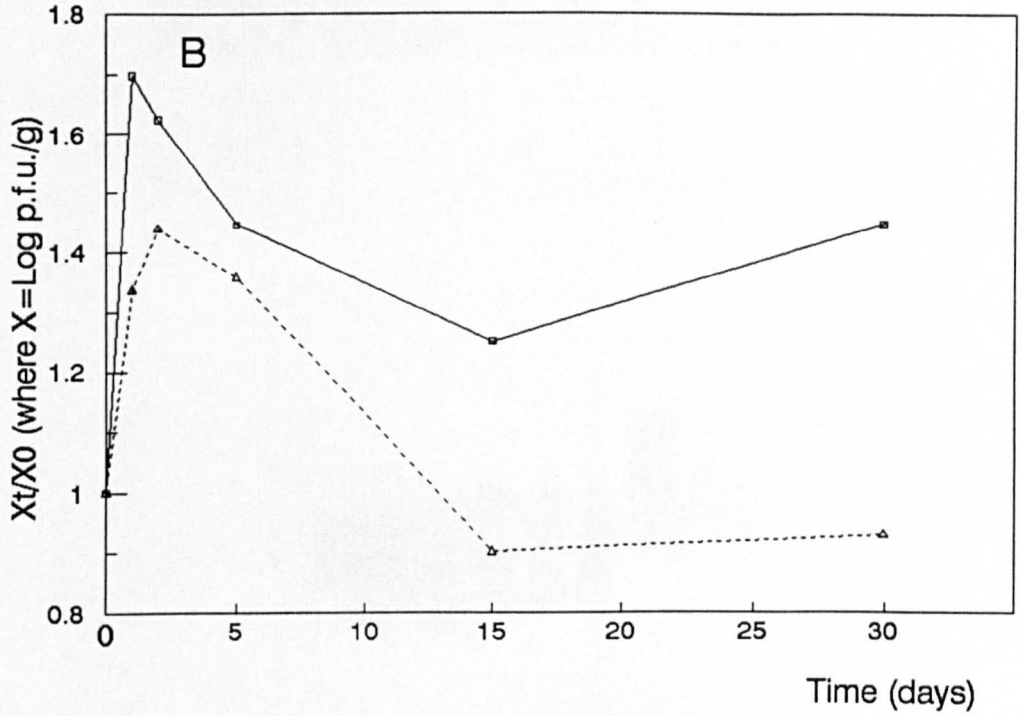
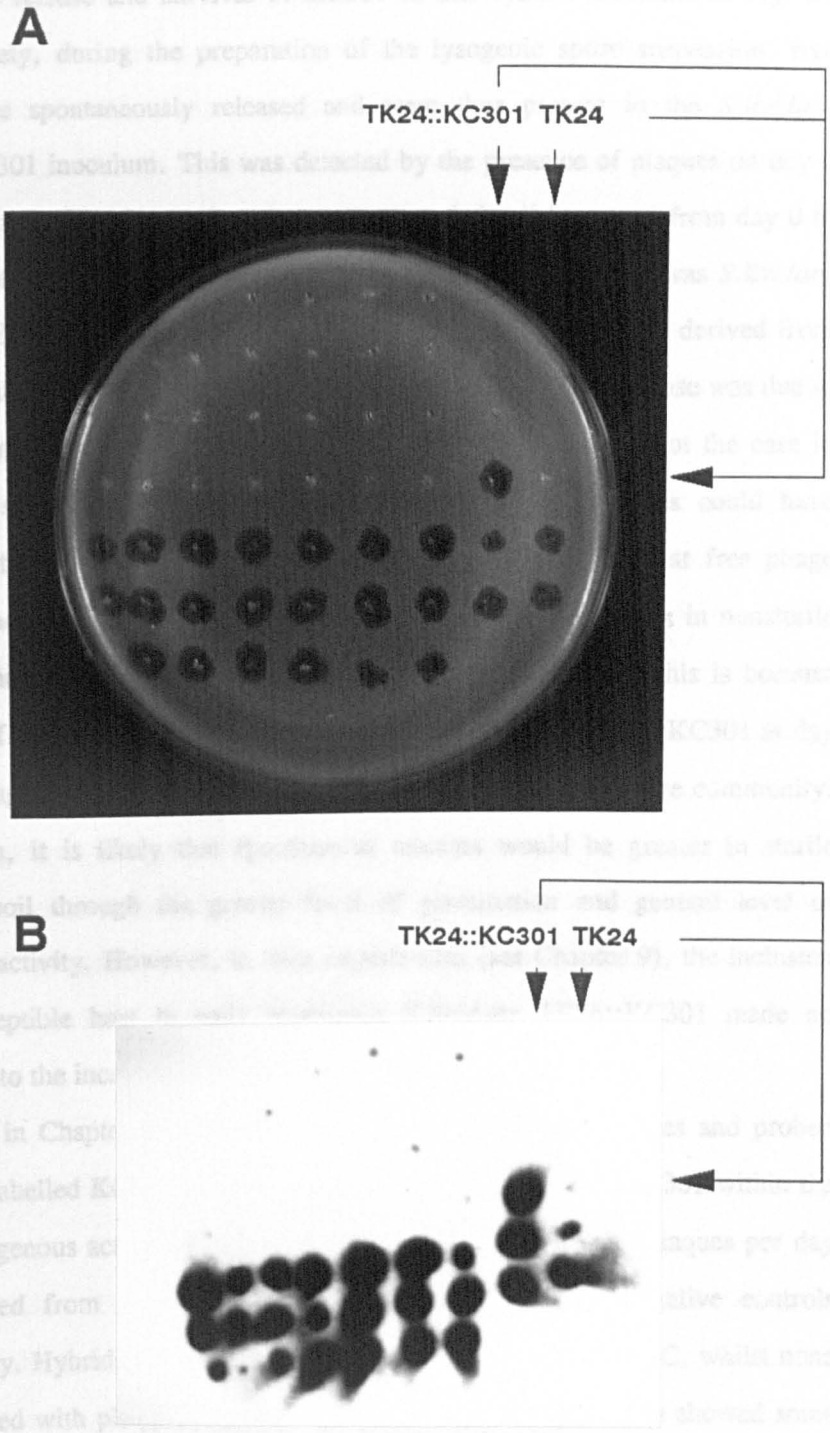

B
Xt/X0 (where X=Log p.f.u./g)
0.8
1
1.2
1.4
1.6
1.8
0
5
10
15
20
25
30
Time (days)

Fig 26. Confirmation of thiostrepton-resistant colonies as lysogens.

Streptomycin-resistant colonies (upper half of photographs) and thiostrepton- and streptomycin-resistant colonies (lower half of photographs) were picked onto SNA seeded with *S.lividans* TK24 (A) and also subjected to colony hybridisation with KC301 DNA (B). *S.lividans* TK24 and *S.lividans* TK24::KC301 from stock cultures were also included as negative and positive controls respectively.


A
TK24::KC301
TK24
B
TK24::KC301
TK24

The release and survival of KC301 in this system is shown in Fig. 25 Unfortunately, during the preparation of the lysogenic spore suspension, free phage were spontaneously released and were thus present in the *S.lividans* TK24::KC301 inoculum. This was detected by the presence of plaques on day 0 isolation plates. KC301 numbers in sterile amended soil increased from day 0 to day 1. As the only streptomycete present in sterile amended soil was *S.lividans* TK24::KC301, the increase in free phage numbers must have been derived from the lysogenic inoculum. Although it is not known whether this release was due to prophage induction, or due to processing of the soil. This was not the case in nonsterile soil where the presence of indigenous streptomycetes could have facilitated the increase in free phage numbers. Even the fact that free phage numbers increased to a greater extent in sterile amended soil than in nonsterile soil does not point conclusively to spontaneous phage release. This is because numbers of indigenous actinophage increased to a similar level as KC301 at day 1, indicating infection of streptomycetes by the resident actinophage community. In addition, it is likely that spontaneous releases would be greater in sterile amended soil through the greater level of germination and general level of metabolic activity. However, in later experiments (see Chapter 9), the inclusion of a susceptible host in soils containing *S.lividans* TK24::KC301 made no difference to the increase in phage titre after prophage induction.

As in Chapter 5, plaques were picked from isolation plates and probed with ^{32}P-labelled KC301 DNA to determine the proportion of KC301 within the entire indigenous actinophage population (see Fig. 27). Twenty plaques per day were picked from sample sets C and E as positive and negative controls respectively. Hybridisation was detected with all plaques from set C, whilst none was detected with plaques from set E. Plaques from sample set D showed some hybridisation (see Fig. 27). Previously (see Chapter 6), a decrease in the percentage of KC301 within the indigenous actinophage population was seen during the course of the experiment. Fig. 28 does show a decline (as in Fig. 16)

in the proportion of KC301 during the course of the experiment, although it was not as consistent as found previously. This might indicate that fresh KC301 is being added to the free phage population by spontaneous release from *S.lividans* TK24::KC301, although further experiments would have to be carried out to confirm this.

Total soil DNA was extracted by a direct lysis procedure using bead-beating (see section 2.24.2) from 10g microcosms. These extracts were run on an agarose gel, Southern blotted and probed with ^{32}P-labelled KC301 DNA (see Fig. 29 [a] and [b]). Hybridisation was only detected at days 2, 5, 15 and 30 from sample set C (Fig. 29 [b], tracks 2, 3, 4 & 5), although, the recovery of DNA from this sample set at days 0 and 1, does not seem to have been very successful, i.e. hybridisation might have been detected here if the initial extraction had been better (see Fig. 29 (a), tracks 5 & 6). Cresswell *et al.* (1991) reported the detection limit of this method to be *ca.* 10^2 c.f.u./g for a multicopy plasmid pIJ673 in *S.lividans* TK24. However, the KC301 genome, either as free phage or lysogen, can only ever be present as one copy per p.f.u. or c.f.u., so one would assume that phage DNA only becomes detectable in soil when numbers of lysogens had been magnified to rise above the detection limit by germination and sporulation.

8.4 Lysogen versus non-lysogen survival in soil: conclusions

S.lividans TK24::KC301 survived as well as *S.lividans* TK24 in sterile amended soil, but in nonsterile soil carriage of the phage was detrimental to survival. The reason for this difference in survival in both soils is not known. It night well be that in conditions of nutrient limitation that bacteriophage prefer to be in the free state, thus increasing their chance of dispersal and carriage to a different host. Again, the proportion of KC301 within the entire actinophage

population declined during course of the experiment, although this decline was not as consistent as had been found previously. Whether this decline is due to the lack of fitness of KC301 is not known. It must be remembered that in the construction of KC301 significant amounts of its DNA were deleted, and although these deletions did not alter the phage's fitness *in vitro*, bacteriophage make very efficient use of their DNA and thus it is unlikely that this DNA had no role in the lifestyle of this phage in some shape or form.

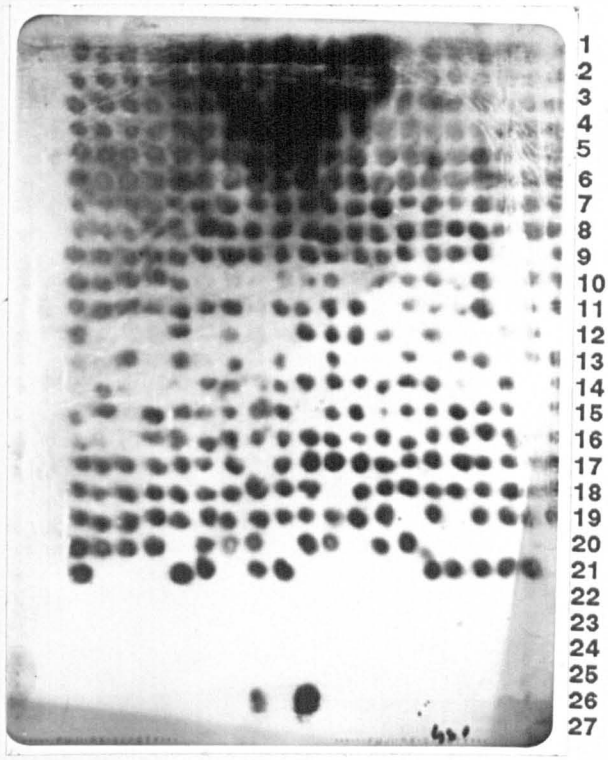
Fig. 27. **Probing of plaques isolated from sterile amended and nonsterile soil with ^{32}P-labelled KC301 DNA.**

Plaques were picked from sample sets C (20/day), D (50/day) and E (20/day) and subjected to plaque hybridisation with KC301 DNA. This allowed the proportion of KC301 within the indigenous actinophage population to be determined as in Chapter 5 (see Fig. 28).

Day	Sample set		
	C	D	E
0	1, A-T	7, A-T 8, A-T 9, A-J	no phage recovered
1	2, A-T	9, K-T 10, A-T 11, A-T	22, A-T
2	3, A-T	12, A-T 13, A-T 14, A-J	23, A-T
5	4, A-T	14, K-T 15, A-T 16, A-T	24, A-T
15	5, A-T	17, A-T 18, A-T 19, A-J	25, A-T
30	6, A-T	19, K-T 20, A-T 21, A-T	6, A-T

(Cont. overleaf)

The number in the table represents the row at which those plaques


A B C D E F G H I J K L M N O P Q R S T
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27

can be found and the letter the column at which they can be found on the autoradiograph.

Plaques at 27, H and 27, J are caused by ΦC31 c1 and KC301 respectively (positive controls).

Fig. 28. **Proportion of plaques on *S.lividans* TK24 caused by KC301 surviving within the indigenous actinophage population when inoculated as a lysogen.**

By probing plaques (^{32}P-labelled KC301 DNA) isolated from nonsterile soil (50 plaques/day) inoculated with *S.lividans* TK24::KC301 (see Fig 27) it was possible to determine what proportion of plaques at each day were caused by KC301 (Sample set D).

Day	Percentage showing hybridisation (from Fig. 27.)
0	100
1	80
2	44
5	84
15	96
30	42

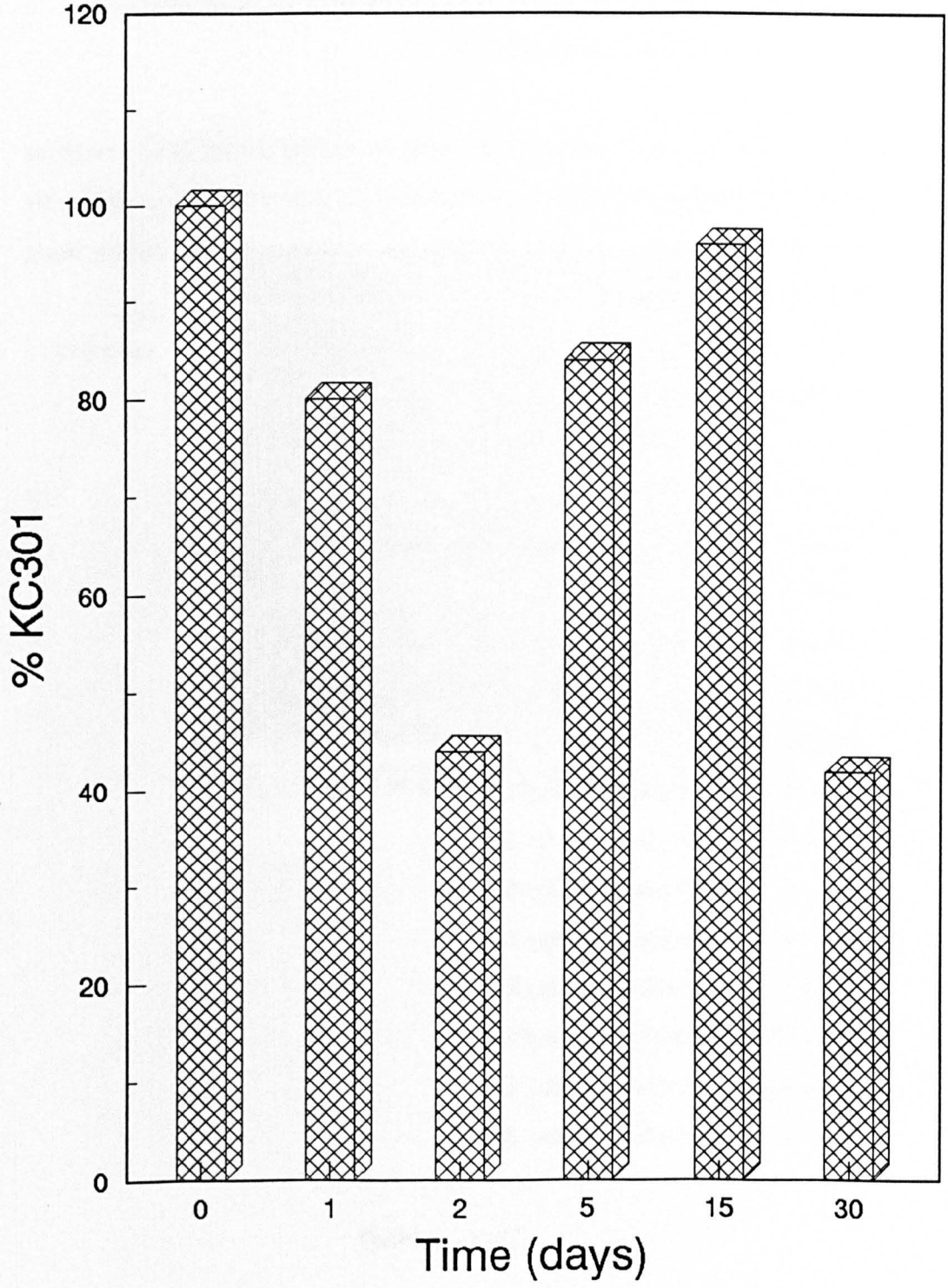

120
100
80
60
40
20
0
% KC301
0
1
2
5
15
30
Time (days)

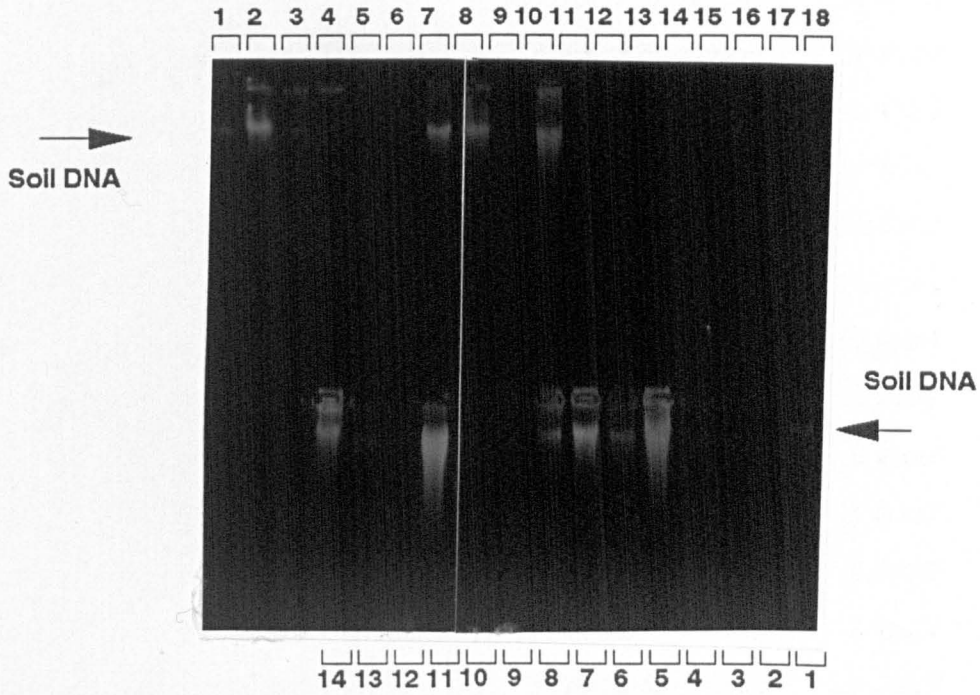
Fig. 29 (a). Tracking of KC301 in soil using DNA extraction by bead beating (gel photograph)

Each 10g sample was extracted using the method for soil DNA extraction using bead-beating described in Section 2.24.2. After this the sample were subjected to agarose gel electrophoresis and southern blotting, before being probed with KC301 DNA.

Lower row of wells.

Track 1. KC301 DNA.
Track 2. Lambda *Hin*DIII (not visible).
Track 3. Sample set D, day 0.
Track 4. Sample set D, day 1.
Track 5. Sample set D, day 2.
Track 6. Sample set D, day 5.
Track 7. Sample set D, day 15.
Track 8. Sample set D, day 30.
Track 9. Sample set E, day 0.
Track 10. Sample set E, day 1.
Track 11. Sample set E, day 2.
Track 12. Sample set E, day 5.
Track 13. Sample set E, day 15.
Track 14. Sample set E, day 30.

(Cont. overleaf)


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Soil DNA
Soil DNA
14 13 12 11 10 9 8 7 6 5 4 3 2 1

Upper row of wells

Track 18. Sample set A, day 0.

Track 17. Sample set A, day 1.

Track 16. Sample set A, day 2.

Track 15. Sample set A, day 5.

Track 14. Sample set A, day 15.

Track 13. Sample set A, day 30.

Track 12. Sample set B, day 0.

Track 11. Sample set B, day 1.

Track 10. Sample set B, day 2.

Track 9. Sample set B, day 5.

Track 8. Sample set B, day 15.

Track 7. Sample set B, day 30.

Track 6. Sample set C, day 0.

Track 5. Sample set C, day 1.

Track 4. Sample set C, day 2.

Track 3. Sample set C, day 5.

Track 2. Sample set C, day 15.

Track 1. Sample set C, day 30.

Fig. 29 (b). Tracking of KC301 in soil using DNA extraction by bead beating (autoradiograph)

This Figure represents the autoradiograph of the gel displayed in Fig. 29 (a).

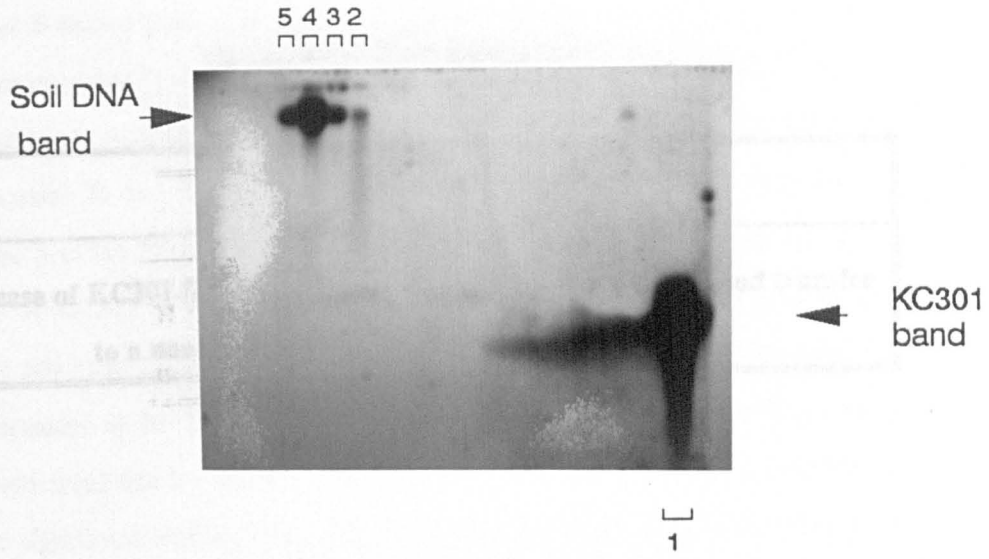
Track 1. KC301 control

Track 2. Sample set C, day 2.

Track 3. Sample set C, day 5.

Track 4. Sample set C, day 15.

Track 5. Sample set C, day 30.


5 4 3 2
Soil DNA band
KC301 band
1

Chapter 9.

Release of KC301 from a lysogenic strain of *S.lividans* TK24 and transfer to a non-lysogenic recipient in sterile amended soil.

9.1 Phage conversion of *S.lividans* TK23 with *tsr*: aims

Previous work (see Chapters 4 and 5) had shown that KC301 was able to infect and lysogenise *S.lividans* in sterile amended soil; work described in Chapter 8 demonstrated that KC301 was released from *S.lividans* TK24::KC301 in large numbers in sterile amended soil. If both of these events could take place in a separate system it seemed likely that they could take place in the same microcosm. It was therefore intended to repeat the experiment described in Chapter 8 in the presence of a marked recipient (*S.lividans* TK23), to assess the ability of KC301 to facilitate transfer of the thiostrepton-resistance gene *in situ* (phage conversion). Unlike the experiment described in Chapter 8, in order for such transfer to be demonstrated it would be critical that all free phage were removed from the lysogenic inoculum. Previously it had proved impossible to detect significant numbers of lysogens in nonsterile soil after inoculation with free KC301, thus, it was decided to confine this experiment to sterile amended soil as it was predicted that the level of phage spontaneously released from a lysogen would be less than that required to cause significany lysogenisation of an inoculated host.

9.2 Monitoring of phage, donor, recipient and convert populations *in situ*

Soil was amended and weighed out into 20g and 100g microcosms as described in Chapter 5 and Section 2.21, except that a set of 10 g microcosms was also used (see Section 2.21). Lysogens were prepared as described in section 2.8.2. Prior to inoculation *S.lividans* TK23 was plated onto R5 containing streptomycin and *S.lividans* TK24 & *S.lividans* TK24::KC301 onto R5 containing spectinomycin to check that no spontaneous mutants had arisen in the inocula. No colonies were detected. Hosts were added to aliquots of SDW, that

when added to soil, gave a final percentage moisture of 15% (see Table 15). In this experiment, the samples were not mixed, and water was allowed to penetrate the soil through diffusion. From the set of 10 g microcosms, three 1 g aliquots were removed and extracted with ¼ strength Ringer's solution per sample set per sample day (see Section 2.22.1). The remaining soil was then used to calculate the % moisture of the soil at each sample day. These 1g samples were serially diluted and plated out in triplicate. One 100g microcosm was extracted using the Chelex-100 method per sample set per sample day, serially diluted and plated out in triplicate for enumeration of hosts and lysogens (see Section 2.22.2). Phage were extracted as in Section 2.23.1. One 20 g microcosm was extracted per sample set per sample day for the enumeration of KC301. Three aliquots of the centrifugation supernatant (see Section 2.23.1) were filtered, serially diluted and assayed for KC301 in triplicate as described in Section 2.7. *S.lividans* TK24 was enumerated on RASS (see Table 6), containing rifampicin, streptomycin, cycloheximide and nystatin (see Table 7). *S.lividans* TK23 was enumerated on the same medium except that spectinomycin was used instead of streptomycin (see Table 7). *S.lividans* TK24::KC301 inoculants and *S.lividans* TK23::KC301 lysogens were counted by incorporating thiostrepton in the medium, the presence of thiostrepton-resistant colonies was also examined in sample sets A, B and C by plating soil extracts onto the relevent media supplemented with thiostrepton. Soil extracts were also plated out on RASS containing basic antibiotics (rifampicin, cycloheximide and nystatin) and streptomycin (sample set B) and spectinomycin (sample sets A and E) to check for the appearance of spontaneous resistance mutants. Extracts from sample set C were plated out onto basic RASS containing streptomycin and spectinomycin to check if chromosomal transfer had taken place.

Table 15. Inocula

Sample set	S.lividans strain (c.f.u./g)		
	TK24	TK23	TK24::KC301
A	$1.6x10^3$	-	-
B	-	$1.3x10^3$	-
C	$1.6x10^3$	$1.3x10^3$	-
D	-	$1.3x10^3$	$1.25x10^3$
E	-	-	$1.25x10^3$

9.3 Transfer of KC301 from *S.lividans* TK24 to *S.lividans* TK23 in sterile amended soil

The growth of *S.lividans* TK23, TK24 and TK24::KC301 (sets A, B and E respectively) is shown in Fig. 30. As before, extensive germination and sporulation was seen in this system, with total numbers rising between 4 and 5 logs. The numbers of *S.lividans* TK24 propagules appeared to increase considerably more than those of the other two strains (see graph B). However, this series of microcosms displayed a low recovery at day 0 in all 10g microcosms containing *S.lividans* TK24 (*ca.* 10^2 for *S.lividans* TK24 compared to *ca.* 10^4 for *S.lividans* TK23 and *S.lividans* TK24::KC301). Any increase on the scaled graphs is dependent on the inoculum size, so it is unlikely that the large rise in relative titre was a reflection of greater fitness of *S.lividans* TK24. The growth of the two parent strains was also assessed when they were inoculated into the same microcosm series (sample set C). This is shown in Fig. 31. Germination and sporulation of both strains occurred to a similar extent to that in monoculture (Fig. 30). No streptomycin- and spectinomycin-resistant colonies were detected in sample set C. Both *S.lividans* TK23 and *S.lividans* TK24 have been cured of known plasmids (Hopwood *et al.*, 1985), so chromosomal recombination could not occur (Rafii & Crawford, 1989a). The appearance of *S.lividans* TK23 lysogens is shown in Fig. 32, along with the growth of donor and recipient. Again, spore counts indicated marked germination in both inoculated strains before extensive sporulation took place. *S.lividans* TK23::KC301 lysogens were first detected at day 2, using ¼ strength Ringer's solution, and at day 5 using the spore extraction method. This confirms that lysogenic mycelia are first found in sterile amended soil at day 2 and spores at day 5 (see Chapters 4 and 5). Spectinomycin- and thiostrepton-resistant colonies from sample set D were confirmed as lysogens by screening for phage release (Fig. 34 [a]) and by probing with ^{32}P-labelled KC301 DNA (Fig. 34

[b]). These colonies were also picked onto R5 containing streptomycin to check that the parental strain was not a spectinomycin-resistant mutant of *S.lividans* TK24. No growth was detected. The appearance of these colonies shows that KC301 had been released from its lysogenic association with *S.lividans* TK24 and infected and lysogenised *S.lividans* TK23. This method of gene transfer is analogous to that described by Zeph *et al.* (1988), who demonstrated the transfer of Tn*501* between strains of *E. coli* in this manner using derivatives of P1. No thiostrepton-resistant colonies were detected in soils uninoculated with lysogens; also no streptomycin- or spectinomycin-resistant colonies were detected in soils uninoculated with *S.lividans* TK24 strains or *S.lividans* TK23 strains respectively. Colonies resistant to both of these antibiotics were not detected at all.

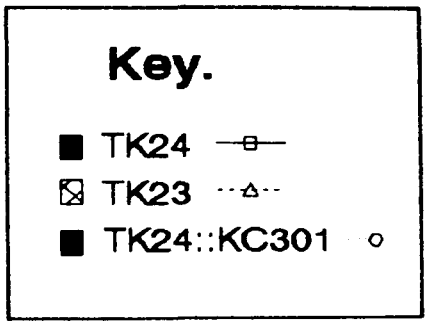
The washing procedure, for the preparation of phage free spore suspensions, described in Section 2.8.2 gave a final spore pellet of *S.lividans* TK24. The supernatant of which contained 67 p.f.u. in 10ml. It was assumed that the pellet contained no more free phage than this. If this was true, then $6.7x10^{-3}$ p.f.u. of free KC301 were inoculated into each gram of soil, *S.lividans* TK24::KC301 stock spore suspension was $1.25x10^{7}$ c.f.u./ml, to obtain an inoculum density of $1.25x10^{3}$ c.f.u./g a 10000 fold dilution was performed. Thus, no microcosm (the largest was 100 g) could be inoculated with more than 1 p.f.u. (0.67 p.f.u.), hence any increase in free phage numbers could be attributed to spontaneous phage release. As was expected from the free phage inoculum size, no plaques were detected at day 0 (see Fig. 33). However, extensive free phage numbers were detected at day 1 (*ca.* 10^{4} p.f.u./g), before declining slightly during the course of the experiment, presumably due to inactivation on soil particles coupled with little replacement from the rapidly sporulating donor.

9.4 Phage-mediated gene transfer in soil: conclusions

Spontaneous phage release occurred during the first 24 hours of the experiment (i.e. phage appeared at day 1, where none had been detected at day 0). Subsequently KC301 infection of *S.lividans* TK23 took place prior to the onset of sporulation (between days 2 and 5), with detectable numbers of lysogenic *S.lividans* TK23 mycelia appearing at day 2. It is suspected that lysogens were present before this date, but were simply below the detection limit of the ¼ strength Ringer's solution method *(ca.* 10^2 c.f.u./g). This demonstrates that phage mediated gene transfer between streptomycetes (analogous to Zeph *et al.*, 1988) can take place in sterile amended soil. Although these events were not examined in nonsterile soil, it is thought that the potential exists in individual microsites, provided that the levels of phage and host are sufficiently high (see Section 10.4).

Fig. 30. **Growth and survival of *S.lividans* TK23 (Sample set B), *S.lividans* TK24 (Sample set A) and *S.lividans* TK24::KC301 (Sample set E) in sterile amended soil (monocultures).**

Graphs A and C give the logged counts at each day, whilst graphs B and show the scaled counts. Graphs A and B represent total numbers of streptomycetes, whilst graphs C and D represent spore counts.



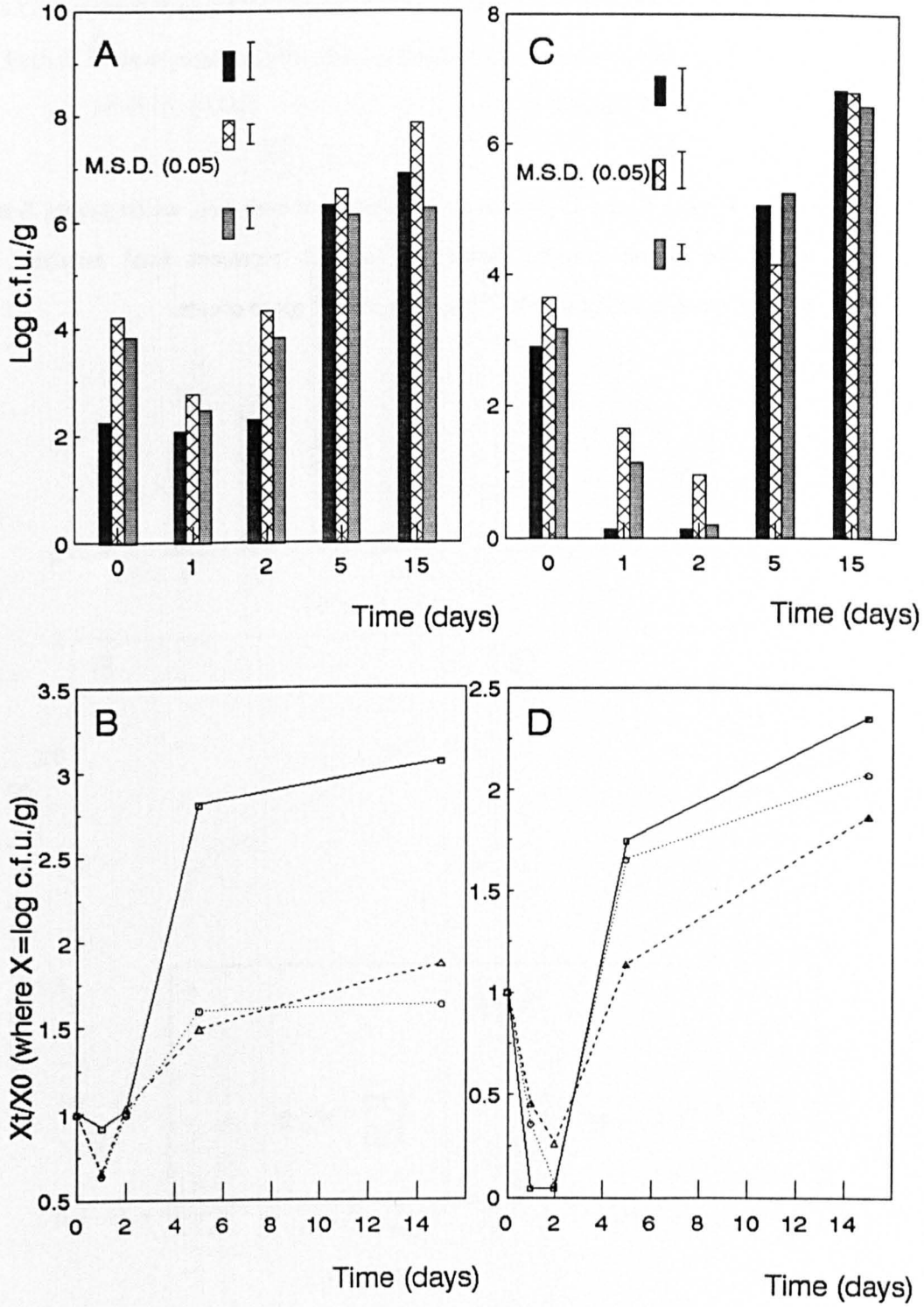
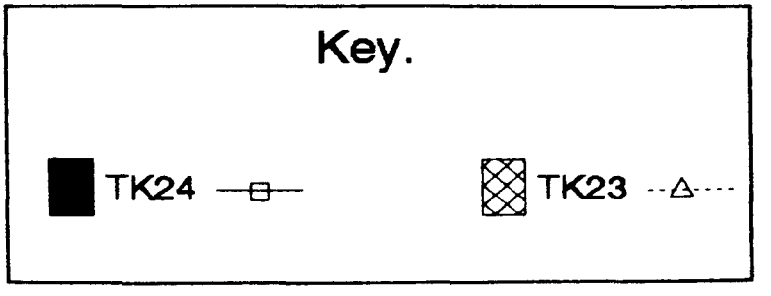

A
M.S.D. (0.05)
Log c.f.u./g
Time (days)
C
M.S.D. (0.05)
Time (days)
B
Xt/X0 (where X=log c.f.u./g)
Time (days)
D
Time (days)

Fig. 31. **Growth and survival of *S.lividans* TK23 and *S.lividans* TK24 in a sterile amended soil mixed culture (Sample set C [mixed cultures]).**

Graphs A and C give the logged counts at each day, whilst graphs B and show the scaled counts. Graphs A and B represent total numbers of streptomycetes, whilst graphs C and D represent spore counts.



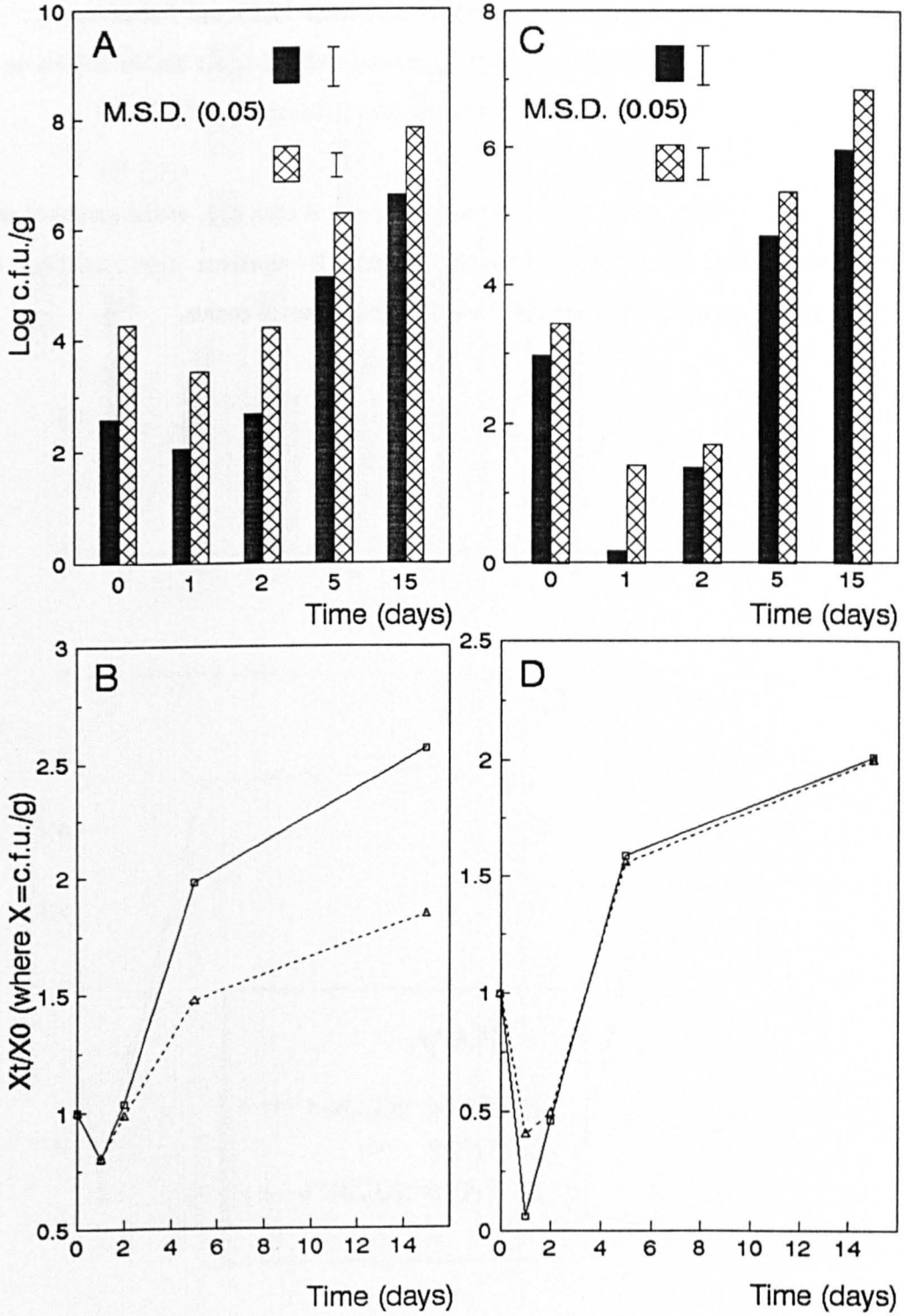
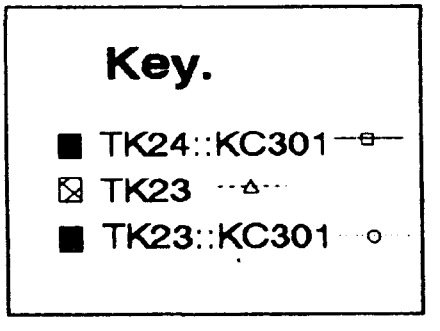

A
M.S.D. (0.05)
Log c.f.u./g
Time (days)
C
M.S.D. (0.05)
Time (days)
B
Xt/X0 (where X=c.f.u./g)
Time (days)
D
Time (days)

Fig. 32. **Growth and survival of *S.lividans* TK23 and *S.lividans* TK24::KC301 and appearance of *S.lividans* TK23::KC301 in sterile amended mixed culture (Sample set D).**

Graphs A and C give the logged counts at each day, whilst graphs B and show the scaled counts. Graphs A and B represent total numbers of streptomycetes, whilst graphs C and D represent spore counts.



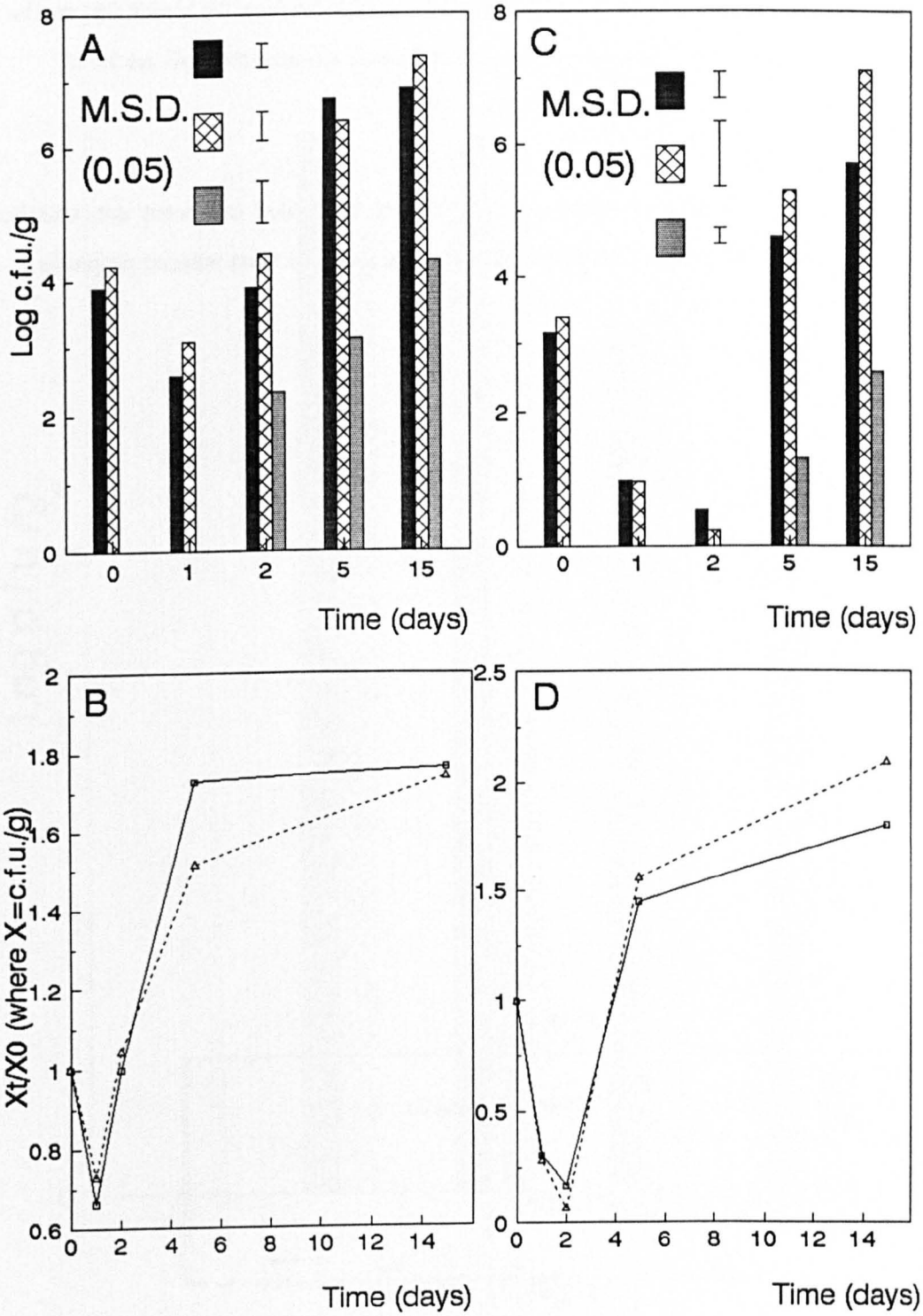
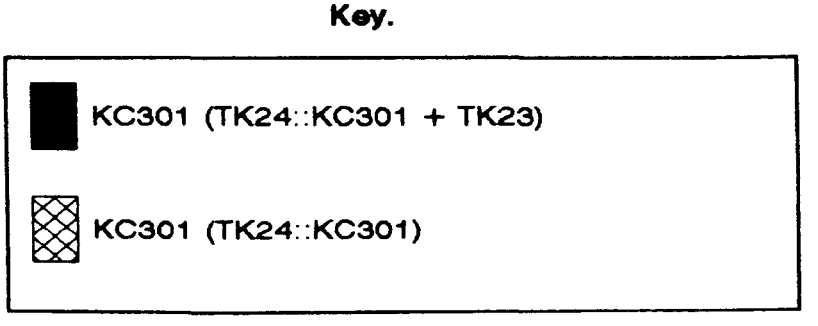

A
M.S.D.
(0.05)
Log c.f.u./g
Time (days)
C
M.S.D.
(0.05)
Time (days)
B
Xt/X0 (where X=c.f.u./g)
Time (days)
D
Time (days)

Fig. 33. **Appearance of KC301 from *S.lividans* TK24::KC301 in the presence (Sample set D) and absence (Sample set E) of *S.lividans* TK23.**

In this experiment lysogens were inoculated that were uncontaminated with free phage, thus all phage in the system came from induced prophage.



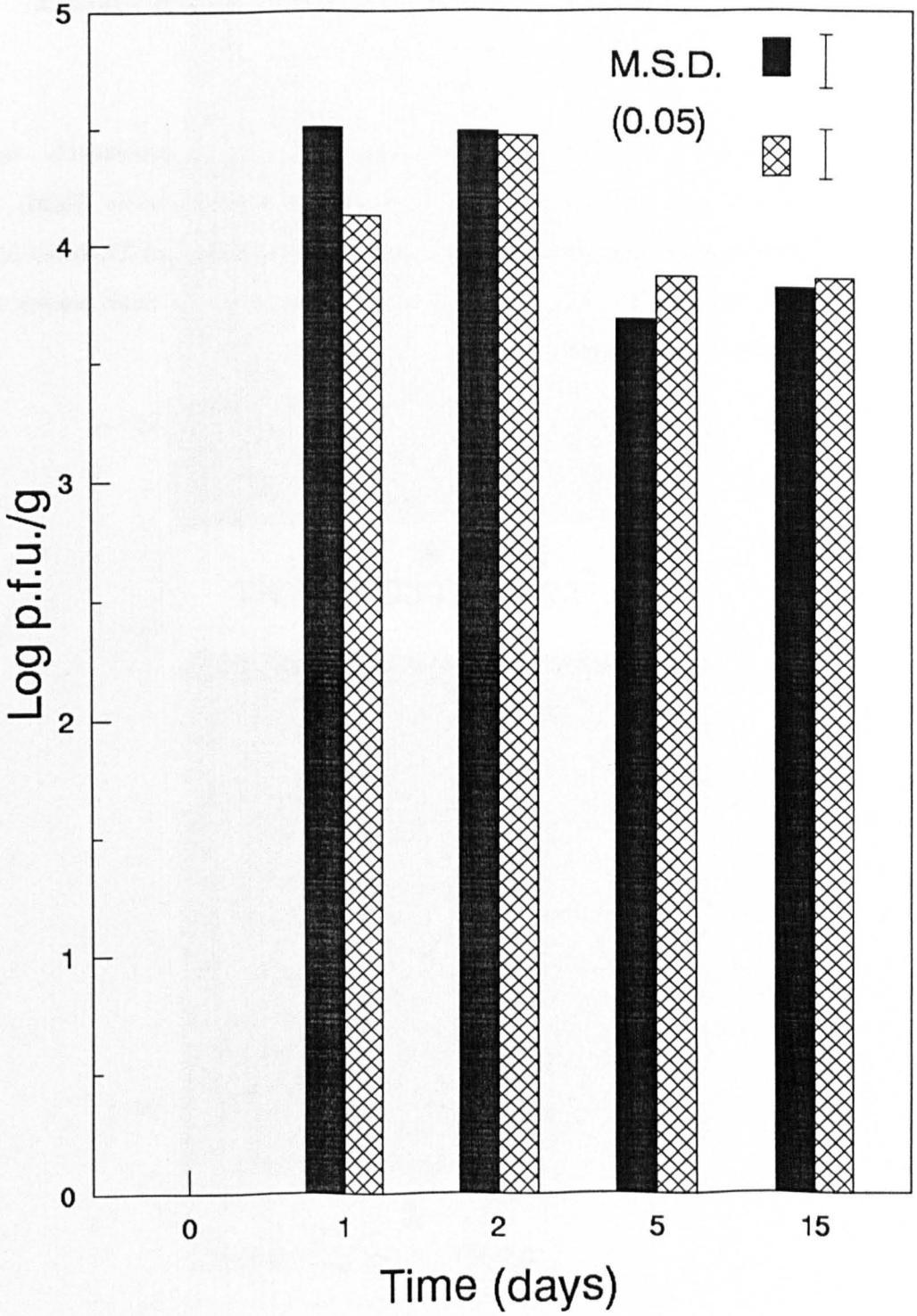

M.S.D.
(0.05)
Log p.f.u./g
0
1
2
3
4
5
0
1
2
5
15
Time (days)

Fig. 34 (a). Confirmation of thiostrepton- and spectinomycin-resistant colonies as *S.lividans* TK23::KC301.

Colonies resistant to spectinomycin (A) and spectinomycin and thiostrepton (B) were picked onto SNA seeded with *S.lividans* TK24, to demonstrate that these colonies were indeed lysogens. *S.lividans* TK24::KC301 and *S.lividans* TK24 stock cultures were also treated in the same manner as positive and negative controls respectively.

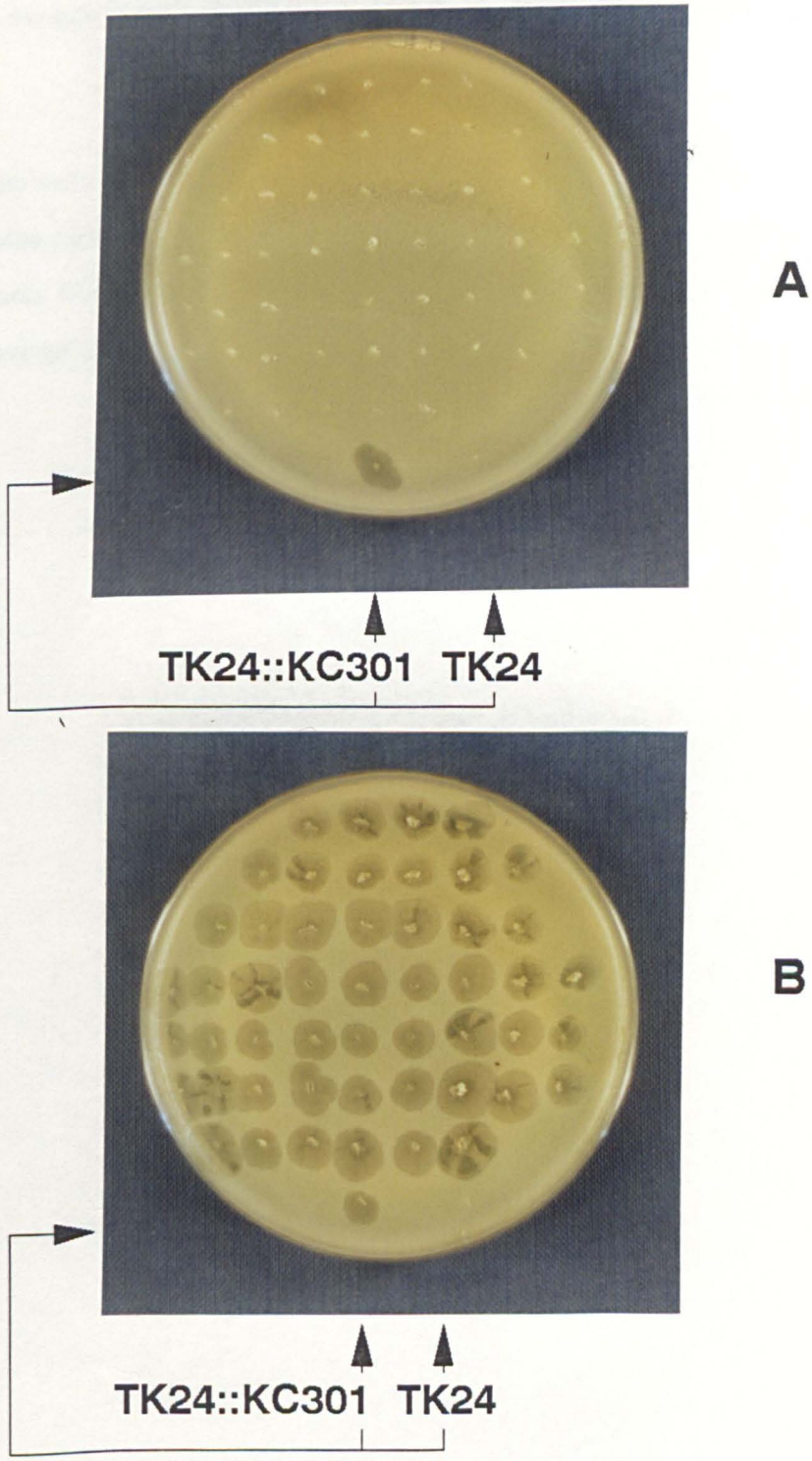
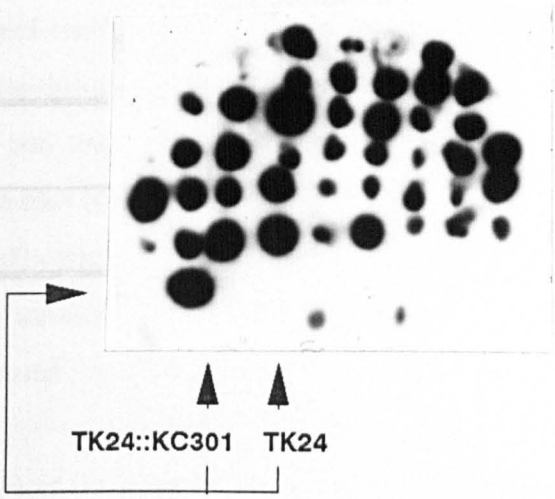

A
TK24::KC301
TK24
B
TK24::KC301
TK24

Fig. 34 (b). Probing of thiostrepton- and spectinomycin-resistant colonies with ^{32}P-labelled KC301 DNA.

Colonies resistant to spectinomycin and thiostrepton were subjected to colony hybridisation with KC301 DNA to demonstrate that these colonies were indeed lysogens. *S.lividans* TK24::KC301 and *S.lividans* TK24 stock cultures were also treated in the same manner as positive and negative controls respectively.


TK24::KC301
TK24

Chapter 10

Discussion

10.1 Method development

The method available for extraction and enumeration of host numbers in soil (¼ strength Ringer's [Wellington *et al.*, 1990]) at the beginning of this work had two drawbacks; firstly the percentage recovery varied from as low as twenty to up to several hundred percent (but was mostly around 100%) and secondly, the method was only capable of detecting numbers greater than *ca.* 10^2 c.f.u./g. Routinely, 100 μl of soil suspension was spread onto one agar plate. If one colony was detected on each replica plate, then the entire (10 ml) soil suspension must have contained 100 cells. Detection could be enhanced by spreading 1ml of the suspension over a series of three plates, however the abundance of liquid added to the plates caused proliferation of motile bacteria resulting in heavily contaminated plates.

The development of the spore extraction method was aimed at removing both of the drawbacks of the ¼ strength Ringer's method. Although initial results indicated that percentage recoveries using this method were as high as 100%, later work showed that extraction efficiencies were more commonly around 20-30% of the inoculum added. Thus, this method did not give any real improvement over the ¼ strength Ringer's method in terms of variation in, and efficiency of, recovery. The real advantages of the spore extraction came in the provision of lower levels of detection and the ability to differentiate between spores and mycelia. As the final suspension was derived from 100g of soil (compared to 1g), even if only 20% of the soil streptomycete population was recovered, more colonies would appear on isolation plates. This effect is best illustrated during the experiment described in Chapter 7, where, even though numbers of *S.lividans* TK24 recovered were consistently lower using the spore extraction method than those derived from the ¼ strength Ringer's procedure, colonies were still detectable at day 60 using the former method. MacDonald (1986) used an ion exchange resin for the dispersion of soil particles to enable

this to be done in a gentle fashion. It was hoped that the problem of fractionation of spore chains during the ¼ strength Ringer's solution method (resulting in very high percentage recoveries) could be negated using such an approach. This proved possible at low shaking speeds, but fractionation of spore chains was detected at higher shaking speeds. Thus, the lower speed was adopted for use in actual soil cross experiments. This point, however, highlights the problems of counting mycelial organisms; even in the inoculum, the presence of spore chains and mycelial fragments meant that the number of c.f.u. bore only a relative resemblance to the actual numbers of propagules. Alternative methods for the preparation of spore suspensions could be developed that allowed the counted c.f.u. to be more indicative of the number of cells e.g. a procedure based on immunological direct counting. In soil, this distinction between c.f.u. and propagules becomes even more unclear; if one imagines a purely copiotrophic streptomycete microcolony (analogous to the colony described by Ensign [1978]) in soil consisting of (for arguments sake) 10 actively growing hyphal tips (Gray *et al.*, 1990) emanating from an inactive mycelial mass, containing 100 nuclei and 50 spores at the centre of the microcolony (giving a total of 160 propagules). If this is then extracted from the environment and spread plated, a number of possibilities exist: (a) the entire cell mass is not disrupted and one colony appears on an isolation plate; (b) the entire mass is disrupted allowing 160 colonies to be counted; (c) the microcolony is partially disrupted allowing somewhere between 1 and 160 colonies to be counted. In fact all of these counts are essentially meaningless (except possibly [b]), as an accurate measure of the total number of propagules because the only section of this microcolony exerting any biological activity would be the hyphal tips, which would be virtually impossible to count using a viability-based method. Although methods are becoming available making use of 16S rRNA probes for direct counting (Amann *et al.*, 1990a) that have the potential to tackle this problem. If one considers the probability that this microcolony is derived from a facultative oligotroph (Williams, 1985) the

problems of reconciling activity with c.f.u. become even more apparent.

The spore extraction method selectively isolates spores from soil in a way analogous to the method of Fægri *et al.* (1977). It is assumed that mycelia are either sedimented during the first series of centrifugations through their attachment to large soil particles or remain in the supernatant during the second (high speed) spin due to their relatively low density. This effect was not predicted during the development of the method, and enabled a great deal of information about the state of streptomycetes in soil to be discerned (see Section 10.2). The spore extraction methods differed from the methods of Skinner (1951) and Mayfield *et al.* (1972) that used mycelial killing by agitation with sand particles or homogenisation, respectively.

The recovery of phage from soil, almost without exception, proved to be very efficient, with extraction efficiencies around 80-100% of the inoculum. Occasionally, reduced recoveries (lowest *ca.* 50%) were detected, but these tended to occur when contamination of overlay plates forced the phage extracts to be reassayed. SNA overlay plates are very susceptible to contamination and consequent masking of plaques - any damaged filters used for phage isolation from nonsterile soil usually resulted in heavy growth of bacteria in the overlay. The use of a chloroform-tolerant actinophage, enabling method D (see Section 2.23.2) of Lanning & Williams (1982) to be used, might alleviate this problem.

Two methods of DNA extraction were used to monitor phage numbers in soil. Both methods (SDS lysis and bead-beating) were those of Cresswell *et al.* (1991). SDS lysis was capable of detecting *ca.* 10^6 p.f.u./g. The detection limit of the bead-beating method was not investigated, but when used in the experiment described in Chapter 8 this method was able to detect KC301 in sterile amended soil from day 2 onwards. At this point there were *ca.* 10^4 p.f.u./g and *ca.* 10^5 c.f.u./g of *S.lividans* TK24::KC301 propagules. As the combined numbers of free phage and lysogens in nonsterile soil were around one log less at this point (ca. 10^4 and 10^3 respectively), but KC301 DNA was

undetectable, it is possible to estimate that the detection limit of the bead-beating method for actinophage DNA would be around this 10^4 level. Cresswell *et al.* (1991) reported that the detection limit for pIJ673 in spores and mycelia using the SDS lysis and the bead-beating methods respectively were *ca.* 10^2 c.f.u./g.. This difference between detection of phage and the plasmid (pIJ673, used by Cresswell *et al.*, 1991) is not necessarily reflective of differences in efficiency of extraction of plasmid/phage DNA, because one p.f.u. is more likely to correspond to one phage genome than one c.f.u. is to one plasmid copy. pIJ101, and hence pIJ673, is a multicopy plasmid (Kieser *et al.*, 1982) and one c.f.u. on an isolation plate is almost certainly not derived from one propagule (see above and Mayfield *et al.*, 1972).

SDS lysis is used in the preparation of ΦC31 DNA (see Section 2.9.2 and Hopwood *et al.*, 1985), so one would imagine that such a procedure would bring about efficient release of DNA from phage particles *in situ*. If the estimate for the detection limit of the bead-beating method is correct it would seem that this is a more sensitive method for detection of actinophage DNA in soil. However, it seems unlikely that beads as large as those used in this procedure (100-110 μm in diameter) should be able to disrupt ΦC31 particles of very much smaller size (153nm x 53nm [Suárez *et al.*, 1984]) to a greater extent than would treatment with SDS. It must be remembered, that the bead-beating method has only been used for phage DNA extraction in the presence of *S.lividans* TK24, so it might well be that this method can only extract KC301 DNA in a lysogenic state. SDS lysis can detect DNA from KC301 spiked soil.

10.2 Growth and survival of hosts

The results on growth and survival of hosts can be divided into two areas; firstly, in sterile amended soil and secondly, in nonsterile/nonsterile amended soil. In sterile amended soil *S.lividans* strains showed a marked germination and sporulation cycle when inoculated as a spore suspension. Almost total germination was seen at day 1 (i.e. few c.f.u. were recovered using the spore extraction method), with little sign of sporulation by day 2. Following this, rapid sporulation occurred, which reached a plateau sometime after day 5. The plateau was *ca.* 10^8 c.f.u./g and was independent of the host inoculum size, i.e. 10^3-10^6. This agreed with the results of Wellington *et al.* (1990) and the *S.lividans* results of Bleakley & Crawford (1989. However the latter authors found that *Streptomyces parvulus* reached levels of *ca.* 10^{10} c.f.u./g in sterile amended soil. This implies that *S.lividans* grew until the soil could support no more propagules. Whether this effect is due to nutrient depletion, that the soil could physically contain no more microorganisms or some other effect, is not clear. Once sporulation had occurred in sterile amended soil, *S.lividans* was capable of surviving for up to 60 days (see Chapter 5). Under conditions of drought (see Chapter 4) there did, however, appear to be a drop in recoverable *S.lividans* 1326.

Amendment of nonsterile soil appeared to have a slight effect on the survival of the bacterium, in that, *S.lividans* seemed to grow a little more extensively in nonsterile amended soil than nonsterile soil (see Chapters 5, 6 and 8); although the difference was by no means great. The small amount of germination in nonsterile soil was also found by Lloyd (1969), who discovered that most conidia (spores) did not germinate in nonsterile soil (percentage germination 1-14%), but in sterile soil extensive germination was seen (75-91%). This failure to germinate is closely associated with the presence of other microorganisms, and is perhaps due to nutrient depletion by indigenous bacteria

resulting in insufficient nutrient levels in order for *Streptomyces* spores to germinate (Lloyd, 1969). The failure of hosts to germinate can not be conclusively blamed on nutrient depletion, but could equally due to the action of germination inhibitors or other unknown factors. Thus, all of this work agrees with Skinner (1951), Lloyd (1969) and Mayfield *et al.* (1972), that in natural soil, *Streptomyces* spp. exist mainly as spores that are able to survive environmental adversity. Thus any drop in spore numbers may be counterbalanced by small amounts of germination (and subsequent sporulation) at sites of nutrient availability. However the reason why amendment did not appear to cause higher levels of germination is not understood. Mayfield *et al.*, 1972 found that amendment of soil with glucose caused a significant increase in the percentage of spores germinating. Wang *et al.* (1989) released *Streptomyces* strains (including *S.lividans* TK23 and *S.lividans* TK24) into sterile and nonsterile soil and found no significant increase or decrease in c.f.u. in sterile soil, but observed a decrease in nonsterile soil. Survival was enhanced when these strains were inoculated as spores.

Inocula were relatively high in this study (*ca.* 10^8 c.f.u./g, *S.lividans* TK24; *ca.* 10^7 c.f.u./g, *S.lividans* TK23), and the organisms were perhaps close to the carrying capacity, which might account for the absence of a discernible increase in sterile soil. Even though the soil was not amended, the act of autoclaving soil does provide a means for amending soil (see Section 1.3.6) by releasing dissolved nutrients as well as those trapped inside previously living organisms (Salonius *et al.*, 1967), allowing germination of spores (Lloyd, 1969).

The fed-batch experiment (see Chapter 7) yielded some useful, if disappointing information, in that, *S.lividans* TK24 was diluted out of the system by continuing rounds of addition of fresh soil. It is interesting to note the extent to which total streptomycete numbers increased with respect to *S.lividans* TK24, presumably due to continued re-inoculation of these streptomycetes from fresh soil. It was hoped that growth in between dilutions of the soil would replenish

that which was removed. However, it seems that *S.lividans* TK24 was not able to increase its numbers by 100% during this period. Work by Wellington *et al.* (1990) had indicated that *S.lividans* TK24 could proliferate in the same nonsterile amended soil, the reason why the organism was not able to do this in this study is not clear. This experiment does, however, illustrate the problems a genetically-engineered microorganism would have in establishing itself in a dynamic environmental system. There are no reports in the literature of similar systems that make use of such a fed-batch system, which is, after all, a more realistic organisation than a simple batch culture favoured by most workers in this field.

It did not appear in any of the soil crosses attempted in this study that KC301 had any significant effect on the numbers of *Streptomyces lividans in situ*. This was presumably due to the fact that the initial multiplicity of infection was generally around 1. According to Miller & Sayler (1991) bacteriophage are only capable of controlling host populations in aquatic systems when the phage is in a large excess. Even if this had been the case, it is unlikely that the entire streptomycete population would have been eliminated by the phage through a number of mechanisms that prevent this occurring (see review by Alexander, 1981); e.g. predator/parasites under biological control, genetic feedback (resistance), refuge in microsites and reduction of available hosts to levels where the phage is physically unable to collide with them within a realistic timescale (Wiggins & Alexander, 1985).

10.3 Survival of phage

KC301 was able to survive for up to 60 days in the free state in both sterile and nonsterile soil (see chapter 5), the survival being greater in nonsterile soil. This contradicts Williams & Lanning (1984) who claimed for other

actinophage the reverse was true. This conclusion is based on total numbers of free phage (indigenous + KC301) so it is important to remember that the difference in phage titre might well be due to the non-KC301 portion of this population. It seems that KC301 numbers undergo an initial burst of activity in all soil types as long as a viable host is present; following this burst there is a gradual decrease in actinophage titre. When the soil was allowed to dry out the phage dropped to undetectable levels, this may be due to the virus being subjected to strong forces of desiccation, aeration and surface tension during the period of drying (Cheo, 1980). This burst of phage numbers after inoculation indicates that there was a brief period of host activity (germination) brought about by a nutrient flush allowing phage infection to take place. Germida (1986) found that nutrient amendment of soil caused an increase in *Azospirillum brasilense* numbers coupled with a simultaneous increase in phage titre. This concurs with the conclusions drawn in Section 10.2, that there was a short interval of germination during the first few days after inoculation (the length and extent of this germination being dependent on the amendment and sterility of the soil). After sporulation there was no longer a susceptible host for phage to propagate on, and thus KC301 numbers fell through adsorption or degradation.

As the phage titre was only at a relatively high level when the host was in the mycelial state this indicates that actinophage exist in a condition of dynamic interaction with their host, existing for only short periods in this state. Mayfield *et al.* (1972) proposed that streptomycete growth in soil takes place in brief periods of activity. Streptomycetes are apparently copiotrophic organisms; so one would imagine that phage would mirror population changes of their host. In nonsterile soils inoculated with KC301 alone, phage numbers increased, so there were obviously susceptible hosts in the soil. ΦC31 infects about 50% of the 137 streptomycete strains tested (Voeykova, 1979; Chater, 1986). As described above, streptomycetes exist mainly as spores, therefore the process of storing (and hence drying) the soil, followed by rewetting at inoculation must have

resulted in germination of spores in order for phage infection to have taken place as ΦC31 only infects newly germinated mycelia (Lomovskaya *et al.*, 1972).

The best picture of *Streptomyces*-phage interactions in soil was provided by a mathematical model (Manchester, 1986; Williams *et al.*, 1986). The basic equation was derived by substituting measured parameter values into a Nicholson-Bailey model of insect-parasitoid interactions; a number of parameters were measured for phage-host interactions, that were then incorporated into the equation and used to predict the performance of phage and host (e.g. burst size, infection efficiency, host generation time, phage generation time and carrying capacity of the soil [see below]). The equation could then be used to predict host and phage numbers. This model assumed that resources (i.e. nutrients) were patchily distributed in space and time and were continuously being depleted and renewed. A successful theoretical treatment of the dynamic relationship between phage and host was only set up when the value of infection efficiency was set at *ca.* two logs below the observed value in soil (*ca.* 10^{-8} rather that *ca.* 10^{-6}). It is interesting that the only suggestion of a dynamic interaction between *S.lividans* and KC301 occurred when fresh nutrients were added to the system (see Chapter 7. In all other batch microcosm experiments (see Chapters 4, 5, 6, 8 & 9) phage numbers simply declined after an initial burst. The model proposed by Manchester (1986) assumes constant renewal of resources.

$$H_{t+1}=sH_t\exp[r((1-H_t/K)-aP_t)]$$

$$P_{t+1}=zcH_t[1-\exp(-aP_t)]$$

Where: H_t and H_{t+1} = host population densities measured as infectable units per unit volume over one generation

r = intrinsic rate of natural increase of the host (5.76)

K = equilibrium host density (carrying capacity)

s = fraction of spore population that survives to the next

generation (0.13)

a = infection efficiency of phage ($2.1x10^{-5}$ - $3.5x10^{-6}$)

P_t and P_{t+1} = numbers of phage present at time t and t+1

c = mean burst size (19-100)

z = fraction of virion bank that survives to the next generation (0.66)

Figures in parenthesis represent the observed parameter values of *Streptomyces* sp. MX1 and Φmx1.

10.4 Appearance of lysogens

Lysogens of KC301 in *S.lividans* TK23, *S.lividans* TK24 and *S.lividans* 1326 were readily found in sterile amended soil (see Chapters 4, 5 and 9) upon co-inoculation of phage and hosts. The ¼ strength Ringer's method allowed detection of thiostrepton-resistant colonies at day 2 and the spore extraction method at day 5; this indicates that lysogenic mycelia were found at day 2 and spores at day 5. As the ¼ strength Ringer's method has a detection limit of *ca.* 10^2 c.f.u./g, one would imagine that KC301 infection had taken place prior to day 2, but was undectable due to an inadequate number of lysogenic infections having taken place or that insufficient growth of newly formed lysogens had occurred in order to raise their titre above this threshold of detection. Obviously spores detected at day 5 resulted from the sporulation of infected mycelia, as actinophage are unable to infect spores.

In nonsterile or nonsterile amended soil two lysogenic colonies were detected on isolation plates (see Chapters 6 and 7). The only conclusion that one can draw from this is that lysogenic infections by ΦC31 of *S.lividans* in natural soil is a rare occurrence. Saye *et al.* (1987) demonstrated that the presence of an

indigenous microflora reduced the number of transductants (hence phage infections) found in lake water. One would imagine that the less fertile conditions found in nonsterile compared to sterile amended soil would favour lysogeny rather than lysis as this would provide a refuge for the phage. It is possible that the physiological state of the host cell in nonsterile soil is not conducive to the formation of a lysogenic association between phage and host (Lenski, 1988), this would provide an anthropomorphic situation where the phage decides it is better to leave the nutrient depleted host in search of a host living under more fertile conditions. However, it is thought that the probable reason for this dearth of lysogens in nonsterile soil is simply because not enough phage and hosts were present in the system.

This can be best illustrated with data from chapter 5. The inocula of sample set F (KC301 x *S.lividans* TK24 [see Table 11]) were counted as $9.26x10^3$ p.f.u./g and $1.41x10^3$ c.f.u./g respectively at day 0. Phage numbers increased to $6.94x10^4$ p.f.u./g at day 1; an increase of *ca.* $6x10^4$ p.f.u./g. If one considers that ΦC31 has a burst size of between 100 and 200 (Rodríguez *et al.*, 1986) and one assumes that KC301 *in situ* performs as ΦC31 does *in vitro*, this increase may have been derived from 600 to 300 infections (Manchester, 1986). Between 20 and 40% of ΦC31 infections result in lysogeny (Lomovskaya *et al.*, 1972), thus, in this soil system, at least 60 and as many as 240 lysogenic infections must have resulted. Using viable counting (with its obvious limitations) it was determined that the soil used in this experiment contained $9.89x10^5$ c.f.u./g indigenous streptomycetes, of which ΦC31 may be able to infect about 50% (Voeykova *et al.*, 1979; Chater, 1986), if the strains tested by Voeykova *et al.*, (1979) were representative of those present in the soil used in this study. Thus a total of $4.96x10^5$ c.f.u./g (50% of total streptomycetes + *S.lividans* TK24 inoculum) should be available for infection. The germination efficiency of *S.lividans* TK24 for this soil sample set between days 0 and 1 was determined to be 77.09% (germination efficiency=$[(X_0 - X_1)/X_0] \times 100$; where

X=c.f.u./g), therefore if the indigenous streptomycetes germinate with the same efficiency as *S.lividans* TK24, the total number of newly germinated mycelia would be 3.82×10^5 c.f.u./g (4.96×10^5 x 0.7709). The germinated portion of the *S.lividans* TK24 population can also be calculated in the same manner; 1.41×10^3 x $0.7709 = 1.09 \times 10^3$ c.f.u./g. Thus, *S.lividans* TK24 propagules comprise 0.29% of the indigenous streptomycete population available for KC301 infection; as such, 0.29% of resulting lysogenic infections should occur in *S.lividans* TK24. The number of lysogens could vary, therefore, from 0.17 to 0.68/g, which are below the detection limit of the spore extraction method; *ca.* 1 c.f.u./g in nonsterile soil (see section 3.1.5). Due to the presence of greater numbers of indigenous streptomycetes, one would expect to detect countable numbers of lysogens in that population (unfortunately indigenous lysogenic streptomycetes were not screened for during the course of this study, although ongoing work has since detected this in nonsterile soil [P.Marsh, unpublished data]). This would suggest that there is a minimum host density required for phage replication in soil, unlike the aquatic environment (Kokjohn *et al.*, 1991). Saye *et al.* (1987) found that the number of transductants obtained in lake water microcosms was proportional to the numbers of donors and recipients. Presumably, this is due to the length of time that a bacteriophage takes to find a susceptible host. Wiggins & Alexander (1985) calculated that with an *E. coli* density of 100 c.f.u./ml, 4000 minutes would be required for one of 1000 ralp.f.u./ml bacteriophage particles to contact one *E. coli* cell and suggested that under such circumstances the initiation of replication would be delayed, but at a host density of 10^5 c.f.u./ml, the initiation of replication would take only 4 minutes. It must be stressed that this study and a similar one by Kokjohn *et al.* (1991) was carried out in liquid systems where the chances of phage/host contact would be much greater than in a broadly static terrestrial one. It would seem, therefore, that in the case described above, the total host density (*ca.* 10^5 c.f.u./g) was of sufficient density to produce phage propagation, but the

S.lividans TK24 population was not adequate for the production of detectable lysogeny. Germida (1986) found that a host titre of 100-1000 p.f.u./g was the minimum that would allow propagation of an *Azospirillum brasilense* phage in soil, although a titre at which lysogeny could be found was not presented. The fact that lysogeny was not found within the *S.lividans* TK24 population illustrates the ecological advantage of temperate bacteriophage having a wide host range. The ability to lysogenise a host has obvious merits in order for a phage to survive environmental adversity (such as drought as in Chapter 5). Stewart & Levin (1984) calculated that whilst virulent phage numbers would increase to a greater extent than those of temperate phage in a (theoretical) aquatic model system in time of feast, the temperate phage would be more able to survive periods of famine. In times of such hardship the greater the size of the lysogenic population the greater the chances of survival of that phage. By examining the case of KC301 above. It is possible to illustrate this point, if ΦC31 had a host range of *S.lividans* TK24 alone, the chances of (detectable) lysogeny occurring would be very small, however, as this phage has a wide host range, many more lysogens can potentially be formed (P. Marsh, unpublished data). With hindsight, therefore, in planning these nonsterile experiments it might have been better to have used a higher host inoculum. However, it is not known what the natural population of *S.lividans* in soil actually is, and by using too high an inoculum the ecological validity of the experiments would have been reduced, i.e. if an inoculum of 10^8 c.f.u./g had been used, lysogens may well have been detected, but would it have been relevant to the numbers of a single streptomycete species actually found in soil ? It is important to remember that soil is a heterogeneous environment, and although theoretically the chances of phage infection are very small, the possibility exists that microsites occur where the relevant titres are locally sufficient for this to take place.

S.lividans TK24::KC301 survived as well as its parent host strain in

sterile amended soil (see Chapter 8). However in nonsterile soil, whilst *S.lividans* TK24 numbers did not decrease significantly during the 30 days of the experiment, *S.lividans* TK24::KC301 titre dropped by *ca.* 2 logs over the first few days of the experiment. This indicates that growth of the organism in sterile amended soil was able to negate that proportion of the population lost through induction of KC301 and subsequent lysis of the host. Chater & Sykes (1976) demonstrated that sporulation could induce lysogens of VP5 (a heteroimmune actinophage to ΦC31 [Dowding & Hopwood, 1973]) in S.*coelicolor* A3(2), whilst Lomovskaya *et al.* (1972) showed that 1% of germinating lysogenic spores spontaneously released ΦC31. However, it is not thought that these events could alone (numerically) account for the drop in the lysogenic population in nonsterile soil.

The transfer of KC301 was detected from a lysogenic donor (*S.lividans* TK24::KC301) to a nonlysogenic recipient (*S.lividans* TK23) in sterile amended soil. This was not attempted in nonsterile soil because it was predicted that the low level of lysogeny found in this system would not allow the detection of such an event, assuming that realistic inoculum levels were used. This experiment was analogous to that of Zeph *et al.* (1988), who demonstrated the transfer of Tn*501* between strains of *E. coli* by derivatives of P1 in sterile and nonsterile soil. Transfer was readily detected in sterile soil, but was only found at day 1 in nonsterile soil (*ca.* 10^2 c.f.u./g). The method used for extraction was similar to the ¼ strength Ringer's solution discussed above, and thus the number of recombinant cells found were only just above the detection limits of this method. However, putative converts were confirmed by probing with labelled Tn*501* (Zeph & Stotzky, 1989). If *E. coli* can undergo phage conversion in an alien environment, it is likely that by the use of high enough inocula, such an event could be demonstrated in nonsterile soil with *Streptomyces*. As demonstrated above, the detection of lysogeny in an inoculated host is dependent on the size of

that inoculum, thus, the use of a system whereby the phage was transferred from a lysogenic donor to non-lysogenic recipient indigenous streptomycetes might prove more facile.

10.5 General conclusions

The extraction procedure developed during the course of this project, whilst not as efficient at recovering streptomycete propagules from soil as traditional methods, is able to detect very low numbers of this genus in soil. This method can differentially isolate spores from an environmental sample, thus allowing a greater insight into the ecology of *Streptomyces in situ*. DNA extraction techniques developed by Cresswell *et al.* (1991) are able to detect actinophage in soil, but do not work as efficiently as when used for the extraction of plasmid DNA. An introduced phage, based on ΦC31, was able to propagate in soil, but was only able to produce significant lysogeny in the absence of the indigenous soil microflora. It is thought that the frequency with which KC301 lysogenises *S.lividans* TK24 in nonsterile soil is dependent on the inoculum titre. KC301 is only present in raised levels when its host is in the mycelial state; indicating a high turnover of the free phage population. KC301 is able to survive in sterile and nonsterile soil, although the titre falls with time.

10.6 Future experiments

There are obviously several experiments that should be attempted with respect to method development. Firstly, the improvement of the percentage recovery of the spore extraction method. If this could be increased to *ca.* 100%, the calculation of the relative proportions of spores and mycelia could be

determined in conjunction with ¼ strength Ringer's counts. At present recoveries of *ca.* 30% mean that differences between the two subpopulations are as a result of a disparity of extraction efficiences. This would allow greater insight into the ecology of the organism, and survival of hyphae in particular. It might be possible to improve the efficiency of DNA extraction protocols, perhaps by developing procedures that are more selective for phage DNA. One way of doing this might be to extract actinophage from soil using the highly efficient method of Lanning & Williams (1982). Prior to the filtration of aliquots from the DNB soil extract, phage could be concentrated by centrifugation and then subjected to the method of Hopwood *et al.* (1985) for small scale phage DNA isolation (see Section 2.9.2). This method commences with a soak out of phage from plaques using DNB and subsequent concentration by centrifugation, so the initial steps are not too dissimilar.

Whilst, the ecology of KC301 in soil has to some extent been characterised, this study has outlined huge gaps in the physiology of the life style of this phage. Whilst a certain amount is known about the genetics of this phage, a great deal more work needs to be done to investigate the regualtion of lysis and lysogeny *in vitro*. As regards the ecology of KC301, the next step would be to quantify the interactions of this phage with its host. Wiggins & Alexander (1985) showed that there was a threshold of host numbers required to bring about the initiation of phage replication in the time period of the experiment. However, Kokjohn *et al.* (1991) found that the reverse was true and there was no threshold value of host cell density for phage replication in the aquatic system. Both of these studies were in aquatic systems, where, through their homogeneous nature, phage-host contact would be expected to occur after a period of time irrespective of the density. However, this is not true of the terrestrial environment where the heterogeneity and solid nature of the soil would cause a threshold density of phage and host below which phage replication could not occur. If this could be determined for KC301 *in situ* it would be possible to learn the scope of this

phage's potential for establishing itself within the natural actinophage community. This could be done by inoculating sterile soil with a known amount of KC301 and adding varying levels of *S.lividans* TK24. The titre could then be determined at which, firstly phage propagation begins and secondly detectable lysogeny is found. The reverse of this could also be attempted using a set amount of *S.lividans* TK24 and varying levels of KC301. In nonsterile soil the situation is a little more complex due to the presence of indigenous streptomycetes and actinophage. These experiments could still be undertaken by screening for lysogeny in a *S.lividans* TK24 population when varying host titre and in the indigenous population when varying phage titre. The characterisation of native streptomycetes in Warwick soil needs to be addressed, with respect to the host range of ΦC31 (previously it has assumed to be *ca.* 50% [Voeykova, 1979]) as this has self-evident implications for calculating the potential number of hosts. In order for this to be worthwhile the entire population must be cultured; this could well prove impossible due to the problems outlined in Chapter 1.

In summary, therefore, this is a highly complex system which this study has just begun to unravel. Thus, there are still many experiments to be carried out before any insight is gained that provides real predictive value into the interactions of streptomycetes and actinophage *in situ*.

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