INTERACTIONS BETWEEN ACTINOPHAGE AND STREPTOMYCETES IN SOIL AND THE FATE OF PHAGE-BORNE GENES

By

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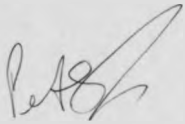
Signed: 

SUMMARY

Host activity is essential for bacteriophage survival. Bacterial growth in soil is sporadic and discontinuous in time and space. Phages must therefore adopt strategies to overcome periods of host inactivity, such as the lysogenic life-cycle, for long-term survival in soil.

KC301 (a derivative of øC31) which contains the thiostrepton resistance gene *tsr* was inoculated into soil microcosms as a lysate or via lysogenic hosts, together with combinations of *Streptomyces* spp. populations. Phage and host populations were monitored over periods of incubation of between 12 and 47 days. The possibility of KC301 lysogenizing indigenous streptomycetes in nonsterile soil was investigated by selection for thiostrepton resistance and molecular analysis of lysogen DNA. The effects of selection for phage-borne *tsr* was examined by adding thiostrepton to soil microcosms before inoculation of soil with resistant lysogenic and sensitive uninfected strains of *Streptomyces lividans*. Correlation between metabolic activity of *S. lividans* and infection by KC301 in soil was also assessed.

KC301 lysogenized indigenous streptomycetes in nonsterile soil when added as a lysate, but not as a prophage in lysogenic spores. A sample of indigenous lysogens proved to contain integrated KC301 DNA. Most of the isolates were stable lysogens, retaining thiostrepton resistance after several rounds of sub-culturing.

Interactions between *S. lividans* and KC301 in soil were limited by the densities of both host and phage populations. In most cases, host population densities in soil were not reduced by lytic KC301 infections. When reduction by lytic KC301 infections of the mycelial stage occurred in soil, sporulation compensated for loss of total viable propagules.

KC301 infected hosts in soil, and established itself as a prophage in lysogenic spores in soil. Turnover of soil at defined times stimulated a small proportion of lysogenic spores to germinate and release KC301 into the soil. KC301 released from germinating lysogenic spores could infect and lysogenize previously uninfected soil inoculants of the same or different *Streptomyces* spp. Lysogenic populations reached lower levels than those of uninfected counterparts, whether growing in the presence or absence of those counterparts.

Thiostrepton in soil caused reduction in the mycelial stage during vegetative growth of sensitive strains following inoculation, but as with phage infections, this was compensated by sporulation. The KC301 lysogen were not affected by thiostrepton in soil, although no selective advantage was conferred on the lysogen population by the presence of the antibiotic.

The majority of the KC301 population in soil existed in the prophage state when soil was inoculated with lysogenic hosts. Although lysogenic hosts were competitively compromised, inputs into the free phage pool could occur without further detriment to host density. Sporulation of hosts compensated for killing of mycelium in soil, and facilitated long-term persistence of KC301.

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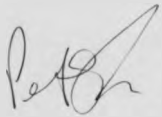
ABBREVIATIONS

AIDS	auto-immune defficiency syndrome
APB	phage buffer
att	phage attachment site
c	repressor gene of phage
CAP	catabolite activator protein
CEC	cation exchange capacity
c.f.u.	colony forming units
cos	cohesive end site of phage genome
DCV	*Drosophila* C virus
cpm	counts per minute
DIG	digoxigenin
dpm	disintegrations per minute
EDTA	diaminoethanetetraacetic acid
ELISA	enzyme linked immunosorbent assay
x g	centrifugal force
G+C	guanine and cytosine
Hfl	high frequency of lysogenization
H.S.D.	honestly significant difference
int	integrase gene
J1501	*S. coelicolor* J1501
kb	kilobases
mol. %	molar percentage
MOI	multiplicity of infection
M.S.D.	minimum significant difference
NB	nutrient broth
NA	nutrient agar
PCR	polymerase chain reaction

PEG	polyethylene glycol
p.f.u.	plaque forming units
Pgl	phage growth limitation
PVPP	polyvinyl pyrrolidone
RASS	reduced arginine starch salts agar
RM	restriction-modification
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
SEM	scanning electron microscopy
SNA	soft nutrient agar
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TEM	transmission electron microscopy
TES	*N*-Tris (hydroxymethyl) methyl-2 aminoethane sulphonic acid
TK23	*S. lividans* TK23
TK24	*S. lividans* TK24
Tn	transposon
Tris	Tris (hydroxymethyl) aminoethane
tsr	thiostrepton-resistance ribosomal methylase gene
xis	excisionase gene

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. Marsh 

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CHAPTER 1

Introduction

1.1 Soil

To study the ecology of soil microorganisms requires an integrated approach, encompassing knowledge of the chemical and physical nature of soil in addition to observations of microflora in that habitat (Lynch, 1983). A major feature of the soil environment, in contrast to aqueous environments, is the abundance of solids which leads to a greater restriction in the movements of organisms, gases, water and nutrients and results in environmental heterogeneity (Nedwell and Gray, 1987).

1.1.1 Soil structure

Soil is made up of minerals, organic matter, liquid material, gaseous material and living organisms (Gray and Williams, 1971; Baver *et al.*, 1972; Alexander, 1977). The mineral fraction (approximately 50 % of the total volume) is comprised of sand (50-2000 μm) and silt (2-50 μm) which are primary aluminosilicates originating from parental rock, and clay particles (<2 μm) which are secondary aluminosilicates such as kaolinite, montmorillonite and illite (Gray and Williams, 1971; Alexander, 1977; Lynch, 1983). Clay particles are extremely important with respect to their influence on chemical and physical properties of soil, due to the net negative charge of clay particle surfaces and their large specific surface area (*e.g.* 50 $m^2 . g^{-1}$ of kaolinite). The presence of these negatively charged particles affects the distribution of soil ions. Cations such as H^+, K^+, Na^+, Ca^{++} and Mg^{++} form a diffuse layer of positive charge around clay particles and thus facilitate binding to negatively charged bacterial surfaces. These

cations (usually low valency) can move away from clay particles and constitute the exchangeable cations of a soil. For kaolinite, illite and montmorillonite, the cation exchange capacities (CEC) are about 5, 20 and 100 milliequivalents 100 g^{-1}, respectively (Lynch, 1983). Clay minerals hold water against the pull of gravity due to their surface activity, the degree of retention decreasing the further water molecules are from the clay surface, until at some point (dependent on clay type) gravitational pull exerts its influence. Clays exist as coatings on larger sand and silt particles, and as microaggregates and aggregates which are stabilized by organic matter and precipitated inorganic material (Stotzky *et al.*, 1990). Aggregates (usually 0.5 to 5 mm in diameter) retain water and form microhabitats for soil microbes. Spaces between microhabitats make up the pore space.

Organic matter (3-6 % of the total volume) is comprised of dead plant, animal and microorganism material in various stages of decomposition, as well as humus (namely humic acid, fulvic acid and humin) which represents incompletely decomposed organic materials. Humus is extremely resistant to decomposition, and is colloidal, able to exchange and hold basic cations and therefore form close associations with secondary aluminosilicates, resulting in colloidal clay, the most chemically reactive component of soil. Organic matter is unevenly distributed throughout profiles of podsolic soils whereas in brown earths they are more uniformly dispersed.

Soil water forms a liquid nutrient medium for microbial growth, and varies in its availability for growth depending on soil porosity, volume of water in the soil and degree of adsorption to clay particles. As soil becomes drier, water in the pore spaces is replaced by air. Generally, CO_2 concentration increases with depth while O_2 decreases, although concentrations in a given volume do not reflect those at particular microsites, as with all soil components.

1.1.2 Nutrient status of soil and effect on microbial communities

The major input of soil organic matter is plant litter which provides a relatively poor balance of the nutrients C, N and P in terms of microbial nutrition. The proportion of N and P compared to C depletes with soil depth, although microbial degradation of plant litter is only nitrogen-limited during the early stages of detrital decay (Nedwell and Gray, 1987). The nutrient status of soil, despite large intermittent inputs of organic carbon, is energy-limited with regard to microbial growth, a consequence of which is that net microbial growth and production rates are slow. Microorganisms capable of predominating in these nutrient-poor conditions are defined as oligotrophic, which require far lower concentrations of energy-yielding carbon compared to copiotrophs, which require high nutrient concentrations (Williams, 1985). Oligotrophs (*e.g. Arthrobacter* and *Caulobacter* spp.) typically have a low substrate saturation constant (K_s) and a low maximum specific growth rate (μ_m), whereas copiotrophs (*e.g. Bacillus*, *Pseudomonas* and *Streptomyces* spp.) usually have a high K_s and μ_m. Hence soil oligotrophs grow slowly with long doubling times, whereas soil copiotrophs multiply quickly at times of relatively high nutrient availability. Analysis of bacteria from nutrient-poor paddy field soil and nutrient-rich grassland soil has shown oligotrophs to be numerically dominant in the nutrient-poor soil, whilst only 50 % of the bacterial population of the nutrient-rich soil comprised of oligotrophs (Kasahara and Hattori, 1991). It is often difficult to ascribe the term oligotrophy to certain soil isolates recovered on nutrient-poor media, as they may have, or gain the ability to grow on nutrient-rich media. Semenov (1991) proposed the separation of heterotrophic soil microorganisms into three groups according to their reaction norma (*i.e.* limits of optimal environmental conditions within which particular populations may grow). Reaction norma were based on organic carbon concentration and structure of assimilated organic compounds, and variability of limits of efficiency of metabolism, substrate affinity and a metabolism rate-determining step. These three

microorganism groups were two groups which had narrow reaction norma at either extreme of organic carbon concentration, and a third group representing the majority of soil microorganisms with wide reaction norma spanning the two extremes. The existence of these three groups represents a soil community capable of utilizing nutrients at all stages of decomposition.

1.1.3 Soil microflora

The soil microflora includes all known types of microorganisms, the general groups of which are the protozoa, algae, fungi, actinomycetes, unicellular bacteria, and viruses (Lynch, 1983). The proportions and species of each group vary from soil to soil and, within a given soil, from microsite to microsite. The proportions and presence or absence of groups and diversity will depend on the environmental factors of a particular soil, as well as variations in conditions within that particular environment. Fungi usually form a major part of the soil biomass, accounting for about 70 % by dry weight, with bacteria (including actinomycetes) being the next most abundant microorganisms. Soil microorganisms contribute significantly to the release of minerals and carbon dioxide for plant growth, as well as being largely responsible for nutrient turnover and hence soil fertility. Therefore they are important factors for plant growth in soil, which accounts for the majority of mankind's food supply (Gray and Williams, 1971). Nutrient turnover includes the cycling of essential elements, and as examples, the nitrogen, carbon and sulphur cycles are outlined below.

The nitrogenous material of dead plant and animal matter fallen onto soil undergoes ammonification due to saprophytic microorganism activity (or putrefaction in anaerobia). The resulting NH_4^+ ions, which are exchangeable with clay complexes, are then nitrified to NO_2^- (*e.g.* by *Nitrosomas* spp.), then oxidized to NO_3^- (*e.g.* by *Nitrobacter* spp.) (Atlas and Bartha, 1981; Lynch, 1983). In anaerobic conditions, NO_3^- is denitrified to N_2, for example by *Pseudomonas*

denitrificans. NO_3^- (or N_2 via nitrogen fixation bacteria such as free-living *Azotobacter* spp. and symbiotic *Rhizobium trifolii*) can then be utilized by plants. Nitrogen limitation in soil is caused by competition for NO_3^- between plants and nitrophilic microorganisms such as cyanobacteria which can store nitrogen as cyanophycin and phycocyanin (Dawes, 1985).

Carbon is most actively cycled through reservoirs of atmospheric CO_2, the dissolved forms of CO_2 (H_2CO_3, HCO_3^- and CO_3^{--}) and organic matter. The involvement of soil microorganisms in carbon cycling is best discussed in terms of a food web. All food webs are based on primary producers (*i.e.* autotrophs, able to convert CO_2 to organic matter), which among microorganisms include algae, cyanobacteria, and the green and purple photosynthetic bacteria. However, the main source of primary production in soil is plant activity, and microbial biodegradation of plant polymers is the major factor in carbon cycling in soil (Atlas and Bartha, 1981). Biodegradative soil microorganisms reintroduce CO_2 into the atmosphere by respiration, immobilize carbon in the form of organic soil substances such as humic acid, and produce simpler organic molecules which can be utilized by other microbial populations. The major plant polymers degraded by microbial action in soil are cellulose, hemicelluloses, starch and lignin. Cellulolytic activity in soil is significantly exhibited by fungi (*e.g. Aspergillus*, *Fusarium* and *Trichoderma* spp.) and bacteria (*e.g. Cytophaga*, *Cellulomonas*, *Streptomyces* and *Nocardia* spp.). Cellulolytic activity in soil is very pH dependent: at pH <5.5, filamentous fungi predominate, whereas at higher pH values, bacteria can predominate. Predation of biodegraders by protozoans, viruses, *Bdellovibrio* spp. and other predatory bacteria such as *Ensifer adhaerans* also contributes to cycling carbon back into the main active reservoirs.

Most of the reactions that cycle sulphur through the ecosphere are performed by microorganisms (Atlas and Bartha, 1981). A wide variety of microorganisms can de-sulphurize organic sulphur-containing compounds. The final product of decomposition of organic sulphur compounds under aerobic conditions is SO_4^{--},

and under anaerobic conditions is H_2S. H_2S can be nonbiologically oxidized to SO_4^{--} in the presence of O_2, or biologically via a series of chemolithotrophic oxidations (*e.g.* by *Thiobacillus* spp.). SO_4^{--} can be utilized by some microorganisms as the terminal electron acceptor in anaerobic respiration. These microorganisms which include the genera *Desulfovibrio*, *Desulfuromonas*, *Desulfotomaculum* and *Desulfomonas* (Lynch, 1990) carry out dissimilatory SO_4^{--} reduction and are obligate anaerobes. They produce H_2S which causes the characteristic rotten egg-like odour of anaerobic soils and sediments. Many plants and microorganisms perform assimilatory reduction of SO_4^{--}, resulting in the incorporation of sulphur into proteins and other sulphur-containing biochemicals.

1.2 The genus *Streptomyces*

Actinomycetes are important both numerically in natural ecosystems, and as producers of many important secondary metabolites, notably antibiotics and enzymes (Locci, 1989). The order Actinomycetales is traditionally classified as a group of aerobic Gram-positive bacteria whose vegetative growth form is a mycelium of branching hyphae, which at some stage of development may produce spore-bearing hyphae. This definition has problems, for example application of molecular and chemical techniques to classification shows discontinuous correlation of the composition and boundaries of the order when compared to classification using morphological characteristics (Goodfellow, 1989). Suprageneric classification of actinomycetes has been achieved using partial sequencing of 16S ribosomal RNA (rRNA), a ubiquitous genetically stable molecule with highly conserved sequences (Goodfellow, 1989). This involves digesting rRNA and sequencing resulting oligonucleotides, which are then compared to oligonucleotide sequences of known isolates in a computer-stored rRNA catalogue. Similarity between two organisms is calculated as the number of unique oligonucleotides that they share divided by the mean of the total number of unique oligonucleotides (Johnson, 1989). The resulting

value can be applied to clustering programmes to produce a matrix of taxanomic clusters based on degrees of rRNA homology. Streptomycetes have been clustered in this way, and include *Streptoverticillium* and *Streptomyces* spp., and moreover, rRNA based homology studies have reliably classified the family *Streptomycetaceae* as a genetically defined suprageneric group (Goodfellow, 1989; Locci, 1989). Sub-generic classification presently relies largely on numeric taxonomy, involving the comparison of 139 phenotypic character units (chemical and morphological), and using clustering programmes to assign *Streptomyces* type strains to 23 major clusters (Locci, 1989). *Streptomyces* spp. exhibit vegetative hyphae which are highly branching and rarely fragment. The aerial mycelium develops into chains of three to many spores (Locci, 1989), which are bound in a hydrophobic bi-layered sheath (Ensign, 1978). They are heterotrophic with an oxidative type of metabolism, and their DNA has a relatively high guanine and cytosine (G+C) content (69-78 mol. %). Streptomycetes are the most likely actinomycetes to be isolated from any habitat, and *Streptomyces* is the dominant genus in almost all soils (Locci, 1989; McCarthy and Williams, 1990). Over 90 % of the known natural antibiotics are produced by the actinomycetes, in addition to pigments, extracellular enzymes, vitamins and the terpenoid compounds which give soils their characteristic odour (Ensign, 1978). In connection to the production of antibiotics, they are also resistant to many antibiotics including rifampicin, neomycin and penicillin G (Locci, 1989). Numerical importance in soil is underlined by their production of many important enzymes, which include ureases, amylases, xylanases and chitinases (Locci, 1989), revealing their role in nutrient cycling in soil.

1.2.1 Physiology and ecology of streptomycetes in soil

Streptomycetes exist largely as spores unevenly distributed in soil, usually being found close to concentrated areas of organic matter where they were formed following mycelial colonization (Williams, 1978). Spores are a desiccation-resistant

semi-dormant (CO_2 evolution from ungerminated spores has been detected) stage which can persist in soil for long periods (Mayfield *et al.*, 1972; Ensign, 1978). Spores enable survival of streptomycete communities throughout periods of nutrient deficiency or famine. The survival capacity of spores is greater than that of the mycelium. Spores added to nonsterile soil have a very low efficiency of germination, probably due to competitive inhibition by indigenous soil microorganisms. Pre-germinated spores added to nonsterile soil will grow for a short time and then sporulate, followed by the general disappearance of the mycelium (Lloyd, 1969). Spores have thicker walls and the presence of an external sheath gives them greater hydrophobicity than hyphal fragments, hence they can be distributed throughout soil ecosystems by water movement as well as by arthropod disturbance (Goodfellow and Simpson, 1985). The presence of nutrients allows germination (the presence of Ca^{++} is essential) and rapid colonization of organic substrates such as fragments of dead fungal hyphae and plant root ensues. Spore germination is related to local spore density and nutrient concentration, as intrapopulation regulation of spore germination frequency in nonsterile soil is dependent on spore density, although this regulation is not interspecific (Triger *et al.*, 1991). The sites of hyphal growth are restricted in size, limited by local nutrients which on exhaustion influence the formation of aerial hyphae and spores. This cellular differentiation is closely regulated by a complex of genetic signals (Chater *et al.*, 1988; Davis and Chater, 1992), and the nutrient status of a particular growth medium will only influence the timing of sporulation following hyphal growth, *i.e.* streptomycetes will sporulate on nutrient-rich media given time. Spore ribosomes are more stable than hyphal ribosomes, and as the spore cytoplasmic constituents are very similar to those of mycelium, this suggests that spores are essentially a dispersal phase, fully prepared for rapid germination when opportunities to colonize organic matter arise (Ensign, 1978). Dormant spores of *Streptomyces antibioticus* contain about one third of the number of ribosomes found in mycelium. Although spore ribosomes display lower activity (protein synthesis)

than substrate hyphae ribosomes, this activity quickly rises to that of a fully grown substrate mycelium during initiation of germination (Quiros *et al.*, 1989). When compared to other natural bacterial groups, the mycelium is a unique adaptation to growth on solid media, primarily soil, and is a method of increasing the size of growth propagules by elongation. The life-cycle of actinomycetes is a good example of convergent evolution when compared to the filamentous fungi. The mycelium facilitates greater coordination of substrate utilization, nutrient translocation and spread between aggregates than seen with unicellular bacteria (Locci, 1988). The filamentous growth kinetics of *Streptomyces* spp. are similar to those of fungi in that total mycelial length and branch number increase exponentially at the same specific rate (Allan and Prosser, 1983). Allan and Prosser (1985) also suggested that the production of secondary metabolites products was equally as important as nutrient limitation in controlling colony development.

Streptomycetes perform an important role in soil in transformation of recalcitrant polymers such as chitin, starch, lignin and hemicelluloses, and secrete a range of extracellular enzymes important for soil biodegradation (McCarthy and Williams, 1992). Although the group is generally regarded as neutrophilic and predominates in neutral soils, a survey of a wide range of acidic soils and man-made wastes showed that there are many acidophilic streptomycetes (predominantly *Streptomyces* spp.), whose physiological characteristics, apart from pH optima, differ significantly from those of neutrophilic streptomycetes (Williams *et al.*, 1971; Khan and Williams, 1975). Acidophilic streptomycetes appear to be important also in the successional development of soils, in that they can grow in direct competition with acidophilic fungi, and due to ammonification by chitinolytic activity, raise the soil pH beyond the pH optima of predominant fungi (Williams and Robinson, 1981). This can lead to neutrophilic streptomycetes becoming more numerous at microsites where there is ammonia adsorption to organic particles (Williams and Mayfield, 1971), and eventually leads to the development of neutral soils which can support broader diversities of microflora. Water tension is important in the success

of streptomycete populations in soil. Growth tends to be optimal in humid pore spaces containing air, whereas waterlogging tends to suppress growth of these aerobes (Williams *et al.*, 1972).

The production of antibiotics by many streptomycetes, a vast majority of which were isolated from soil, has prompted much research and speculation into the possible production and role of antimicrobial secondary metabolites in soil (Williams and Vickers, 1986). Although antibiotics have not been isolated from natural untreated soils, production has been detected in nonsterile amended and sterile unamended and amended soils when inoculated with antibiotic-producing strains of streptomycetes and fungi (Williams, 1982). Possible reasons for the lack of detection in untreated soils include instability of the compounds, adsorption to clay colloids in an unextractable form, inadequate sensitivity of detection techniques and the oligotrophic nature of soil resulting in sporadic and limited growth of producers. If they are produced in natural soil ecosystems, antibiotics could have several roles. Firstly, their antimicrobial action would give selective advantages to producing strains. There is evidence to support the occurrence of antibiosis in soil, for example survival of a *Salmonella* sp. in soil was inhibited by the presence of streptomycin-producing *Streptomyces bikiniensis* (Turpin, *et al.*, 1992). Thiostrepton induces the production of several proteins via its effect on a thiostrepton sensitive promoter (*tipA*) in *Streptomyces lividans* (Murakami *et al.*, 1989). These proteins may be capable of thiostrepton degradation (C.J. Thompson, personal communication), and therefore may indicate the presence of a second type of resistance in strains which do not contain the thiostrepton resistance gene *tsr*. Secondly, it is possible that streptomycete secondary metabolites play a role in cellular differentiation, for example A-factor from *Streptomyces griseus* induces it to form aerial mycelium (Murakami, 1988; Beppu, 1992). Secondary metabolites may also play a role in establishing stable microbial communities via their influence on differentiated growth in other microbes, such as stimulation of fruiting body formation of basidiomycetes, *i.e.* influence the development of populations within

communities (Beppu, 1992). However, the defence mechanism theory is supported by the fact that many antibiotics are produced during nutrient depletion and onset of sporulation, and would therefore suppress other opportunistic microbes which could take advantage of streptomycete dormancy phases. The production of antibiotics which are insoluble in water, such as thiostrepton, may result in mycelium becoming coated with a layer of antibiotic because the insoluble nature of the molecule would hinder diffusion away from producing mycelium. This coating could inhibit utilization of nutrients from dead mycelium by other bacteria, allowing re-colonization of that microsite by a germinating producer of the antibiotic.

During and following periods of activity in soil, streptomycetes have the potential to influence the ecology of other soil organisms (including plants via rhizosphere interactions) due to the production of a plethora of enzymes and possibly antibiotics. They may also affect soil organisms by producing siderophores, which chelate iron and thus antagonize growth of non-siderophore-producing organisms in low iron environments, for example *Actinomyces* spp. produce ferrioxamine siderophores (Lynch, 1990). The discontinuous periods of rapid growth experienced by streptomycetes in soil allow for a high probability of genetic interactions. Genetic interactions in soil have many implications with regard to evolutionary changes in soil microorganisms as well as influencing their ecology, and therefore the subject warrants a detailed overview before considering the streptomycetes in particular.

1.2.2 Genetic interactions in soil

In a diverse community of microorganisms in the soil ecosystem, there is the potential for different types and varying degrees of interactions, both within species in addition to between different species and different major groups. These interactions include competition, predation, parasitism, commensalism, mutualism and signalling by specific molecules. Genetic interactions in natural ecosystems have

received much interest over recent years (reviews: Stotzky and Babich, 1986; Trevors *et al.*, 1987; Stotzky *et al.*, 1990), largely because of concerns over the release of genetically engineered microorganisms into the environment.

The nature of bacterial gene transfer mechanisms has been analysed using laboratory strains, and whilst these may not be representative of organisms in their natural habitat, use of these strains in model environments demonstrates how genetic interactions may occur in natural microbial communities (Trevors *et al.*, 1987; Stotzky *et al.*, 1990). Three types of gene transfer in natural environments are recognized; transformation, conjugation and transduction (protoplast fusion may occur at very low frequencies in the environment, but this type of transfer is as yet uninvestigated).

Transformation is the uptake of naked double stranded DNA by competent cells (cells which can be transformed). The incoming DNA is integrated into the host chromosome, with which it also replicates. Many bacterial genera are naturally competent, being part of the organism's life cycle, whereas others can be induced into competency. There is potential for transformation to occur in soil because of the presence of DNA released from dying cells or secreted from living cells. Naked DNA may persist in soil for long periods by adsorption to particulates, especially clays, although it may be also rendered biologically inaccessible by its adsorption or become quickly degraded, depending on local conditions. Khanna and Stotzky (1992) reported that DNA from *Bacillus subtilis* could transform that species in soil when bound to montmorillonite, and that binding did not alter the transforming abilities of the DNA. Furthermore, frequency of transformation was found to be influenced by local conditions such as pH and temperature, and binding to the clay protected the DNA from DNase action. Naturally competent *Acinetobacter calcoaceticus* will take up free high molecular weight DNA (including chromosomal and plasmid DNA) regardless of the homology or heterology of that DNA, implying a potential for intergeneric genetic interactions (Lorenz *et al.*, 1992).

Conjugation is the process by which plasmids mediate their own transfer from donor to recipient. Transfer occurs via sex pili on donor cells which interact with specific sites on the surface of recipient cells. The transfer operon of conjugative plasmids codes for all the conjugative functions required for transfer, and these plasmids can also mediate transfer of non-conjugative plasmids, *i.e.* by mobilization. Conjugative plasmids may also integrate into host chromosomes and cause transfer of chromosomal genes during conjugation. Plasmids are ubiquitous in microbial communities and are often present in multiple copies in cells, the copy number being generally higher for smaller plasmids and lower for larger plasmids (the general size range is between 1 and 300 kb). Many phenotypes are determined by plasmids (*e.g.* antibiotic resistance, biodegradation of simple and complex compounds, phage restriction and many others). Plasmids, which can exert metabolic burdens on their hosts due to encoding of various functions, can be lost from cells. This may occur especially if selection for a particular plasmid-borne gene such as a heavy metal resistance gene is lost (Freter, 1984; Saunders, 1984, cited by Stotzky and Babich, 1986). Conjugation is the most widely studied mode of genetic interaction in soil. An essential requirement for conjugative interactions is cell-to-cell contact, so the semi-static nature of soil is limiting in this respect, with spatial positioning governing the probability of contact. Soil nutrient status generally causes reduced metabolic rates in soil microbes (Trevors *et al.*, 1987; section 1.1.2) and therefore affects conjugation which depends on the metabolic state of donors and recipients.

Transduction is the bacteriophage-mediated transfer of genetic material between bacterial cells (Kokjohn and Miller, 1992). Two types are recognized, the first being generalized transduction, whereby any genetic element (chromosomal or plasmid) can be accidentally packaged into the phage capsid instead of the phage genome. This occurs when a transducing phage infects a host via the lytic life-cycle (section 1.3.1). No more than 5 % of host chromosome, which may include any gene or genes, can usually be packaged in this way and the resulting transducing

particle is capable of adsorbing to susceptible hosts and injecting the DNA. This DNA may then integrate into the new host's chromosome, although because of the lack of phage DNA in this particle, no further infection functions can occur (Stotzky *et al.*, 1990). The second type of transduction is referred to as specialized transduction, which can only occur via the lysogenic life-cycle of temperate phages (section 1.3.2). Specialized transduction starts during induction of the prophage from the host chromosome, when a segment of the host DNA adjacent to the phage integration site is accidentally excised with the phage DNA. The combined phage and host DNA is then packaged into the phage capsid as in the normal lytic cycle, and the resulting transducing particle can then infect and lysogenize a new host, and integrate the old host DNA into that of the new host along with the prophage. The resulting transductants contain a defective prophage, often immune to superinfection. Another mode of phage-mediated genetic interaction between bacteria is known as lysogenic conversion, by which cells exhibit phenotypes specifically encoded by temperate phages which have lysogenized them (Kokjohn and Miller, 1992). This phenomenon has been most commonly demonstrated by the expression of antibiotic or heavy metal resistance genes present on temperate phages which lysogenize hosts, and this system has been used to evaluate the possibilities of transduction in soil (Germida and Khachatourians, 1988; Zeph *et al.*, 1988; Zeph and Stotzky, 1989; Herron and Wellington, 1990). Nucleic acid packaged in phage particles is protected from environmental attack compared to naked DNA, and persistence may be imparted by the adsorption of phage to clay particles (Zeph *et al.*, 1988). Phage particles may be carried between aggregates via water movement through soil fissures (McKay *et al.*, 1993), and therefore may facilitate wide zones of genetic interaction in soil ecosystems compared to conjugation or transformation. Transduction and lysogenic conversion may therefore be the most important factors in genetic interactions in soil. The biology and ecology of bacteriophages will be further discussed in sections 1.3 and 1.4.

1.2.3 Genetic interactions between streptomycetes in soil

Genetic interactions between *Streptomyces* spp. *in vitro* have been well documented, particularly with regard to plasmid and chromosomal transfer which requires fusion of the hyphae of recipient and donor, analogous to fusion between donor and recipient cells via pili in conjugation in Gram-negative bacteria (review: Hopwood *et al.*, 1986). Furthermore, *Escherichia coli* is capable of transferring plasmids via conjugal transfer to *Streptomyces* spp. (Mazodier *et al.*, 1989). Transduction of *Streptomyces* spp. *in vitro* by at least two known transducing actinophages is possible (Stuttard, 1979; Süss & Klaus, 1981), and interspecific transfer of cosmids mediated by a temperate actinophage has also been reported (Morino *et al.*, 1988).

Conjugation and lysogenic conversion (but not transduction or transformation) have so far been studied as modes of genetic interactions between *Streptomyces* spp. in soil. The copiotrophic life-style *Streptomyces* spp. in soil (Williams, 1985) leads to short periods of high rates of metabolic activity during intermittent nutrient availability, and these periods of comparatively intense growth permit a high probability for genetic interactions (Trevors *et al.*, 1987). The presence of common antibiotic resistance and synthesis genes in a wide variety of streptomycetes in diverse soil ecosystems suggests gene transfer has occurred many times throughout their history (Wellington *et al.*, 1992). Many of these genes are borne on plasmids of *Streptomyces* spp., and provide useful markers in studying conjugative interactions between streptomycetes, as well as being useful tools in studying their life-cycle *in situ*. Wellington *et al.* (1990) reported interspecific transfer of the plasmid pIJ673 between *Streptomyces violaceolatus* ISP5438 (pIJ673) and *S. lividans* TK24 in nonsterile soil. Use of SEM observations showed that mycelial growth and therefore conjugation had occurred within two days of spore germination in soil, after which re-sporulation had occurred and no further conjugative transfers took place. Frequency of plasmid transfer was increased

dramatically when the soil was nutrient amended, reflecting a correlation between metabolic activity in soil and genetic interactions. Peak transconjugant numbers and plasmid DNA in soil correlated with peak abundance of parental strains and mycelium at day fifteen, as observed in a sixty day study on conjugative transfer between *S. violaceolatus* ISP5438 (pIJ673) and *S. lividans* TK24 in nonsterile soil (Cresswell *et al.*, 1992). This corresponded to lysogenic conversion of inoculated *S. lividans* TK24 by KC301 occurring at the same time in a similar experiment, illustrating that genetic interactions were only occurring at peaks of host activity. Temperate actinophages can be readily isolated from soil (Dowding and Hopwood, 1973; Stuttard and Dwyer, 1981; Ogata *et al.*, 1985; Foor *et al.*, 1985; Tilley *et al.*, 1990), suggesting the potential for transduction as a natural aspect of interactions in the soil ecosystem. Lysogenic conversion of *S. lividans* strains in sterile and nonsterile soil by the temperate actinophage KC301 can occur, and intraspecific gene transfer between *S. lividans* strains mediated by KC301 has also been reported in sterile soil (Herron and Wellington, 1990; Herron, 1991). The ecology of actinophage and streptomycete communities in soil will be further discussed in section 1.4.5.

1.3 Bacteriophages

Bacteriophages are viruses that infect bacteria. Since their discovery in 1917 they have been used extensively in the study of molecular biology, mainly by virtue of their quick and easy propagation in laboratory bacterial cultures and the small genome size (Douglas, 1975; Freifelder, 1987). Morphologically, the most common types of phages amongst all bacteria are those belonging to the A and B groups (of which types belonging to the A1 an B1 groups are the most common), which consist of isometric heads attached to tails which are respectively sheathed or unsheathed, all of which comprise of protein subunits (Bradley, 1967; Reanney and Ackermann, 1982). Other main types are the tail-less minute head phages and the filamentous

phages. Most knowledge of phages is based on research involving coliphages, notably the T-even series and phage-lambda, so although most of the following review is based on these phages, it can be tentatively applied to the majority of phages, especially those of the A and B groups.

The protein head-sheath or capsid of a phage is a hollow case of polyhedral (isopolyhedral in the case of A1 and B1 groups) plates which are made up of protein subunits called capsomeres. Head size is generally between 50 and 100 nm depending on type (Douglas, 1975; Chater, 1986). The tail, attached to the head via the protein collar, is a noncontractile helical arrangement of protein monomers, around which (in all A type but not B type phages) is a contractile helical arrangement of protein monomers called the protein sheath. Tails vary greatly in length depending on type, and can be up to 200 nm. At the end of the tail is the attachment apparatus required for infection of host cells, which consists of the end plate (usually a hexagon of protein), below which are the tail pins which assist in securing the phage to the host cell surface. Some types have long fibres attached to the end plates which assist in anchoring the phage to bacterial surfaces when they first come into contact. The phage genome is contained within the head, and is double-stranded DNA in the case of A and B types, or single stranded DNA or RNA in the case of the tail-less head and filamentous phages.

Phages may be regarded as bacterial parasites which can persist in the environment outside their hosts, but have the absolute requirement of infecting bacterial cells within which to replicate if they are to multiply and survive as dynamic populations (Freifelder, 1987). In order for multiplication to occur, a phage must be able to protect its genome from environmental damage, deliver that genome into the host bacterium, convert the host into a phage-producing system to produce multiple progeny phage, and then release that progeny back into the environment where future infections may take place. Depending on phage type, two types of infection cycle are possible. Virulent phages follow the lytic cycle only (section 1.3.1), in which the host is lysed. Temperate phages can either follow the

lytic cycle or the lysogenic cycle (section 1.3.2) depending partly on environmental conditions, where lysis of the host does not occur and the phage genome exists as an integrated replicable entity in the host chromosome. Filamentous phages follow neither cycle, but attach to the pili of F^+ Gram-negative cells, which continue to grow and produce progeny phages at the same time which are secreted from the cell without lysis (Duckworth, 1987).

1.3.1 The lytic cycle

Adsorption of the phage to the host cell surface occurs in three stages (Duckworth, 1987). Initial contact occurs due to free movement of the host cell in a liquid medium and by diffusion and Brownian motion of the phage. Reversible binding follows contact (aided by tail plate fibres where present) caused by electrostatic forces which may require the presence of divalent cations such as Ca^{++} and Mg^{++}. This is followed by irreversible binding to specific receptors (the specificity for which is determinative of host range), mediated by the tail plate pins. Enzymes in the tail plate act on the host cell membrane to allow piercing by the tail, caused by contraction of the sheath in the case of A phages (*e.g.* T4). The phage DNA is probably drawn into the cell cytoplasm by interaction with molecules there moving at greater velocities than encountered in the capsid (Douglas, 1975), although there is some evidence, in the case of T-even phages, that the tail does not actually penetrate into the lumen of the host, so that the phage DNA is delivered to the outside of the membrane then taken up by some other mechanism such as transformation (Simon and Anderson, 1967, cited by Duckworth, 1987). Phage multiplication then proceeds as follows. Early mRNA encoded by the phage genome (which requires host RNA polymerase) causes the host ribosomes to make early proteins, enzymes which shut-off the cell's RNA and protein synthesis, degrade the cell DNA to subunits ready for phage genome assembly, and construct copies of the phage genome. With double-stranded DNA phages, the original genome and new

copies are copied through several generations of replication, during which transcription of phage genes encoding structural proteins occurs. These proteins and phage genome copies are then assembled into infectious mature progeny particles called virions, at which point phage-induced lysozyme production causes breakdown of cell wall components such as murein. As a consequence the cell bursts, liberating the progeny virions into the environment. Substantial amounts of lysozyme are also released into the environment at this point.

1.3.2 The lysogenic cycle

Environmental conditions which can cause lysogeny are depleted nutrient concentrations and high multiplicities of infection (infecting phage-to-bacteria ratio). Therefore conditions in which host growth and multiplication is under threat increase frequencies of lysogeny, *i.e.* to be contained within possibly dormant hosts for long periods of unfavourable environmental conditions increases the chances of the survival of phage populations. The recognized factors which influence the choice between lytic or lysogenic cycles once the phage genome is inside the host cytoplasm are viral and host genotypic expression and environmental conditions, which also affect host physiology and genotypic expression (Herskowitz and Hagen, 1980; Herskowitz and Banuett, 1984; Echols, 1986). Central to the choice in pathway (in phage lambda) seems to be the cII protein, encoded by the phage, which controls the expression of the *int* and *xis* genes necessary for phage integration and excision, and continued expression of the repressor protein cI, the *c* gene product (see later). Low levels of cII protein result in lysis, whereas high levels result in lysogeny. The host Hfl protein antagonizes the establishment of lysogeny by inhibiting cII activity, and other host proteolytic activities are probably responsible for cII degradation to a lesser extent. Hence the lysis-lysogeny switch is largely governed by the balance of the Hfl-cII relationship. Protein cIII, another phage-encoded protein required for lysogenization is known to inhibit Hfl, and is

therefore an important factor in monitoring multiplicity of infection (MOI), *i.e.* a high MOI leads to high cIII levels, Hfl is inhibited and lysogeny can ensue. There are other possible influences on Hfl activity, for example there is strong evidence that cAMP-CAP is a negative regulator (*i.e.* harsh environmental conditions such as starvation and lack of utilizable carbon source leads to lysogeny in this way by stimulating increased cAMP-CAP levels).

Some infections of a given population of host bacteria by temperate phage will be lytic (section 1.3.1) but others, due to the conditions discussed above, will be lysogenic. Adsorption and delivery of phage DNA into the host cytoplasm occurs as in the lytic cycle, after which the course of lysogenic infections takes a different route. The phage genome first circularizes by joining at the *cos* site, the cohesive ends of the phage genome. At the start of the lysogenic cycle, a repressor gene (*c*) on the phage genome is transcribed, as well as the *int* gene which encodes the recombinase enzyme, necessary for the integration of the prophage into the host chromosome. The product of the *c* gene is a repressor protein(s) which binds to specific sites on the phage genome and prevents the synthesis of mRNA encoding lytic functions (Freifelder, 1987; Campbell, 1993). The *int* gene product is a site-specific recombinase which catalyses a precise cross-over reaction between the phage and host attachment sites (*attP* and *attB* respectively, which are homologous sequences), the result of which is the integration of the phage genome into the host chromosome. For most temperate phages, there is only one integration site. Known as the prophage, the phage DNA (flanked by the combined *attP* and *attB* sites) continues to express only the *c* gene, which maintains the prophage state as well as preventing superinfection by homologous phage or heterologous phages with areas of genome homologous to the *c* gene product binding site. The now lysogenized host is termed lysogenic (Lwoff, 1953), and is not harmed by the prophage, which is replicated along with the host chromosome during cell division. A certain proportion of a multiplying lysogenic population will spontaneously release phage at a very low frequency, *i.e.* the prophage of some lysogens reverts to the lytic cycle.

This prophage induction is usually brought about by cellular damage, especially host DNA damage by agents such as U.V. light and mitomycin C, which signals derepression (*i.e.* halts expression of the *c* gene), although induction naturally occurs at a very low frequency in healthy cells, the reason for which is unknown. Loss of the repressor protein allows expression of the *xis* gene, the product of which is excisionase, which catalyses the excision of the prophage from the *attB* site (Freifelder, 1987). With the absence of the *c* gene product, the lytic cycle can proceed as outlined in section 1.3.1.

The presence of prophages in lysogenic hosts can have effects on the host phenotype, a phenomenon termed as lysogenic conversion. This is most commonly manifested as superinfection immunity caused by the presence of the *c* gene product as reviewed above. Prophages can also cause superinfection immunity, or alter phage susceptibility, by encoding changes in the structure of phage-specific binding sites on the cell surface such as encountered in lysogenic *Salmonella* spp. and *E. coli* (Duckworth, 1987). Other types of lysogenic conversion include the encoding of toxins in strains of *Corynebacterium diphtheriae*, rendering them pathogenic, and the expression of heavy metal and antibiotic resistance genes (section 1.2.2). The presence of prophage DNA can be a burden to the host, by causing the diversion of cellular resources to the synthesis of foreign DNA and for the expression of prophage encoded functions such as resistance and superinfection immunity (Cooper and MacCallum, 1984).

Lysogeny may be an evolutionary advantage for phages, as 90 % of all phages isolated are temperate (Freifelder, 1987). The evolutionary advantage of being temperate has been analysed by mathematical modelling of situations involving various combinations of virulent phage, temperate phage and hosts (Stewart and Levin, 1984). Models demonstrated that temperate phages were maintained in mixed bacterial populations (lysogenic and sensitive), and could coexist with virulent phages. Advantages are conferred on both hosts and temperate phages in comparison to virulent phage-host systems. Temperate phages are

maintained inside hosts when the host population oscillates below the level necessary for phage to survive by the lytic cycle alone, and the usually low rates of prophage induction result in a relatively negligible impact on host density. The production of free phage and also lysozyme liberated from hosts during lysis by a lysogen population can be seen as "weapons", whereby other sensitive bacteria in a particular niche can be lysed and render resources in that niche available to the lysogens. The steady state of lysogen-phage systems is however influenced by environmental conditions, especially nutrient concentration. Other factors which can increase induction frequency, such as temperature shifts, also affect steady state, as Dykhuizen and Hartl (1983) reported an increase in temperature of a chemostat culture of a lysogenic *E. coli* resulted in a 10^{-2}-fold drop in density, although normal density was regained after a certain period at the optimum temperature. These workers also showed selective advantages or disadvantages of lysogens in the presence of isogenic uninfected counter-parts in chemostats, depending on temperate phage type.

Another type of lysogeny, nonintegrative lysogeny, occurs with certain phage types (*e.g.* coliphage P1), which do not integrate into the host chromosome, but exist as a circularized, repressed genome outside the host DNA. This plasmid-like entity replicates at the same rate as the chromosome *i.e.* once at cell division, with each daughter cell inheriting a phage genome copy. Thus nonintegrative lysogens inherit the ability to lyse and produce an infective phage progeny like integrative lysogens.

1.3.3 Survival of bacteriophages

Phages are found in all environments where their hosts exist, and are generally susceptible to the same physical and chemical factors which can harm or kill their hosts (Douglas, 1975). For example, detergents, phenolic substances and alkalis will attack the protein coat of phages, elevated temperatures cause

inactivation (loss of infectivity), and ionizing and non-ionizing radiations cause DNA damage.

When regarding phage populations in terms of their predatory or parasitic relationship with their hosts, one must consider the dynamics which govern the coexistence of predators and the prey populations they require to survive on. The dynamic requirement for long-term survival is that a predator or parasite population must not predate all available hosts in a given habitat, as the sudden loss of hosts (or crash in hosts to a sub-minimal level) would lead to the extinction of the local predator population. This means that in natural predator-prey systems, some mechanism such as density dependent regulation exists to ensure that both populations coexist (Alexander, 1981). Mathematical models have been used to make predictions on the outcome of interacting phage and host populations in simple systems, and these have been partially confirmed by conducting experiments in liquid cultures (Lenski, 1988). A stable coexistence of phage and host populations is represented as oscillations of constant frequency and amplitude of both population densities, with the predator oscillations lagging behind those of the prey. Rises in bacteria density are at first resource-limited, until the density reaches a point where phage can infect and significantly reduce the bacterial density. This results in a rise in phage density and a corresponding drop in bacteria density, which eventually reaches a level below the minimum infective density of the phage population (at least in terms of impact on host numbers). The phage density then falls whilst the bacteria density resumes rising. The stability of these oscillations is affected by various environmental conditions such as resource concentration. For example, increased resource concentration can result in large peaks in bacterial growth, and the subsequently more intense phases of phage infection may lead to ever bigger oscillations until both populations crash. Another important consideration in host-phage interactions is how the evolution of both populations affects interactions. The large population sizes and short generation times of bacteria and phage populations facilitate the occurrence of mutations which may confer temporary advantages to

one population over the other. This is commonly manifest as mutations towards immunity to infection on the part of bacteria, and counter-mutations to overcome new immunities on the part of phages (Lenski, 1984). The result of such mutations leads to polymorphic phage and host populations where originally there was only one phenotype each of predator and prey (Cooper and MacCallum, 1984). A common factor in the mutation of susceptible bacteria to immune clones is that the mutants are in some way disabled compared to the wild-type parents, usually apparent as reduced substrate utilization capacity. Chao *et al.* (1977) reported that such mutants of *E. coli* (compromised in glucose utilization) were out-competed in continuous culture with the wild-type. The presence of a lytic phage (T7) which could infect the wild-type but not the immune mutant allowed the mutant to exist at its normal monocultural density so long as the wild-type and T7 existed in steady state. This is an example of phages stabilizing competing bacterial populations in resource-limited habitats. Caution must be taken when considering these models and experiments, as they are based on homogeneous and/or controlled conditions in liquid culture, and will not be entirely representative of situations in natural ecosystems.

The potential for mutations is always present in communities, especially when considering all the modes of genetic interactions which may take place, as discussed in section 1.2.2. Furthermore, phage genomes are known to contain interchangeable modules or cassettes which may contain part or whole gene(s). Inversion of these occurs regularly during infection cycles, and they can potentially be exchanged between different phage types infecting the same cell, thus creating new strains of phage (Schneider and Kutzner, 1989; Schmeiger, 1990). For example, the G-region of the temperate coliphage Mu is invertible, and depending on its orientation encodes a different type of protein in the phage tail, thus controlling the specificity of binding to different host species. Therefore a population of Mu has two different host ranges depending on the G-region orientation of individuals, hence increasing survival probability.

1.4 Ecology of bacteriophages in natural environments

Phage ecology has received surprisingly little attention in comparison to bacterial ecology, the main body of phage research being directed at the use of phages in molecular biology. A review of the previous sections shows that phages are integral components of bacterial communities, with regard to their ubiquity in addition to the potential they have for mediating interactions between populations, as well as altering and controlling populations by their various impacts. Increasing the understanding of the role of phages in natural environments will enhance knowledge of the ecology of bacterial communities. For example, specific phages have been used to study complex interactions between bacterial predator populations in soil, and host activity in soil (Germida and Casida, 1983; Germida, 1986).

1.4.1 Bacteriophage ecology in freshwater and marine environments

Enumeration of freshwater and marine viruses by counting plaque forming units (p.f.u.) on indicator bacteria plates gave very low densities (1 to 10^3 p.f.u. ml^{-1}; Primrose *et al.*, 1982; Farrah, 1987) in relation to bacterial densities, so it was initially assumed that phages had little ecological importance in these ecosystems (Miller and Sayler, 1992). The continually fluxing environment of aquatic habitats means there is an infinite possibility of a phage particle and host cell meeting at some point, however low the probability (Kokjohn *et al.*, 1991). In natural ecosystems under certain circumstances, this probability may be infinitely low, resulting in a minimum phage and/or host density for interactions to take place (Wiggins and Alexander, 1985). These conclusions were based on observations in aquatic microcosms, where the barriers to host refuge (in the form of spatial avoidance of phage-host contact) set up by microcosm walls would increase probability of phage spread through a host population, and thus result in a host population crash as in clearing of broth cultures in one-step growth experiments in

flasks (Douglas, 1975). An analogy to such phage ecology studies in aquatic microcosms was illustrated by results of infection studies of *Drosophila* C virus (DCV) in vials (Scotti *et al.*, 1981). The virus spread into all susceptible flies in vials when one infected fly was added, given sufficient time for contact between flies to take place. Similar epizootics of *Drosophila* spp. have not been reported in natural environments, probably due to low mean densities of hosts. Therefore DCV epidemics might only occur when the local host density exceeds a minimum value, comparable to bacterial densities in water rising to levels which facilitate phage multiplication (Wiggins and Alexander, 1985) due to increases in local productivity. Recent studies have used direct counting, such as transmission electron microscopy (TEM), to estimate phage numbers, and have shown that phage densities can be as high as 10^6 to 10^{10} p.f.u. ml^{-1} while bacteria can range from 10^3 to 10^7 c.f.u. ml^{-1} (Bergh *et al.*, 1989; Bratbak *et al.* 1990; Hara *et al.*, 1991; Miller and Sayler, 1992). Peaks in phage numbers typically follow peaks in bacterial numbers during periods of relatively high phytoplankton and bacterial activity such as during spring blooms in marine ecosystems. These peaks are then followed by drops in bacterial numbers, and subsequently phage numbers. Therefore it seems that bacterial populations in such habitats may be controlled by phage populations, and that these interactions are dependent on environmental conditions favouring host growth such as optimal temperatures and nutrient status, which are in turn controlled by seasonal variations (Bratbak *et al.*, 1990; Moebus, 1992; Proctor *et al.*, 1993). Proctor *et al.* (1993) also estimated that *in situ*, as much as 31 % of marine *Vibrio* spp. may be infected at any one time, and up to 62 % of the population mortality was due to lytic infections. The extent to which viruses control productivity of marine phytoplankton remains debatable based on the evolution of resistant hosts (Oloffson and Kjelleberg, 1991; Suttle *et al.*, 1991), although resistance mutations of hosts may be limited by the high diversity of host and virus populations in marine communities. This host diversity would allow continued multiplication of polyvalent viruses if one host population evolved resistance. Ogunseitan *et al.* (1990) used lake

water microcosms to demonstrate that *Pseudomonas aeruginosa* and phage populations existed in a state of dynamic equilibrium as described in section 1.3.3., and furthermore, that infection, pseudolysogenization and lysis occurred independently of host density.

Distribution of freshwater and marine phages appears to be directly related to that of their hosts, as might be expected. Hence phages of enterobacteria are readily isolated from freshwater systems and inshore (but not off-shore) marine environments, due to the distribution of faecal material and sewage (Farrah, 1987; Moebus, 1987; Paul *et al.*, 1993). Phages of indigenous bacteria include those which infect *Pseudomonas*, *Aeromonas* and *Hyphomicrobium* spp. in freshwater systems (Farrah, 1987; Ogunseitan *et al.*, 1992), and those which infect *Vibrio* spp. and cyanobacteria in marine systems (Moebus, 1987). Inorganic and organic particulate matter can affect the survival of phages in water, largely by acting as binding sites, and either prolonging the persistence of phages by adsorption or rendering them inaccessible to hosts. The presence of organic matter, such as humic and fulvic acids in freshwater can also affect phage multiplication by providing suspended nutrient substrates for hosts. The ionic composition of water appears to be fundamentally important in phage survival, especially with regard to Ca^{++} and Mg^{++} which aid adsorption to cells, and this factor is strongly influenced by ambient pH. Suttle and Chen (1992) showed that solar radiation was the most important factor in inactivation of marine phages in shallow coastal waters, although this depended on clarity of defined water columns and depth of sampling (*i.e.* protection from U.V. radiation).

Transduction (section 1.2.2) has been investigated in freshwater systems (review; Kokjohn and Miller, 1992), and Saye *et al.* (1987) demonstrated the transfer of *P. aeruginosa* plasmid Rms149 by the generalized transducing phage øDS1. No studies have so far established the occurrence of phage-mediated genetic interactions in marine systems, although it is very likely transduction is possible in

this ecosystem given the large numbers of hosts and phages, and the high rates of infection.

1.4.2 Bacteriophage ecology in soil

The infectivity of phages in liquid environments such as lake water can be enhanced by the presence of clay minerals, due to adsorption to mineral surfaces causing stabilization (Babich and Stotzky, 1980). In soil, free phages are in comparatively closer relationships with the solid elements, and therefore the physical interactions between soil solids and phages are fundamental considerations in their ecology. Depending on type, clays can exert beneficial or deleterious effects on phages in soil. Different phages can have different binding sites on clays (Lipson and Stotzky, 1985a), facilitating the coexistence of several free phage populations. Sykes and Williams (1978) reported that adsorption to calcium and sodium montmorillonite expanded the pH optima of actinophages, whereas adsorption to sodium (but not calcium or aluminium) treated kaolin reduced this range. Adsorption rates to sodium treated kaolin were much higher than to montmorillonite, probably due to strong positive end charges on kaolin, and adsorption can prolong infectivity depending on clay and phage type (Lipson and Stotzky, 1985b). All soil actinophages (adsorbed or free) are inactivated below about pH 5.0 however, and no actinophage can be isolated from acidic soils, although acidophilic streptomycetes can be infected by actinophages from neutral soils (Sykes *et al.*, 1981). Adsorption to clays is directly related to the clay cation exchange capacity (CEC) and is strongly influenced by the presence of cations. Lipson and Stotzky (1983) noted that montmorillonite (high CEC) adsorbed reoviruses at a much higher rate than kaolin (low CEC), although the presence of divalent cations greatly enhanced the adsorption of both. The type of cation saturating the exchange complex of a clay greatly affects the adsorption of phages, as shown by the different rates of adsorption by kaolin homoionic to different

cations described above (Sykes and Williams, 1978). Proteins in soil bind to clay particles depending on clay type, cation type in the colloid bilayer and protein type. As with phages, montmorillonite binds proteins to a greater extent than kaolin (Dashman and Stotzky, 1984), and the binding of proteins can cause physical changes to clay particle properties such as expansion of montmorillonite (but not kaolinite) particles by proline and arginine (Dashman and Stotzky, 1985). The binding of proteins to clays renders them available as local nutrients for bacteria also bound to clays, although utility of the proteins by bacteria depends on location and degree of protein binding (Dashman and Stotzky, 1986). Thus proteins (and other nutrients) bound to clays directly influence phages both in competition for binding sites on particles (Lipson and Stotzky, 1984), and by facilitating close associations between potential hosts and clay particles to which phages are bound. Phages adsorbed to clays may also act as substrates for nutrition of adsorbed bacteria (Lipson and Stotzky, 1985c).

Temperature, apart from its known effects on the infective duration of free phages (Douglas, 1975), also influences the proliferation of phages in relation to host growth optima in soil. Hence Reanney and Marsh (1973) deduced that because proliferation of phages attacking *Bacillus stearothermophilus* in soil was higher at 45°C than at 55°C, the host's optimal growth temperature in soil must be in a mesophilic range rather than thermophilic as when grown *in vitro*. The nutrient status of soil results in unevenly alternating periods of growth and inactivity because of discontinuous substrate availability, and this causes a problem for virulent phages which require metabolically active hosts for proliferation (Williams *et al.*, 1987). The wider host range of virulent phages compared to temperate phages isolated from soil may be one adaptation to this problem. Virulent phages can however survive for long periods of host inactivity in soil, as Germida (1986) demonstrated that although *Azospirillum brasilense* phage became undetectable in soil after a long period of host inactivity, addition of viable host cells and nutrients caused a burst in phage numbers, indicating the free phage was still present in unrecoverable form.

Pantastico-Caldas *et al.* (1992) found a similar trend with a temperate phage of *B. subtilis* when adding nutrients to sterile soil previously seeded with host and phage, and furthermore noted that the temperate phage population had no impact on the host density in soil, whereas a virulent phage population in the same conditions caused a 10^{-1}-fold decrease. Although virulent phages can persist for long periods in the free state in soil, it may be postulated that temperate phages are selected for in soil because of their negligible impact on host density, and ability to survive protected within lysogenic host populations for long periods of time (section 1.3.2). Hence the majority of soil phages might be lysogenic or pseudolysogenic (Williams *et al.*, 1987).

1.4.3 The actinophage øC31

Phages capable of infecting streptomycetes can be readily isolated from most soils, and those infecting *Streptomyces* spp. are the most common, reflecting their ubiquity in soil ecosystems (Williams and Lanning, 1984). *Streptomyces* spp. phages can be polyvalent or specifically infect individual species (Wellington and Williams, 1981). The isolation of temperate phages capable of infecting *S. coelicolor* A3(2) (Lomovskaya *et al.*, 1970, cited by Lomovskaya *et al.*, 1980; Dowding and Hopwood, 1973), the best studied *Streptomyces* sp., enabled the study of relationships between phages and differentiated bacteria. The most extensively studied actinophage is øC31 which was isolated from *S. coelicolor* A3(2) by induction (reviews: Lomovskaya *et al.*, 1980; Chater, 1986), and is widely used as a cloning vector in *Streptomyces* spp. genetics. Lomovskaya *et al.* (1971) proved that it was a true temperate phage by demonstrating segregation of the prophage in crosses between lysogenic and uninfected strains (in precisely the same way as other genetic markers do). About 30 to 40 % of cells infected by øC31 will be lysogenized (section 1.3.2), the mean burst size (in *S. lividans* 66) is 55, and 1 % and 10 % of lysogenic spores of *S. coelicolor* A3(2) and *S. lividans* 66 will

spontaneously release phage on germination respectively (Lomovskaya *et al.*, 1972; Lomovskaya *et al.*, 1980; Chater, 1986). It has a wide host range and infects about half the 137 strains it has been tested against (Voeikova *et al.*, 1979; Chater, 1986). Its physical structure, similar to most actinophages, places it in group B1 of the phage classification cited in section 1.3. It has a polyhedral head 53 nm wide, a noncontractile tail of 5 by 100 nm, and a basal plate of 15 nm with at least one tail pin (Suárez *et al.*, 1984). The phage DNA is 41.5 kilobases (kb) long, has a G+C content of 63 mol. % and cohesive ends of short single stranded complementary termini (Chater, 1986). Based on molecular weight only, the virion appears to have three major proteins in common with those of phage lambda, although these are not necessarily identical in primary structure (Chater, 1986). The phage genome is a linear molecule with the 3 kb *c* gene (repressor gene, section 1.3.2) located at the centre, to the left of which is a region which encodes essential late functions. To the right is about 10 kb of essential DNA encoding early functions, beyond which is 8 kb of inessential (for the lytic cycle) DNA which includes the *attP* site required for prophage integration, and at least two functions which interact with the host restriction-modification (RM) function of *S. coelicolor* A3(2) (Chater *et al.*, 1985). The øC31 prophage is integrated between *uraA* and *pheA* on the *S. coelicolor* chromosome (Lomovskaya *et al.*, 1980). The *c* gene encodes three distinct proteins involved in the repressor function, possibly autoregulated by the smallest of these proteins binding to several loci throughout the øC31 genome (Smith *et al.*, 1992). Hyphal fusion between growing cultures of lysogenic and uninfected strains can result in zygotic induction, caused by plasmid-reliant crossover of lysogenic host DNA to the recipient, the resulting drop in the *c* gene product's concentration leading to phage induction (Emel'yanova *et al.*, 1973; Lomovskaya *et al.*, 1980). Although *S. lividans* and *S. coelicolor* are closely related (Davis and Chater, 1992; Leblond *et al.*, 1993), one major difference is the phage growth limitation (Pgl) system of *S. coelicolor* A3(2) which encodes the RM function, of which there is no homologous DNA in *S. lividans*. If øC31 infects a Pgl^+ host such as *S. coelicolor*

A3(2), the phage is modified in some way (the mechanism is still unknown), so that after lysis, the progeny phages cannot reinfect a Pgl$^+$ host, as they can now be recognized due to the modification and thus be restricted on entry of the genome into the cytoplasm. Modified øC31 can still infect Pgl$^-$ strains such as *S. lividans* and Pgl$^-$ mutants of *S. coelicolor* (*e.g. S. coelicolor* J1501), although once propagated by a Pgl$^-$ strain, the progeny øC31 will have lost the modification of its progenitor, and can now infect Pgl$^+$ strains (Laity *et al.*, 1993). The RM function also works against phages homoimmune but not heteroimmune to øC31. The homologous sequence in the host and phage *att* sites is only three bases (TTG), which is the shortest known region for site specific recombination in phages and integrative streptomycete plasmids (Rausch and Lehmann, 1991). The coding region for the probable øC31 recombinase, protein p68, is close to *attP*, and becomes separated from its promoter upon integration. Additionally, p68 has no similarity to any of the known family of site-specific recombinases (Kuhstoss and Rao, 1991). Therefore, although øC31 bears many similarities to the B1 phages infecting Gram-negative hosts, the presence of fundamentally differing functional proteins (p68 and the three *c* encoded proteins) suggests an early divergence in the evolution streptomycetes from the unicellular eubacteria.

1.4.4 Ecology of actinophages in soil

There have been very few studies on the ecology of actinophages in soil, most being qualitative observations of phage persistence in relation to the various physical factors of soil (review: Williams *et al.*, 1987; section 1.4.2). Estimates of dynamics in soil related to host and actinophage densities, based on a mathematical model, predicted that high efficiencies of infection (section 2.21) would result in over-loading of the host population by phages. This would cause a crash in hosts followed by the extinction of the phage population. Lower efficiencies of infection would allow a steady state of coexistence between hosts and phage (Williams *et al.*,

1987). Herron and Wellington (1990) used a mutant of øC31, KC301 (Chater *et al.*, 1982) which carries the thiostrepton resistance gene *tsr*, to study interactions with *S. lividans* in sterile and nonsterile soil. Bursts in free KC301 titre in soil directly corresponded to periods of germination of host spores, and lysogenic thiostrepton resistant populations due to infections in soil were readily established. Furthermore, KC301 mediated genetic interactions between different *S. lividans* strains (Herron, 1991), manifested as lysogenic conversion of a sensitive strain by KC301 released from the lysogen in soil.

1.5 Aims

The general aim of this project was to expand knowledge of the ecology of actinophages with respect to interactions with hosts in soil, based on the initial findings of Herron and Wellington (1990) and Herron (1991). The specific aims were:

1. To establish the potential for density dependence to influence interactions and therefore the impact of phage populations on hosts in soil.
2. To determine whether the temperate life-style enables long-term survival of temperate phages in soil.
3. To evaluate the potential impact caused by the presence of a prophage and phage-borne genes on lysogenic and uninfected hosts.
4. To determine the survival capacity of a genetically engineered actinophage (KC301) in natural soil, and ability to spread into indigenous hosts.

CHAPTER 2

Materials and methods

2.1 Bacterial strains

Streptomycete strains used as hosts for phage propagation and in soil microcosm studies were *S. lividans* TK24 and *S. lividans* TK23 (streptomycin and spectinomycin resistant mutants of *S. lividans* 1326 respectively; no known plasmids, Hopwood *et al.* (1985). Source; T. Kieser), and *S. coelicolor* J1501 (streptomycin resistant *pgl*$^-$ mutant of *S. coelicolor* M130, histidine and uracil auxotroph, no known plasmids, Chater *et al.* (1982). Source; D.A. Hodgson). *Staphylococcus aureus* (ATCC 6538P) was used for bioassays of thiostrepton extracted from soil.

2.2 Phage

Actinophage used were øC31 (temperate wild type, Lomovskaya *et al.*, 1970, cited by Lomovskaya *et al.*, 1980. Source; M.J. Buttner) and KC301 (Chater *et al.*, 1982. Source; K.F. Chater). KC301 is a mutant of øC31 which carries the thiostrepton resistance gene *tsr* (Thompson *et al.*, 1980). Thiostrepton resistance is expressed by hosts when lysogenized by KC301.

2.3 Media

All media were made up with distilled water and sterilized by autoclaving at 121°C for 15 minutes unless otherwise stated. Media and their constituents not listed in Hopwood *et al.* (1985) are given in Table 2.1, and a list of antibiotics is given in Table 2.2.

Table 2.1 Media

Medium (reference)	Constituents per litre[1] (supplier) unless otherwise stated	
R2.5[2]	sucrose	51.5 g
(Hopwood *et al.*, 1985)	K_2SO_4	0.125 g
	$MgCl_2.6H_2O$	5.05 g
	glucose	5 g
	casamino acids (Difco)	0.05 g
	yeast extract (Oxoid)	2.5 g
	LabM agar (Amersham)	22 g
	TES buffer (BDH)	2.87 g
	trace elements solution	1 ml
	(5 ml 0.5% (w/v) KH_2PO_4)	
	(2 ml 5 M $CaCl_2.2H_2O$)	
	(7.5 ml 20% (w/v) L-proline	
	(FSA))	
	(3.5 ml 1 N NaOH)	
trace elements solution	$ZnCl_2$	40 mg
(Hopwood *et al.*, 1985)	$FeCl_3.6H_2O$	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

Table 2.1 Media (continued)

Medium (reference)	Constituents per litre[1] (supplier) unless otherwise stated	
oatmeal agar (OMA)	fine oatmeal[3]	20 g
	yeast extract (Oxoid)	5 g
	LabM agar (Amersham)	15 g
reduced arginine starch salts agar (RASS) (Herron & Wellington, 1990)	L-arginine (Sigma)	0.1 g
	soluble starch (BDH)	12.5 g
	K_2HPO_4	1 g
	NaCl	1 g
	$MgSO_4.7H_2O$	0.5 g
RASS (continued)	(1 ml 1 % (w/v) $Fe_2(SO_4)_3.6H_2O$)	
	(1 ml 0.1 % (w/v) $CuSO_4.5H_2O$)	
	(1 ml 0.1 % (w/v) $ZnSO_4.7H_2O$)	
	(1 ml 0.1 % (w/v) $MnSO_4.4H_2O$)	
	LabM agar (Amersham)	15 g
	pH 8.0	
phage buffer (APB) (Stuttard, 1979)	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

Table 2.1 Media (continued)

Medium (reference)	Constituents per litre[1] (supplier) unless otherwise stated	
peptone broth (Lomovskaya *et al.*, 1972)	Peptone (Oxoid)	10 g
	NaCl	5 g
	glucose	10 g
	NaH_2PO_4	1.25 g
	KH_2PO_4	0.42 g
	L-tryptophan (Sigma)	0.02 g
	LabM agar (Amersham)[4]	2 g
	pH 7.0	
	(1 ml 0.05 g/ml $CaCl_2$)	
	(1 ml 0.042 g/ml $MgSO_4$)	

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

[2]The amounts of the constituents for R2.5 were exactly half those of R5 (Hopwood *et al.*, 1985).

[3]The fine oatmeal was added to the distilled water, boiled, and left to stand at 70°C for 30 minutes before adding the agar and yeast extract, and autoclaving.

[4]0.2 % (w/v) agar was included in peptone broth when germinating spores to prevent cohesion.

Table 2.2 Antibiotic stocks

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

All antibiotics were supplied by Sigma, except nystatin which was supplied by BDH.

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

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

[3]Dissolved in methanol

[4]Dissolved in distilled water and filter sterilized through a 0.22 μm nitrocellulose filter (Minisart, Sartorius).

[5]Dissolved in chloroform

[6]Defined media refers to RASS; complex to R5, R2.5 and OMA, and liquid to TSB.

2.4 Buffers, reagents and solutions

Buffers, reagents and solutions were made up with distilled water and sterilized at 121°C for 15 minutes where appropriate. Buffers, reagents and solutions not listed in Hopwood *et al.* (1985) are given in Table 2.3.

2.5 Growth and maintenance of *Streptomyces* spp. cultures

S. lividans strains were grown on OMA (Table 2.1) plates, and *S. coelicolor* J1501 was grown on R2.5 plates containing appropriate antibiotics at 30°C for five to seven days. Spores were harvested from a well sporulated plate by flooding it with 9 ml of SDW and scraping the spores off the substrate mycelia with a sterilized inoculating loop. The spore suspension was transferred to a sterile universal bottle and vortexed for about one minute and filtered through sterile non-absorbent cotton wool. Following this, the suspension was centrifuged at 1600 x g for ten minutes. The supernatant was discarded and the pellet was vortexed briefly after being resuspended in 20 % (v/v) glycerol and stored at -20°C (Hopwood *et al.*, 1985). Stocks were sub-cultured every six months.

Liquid cultures of *S. lividans* were grown in 50 ml of TSB with appropriate antibiotics in 250 ml flasks (fitted with springs) using a Gallenkamp Orbital Shaker (180-200 rpm) at 30°C.

2.6 Growth and maintenance of actinophage

Phage stocks were grown according to Hopwood *et al.* (1985), and stored at 4°C in nutrient broth or APB. The lysate was serially diluted in NB and 100 µl spotted onto 5 cm NA plates. SNA (0.8 ml cooled to 45°C) containing spores of *S. lividans* TK24 (*ca.* 10^7 c.f.u. ml^{-1}) was then mixed with the lysate. The plates were

incubated at 30°C for twenty-four hours. Lysates were sub-cultured every six months.

Table 2.3 Buffers, reagents and solutions

Reagent (reference)	Constituents (supplier)	
buffers for DIG DNA Labeling and Detection Kit Nonradioactive (Boehringer Mannheim):		
buffer 1	2 M Tris-HCl, pH 7.5	50 ml
	5 M NaCl	30 ml
	distilled water to	1000 ml
buffer 2[1]	buffer 1	100 ml
	blocking reagent	1.5 g
	(vial 11 of kit)	
buffer 3	1 M Tris-HCl, pH 9.5	100 ml
	5 M NaCl	20 ml
	1 M $MgCl_2$	50 ml
	distilled water to	1000 ml
buffer 4	TE buffer	

Table 2.3 Buffers, reagents and solutions (continued)

Reagent (reference)	Constituents (supplier)	
hybridization solution[1] for DIG DNA Labeling and Detection Kit Nonradioactive (Boehringer Mannheim)	20 x SSC	25 ml
	blocking reagent (vial 11 of kit)	1.5 g
	N-lauroylsarcosine, Na-salt (Sigma)	100 mg
	10 % (w/v) SDS	200 µl
	distilled water to	100 ml
nutrient broth, egg albumin (Lanning & Williams, 1982)	nutrient broth (Difco)	8 g
	distilled water to	1000 ml
	pH 8.0	
	autoclave in 50 ml portions, cool to room temperature, then add 1 ml of 5 % (w/v) egg albumin (Sigma) per portion	

[1]Prepare at least one hour in advance by dissolving the blocking reagent in the solutions at 70°C.

2.6.1 Preparation of phage lysates

Lysates were prepared by the method of Hopwood *et al.* (1985). A well isolated single plaque grown as in section 2.6 was transferred to 2 ml of NB using a sterile Pasteur pipette and soaked at 22°C for two hours. The NB was then sterilized by filtration through a 0.45 μm nitrocellulose filter (Minisart, Sartorius), and the filtrate was assayed as in section 2.6. This method typically yielded *ca.* 10^8 p.f.u. ml^{-1} lysate.

2.6.2 Preparation of high titre lysates

High titre lysates were prepared using the method based on Stuttard (1979). About 2 x 10^4 p.f.u. were spotted onto each of two 9 cm NA plates, which were then inoculated with SNA (2.5 ml, seeded with *ca.* 10^7 c.f.u. ml^{-1} of *S. lividans* TK24 spores) and incubated at 30°C for twenty-four hours. Each plate was flooded with 13 ml of APB and soaked at 22°C for two hours. The APB from both plates was combined and centrifuged at 110,000 x g at 4°C for seventy five minutes. The pellet was resuspended in 2 ml of APB and sterilized by filtration through a 0.45 μm nitrocellulose filter (Minisart, Sartorius) and assayed as described in section 2.6.

2.7. Growth and maintenance of lysogenic spores

The method of Hopwood *et al.* (1985) was used to grow and maintain lysogenic stocks. R5 plates containing streptomycin were spread with 100 μl of spore suspension (*ca.* 10^9 c.f.u. ml^{-1}) of *S. lividans* TK24 or *S. coelicolor* J1501. After being allowed to dry thoroughly, *ca.* 20 μl of KC301 lysate prepared as in section 2.6.1 was spotted onto the plate, which was then incubated at 30°C until the area within the spot was sporulating. The plate was replicated to OMA (*S. lividans* TK24) or R2.5 (*S. coelicolor* J1501) containing streptomycin and thiostrepton, and

incubated at 30°C until sporulation occurred in the area corresponding to the original spot. Lysogens could then be purified by single colony isolation, again on OMA or R2.5 containing the two antibiotics. Spore suspensions in 20 % (v/v) glycerol were prepared from single colony isolates as described in section 2.5, although the spores were harvested from the sporulated OMA or R2.5 plates using 25 mM tetrasodium pyrophosphate rather than SDW. The spores were allowed to stand in the tetrasodium pyrophosphate for thirty minutes before being centrifuged at 1600 x g for ten minutes, and then resuspended in 20 % (w/v) glycerol. These lysogenic stocks were checked by spotting onto SNA overlays containing *S. lividans* TK24 on NA and incubating at 30°C for twenty-four hours. Turbid plaques caused by spontaneous phage release from lysogenic colonies resulted. The lysogenic stocks were also checked by growth on R5 containing streptomycin and thiostrepton. These stocks were checked for free phage by filtering 200 μl through a 0.45 μm membrane filter (Ministart, Sartorious), and assaying for free phage as described in section 2.6. This check was carried out immediately after preparation of lysogenic spore suspensions, and immediately before their use in experiments.

2.8 Preparation of KC301 DNA

Phage DNA was prepared and stored using the small scale preparation of *Streptomyces* spp. phage DNA method of Hopwood *et al.* (1985). About 2 x 10^4 p.f.u. were spotted onto each of three 9 cm NA plates and overlaid with 2.5 ml of molten SNA (cooled to 45°C, containing *ca.* 10^7 c.f.u. ml^{-1} of *S. lividans* TK24). These were incubated at 30°C for twenty-four hours, after which the SNA was scraped into 12 ml of NB in a sterile Oakridge 50 ml polycarbonate tube. The mixture was sucked up and down a sterile 10 ml pipette twice, and then soaked at 22°C for two hours. The mixture was centrifuged at 14000 x g to remove the SNA. Phage were pelleted by centrifuging the supernatant at 110,000 x g at 4°C for seventy five minutes. The supernatant was discarded, and 400 μl of RNase solution

was added to the pellet, which was then incubated in a Gallenkamp orbital shaker at 37°C for twenty minutes. This mixture was transferred to a 1.5 ml Eppendorf tube, 80 μl of SDS mix was added, and it was incubated at 70°C for thirty minutes. Immediately, 100 μl of 8M potassium acetate was added and the mixture placed on ice for fifteen minutes. The mixture was sedimented by centrifuging at 12000 x g for ten minutes, and the supernatant transferred to a fresh Eppendorf tube. An equal volume of isopropanol was mixed in and this was left to stand at room temperature for five minutes. The DNA was pelleted by centrifuging at 12000 x g for five minutes, and the supernatant was discarded. The pellet was then dissolved in 700 μl of 300 mM sodium acetate, mixed with 700 μl of isopropanol, left at room temperature for five minutes. The DNA was again pelleted by centrifuging at 12000 x g for five minutes, and the supernatant discarded. This pellet was dissolved in 50 μl of 300 mM sodium acetate and 50 μl of isopropanol, before being finally centrifuged at 12000 x g for five minutes, and the supernatant discarded. The DNA pellet was resuspended in 50 μl of sterile TE buffer, and stored at 4°C.

2.9 Preparation of *Streptomyces* spp. DNA

Streptomyces spp. (laboratory strain and wild type isolates) DNA was prepared and stored using the rapid small scale isolation of *Streptomyces* spp. total DNA procedure of Hopwood *et al.* (1985). Spores (*ca.* 10^7 c.f.u.) were inoculated into 50 ml of TSB plus appropriate antibiotics in a 250 ml conical flask, and incubated in a Gallenkamp orbital shaker (200 rpm) at 30°C for twenty-four hours. Mycelium (50 mg) was resuspended in a 1.5 ml Eppendorf tube in 500 μl of lysozyme solution, and incubated at 37°C until the mixture became translucent. SDS (250 μl) was then added and vortexed to reduce the viscosity of the solution. Neutral phenol chloroform (250 μl) was added, vortexed for thirty seconds, and then centrifuged at 12000 x g for two minutes. The upper aqueous layer was removed and re-washed in neutral phenol chloroform as above until the white

protein interface between the two liquid layers disappeared. The aqueous layer of the final wash was added to a clean sterile Eppendorf tube, and 0.1 vol of 3M sodium acetate, pH 4.8 was mixed in before mixing in 1 vol of isopropanol and standing at room temperature for five minutes. The mixture was centrifuged at 12000 x g for two minutes, and the supernatant removed. The DNA pellet was redissolved in 500 μl of TE buffer and 25 μl of spermine-HCl (Sigma) and then stood at room temperature for five minutes. The mixture was centrifuged at 12000 x g for two minutes and the supernatant removed. The pellet was vortexed in 300 μl of 0.3 M sodium acetate, 10 mM $MgCl_2$, and then 700 μl of ethanol was mixed in before standing at room temperature for one hour. The DNA was pelleted by centrifuging at 12000 x g and the supernatant removed. The pellet was resuspended in 100 μl of TE buffer and stored at 4°C. Preparations were checked by mixing 2 μl of DNA with 2 μl of 5 x loading buffer and 6 μl of SDW, and electrophoresing through a 25 ml 1 % agarose (w/v) in TBE mini-gel containing 0.5 μg. ml^{-1} of ethidium bromide at 80 volts for thirty minutes (Hopwood *et al.*, 1985). A 1 kb DNA size marker (Bethesda Research Laboratories) mixed using the same conditions as the test DNA was also run on all gels to check band sizes.

2.10 Restriction endonuclease digestion of phage and *Streptomyces* spp. DNA

DNA restriction digestions were carried out by mixing about 5 μg of DNA (in TE buffer), 3 μl of 10 x enzyme buffer, 2 μl of enzyme (Gibco BRL). This was made up to 30 μl with SDW, and incubated at 37°C for two hours. Digestions were checked on a mini-gel as described in section 2.9. For Southern blotting analysis, a volume of the digestion mix corresponding to about 0.3 μg of restricted DNA was mixed with 5 μl of 5 x loading buffer and made up to 30 μl with SDW and run on a 100 ml 1 % agarose (w/v) in TBE gel containing 0.5 μg. ml^{-1} of ethidium bromide at 25 volts for sixteen hours. A 1 kb DNA size marker was also run on this gel. The DNA was transferred to nylon membrane (HybondN) using the method of Hopwood

et al. (1985). DNA was fixed to the filter by exposing it (DNA side) to short wave ultraviolet radiation for three minutes. The filter was sealed in a plastic bag and stored ready for hybridization.

2.11 Preparation of filters for colony hybridization

Filters for colony hybridization were prepared by the method of Hopwood *et al.* (1985). A nylon (HybondN) filter (sterilized by autoclaving) was aseptically placed on a R5 plate (Hopwood *et al.*, 1985) supplemented with appropriate antibiotics. Colonies for hybridization (including positive and negative controls) were picked onto the filter, and incubated at 30°C for twenty-four hours. The filter was then placed on three sheets of 3M (Whatman) paper soaked in lysozyme solution (4 mg. ml^{-1} in TE buffer), and incubated at 37°C for forty five minutes. The filter was then placed on three sheets of 3M paper soaked in 1 % SDS in 1 M NaOH for twenty minutes. The filter was then dried on a sheet of 3M paper, and transferred to a further three sheets soaked in 7 vols 1 M Tris-HCl, pH 7.5, 3 vols 5 M NaCl for five minutes. This stage was repeated three times. The filter was briefly placed on a sheet of 3M paper and then dried at room temperature. DNA was fixed to the filter as described in section 2.10.

2.12 Preparation of filters for plaque hybridization

The method of Hopwood *et al.* (1985) was followed during this procedure. Plaques (including positive and negative controls) were picked onto a 23 cm bioassay plate of NA overlaid with SNA containing *ca.* 10^7 c.f.u. ml^{-1} of *S. lividans* TK24, and incubated at 30°C for twenty-four hours. The plate was kept at 4°C for two hours to harden the SNA. A sterile nylon (HybondN) filter was placed on the surface of the plate and left at room temperature for ten minutes. The filter was then transferred to three sheets of 3M paper soaked in 0.5 M NaOH for ten

minutes. After this it was put on three sheets of 3M paper soaked in 1 M Tris-HCl, pH 7.5 for two minutes (this stage was repeated once). The filter was finally placed on three sheets of 3M paper soaked in 1 M Tris-HCl, pH 7.5, 1.5 M NaCl for five minutes. The filter was dried at room temperature and the DNA was fixed to the filter as described in section 2.10.

2.13 Hybridization with ^{32}P-labelled DNA probes

Hybridization with ^{32}P-labelled DNA probes was used for colony hybridizations because DIG labelling (see section 2.14) of colony DNA resulted in non-specific binding of the antibody conjugate to colony proteins, thus making the distinction of positive and negative results impossible. Method B of Hopwood *et al.* (1985) was used with a Random Primed DNA Labelling Kit (Boehringer Mannheim) instead of nick translation, and incorporated label was separated from unincorporated by the method described in section 2.13.3. Prehybridization, hybridization and stringency washes were carried out in a Hybaid Mini Hybridization Oven, rather than using the polythene bag method of Hopwood *et al.* (1985), although conditions and solutions were the same.

2.13.1 Prehybridization

The oven was pre-heated to 70°C. The nylon filters to be hybridized were soaked in 2 x SSC, and stacked, separated by sheets of nylon mesh also soaked in 2 x SSC. The filter/nylon mesh stack was then rolled up and placed in a hybridization bottle. About 20 ml of 2 x SSC was put into the bottle which facilitated unrolling the filter/nylon mesh stack so that it stuck to the inside of the bottle. The 2 x SSC was then replaced with 5 ml (small bottles) or 10 ml (large bottles) of prehybridization fluid (Hopwood *et al.*, 1985). The bottle was then fixed into the rotisserie of the oven, ensuring that when it rotated the filter/nylon mesh stack did

not unroll. Prehybridization was carried out by rotating the bottle at 70°C for at least three hours.

2.13.2 Labelling of probe

DNA to be used as a template was ethanol precipitated as follows. To a measured volume of DNA stock (in TE buffer), 0.1 vol of 3 M sodium acetate and 2 vols of ice cold ethanol were added, mixed and placed at -20°C for sixteen hours. The precipitated DNA was pelleted by centrifugation at 12000 x g for ten minutes and then washed in ice cold 70 % (v/v) ethanol. After further centrifugation at 12000 x g for ten minutes, the DNA was dried under vacuum and resuspended in SDW (Herron, 1991).

A volume of DNA in SDW corresponding to about 5 μg of DNA was made single stranded by boiling for ten minutes and immediately placing on ice. A Random Primed DNA Labelling Kit (Boehringer Mannheim) containing all the necessary solutions (except $dGT^{32}P$) was used to label the probe. The following reaction was set up: 2 μl of 10 x reaction mix (containing hexanucleotide primers), 1 μl dATP, 1 μl dTTP, 1 μl dCTP, 2 μl of single stranded DNA (corresponding to 5 μg), 7 μl of SDW, 5 μl of $dGT^{32}P$ (50 μCi, Amersham) and 1 μl of Klenow enzyme. This mix was incubated at 37°C for thirty minutes. The reaction was stopped by adding 2 μl of 5 x loading buffer.

2.13.3 Separation of incorporated from unincorporated label

A hole was pierced in the bottom of a 0.5 ml Eppendorf tube with a size 25 syringe needle. A 1 ml pipette tip with top and bottom removed was inserted into the top of the tube so as to increase the capacity of the tube. About 20 μl of 0.17 mm diameter glass beads in TE buffer were placed in the bottom of the Eppendorf tube, which was then filled to the top of the 1 ml tip with Sephadex G25 in TE

buffer. This Eppendorf tube was fitted into a 1.5 ml Eppendorf (bottom removed), which in turn was placed in a narrow walled 14 mm diameter glass tube. This was centrifuged at 700 x g for four minutes to compact the gel. After centrifugation, the 0.5 ml Eppendorf tube with resin was placed in a fresh sterile 1.5 ml Eppendorf tube.

The mixture prepared as described in section 2.13.2 was placed onto the surface of the resin in the 0.5 ml Eppendorf tube, and centrifuged as above. About 20 μl of incorporated label was deposited in the 1.5 ml Eppendorf tube, whilst the unincorporated label remained in the resin column with the loading buffer (Herron, 1991). The specific activity of the incorporated label was checked by placing it (in the Eppendorf tube) in a Beckman LS7000 liquid scintillation system, and counting the signal using programme 10 (1 min). This gave a reading in counts per minute (cpm) of the activity of the 20 μl of probe. This reading was converted to disintegrations per minute (dpm) using the following equation:

Equation 2.1 Cerenkov counts per minute (dpm: Phillips, 1992)

(probe count (cpm) - background count (cpm)) x 2.5

The background count was measured using a 1.5 ml Eppendorf tube containing 20 μl of SDW. This method yielded probe of specific activity *ca.* 10^8 to 10^9 dpm.

2.13.4 Hybridization

After three hours of incubation at 70°C, the prehybridization reaction was removed. The incorporated label was made single stranded by boiling for ten minutes, then immediately placed on ice. Fresh prehybridization solution (10 ml in the large bottles, 5 ml in the small bottles) was added to the hybridization bottle,

into which the probe was then added. The bottle was then put in the rotisserie of the oven, and incubated whilst rotating at 70°C for sixteen hours.

2.13.5 Stringency washes

The hybridization mixture was poured out of the bottle. The filter was washed twice with 2 x SSC, 0.1 % SDS at 70°C for thirty minutes (the bottle was approximately half filled with the wash solutions). The filter was then washed twice with 0.2 x SSC, 0.1 % SDS at 70°C for thirty minutes. After, stringency washes, the filter was sealed in a plastic bag.

2.13.6 Autoradiography

The filter was placed in a Harmer film cassette with Fuji X-ray film and two intensifying screens. The cassette was sealed in aluminium foil and left at -70°C for a sufficient time to obtain the best exposure. The exposed film was developed in Kodak LX-24 developer (five minutes) and fixed in Kodak FX-40 fixer (five minutes).

2.14 Hybridization with DIG-labelled probes

Hybridization with DIG-labelled probes was used where possible (see section 2.13) as a less hazardous alternative to labelling with ^{32}P labelled DNA. DNA was random primed labelled in both methods, which were used to show positive or negative hybridization to KC301 DNA, so there was no difference in binding specificity between the two methods. The method used for hybridization with DIG-labelled probes was as described in the instructions accompanying the labelling kit (DIG DNA Labelling and Detection Kit Nonradioactive (Boehringer Mannheim)), with adaptations kindly suggested by C.J. Bruton.

2.14.1 Prehybridization

Filters were placed in freshly made prehybridization fluid (heated to 70°C) in a polythene box, which was placed in a 70°C shaking water bath (Grant SS40-2) for one hour.

2.14.2 Labelling of probe

KC301 DNA was endonuclease restricted with *Eco*RI (Gibco BRL) as described in section 2.10 to produce linear fragments required for this reaction. The restricted DNA was ethanol precipitated and resuspended in SDW as described in section 2.13.2. A volume of this DNA corresponding to 1.5 μg of DNA was boiled for ten minutes to produce single stranded DNA, then immediately cooled on ice. To this, 2 μl of hexanucleotide mixture (vial 5 of the kit) and 2 μl of dNTP labelling mixture (vial 6) were added, and then the total volume was made up to 19 μl with SDW. Klenow enzyme (1 μl, vial 7) was added to the mixture, which was then incubated at 37°C for sixteen hours. The reaction was stopped by adding 2 μl of 0.2 M EDTA, pH 8.0. The labelled DNA was precipitated by adding 2 μl of 4 M LiCl and 60 μl of ice cold ethanol, and placing at -70°C for sixteen hours. The DNA was pelleted by centrifuging at 12000 x g, and then washed in 100 μl of ice cold 70 % ethanol, centrifuged again at 12000 x g. The final DNA pellet was resuspended in 50 μl of TE buffer at 37°C for thirty minutes, and stored at -20°C.

2.14.3 Hybridization

The prehybridization solution was discarded, and the filter was placed in a polythene bag with 2.5 ml per 100 cm^2 of fresh prehybridization solution. The probe (section 2.14.2) was denatured by boiling for ten minutes then cooling on ice. The probe (5 μl per ml of prehybridization solution) was added to the bag which

was incubated at 70°C for sixteen hours. The filter was washed two times with 50 ml of 2 x SSC, 0.1 % SDS for five minutes with gentle shaking (Ika-Vibrax-VXR reciprocal shaker) at room temperature. The filter was then washed two times with 50 ml of 0.1 x SSC, 0.1 % SDS for fifteen minutes at 70°C in a shaking water bath. All further stages were carried out at room temperature with gentle shaking using an Ika-Vibrax-VXR reciprocal shaker. The filter was washed with 50 ml of buffer 1 for one minute, before being washed in 100 ml of buffer 2 (prepared one hour in advance) for thirty minutes. Following this wash, the filter was again washed in 50 ml of buffer 1, after which it was incubated for thirty minutes with antibody conjugate (8 μl of antibody conjugate (vial 8) in 40 ml of buffer 1). This stage was followed by two fifteen minute washes with 100 ml of buffer 1. The filter was equilibrated by washing in 40 ml of buffer 3 for two minutes, after which it was placed in a polythene bag. The colour solution (45 μl of NBT solution (vial 9) and 35 μl of X-phosphate solution (vial 10) in 10 ml of buffer 3) was added to the filter in the bag which was then sealed and placed in the dark to develop (no shaking). Once satisfactorily developed, the reaction was stopped by washing the filter in 50 ml of buffer 4 for five minutes. The filter was stored in a fresh sealed polythene bag containing a small volume of fresh buffer 4.

2.15 Soil microcosms

Soil was taken from a wheat field site at Cryfield Hall, University of Warwick. The soil was air dried to about 1 % moisture and stored at room temperature. Analysis of particle size gave a composition (percent, dry weight) of: 63.6 % sand, 18.4 % silt, 11.7 % clay and 6.2 % loss on ignition (Wellington *et al.*, 1990). About 20 % of the clay fraction was kaolin, 30 % was mica, 14 % was chlorite and 27 % was an expansible phase (Soil Survey and Land Research Centre; profile no. SP 29/6909). For microcosm experiments the soil was pulverized using a pestle and mortar, and passed through a 2 mm sieve. Sterile soil was prepared by

autoclaving it twice at 121°C for fifteen minutes, with a twenty-four hour interval between the two treatments. The pH of sterile and nonsterile sieved soil was checked before experiments by mixing 20 g of soil with 50 ml of fresh distilled water and stirring for fifteen minutes. The pH of this extract was measured using a Jenway 3020 pH meter. Nonsterile soil was pH 7.1; sterile soil was pH 6.8. Soil was amended when required by adding 2 % (w/w) chitin (from crab shell; Sigma) and mixing in before wetting. The soil was wetted to about 15 % moisture (40 % of moisture holding capacity corresponding to -3 bar (-300 kPa)) with SDW. Inoculants of phage and streptomycete hosts were introduced via this wetting stage as described in sections 2.15.1 and 2.15.2. Moisture content was measured at every sampling time by recording the percentage loss of weight from a weighed sample of soil after being placed in an oven (103°C) for sixteen hours. Prior to inoculation, sterile soil was checked for sterility by plating 100 µl of extract from a 1 g sample (section 2.16.1) on NA and incubating at 30°C for three days. Similar checks were carried out throughout sterile soil experiments. Contamination was infrequent except in renewable microcosms containing phage only (Chapter 6), which were susceptible to fungal growth. As there was no apparent effect on the phage, this contamination was disregarded when analysing results.

2.15.1 Batch microcosms

Separate soil microcosms were prepared for each sampling day, and for each extraction type to facilitate destructive sampling. For extraction of total viable propagules, 10 g of soil was added to glass 25 ml universal bottles; for extraction of spores, 100 g or 10 g of soil was added to glass 300 ml or 25 ml universal bottles respectively; and for phage extractions, 20 g or 2 g of soil was added to glass 100 ml or 5 ml bijoux bottles respectively. Inoculants of phage and streptomycete hosts were introduced to these microcosms via prepared volumes of SDW to achieve the moisture content described in section 2.15. Microcosms were incubated at 22°C for

fifteen days with loosened lids to allow gas exchange. No detectable moisture loss was recorded.

2.15.2 Renewable microcosms

For certain experiments (see Chapters 6, 7 and 8) microcosms were required which facilitated repeated sampling and allowed repeated replacement of removed soil with fresh soil. Sterile soil (500 g) was placed in a sterile (swabbed with 70 % ethanol) polythene box (Stewart, size: 6 x 9 x 16 cm) which had an air-tight lid. Two 2 mm holes were made in the centre of the lid (to allow gas exchange) with 3 cm between them, and these were covered in a double layer of muslin to prevent aerial contamination. Inoculants in SDW (as described in section 2.15) were added to the soil which was then thoroughly stirred with a sterilized spatula. Microcosms were incubated at 22°C for nineteen to forty seven days. Non-destructive samples were taken using a sterilized cork borer, taking care not to mix the soil. The soil underwent turnover (*i.e.* replacement of removed soil with fresh wet sterile soil) at fourteen day intervals. The weight of soil replaced was equivalent to 20 % of the soil (wet weight) at the start of the experiment. The soil was stirred thoroughly at these stages. Approximately 0.5 % of the moisture was lost during the fourteen day intervals between turnover, and this was compensated for with the fresh soil additions.

2.16 Extraction and enumeration of soil populations

It was necessary to scale down the sample sizes and eluent volumes of the spore and phage extractions (by a factor of ten) when sampling the renewable microcosms because the recommended sample sizes for these methods would severely deplete the soil of these microcosms. The scaled down volumes and weights are given in parentheses following the recommended weights and volumes

where appropriate in sections 2.16.2 and 2.16.3. All streptomycete and phage counts were expressed as colony forming units (c.f.u.) or plaque forming units (p.f.u.) per gram of dry soil (except when enumerated from liquid culture), and converted to $\log_{10}$ values for plotting on the graphs. Zero plate counts were represented on graphs as <0 ($\log_{10}$ c.f.u. or p.f.u. g^{-1}), *i.e.* where host or phage counts were below the detection limits of extraction methods.

2.16.1 Extraction and enumeration of *Streptomyces* spp. total viable propagules from soil

Soil (1 g wet weight) was placed in 9 ml of ¼ strength Ringers solution and shaken (maximum speed, ten minutes) on a Griffin flask shaker. The supernatant was serially diluted in ¼ strength Ringers solution and spread onto RASS or R2.5 plates containing appropriate antibiotics to enumerate c.f.u. (Herron & Wellington, 1990). Plates were incubated at 30°C for three to five days.

2.16.2 Extraction and enumeration of *Streptomyces* spp. spores from soil

Extraction and enumeration of *Streptomyces* spp. spores from soil was done using the method of Herron & Wellington (1990). Soil (100 g (10 g) wet weight) was placed in 100 ml (10 ml) of 0.1 % (w/v) sodium deoxycholate/2.5 % (w/v) PEG 6000 along with 20 g (2 g) of Chelex-100 ion exchange resin (Bio-Rad). This mixture was gently shaken at 4°C for two hours on a Griffin flask shaker (setting 8), and then centrifuged at 900 x g for thirty seconds. The supernatant was filtered through a 5 cm diameter Millipore filter holder with no membrane filter and stored at 4°C whilst the pellet was re-extracted as before, but for only one hour at 4°C. The re-extracted pellet was centrifuged and filtered as before. The two supernatants were then pooled and centrifuged at 2800 x g for fifteen minutes. The pellet was

resuspended in 10 ml (1 ml) of ¼ strength Ringers solution, and c.f.u. were enumerated as described in section 2.16.1.

2.16.3 Extraction and enumeration of actinophage from soil

Method C of Lanning & Williams (1982) was used for extraction and enumeration of chloroform-sensitive actinophage from soil. Soil (20 g (2 g) wet weight) was added to 50 ml (5 ml) of NB containing 0.1 % (w/v) egg albumin (Sigma) pH 8.0, and was shaken at maximum speed on a Griffin flask shaker at 4°C for thirty minutes. The mixture was then left at 4°C for sixteen hours, before being centrifuged at 1200 x g for thirty minutes. The supernatant was filtered through a 0.45 μm membrane filter (Minisart, Sartorius) and p.f.u. were enumerated as described in section 2.6.

2.17 Determination of phage burst size

The burst size of KC301, wild type øC31 and øC31 c1 in *S. lividans* TK24 was determined by performing one-step growth experiments according to the method described by Lomovskaya *et al.*, (1972) to assay the burst size of wild type øC31 and various mutants in *S. lividans* 66.

2.17.1 Germination of spores and adsorption of phage

A spore suspension of *S. lividans* TK24 in 20 % (w/v) glycerol was washed to remove the glycerol by suspending 100 μl (containing *ca.* 10^8 c.f.u.) in 5 ml of ¼ strength Ringers solution and then centrifuging at 3000 x g for ten minutes. After discarding the supernatant the pellet was resuspended in 900 μl of pre-warmed (50°C) peptone broth, 0.2 % (w/v) agar, and pre-germinated by placing at 50°C for ten minutes (Hopwood *et al.*, 1985). The suspension was then incubated at 30°C

with aeration (using a Heidolph 50300 magnetic stirrer, 400 rpm) for five hours, after which a phage lysate was added to give a MOI of about 0.1 (*i.e. ca.* 10^7 p.f.u. ml^{-1}). The suspension was incubated for a further thirty minutes at 30°C (this gave the maximum phage adsorption rate) with gentle stirring (25 rpm) to allow infective phage adsorption to germ tubes. øC31 antiserum (kindly supplied by M.C.M. Smith; 100 µl of a ten-fold dilution in peptone broth (no agar) of the frozen stock) was added to the suspension, which was incubated at 30°C for ten minutes with gentle stirring to inactivate unadsorbed phage. The suspension was diluted by a factor of one thousand into pre-warmed (30°C) 10 ml aliquots of fresh peptone broth (no agar), which were immediately assayed for p.f.u. as described in section 2.6 to determine the infective centres count.

2.17.2 One-step growth curve

The suspension of infective centres prepared as in section 2.17.1 was incubated with no agitation at 30°C for two hours. Samples were assayed for p.f.u. at specified times throughout incubation to obtain a progeny count. The burst size was calculated by dividing the progeny count by the infective centres count.

2.18 Measurement of thiostrepton activity in soil

Certain microcosms (see Chapter 6) contained thiostrepton which was added to the dried soil before the wetting and inoculation stage. In the renewable microcosms, thiostrepton was included with fresh soil at soil turnover, at the initial concentration, to maintain a biologically active concentration in the microcosms. Active thiostrepton was recovered from sterile soil and bioassayed according to the method of Schilhabel, as described in Marsh *et al.* (1993).

2.18.1 Extraction of thiostrepton from soil

Soil (5 g) sampled from renewable microcosms was added to 2 vols of ethyl acetate and shaken on a Griffin flask shaker at high speed for thirty minutes. The mixture was centrifuged at 1600 x g for fifteen minutes. The supernatant was transferred to the flask of a Büchi Rotavapor-R vacuum rotator, and was desiccated at 54°C. The residue was redissolved in 100 μl of chloroform.

2.18.2 Determination of recoverable thiostrepton activity

Activity of the thiostrepton extract prepared as in section 2.18.1 was determined as follows. Extract (15 μl) was added to an antibiotic assay disc, and the chloroform was allowed to evaporate before being placed on a nutrient agar overlay containing *ca.* 10^7 c.f.u. ml^{-1} of *Staphylococcus aureus* (ATCC 6538P), on a 23 cm nutrient agar plate. The plate was incubated at 37°C for twenty-four hours. Zones of inhibition around the discs were measured in mm, and these measurements were compared to a standard curve. The standard curve was constructed by making a series of known dilutions of thiostrepton in chloroform, and measuring zones of inhibition in a *Staph. aureus* overlay as described above.

2.19 Measurement of metabolic activity of *S. lividans* in soil

Metabolic activity of *S. lividans* in sterile unamended soil was measured by monitoring chitinase activity and respiration (CO_2 evolution) in two experiments described in Chapter 3.

2.19.1 Chitinase activity

Chitinase activity in soil inoculated with *S. lividans* TK24 was measured using an adaptation of the method of Wirth and Wolf (1992). Soil (1 g) sampled from 10 g batch microcosms (section 2.15.1) was added to 4 ml of sodium acetate-acetic acid buffer (pH 8.0, 0.2 M) in a sterile 25 ml plastic universal bottle, and shaken at full speed on a Griffin flask shaker for one hour. The soil was pelleted by centrifugation at 1200 x g for ten minutes. Five replicate samples of supernatant (100 μl) were added to the wells of a microtitre plate (7 x 10 mm cavities, Nunclon), along with 50 μl of sodium acetate-acetic acid buffer (pH 8.0, 0.2 M) and 50 μl of 2 mg. ml^{-1} carboxymethyl-substituted chitin labelled with remazol brilliant violet (CM-chitin-RBV; Blue Substrates). The microtitre plate was incubated, with the lid on, at 37°C for four hours, after which the reaction was stopped by adding 50 μl of 1 N HCl, causing precipitation of the non-degraded high-polymeric substrate. The plate was then centrifuged at 1450 x g for ten minutes to remove the non-degraded substrate, after which 175 μl of supernatant was transferred to clean microtitre plate wells. Chitinase activity was determined by measuring the optical density (at 540 nm) of the supernatant containing the soluble dye-labelled degradation product using a Multiscan Plus Mk II plate reader. Blanks were treated similarly, but with 100 μl of SDW added instead of soil extract. Background activity was measured by making extracts from sterile soil inoculated with SDW only. This activity was subtracted from the readings taken from soil inoculated with *S. lividans* TK24. Based on the extraction and assay conditions, one unit of enzyme activity in 1 g of dry soil was calculated as absorbance x 1000 x h^{-1} (Wirth and Wolf, 1992). These values were converted to $\log_{10}$ numbers prior to analysis by M.S.D. (section 2.20). Relative activities were plotted as % of maximum activity in each microcosm set.

2.19.2 Carbon dioxide evolution

CO_2 evolution by respiring *S. lividans* TK24 and *S. lividans* TK24 (KC301) in soil was measured using an adaptation of the renewable microcosms described in section 2.15.2. The apparatus used is illustrated in Fig. 2.1. Renewable microcosms of 500 g of sterile unamended soil were inoculated with spore suspensions as described in section 2.15.2 and incubated at 22°C, and air was drawn over the surface of the soil by a peristaltic pump (Watson-Marlow 503S) at a rate of about 1 ml per minute, and fed via silicone tubing into a boiling tube containing 20 ml of 1 N NaOH to dissolve the CO_2. Readings were periodically taken by titrating the quantity of alkali which had not reacted with CO_2. This was done by adding excess $BaCl_2$ (a few drops of 3 N $BaCl_2$) to precipitate the carbonate as insoluble $BaCO_3$, and a few drops of phenolphthalein indicator (1 % phenolphthalein in 95 % ethanol), and titrating with 1 N HCl (Anderson, 1982). A control microcosm which consisted of sterile unamended soil inoculated with SDW only was treated in exactly the same way. The amount of CO_2 evolved during incubation was calculated using equation 2.2, and then expressed as mg CO_2. m^{-2}. h^{-1} (the surface area of the microcosm soil exposed to the air was 150 cm^2).

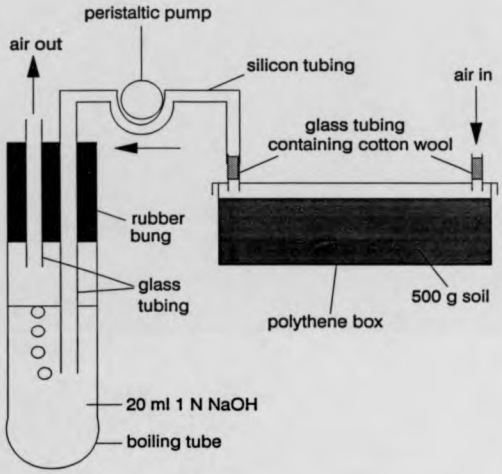
Equation 2.2 Milligrams of CO_2 = (B - V)NE

Where:

B	volume (ml) of 1 N HCl needed to titrate the NaOH from the control microcosm to end point.
V	volume (ml) of 1 N HCl needed to titrate the NaOH from the microcosms inoculated with spores to end point.
N	normality of the acid.
E	equivalent weight (CO_2 = 22).

Fig. 2.1 **Apparatus for measuring carbon dioxide evolution from renewable soil microcosms (not to scale)**

Fig. 2.1


peristaltic pump
air out
silicon tubing
air in
glass tubing
containing cotton wool
rubber
bung
glass
tubing
500 g soil
polythene box
20 ml 1 N NaOH
boiling tube

2.20 Statistical analysis

All points on graphs were means of counts obtained as follows. Spore and mycelia counts and phage counts in soil were made by sampling in triplicate and counting each sample in triplicate (n=9). Spore counts in soil were made by taking single samples and counting in triplicate (n=3). Phage counts in one-step growth experiments were made by diluting the infective centres (after adsorption of phage and inactivation of free phage) into triplicate aliquots of broth, and counting in duplicate (n=6).

Statistical analysis was carried out using the MINITAB software package (Minitab Statistical Software, State College, Pa., USA). Minimum significant differences (M.S.D.) between means were calculated from analysis of variance (ANOVA) using the method of Peterson (1985), when the number of replicates for each sample was not the same. When the number of replicates for each sample was the same, Tukey's honestly significant difference (H.S.D.) was used. The calculations for H.S.D. and M.S.D. are shown in Equations 2.3 and 2.4.

Equation 2.3 Tukey's honestly significant difference (H.S.D.)

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

Equation 2.4 Minimum significant difference (M.S.D.)

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

Where:

Q_a	value from Studentized range (Q) table; 95 % confidence limits. Depending on μ (error degrees of freedom from ANOVA and number of means (*i.e.* sample 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.

2.21 Efficiency of infection

Efficiencies of infection (*i.e.* the probability of an individual phage successfully replicating in a host unit) were calculated using Equations 2.5 and 2.6 (Manchester, 1986, cited by Williams *et al.*, 1987).

Equation 2.5 Hi = (Pt - Po)/c

Equation 2.6 a = Hi/(Ht x Pt)

Where:

Hi	number of hosts infected
Po	phage count at time 0
Pt	phage count at time t
c	mean burst size (øC31 = 55)
Ht	host count at time t
a	efficiency of infection

CHAPTER 3

Relationship between growth of *S. lividans* TK24 and infection by KC301

3.1 Introduction

The physiology of the lytic and lysogenic cycles of wild type øC31 has been extensively studied (reviews: Lomovskaya *et al.*, 1980; Chater, 1986). The wild type had undergone many insertion and deletion mutations before being developed into KC301 (Chater *et al.*, 1982; Hopwood *et al.*, 1985), which may have affected some of the properties of the infection cycle such as burst size, length of rise period and frequency at which lysogenic spores release phage on germination. These were determined by repeating the experiments of Lomovskaya *et al.* (1972) with øC31, KC301 and *S. lividans* TK24 (KC301) using the same experimental conditions, except that *S. lividans* TK24 was used as the host instead of *S. lividans* 66.

3.2 Procedure for measuring burst size, rise period and adsorption frequency of øC31 and KC301, and spontaneous release frequency of KC301 from *S. lividans* TK24 (KC301)

One-step growth experiments were performed on øC31 and KC301 with *S. lividans* TK24 spores (section 2.17). The adsorption period needed to be at least thirty minutes for detectable infective centres to be counted. Together with the ten minute phage inactivation period using øC31 antiserum, the forty minute latent period (Lomovskaya *et al.*, 1980) was therefore complete when samples for progeny counts were first taken.

The infective adsorption frequency of øC31 and KC301 to germinated *S. lividans* TK24 spores was calculated by dividing the infective centre count (prepared as described in section 2.17.1) by the original total phage count. Infective

adsorption frequency of KC301 to *S. lividans* TK24 spores was also measured in conditions designed to simulate the potential chemical environment of sterile soil microcosms. This was done in sterile soil water prepared as follows. Soil was mixed with 2.5 volumes of distilled water (*i.e.* as prepared for pH measurements, section 2.15) then filtered through a 0.22 μm nitrocellulose filter (Millipore). *S. lividans* TK24 spores were germinated in peptone (section 2.17.1), but after the five hours incubation, the spores were pelleted by centrifugation at 3000 x g for ten minutes, and resuspended in 900 μl of sterile soil water. The infective centre count was then determined (section 2.17.1).

To determine the frequency at which *S. lividans* TK24 (KC301) released KC301 on germination, the method described by Lomovskaya *et al.* (1972) was used. *S. lividans* TK24 (KC301) spores (*ca.* 10^6 c.f.u. ml^{-1}) were pre-germinated and treated with øC31 antiserum as described in section 2.17.1. This suspension was diluted 10^{-3}-fold into triplicate 10 ml aliquots of peptone broth (no agar) to render the antiserum inactive. The total *S. lividans* TK24 (KC301) was then measured by plating out samples on NA and incubating at 30°C for three days, and the proportion of propagules which released KC301 was determined by plating out samples on lawns of *S. lividans* TK24 on NA as described in section 2.6.

3.3 Comparisons between the infection-cycles of øC31 and KC301

The burst sizes of øC31 and KC301 in *S. lividans* TK24 (Fig. 3.1) were 80 and 120 respectively. The rise period (period of time between time 0 and the end of increase in phage density) for øC31 was twenty minutes, whereas for KC301 it was sixty minutes. The infective adsorption frequency of øC31 in peptone was 1.1 x 10^{-4}, whereas for KC301 it was 1.0 x 10^{-5} and 2.6 x 10^{-5} in peptone and sterile soil water respectively. The count of *S. lividans* TK24 (KC301) after pre-germination and the subsequent treatments outlined in section 3.2.1 was 2.1 x 10^2 c.f.u. ml^{-1}. From the same sample, number of plaques formed on *S. lividans* TK24

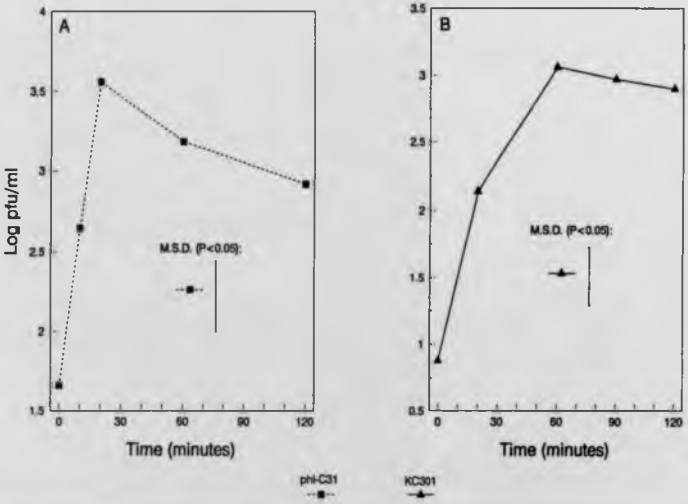
lawns on NA was 2.0 x 10^1 p.f.u. ml^{-1}, *i.e.* 9.5 % of the germinated *S. lividans* TK24 (KC301) spores released KC301.

Fig. 3.1 **One-step growth curves using *S. lividans* TK24 as the host**

(A) øC31: burst size = 80; rise period = 20 min.

(B) KC301: burst size = 120; rise period = 60 min.

Fig. 3.1


A
B
Log pfu/ml
M.S.D. (P<0.05):
M.S.D. (P<0.05):
Time (minutes)
Time (minutes)
phi-C31
KC301

3.4 Relationship between growth of *S. lividans* TK24 and infection by KC301: discussion

The results of the one-step growth experiments suggested that KC301 had a larger burst size (120) than øC31 (80). However the rise period in both cases was under way within the first twenty minutes that samples were taken for progeny counts, so it is possible that the latent period had ended and the rise period commenced before the progeny count samples were taken. Hence there was possibly no difference in burst size between øC31 and KC301. This conclusion is supported by the fact that the burst size of øC31 is quoted as being between 10 and 100 (Chater, 1986), which is a larger range than the difference between 80 and 120. The rise period for KC301 was three times longer than that for øC31 which suggested that the lytic cycle of KC301 was compromised compared to that of the wild type. Rodriguez *et al.* (1986) measured the rise period of øC31 to be between 20 and 30 minutes. The frequency of infective adsorption for KC301 was one order of magnitude lower than that of øC31, which again suggested that KC301 was to some way extent disabled in its infection-cycle compared to the wild-type. The soluble components of sterile soil did not affect the infective adsorption frequency of KC301. *S. lividans* TK24 (KC301) spontaneously released KC301 at the same frequency (10 %) as observed for spontaneous release of øC31 from *S. lividans* 66 (øC31) (Lomovskaya *et al.*, 1980).

CHAPTER 4

Spread of KC301 into indigenous hosts in nonsterile unamended soil

4.1 Introduction

The ultimate fate of DNA introduced into a natural habitat is determined by the establishment and growth of the microbial host in which it is contained (Stotsky & Babich, 1986). Previous work (Herron & Wellington, 1990; Herron, 1991; Cresswell *et al.*, 1992) has shown infection of inoculated *S. lividans* strains in sterile and nonsterile soil by KC301 and, therefore, multiplication of KC301 in soil to be possible. Lysogenization of these inoculants by KC301 was also demonstrated by selecting for the *tsr* gene in selective plating. This experiment was designed to show whether KC301 could lysogenically infect indigenous streptomycete hosts in nonsterile soil, and therefore establish itself in a reproducible form (integrated prophage) so as to ensure its persistence in soil.

4.2 Procedure for monitoring KC301 and indigenous streptomycetes in nonsterile soil

Two sets of batch microcosms of nonsterile unamended soil were prepared as described in section 2.15.1. These were inoculated as shown in Table 4.1. Set B were control microcosms inoculated with SDW only to enable monitoring of indigenous phage and host populations throughout the incubation period without the influence of allochthonous inocula.

Table 4.1 Microcosms used for determining the survival of KC301 in nonsterile unamended soil

Microcosm	KC301 (p.f.u. g^{-1} dry soil)
A	4.0×10^5
B	-

The microcosms were incubated at 22°C for fourteen days. On days 0, 1, 2, 5 and 15, streptomycete spores, total viable propagules and actinophages from both microcosms were extracted and enumerated as described in section 2.16. Indigenous streptomycetes were plated on RASS containing cycloheximide, nystatin and rifampicin (Table 2.2). Thiostrepton resistant streptomycetes (and therefore putative lysogens of KC301) were isolated on RASS containing the above antibiotics plus thiostrepton.

4.3 Survival of KC301 in nonsterile soil

The extraction efficiency of KC301 on day 0 was 35 %. The proportion of p.f.u. enumerated in microcosm set A that were KC301, and the homology between KC301 and indigenous phages in microcosm set B was checked as follows. Fifty plaques per sample day isolated from all microcosms were picked onto SNA overlays containing *S. lividans* TK24 on NA, and incubated at 30°C for twenty-four hours. Plaque DNA was fixed to nylon filters as described in section 2.12, and probed with radio-labelled KC301 DNA (section 2.13) (Fig. 4.1). All phages counted from microcosm set A were KC301 (Fig. 4.2b). Streptomycete spores

Fig. 4.1 **Autoradiograph of KC301 plaque DNA hybridized with ^{32}P-labelled KC301 DNA**

About 50 plaques per sample day were picked from phage assay plates from both microcosm sets. This autoradiograph represents isolates from microcosm set A, days 0 and 1. The number of positive hybridizations out of 50 represented the proportion of p.f.u. on assay plates that were KC301. Positive and negative controls were *S. lividans* TK24 (KC301) and *S. lividans* TK24 respectively.

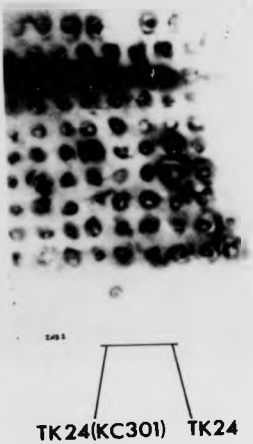


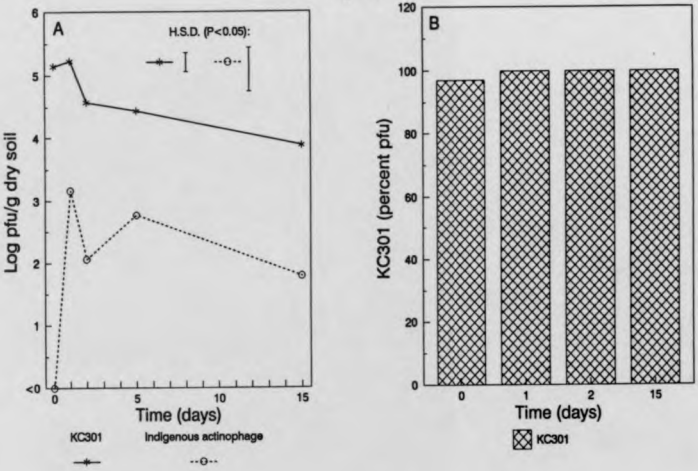
Fig. 4.1

Fig. 4.2 Actinophage populations in nonsterile unamended soil

(A) Population changes in in KC301 and indigenous actinophage.

(B) Proportion of actinophage extracted from microcosm set A which were KC301 (Fig. 4.1).

Fig. 4.2


A
H.S.D. (P<0.05):
Log pfu/g dry soil
6
5
4
3
2
1
<0
0
5
10
15
Time (days)
KC301
Indigenous actinophage
B
KC301 (percent pfu)
120
100
80
60
40
20
0
0
1
2
15
Time (days)
KC301

lysogenized by KC301, and therefore detectable by thiostrepton resistance, were detected from day 2 onwards in microcosm set A (Fig. 4.3a). No thiostrepton resistant colonies were detected in microcosm set B. Lysogens were detectable after a significant phase of sporulation of the indigenous streptomycetes between days 1 and 2 (Fig. 4.3a). This corresponded to a significant drop in KC301 count at the same time. Following this sharp drop in KC301 count (from 1.7×10^5 to 3.8×10^4 p.f.u. g^{-1} over 24 hours), the KC301 count declined less rapidly (from 3.8×10^4 to 7.7×10^3 p.f.u. g^{-1} over thirteen days). This trend was repeated in the indigenous actinophages counts in microcosm set B (Fig. 4.3b). Indigenous actinophages in microcosm set B were only detectable after twenty-four hours' incubation following wetting of the soil (Figs. 4.2a and 4.3b), and the level of indigenous actinophages remained at about two orders of magnitude below that of KC301 in microcosm set A. No lysogens were detected in the total viable propagules extractions, and there was no significant increase or decrease in these counts over the first five days of incubation.

4.3.1 Confirmation of the integration of the KC301 prophage into indigenous hosts chromosomes

The putative lysogens detected in microcosm set A (Fig. 4.3a) were confirmed as being lysogens of KC301 by demonstrating spontaneous phage release (Fig. 4.4a) as described in section 2.7.1. The presence of KC301 DNA in thiostrepton resistant isolates was determined by positive hybridization of radio-labelled KC301 DNA to colony blots (Fig. 4.4b) using the method described in section 2.13. About ten thiostrepton resistant colonies, and five colonies from plates which did not contain thiostrepton from each microcosm were checked for each day sampled.

Twenty-two of the putative lysogenic isolates were stored as spore suspensions as described in section 2.5. The stability of the lysogenic state of these

isolates was tested by repeatedly sub-culturing them on R5 containing thiostrepton (Table 2.3). By the tenth round of sub-culturing, two of the twenty two isolates had lost thiostrepton resistance (Fig. 4.5a).

øC31 lysogenizes *S. coelicolor* by site-specific integration into the host chromosome (Chater *et al.*, 1982; Rausch and Lehmann, 1991). Based on the premise that øC31 exists as a prophage in *Streptomyces* strains (Lomovskaya *et al.*, 1980), three isolates from the putative stable twenty lysogens were tested for integrated KC301 prophage. Total DNA preparations of these isolates and *S. lividans* TK24 (KC301) and *S. lividans* TK24 were made using the method described in section 2.9. These preparations, as well as KC301 DNA, were separately restricted with *Eco*RI or *Cla*I and Southern blotted as described in section 2.10, the aim being to cut the prophage DNA near to its attachment site (*attP*). The Southern blot was then hybridized with DIG-labelled KC301 DNA (Fig. 4.5b) as described in section 2.14. The junction fragment of the KC301 *attP* site and the host chromosome (5.7 kb) in *S. lividans* TK24 (KC301) can be seen in the *Cla*I digest, and this corresponds to the junction fragment of øC31 in *S. coelicolor* J1501 (Chater *et al.*, 1982). The *Cla*I digests also show the junction fragment of KC301 with the three indigenous lysogens' chromosomal DNA at 2.5 kb.

Fig. 4.3 Population changes in actinophages and indigenous streptomycetes in nonsterile unamended soil

(A) Survival of KC301 and lysogeny of indigenous streptomycetes.

(B) Population changes of indigenous actinophages and streptomycetes.

Fig. 4.3

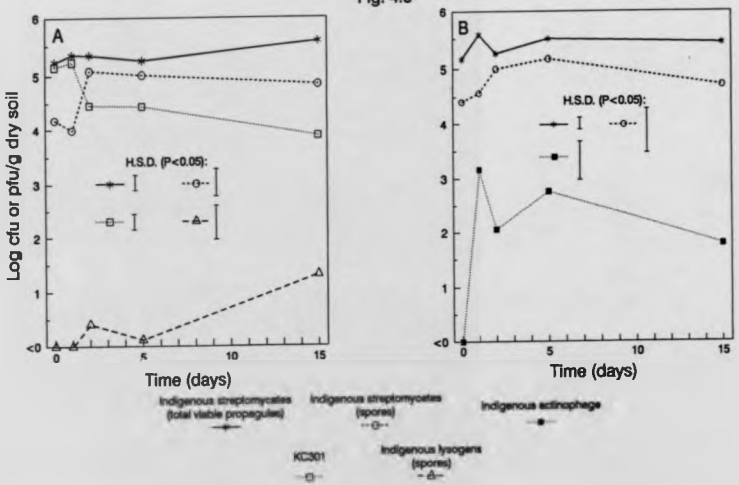
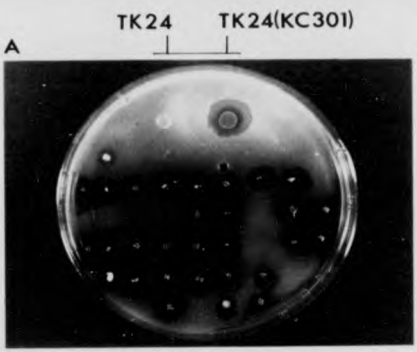

A
B
Log cfu or pfu/g dry soil
H.S.D. (P<0.05):
H.S.D. (P<0.05):
Time (days)
Time (days)
Indigenous streptomycetes (total viable propagules)
Indigenous streptomycetes (spores)
Indigenous actinophage
KC301
Indigenous lysogens (spores)

Fig. 4.4 Confirmatory tests for putative lysogenic isolates from microcosm set A

(A) Spontaneous phage release from colony isolates.

(B) Autoradiograph showing hybridization of ^{32}P-labelled KC301 DNA to colony DNA. These colonies did not directly correspond to those shown in Fig. 4.4a, but represented twenty two putative lysogens. Fifteen of these positively hybridized to ^{32}P-labelled KC301 DNA.

In both cases, positive and negative controls were *S. lividans* TK24 (KC301) and *S. lividans* TK24 respectively.



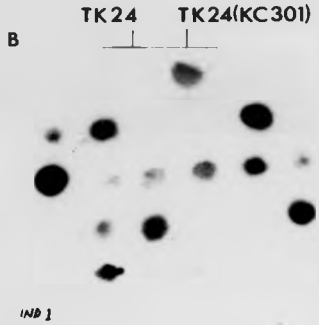


Fig. 4.4

4.4 Lysogenization of indigenous soil populations by KC301: discussion

KC301 was capable of lysogenically infecting indigenous hosts, and as the host range of øC31 rarely extends beyond the genus *Streptomyces* (Lomovskaya *et al.*, 1980), the lysogens were likely to be of this genus. Lysogens were detected by the spore-specific extraction method only which demonstrates higher sensitivity of this method compared to that of the total viable propagules extraction method (Herron and Wellington, 1990). The significant drops in both KC301 and indigenous actinophage numbers which correspond to sporulation were repeated in all soil experiments of this project and have been observed in previous studies (Herron & Wellington, 1990; Herron, 1991; Cresswell *et al.*, 1992). Novikova *et al.* (1973) demonstrated nonspecific adsorption of øC31 to resting spores of *S. coelicolor* A3(2), so it is possible that phage released from lysing mycelia became adsorbed to newly forming spores, thus reducing the free phage pool by a significant proportion.

The existence of KC301 as an integrated prophage in the chromosomal DNA of three of the lysogens, and the stability of thiostrepton resistance of 91 % of the lysogens suggests KC301 has the ability to survive in the presence of indigenous streptomycetes in the soil used in this study. The KC301 prophage could potentially persist in spores over long periods of time, to be re-introduced into the free state by lysis of germinating lysogenic spores at some later stage.

Fig. 4.5 Tests to confirm stable integration of KC301 prophage into indigenous streptomycetes

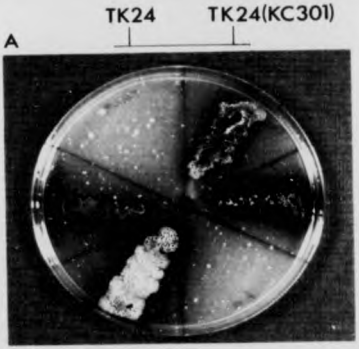
(A) Isolates were repeatedly sub-cultured on R5 containing thiostrepton. This sixth generation sub-culture plate shows one isolate (bottom right-hand segment) which had lost thiostrepton resistance and hence the prophage. Positive and negative controls were *S. lividans* TK24 (KC301) and *S. lividans* TK24 respectively.

(B) Southern blot of DNA from putative lysogens (thiostrepton resistant after ten sub-culturings on selection) hybridized with DIG-labelled KC301 DNA.

Lane 13 = lambda *Pst*I DNA size marker; sizes in kb.

Key:

Lane	Restriction enzyme	Restricted DNA
1	*Eco*RI	KC301
2	*Eco*RI	*S. lividans* TK24
3	*Eco*RI	*S. lividans* TK24 (KC301)
4	*Eco*RI	indigenous lysogen 18
5	*Eco*RI	indigenous lysogen 32
6	*Eco*RI	indigenous lysogen 48
7	*Cla*I	KC301
8	*Cla*I	*S. lividans* TK24
9	*Cla*I	*S. lividans* TK24 (KC301)
10	*Cla*I	indigenous lysogen 18
11	*Cla*I	indigenous lysogen 32
12	*Cla*I	indigenous lysogen 48



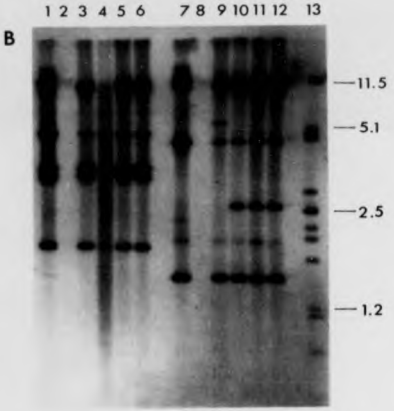


Fig. 4.5

CHAPTER 5

Density dependence of interactions between KC301 and *S. lividans* TK24 in sterile unamended soil

5.1 Introduction

Interactions between populations of microorganisms in natural environments (for example predation, parasitism and gene transfer) require the population densities of potentially interacting microorganisms to be sufficiently high (Stotsky *et al.*, 1990). The spatial and temporal (*i.e.* time periods between physical disturbance and/or nutrient input) limitations of the soil environment influence the probability of a susceptible host coming into contact with an infective phage. Wiggins and Alexander (1985) suggested that in aquatic environments, phages did not interact with hosts (*i.e.* infect and cause an impact on host populations) where the host population density is below about 10^4 c.f.u. ml^{-1}. Kokjohn *et al.* (1991) maintained that there was no minimum limit for phage-host interactions in aquatic environments, due to their homogeneous nature and being in a constant state of flux. Pantastico-Caldas *et al.* (1992) determined 10^6 c.f.u. g^{-1} to be the minimum limit at which bacteriophage infect *Bacillus subtilis* in soil, although this figure was based on infections carried out in liquid culture and comparing results to observations in soil. Germida (1986) showed that phage Ab-1 required a host inoculum (*Azospirillum brasilense*) in soil of at least 10^2 c.f.u. g^{-1} to replicate.

The aim of this study was to determine the minimum population densities of KC301 and *S. lividans* TK24 at which interactions (*i.e.* multiplication of the phage and lysogenization of the host) take place in soil. The study of two interacting populations necessitated the use of sterile unamended soil.

5.2 Procedure for monitoring KC301 and *S. lividans* TK24 in sterile unamended soil

The experiment was split into two experiments. In Experiment 1, a set of batch microcosms (prepared as described in section 2.15.1) were inoculated with 1.6×10^6 c.f.u. g^{-1} of *S. lividans* TK24 and a range of KC301 densities between 1.3×10^1 and 1.3×10^4 p.f.u. g^{-1} (Table 5.1). In Experiment 2, a set of batch microcosms were inoculated with 2.0×10^4 p.f.u. g^{-1} of KC301 and a range of *S. lividans* TK24 densities between 2.0×10^0 and 2.0×10^5 c.f.u. g^{-1} (Table 5.2).

Table 5.1 **Microcosms used to determine the minimum phage density at which interactions occur between KC301 and *S. lividans* TK24 (Experiment 1)**

Microcosms (MOI)	KC301 (p.f.u. g^{-1} dry soil)	*S. lividans* TK24 (c.f.u. g^{-1} dry soil)
A (0.008)	1.3×10^4	1.6×10^6
B (0.0008)	1.3×10^3	1.6×10^6
C (0.00008)	1.3×10^2	1.6×10^6
D (0.000008)	1.3×10^1	1.6×10^6
E (0.0000008)	1.3×10^0	1.6×10^6
F	-	1.6×10^6

Table 5.2 **Microcosms used to determine the minimum host density at which interactions occur between KC301 and *S. lividans* TK24 (Experiment 2)**

Microcosms (MOI)	KC301 (p.f.u. g^{-1} dry soil)	*S. lividans* TK24 (c.f.u. g^{-1} dry soil)
A (10000)	2.0×10^4	2.0×10^0
B (1000)	2.0×10^4	2.0×10^1
C (100)	2.0×10^4	2.0×10^2
D (10)	2.0×10^4	2.0×10^3
E (1)	2.0×10^4	2.0×10^4
F (0.1)	2.0×10^4	2.0×10^5
G	2.0×10^4	-

The microcosms were incubated at 22°C for fifteen days. On days 0,1,2,5 and 15, *S. lividans* TK24 spores, total viable propagules and KC301 were extracted and enumerated as described in section 2.16. *S. lividans* TK24 was plated on RASS containing streptomycin, and putative *S. lividans* TK24 (KC301) lysogens were isolated on RASS containing streptomycin and thiostrepton (Table 2.2).

5.3 Density dependent survival of KC301 in soil containing susceptible hosts

The extraction efficiencies on day 0 for Experiment 1 were: spores, 22 %; total viable propagules, 117 %; phage, 63 %, and for Experiment 2: spores, 9 %; total viable propagules, 103 %; phage, 104 %.

The host population changes in all the other microcosm sets of Experiment 1 (including set F which contained no KC301) were identical to those of microcosm set B, and therefore the population changes of *S. lividans* TK24 plotted in Fig. 5.1 were taken from microcosm set B of Experiment 1. There were no significant changes in population level of KC301 in the absence of *S. lividans* TK24 (Fig. 5.3a).

Significant increases in free phage density (Fig. 5.1a) and lysogenization of *S. lividans* TK24 spores (Fig. 5.1a) and mycelia (Fig. 5.1b) were detected only when the phage density was at least 10^3 p.f.u. g^{-1}, with a host density of 10^6 c.f.u. g^{-1}. When the phage density was 10^4 p.f.u. g^{-1}, lysogenization of *S. lividans* TK24 occurred when the host density was at least 10^3 c.f.u. g^{-1} (Figs. 5.2 and 5.3), although significant increases in phage density were only detected at host densities of 10^4 c.f.u. g^{-1} and above. Lysogenic spores were only detectable from day 5 onwards (Figs. 5.1a and 5.3b), *i.e.* at the end of five day growth cycle of germination and resporulation, whereas lysogens were detected in the extractions of total viable propagules from day 1 onwards (Figs. 5.1b and 5.2b).

Putative lysogens were checked using the method described in sections 2.7.1 and 2.13. Putative lysogens spontaneously released phage (Fig. 5.4a) and positively hybridized to ^{32}P-labelled KC301 DNA (Fig. 5.4b).

These experiments also served to establish detection limits of the three extraction techniques used. The detection limits were: for the total viable propagules extraction method, 10^1 c.f.u. g^{-1}; for the spore-specific extraction method, 10^1 c.f.u. g^{-1}; and for the actinophage extraction method, 10^2 p.f.u. g^{-1}.

Fig. 5.1 Interactions between KC301 and *S. lividans* TK24 in sterile unamended soil at various phage densities

(A) Population changes in *S. lividans* TK24 spores and bursts in KC301 and formation of lysogenic spores when phage density was 1.3×10^3 p.f.u. g^{-1} or higher.

(B) Population changes in *S. lividans* TK24 total viable propagules, and bursts in KC301 and formation of lysogens when phage density was 1.3×10^3 p.f.u. g^{-1} or higher.

Microcosm sets were inoculated with 1.3×10^0, 1.3×10^1, 1.3×10^2, 1.3×10^3 and 1.3×10^4 p.f.u. g^{-1} of KC301. All microcosms were inoculated with 1.6×10^6 c.f.u. g^{-1} of *S. lividans* TK24. The *S. lividans* TK24 population changes shown (from microcosm set B) were identical in all microcosm sets.

Zero plate counts recorded in microcosm sets throughout the experiment are not plotted on the graphs.

Fig. 5.1

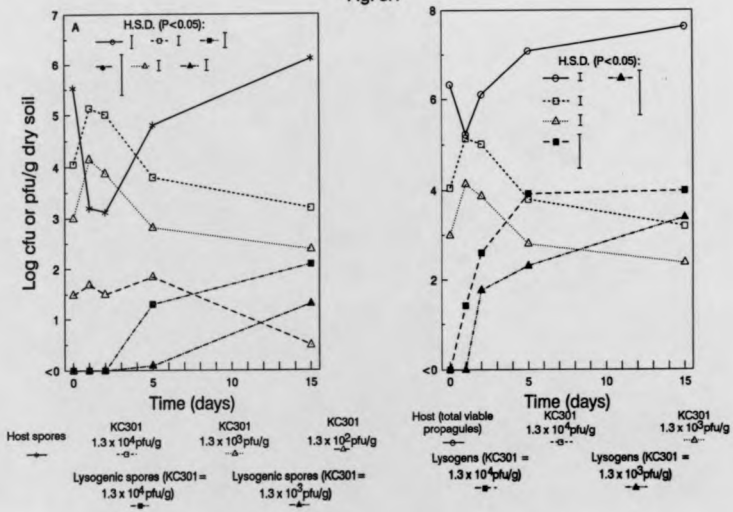

A
H.S.D. (P<0.05):
Log cfu or pfu/g dry soil
Time (days)
Host spores
KC301 1.3 x 10⁴pfu/g
KC301 1.3 x 10³pfu/g
KC301 1.3 x 10²pfu/g
Lysogenic spores (KC301 = 1.3 x 10⁴pfu/g)
Lysogenic spores (KC301 = 1.3 x 10³pfu/g)
H.S.D. (P<0.05):
Time (days)
Host (total viable propegules)
KC301 1.3 x 10⁴pfu/g
KC301 1.3 x 10³pfu/g
Lysogens (KC301 = 1.3 x 10⁴pfu/g)
Lysogens (KC301 = 1.3 x 10³pfu/g)

Fig. 5.2 **Interactions between KC301 and *S. lividans* TK24 in sterile unamended soil at various host densities**

(A) Population changes in *S. lividans* TK24 spores and mycelia.

(B) Bursts in KC301 and formation of lysogens when host density was respectively 2.0×10^4 and 2.0×10^3 c.f.u. g^{-1} or higher.

Microcosm sets were inoculated with 2.0×10^0, 2.0×10^1, 2.0×10^2, 2.0×10^3, 2.0×10^4 and 2.0×10^5 c.f.u. g^{-1} of *S. lividans* TK24. All microcosm sets were inoculated with 2.0×10^4 p.f.u. g^{-1} of KC301. Population changes in KC301 at 2.0×10^2 c.f.u. g^{-1} *S. lividans* TK24 (B) were identical at the lower host densities.

Zero plate counts recorded in microcosm sets throughout the experiment are not plotted on the graphs.

Fig. 5.2

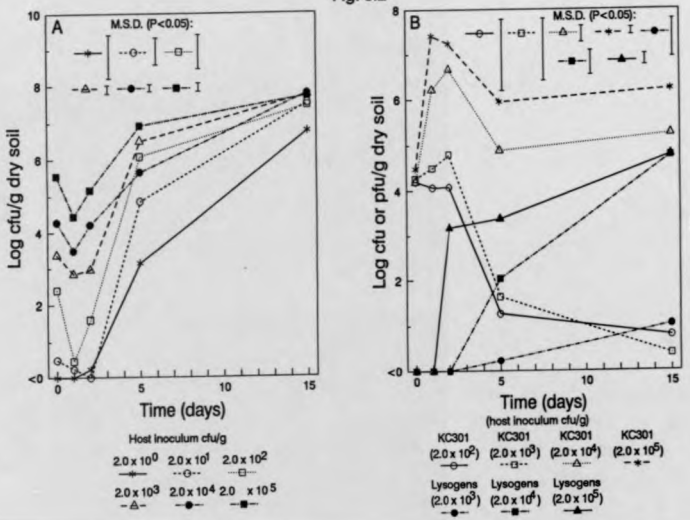

A
M.S.D. (P<0.05):
Log cfu/g dry soil
Time (days)
Host inoculum cfu/g
2.0 x 10^0
2.0 x 10^1
2.0 x 10^2
2.0 x 10^3
2.0 x 10^4
2.0 x 10^5
B
M.S.D. (P<0.05):
Log cfu or pfu/g dry soil
Time (days)
(host inoculum cfu/g)
KC301 (2.0 x 10^2)
KC301 (2.0 x 10^3)
KC301 (2.0 x 10^4)
KC301 (2.0 x 10^5)
Lysogens (2.0 x 10^3)
Lysogens (2.0 x 10^4)
Lysogens (2.0 x 10^5)

Fig. 5.3 **Interactions between KC301 and *S. lividans* TK24 spores in sterile unamended soil at various host densities**

(A) Population changes in *S. lividans* TK24 spores and KC301 in soil containing no *S. lividans* TK24.

(B) Bursts in KC301 and formation of lysogenic spores when host density was respectively 2.0×10^4 and 2.0×10^3 c.f.u. g^{-1} or higher.

Microcosm sets were inoculated with 2.0×10^0, 2.0×10^1, 2.0×10^2, 2.0×10^3, 2.0×10^4 and 2.0×10^5 c.f.u. g^{-1} of *S. lividans* TK24. All microcosm sets were inoculated with 2.0×10^4 p.f.u. g^{-1} of KC301. Population changes in KC301 at 2.0×10^2 c.f.u. g^{-1} *S. lividans* TK24 (B) were identical at the lower host densities.

Zero plate counts recorded in microcosm sets throughout the experiment are not plotted on the graphs.

Fig. 5.3

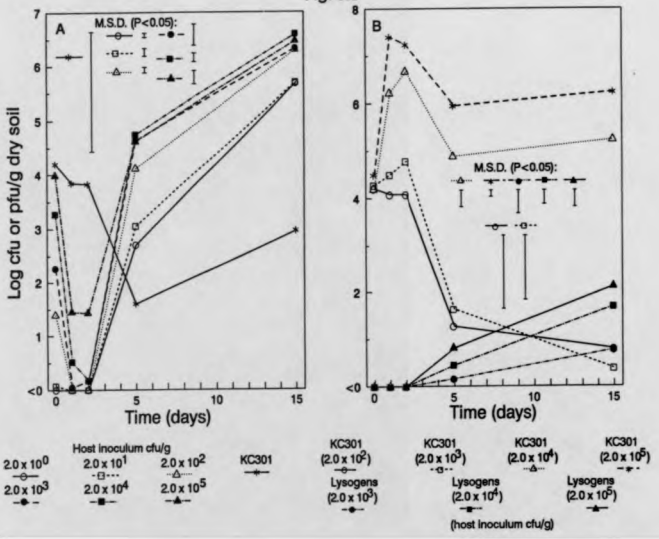
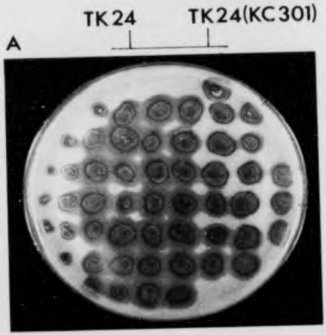

A
M.S.D. (P<0.05):
Log cfu or pfu/g dry soil
Time (days)
B
M.S.D. (P<0.05):
Time (days)
Host inoculum cfu/g
2.0 x 10^0
2.0 x 10^1
2.0 x 10^2
KC301
2.0 x 10^3
2.0 x 10^4
2.0 x 10^5
KC301 (2.0 x 10^2)
KC301 (2.0 x 10^3)
KC301 (2.0 x 10^4)
KC301 (2.0 x 10^5)
Lysogens (2.0 x 10^3)
Lysogens (2.0 x 10^4)
Lysogens (2.0 x 10^5)
(host inoculum cfu/g)

Fig. 5.4 Confirmatory tests for putative lysogens

(A) Spontaneous phage release from colony isolates.

(B) Autoradiograph showing hybridization of ^{32}P-labelled KC301 DNA to colony DNA.

In both cases, positive and negative controls were *S. lividans* TK24 (KC301) and *S. lividans* TK24 respectively.



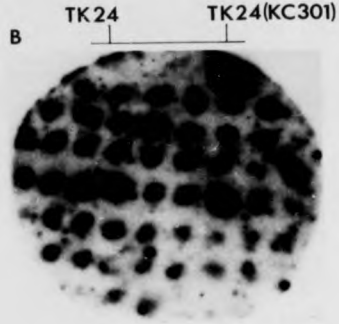


Fig.5.4

5.4 Densities of KC301 and *S. lividans* TK24 in sterile unamended soil at which interactions take place: discussion

Densities of populations of KC301 and *S. lividans* TK24 in soil needed to be above a certain level before interactions could take place. The densities of host and phage at which the largest increases in free phage densities occurred were also those at which the highest frequency of lysogeny was encountered (Experiment 2, microcosm set F), and the MOI here was 0.1. That is, there were no lytic or lysogenic interactions between KC301 and *S. lividans* TK24 when their densities were respectively less than 10^3 p.f.u. and c.f.u. g^{-1}. It is generally held that *in vitro*, a high MOI leads to an increased frequency of lysogeny (Freifelder, 1987). The results of these experiments suggest however that in soil, the frequency of lysogeny is dependent on the densities of both host and phage being sufficiently high to facilitate infective encounters, rather than on a particular ratio between the two populations.

CHAPTER 6

Long-term survival of KC301 and *S. lividans* TK24 in sterile unamended soil

6.1 Introduction

Liquid nutrient amendment of soil causes increases in phage populations which infect *Arthrobacter globiformis*, *Bacillus stearothermophillus* and *Ensifer adhaerans* (Reanney and Marsh, 1973; Tan and Reanney, 1976; Germida and Casida, 1981; Germida and Casida, 1983). Long-term survival of phages which infect *Azospirillum brasilense* and *B. subtilis* in soil has been demonstrated by showing increases of free phage density in soil when the soil received liquid nutrient amendment at defined times. This caused renewed growth of hosts in soil thus facilitating lysis by phage populations (Germida, 1986; Pantastico-Caldas *et al.*, 1992). The results of such studies have not, however, conclusively established the precise mechanism by which phage populations in soil are maintained through long periods of host inactivity, and are then able to reappear in an infective state when renewed growth of hosts occurs. Whilst virulent phages may persist in soil for long periods of time (Williams *et al.*, 1987), relying on the occurrence of susceptible hosts, it is likely that many phages survive in natural ecosystems as prophages. Therefore temperance may be a major factor in the long-term survival of many phages in the natural environment (Schmeiger, 1990).

So far, studies on the interactions of actinophage and host in soil have concentrated on one growth-cycle (Herron and Wellington, 1990). Cresswell *et al.* (1992) used a dynamic soil microcosm system to study the fate of a KC301 and a streptomycete plasmid in nonsterile soil. The aims of this experiment were to show repeated cycles of growth of *S. lividans* TK24 in the presence of KC301 in soil, to demonstrate repeated lysis and therefore several inputs of KC301 into the free phage

pool, and to deduce the mechanism by which a temperate phage such as KC301 may survive over long periods of time in soil.

6.2 Development of a microcosm and sampling system for studying long-term survival of KC301 and *S. lividans* TK24 in soil

The nonsterile dynamic soil microcosms of Cresswell *et al.* (1992) received 50 % soil turnover every fifteen days, and this resulted in a gradual step-wise diminishing of inoculants. This was probably due to competition with indigenous microorganisms compounded by the large amount of soil removed at each turnover diluting out inoculants. It was therefore decided to use sterile unamended soil and replace 20 % of the original wet weight of soil at turnover, *i.e.* the microcosms used in this experiment were the 500 g renewable type described in section 2.15.2. An additional reason for using sterile soil in this and subsequent experiments (Chapters 7 and 8) was to enable specific interactions between defined populations to be examined. Observing survival of inoculants in competition with an unknown array of indigenous microorganisms was not the object. The principle in using large volumes of soil was to facilitate both repeated non-destructive sampling over long periods of time, and replacement of a proportion of the soil with fresh soil to emulate nutrient turnover and physical disturbance of the soil ecosystem at defined times. The sample and eluent volumes of the spore-specific and phage extraction methods (sections 2.16.2 and 2.16.3) were scaled down by a factor of ten to allow repeated sampling from the renewable microcosms. These adapted methods were checked (data not shown) to ensure the trends observed using the original versions could still be seen when the extraction methods were scaled down.

6.3 Procedure for monitoring long-term survival of KC301 and *S. lividans* TK24 in sterile unamended soil

Five renewable microcosms of sterile unamended soil were prepared as described in section 2.15.2. These were inoculated with KC301 and/or *S. lividans* TK24 as shown in Table 6.2. and incubated at 22°C for forty-seven days.

Microcosms A, B and C received 20 % soil turnover (*i.e.* replacement of the original wet weight of soil with fresh, wet uninoculated soil) at fourteen day intervals (see section 2.15.2). Microcosms D and E received no soil turnover, but were thoroughly stirred at the same times that microcosms A, B and C underwent turnover. This was to check whether physical disturbance (and therefore redistribution of the nutrients present in the original microcosm soil), rather than addition of fresh nutrients and stirring, would cause changes in the two populations. Microcosms C and E were used to survey the survival of KC301 in soil in the absence of susceptible hosts, and microcosm B was used to survey the survival of *S. lividans* TK24 in soil in the absence of infective phage.

Table 6.1 **Microcosms used for studying long-term survival of KC301 and *S. lividans* TK24 in soil**

Microcosms (MOI)	KC301 (p.f.u. g^{-1} dry soil)	*S. lividans* TK24 (c.f.u. g^{-1} dry soil)
A (0.3)	5.6×10^4	1.9×10^5
B	-	1.9×10^5
C	5.6×10^4	-
D (0.3)	5.6×10^4	1.9×10^5
E	5.6×10^4	-

6.4 Long-term interactions of KC301 and *S. lividans* TK24 populations in soil

The mean extraction efficiencies were: for total viable propagules, 248 %; for spores, 136 %; and for actinophage, 31 %. These values were generally different from those observed in batch microcosms (Chapter 5), probably because the soil was inoculated by mixing in the SDW plus inoculants rather than by pouring it onto the soil which was not mixed in batch microcosms.

Putative lysogens were shown to spontaneously release phage and positively hybridize to ^{32}P-labelled KC301 DNA, by using methods described in sections 2.7.1 and 2.13.

Significant increases in free phage density (in the presence of *S. lividans* TK24) occurred at the start of incubation, when soil received turnover and when it was only stirred (Fig. 6.1). This repeating trend correlated with germination of both *S. lividans* TK24 and *S. lividans* TK24 (KC301) spores, which had arisen from infections, although these were not always significant. The lysogen population did not increase beyond 10^3 c.f.u. g^{-1} throughout incubation. The presence of KC301 did not appear to affect *S. lividans* TK24 populations (compare Fig. 6.1 to Fig. 6.2a). The efficiencies of infection (section 2.21; Table 6.2) were based on maximum increases in phage count which occurred between days 0 and 1 for day 1, 14 and 15 for day 15, 28 and 30 for day 30, and 42 and 44 for day 44, and the respective total viable host counts at days 1, 15, 30 and 44. Frequencies of lysogeny were about 10^{-5} when based on host counts at time t, and about 10^{-3} (after day 1) when based on the original host

Fig. 6.1 **Long-term survival of *S. lividans* TK24 and KC301 in sterile unamended soil, and formation of lysogens**

(A) Soil received 20 % turnover every fourteen days.

(B) Soil was stirred only every fourteen days.

Fig. 6.1

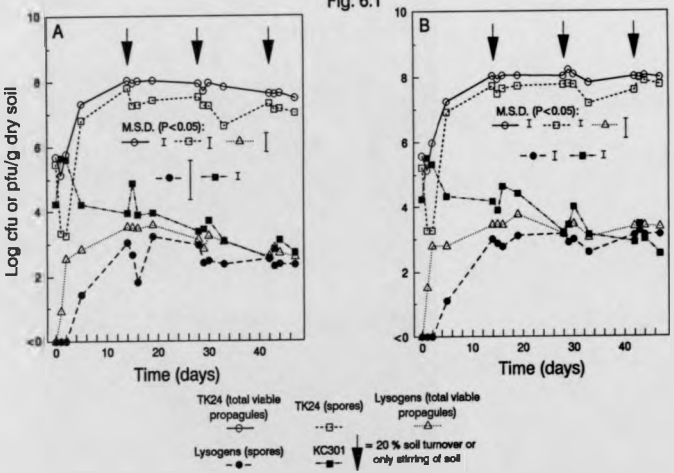
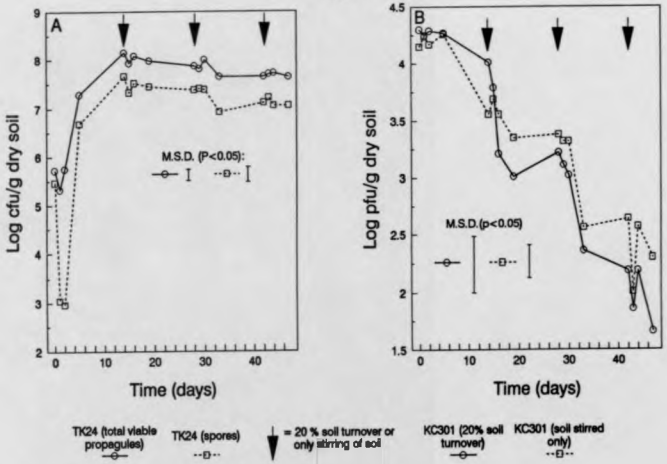

A
B
Log cfu or pfu/g dry soil
M.S.D. (P<0.05):
Time (days)
TK24 (total viable propagules)
TK24 (spores)
Lysogens (total viable propagules)
Lysogens (spores)
KC301
= 20 % soil turnover or only stirring of soil

Fig. 6.2 **Long-term survival of separate populations of *S. lividans* TK24 or KC301 in sterile unamended soil**

(A) *S. lividans* TK24 in soil which underwent 20 % turnover every fourteen days.

(B) KC301 in soil which underwent either 20 % turnover or stirring only every fourteen days.

Fig. 6.2


A
Log cfu/g dry soil
M.S.D. (P<0.05):
Time (days)
B
Log pfu/g dry soil
M.S.D.(p<0.05)
Time (days)
TK24 (total viable propagules)
TK24 (spores)
= 20 % soil turnover or only stirring of soil
KC301 (20% soil turnover)
KC301 (soil stirred only)

inoculum total viable count at day 0 (Table 6.2). The latter expression of lysogeny frequency is usually preferred, as *in situ* cell division during incubation can increase apparent frequencies of lysogeny when no actual further infections have occurred (Stotsky *et al.*, 1990).

In the absence of *S. lividans* TK24, KC301 did not increase in density, but its level gradually decreased with time from 10^4 p.f.u. g^{-1} at day 0 to 10^2 or 10^1 p.f.u. g^{-1} (depending on soil treatment) at day 47 (Fig. 6.2b). A similar pattern was followed in the microcosms which contained KC301 and *S. lividans* TK24, *i.e.* a diminishing of free phage density from 10^4 p.f.u. g^{-1} at day 0 to 10^2 p.f.u. g^{-1} at day 47. The only difference was the bursts in free phage density at turnover or stirring (Fig. 6.1).

Table 6.2 Mathematical analysis of some of the data from Fig. 6.1

Day	a	Frequency of lysogeny (Ht)	Frequency of lysogeny (Ho)
1	1.2×10^{-7}	5.9×10^{-5}	1.6×10^{-5}
15	1.7×10^{-10}	3.4×10^{-5}	6.2×10^{-3}
30	1.0×10^{-10}	1.9×10^{-5}	3.4×10^{-3}
44	2.8×10^{-10}	1.2×10^{-5}	1.0×10^{-3}

a = efficiency of infection (section 2.21).

Frequencies of lysogeny were based on Ht (total host count at time t) or Ho (total host count at time 0).

6.5 Long-term survival of KC301 and *S. lividans* TK24 in sterile unamended soil: discussion

Populations of KC301 and *S. lividans* TK24 interacting in soil resulted in the establishment of a lysogenic population which maintained a level about five orders of magnitude below that of the uninfected parent strain. The presence of a susceptible host population made little difference to the level of free phage in soil compared to the absence of susceptible hosts. This suggested that the host population was not actively growing except immediately after inoculation and at subsequent turnover. This was indicated by repeated peaks in phage density at these times. The KC301 population was capable of establishing itself in a population of lysogens, thus persisting over a long period of time. Furthermore, at soil turnover, lysis of hosts occurred resulting in fresh input into the free phage pool. This may have been due to lysis of either germinating *S. lividans* TK24 (by free KC301) or induction of *S. lividans* TK24 (KC301) or a combination of the two. It could be speculated that if the microcosms had been left for long enough for the detectable free phage population to entirely disappear due to prolonged host inactivity, KC301 would still persist in the prophage form. The efficiencies of infection fall below the level predicted (Williams *et al.*, 1987) to cause a numerical impact on host populations in soil. This factor may explain (in addition to the temperate nature of KC301) why no numerical impact on *S. lividans* TK24 in soil was observed.

CHAPTER 7

Competition between lysogenic and uninfected populations of *S. lividans* in sterile unamended soil

7.1 Introduction

Lysogenic conversion is the alteration of host phenotype by the expression of a gene carried on prophage DNA (Kokjohn and Miller, 1992). This phenomenon was demonstrated in lysogens of KC301 used in this project by the expression of thiostrepton resistance. The presence of prophage can exert other influences on the host. For example, Abebe *et al.* (1992) observed that lysogenization of *Bradyrhizobium japonicum* prevented it from forming nodules in soybean plants. Herron (1991) showed that *S. lividans* TK24 (KC301) did not survive as well as *S. lividans* TK24 in nonsterile soil, possibly due to lysis of germinating lysogenic spores. A hypothesis could be made that an uninfected host would have a competitive advantage over a lysogenic host of the same strain in soil, unless the soil contained a selective substance which favoured a gene expressed by the prophage, in which case the competitive advantage would be reversed. One aim of this experiment was to determine the competitive relationship between *S. lividans* TK23 and *S. lividans* TK24 (KC301) in soil. Thiostrepton is produced by *S. azureus* (Glasby, 1976). Although *S. azureus* is a natural soil streptomycete, production of thiostrepton in natural soil has not been detected. Production of thiostrepton in sterile soil has been demonstrated (M. Meijer and M.B. Schilhabel, personal communication) so it is possible this antibiotic is produced in natural soil ecosystems. It is generally assumed that antibiotic production in soil plays a role in competitive interactions, and there is evidence of antibiotic production at sites of high nutrient content in soil, for example the rhizosphere, decaying organic matter and seeds (Williams, 1982; section 1.2.1). Turpin *et al.* (1992) showed that reduced

survival of *Salmonella dusseldorf* (streptomycin sensitive) in sterile amended soil was caused by the presence of streptomycin or by the presence of coinoculated *Streptomyces bikiniensis*, a known streptomycin producer. The presence of an integrated prophage may be regarded as a nuisance to the host, which must divert resources towards the synthesis of the extra phage DNA (Cooper and MacCallum, 1984; section 1.3.2). Although this is compensated to some extent by the *tsr* gene of KC301 and superinfection immunity, the expression of these functions also requires host resources, possibly resulting in lysogens being metabolically disadvantaged compared to uninfected counterparts. Thus it was the aim of this experiment to investigate the role of selection in reversing the competitive advantage of *S. lividans* TK23 over *S. lividans* TK24 (KC301), therefore emulating the influence of the product of a soil streptomycete on the growth of other species in soil.

7.2 Procedure for comparing survival of mixed populations of *S. lividans* TK23 and *S. lividans* TK24 (KC301) in sterile unamended soil

Wellington *et al.* (1993) established that thiostrepton remained stable in soil in an extractable active state for seventy days, and used batch microcosms of sterile unamended soil to determine the effect of a range of thiostrepton concentrations (between 2 and 50 $\mu g. g^{-1}$) on the sensitive strain *S. lividans* TK23 when growing in soil. Thiostrepton at between 2 and 50 $\mu g. g^{-1}$ caused a drop in viable count. To check that thiostrepton at 50 $\mu g. g^{-1}$ had a deleterious effect on the sensitive strain, but no effect on the resistant lysogen in soil, this experiment was repeated using *S. lividans* TK23 and *S. lividans* TK24 (KC301) in separate batch microcosm sets (data not shown). Thiostrepton reduced the *S. lividans* TK23 total viable count in soil whilst having no effect on *S. lividans* TK24 (KC301), and therefore it was decided to add 50 $\mu g. g^{-1}$ of the antibiotic to the soil of three microcosms in this experiment (Table 7.1).

Six renewable microcosms were prepared as described in section 2.15.2 and were inoculated as described in Table 7.1.

The microcosms were incubated at 22°C for forty-seven days and underwent soil turnover every fourteen days as described in section 2.15.2. Thiostrepton was added to three of the microcosms (Table 7.1) at 50 μg. g^{-1} dry soil before wetting and inoculation, and was also included in the fresh soil at turnover times at the same level. All microcosms were sampled for spores, total viable propagules and actinophage as described in section 2.16. *S. lividans* TK24 was enumerated on RASS containing streptomycin; *S. lividans* TK24 (KC301) was enumerated on RASS containing thiostrepton and streptomycin; *S. lividans* TK23 was enumerated on RASS containing spectinomycin; and *S. lividans* TK23 (KC301) arising from infections was enumerated on RASS containing thiostrepton and spectinomycin (Table 2.2). Extracts from microcosms A and B were also plated on RASS containing spectinomycin and thiostrepton to check for spontaneous resistance mutations. Thiostrepton activity was monitored as described in section 2.18.

Table 7.1 **Microcosms used for determining the competitive relationships between *S. lividans* TK23 and *S. lividans* TK24 (KC301)**

Microcosms	*S. lividans* TK23 (c.f.u. g^{-1} dry soil)	*S. lividans* TK24 (KC301) (c.f.u. g^{-1} dry soil)
A*	-	2.8×10^5
B	-	2.8×10^5
C*	3.3×10^5	-
D	3.3×10^5	
E*	3.3×10^5	2.8×10^5
F	3.3×10^5	2.8×10^5

* = 50 μg. g^{-1} of thiostrepton included in soil.

7.3 Competition between *S. lividans* TK23 and *S. lividans* TK24 (KC301) in soil and selection for the phage-borne gene

The mean extraction efficiencies for the total viable propagules method for *S. lividans* TK23 and *S. lividans* TK24 (KC301) were 313 and 167 % respectively, and for the spore-specific method, 189 and 86 % respectively. No spontaneous mutants resistant to thiostrepton and spectinomycin were found in extracts from microcosms A and B.

In the microcosms inoculated with *S. lividans* TK23 alone, thiostrepton caused a significant drop in the total viable propagules count between days 1 and 2 (Fig. 7.1a) whereas in its absence, this count significantly increased over the same period (Fig. 7.1b). Conversely, in the microcosms inoculated with *S. lividans* TK24 (KC301) alone, there was a significant increase in the total viable propagules count of *S. lividans* TK24 (KC301) in the presence of thiostrepton between days 1 and 2 (Fig. 7.2a), whereas in its absence, this count significantly dropped over the same period (Fig. 7.2b). This trend was not repeated at subsequent soil turnovers. After thirty days, the maximum population level of *S. lividans* TK24 (KC301) was about one order of magnitude lower than that of *S. lividans* TK23 when these populations were grown separately (Figs. 7.1 and 7.2).

When *S. lividans* TK23 and *S. lividans* TK24 (KC301) were inoculated into the same microcosms, they achieved population densities which were lower than when they were grown separately (Figs. 7.1, 7.2, 7.3 and 7.4). The maximum population density of *S. lividans* TK24 (KC301) remained at about one order of magnitude below that of *S. lividans* TK23, whether in the presence or absence of thiostrepton (Figs. 7.3 and 7.4).

Fig. 7.1 **Long-term survival of *S. lividans* TK23 in sterile unamended soil**

(A) Soil contained 50 $\mu g.\ g^{-1}$ of thiostrepton.

(B) Soil contained no thiostrepton.

Fig. 7.1

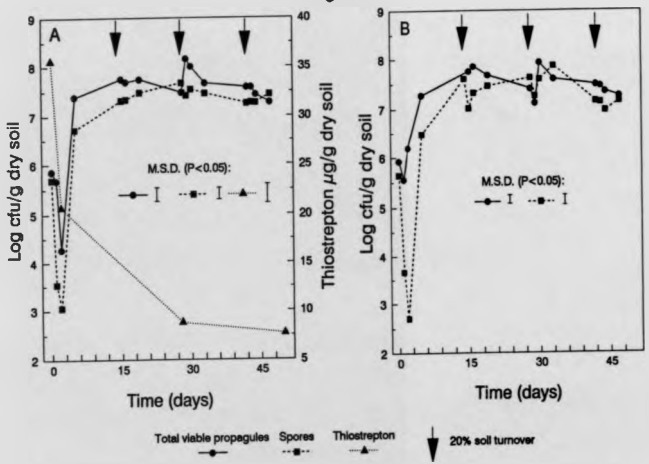

A
B
Log cfu/g dry soil
Thiostrepton μg/g dry soil
M.S.D. (P<0.05):
Time (days)
Total viable propagules
Spores
Thiostrepton
20% soil turnover

Fig. 7.2 **Long-term survival of *S. lividans* TK24 (KC301) and release of KC301 in sterile unamended soil**

(A) Soil contained 50 $\mu g. g^{-1}$ of thiostrepton.

(B) Soil contained no thiostrepton.

Fig. 7.2

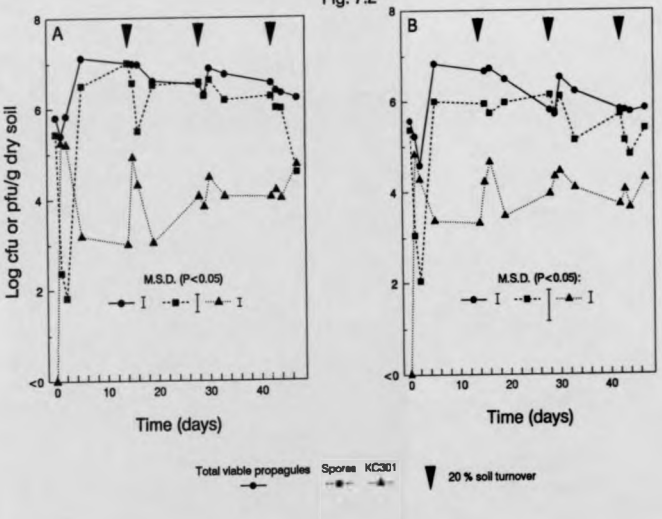

A
B
Log cfu or pfu/g dry soil
M.S.D. (P<0.05)
M.S.D. (P<0.05):
Time (days)
Time (days)
Total viable propagules
Spores
KC301
20 % soil turnover

Fig. 7.3 **Long-term survival of *S. lividans* TK23 and *S. lividans* TK24 (KC301) in sterile unamended soil containing 50 $\mu g. g^{-1}$ of thiostrepton**

(A) Population changes in *S. lividans* TK23 and *S. lividans* TK24 (KC301).

(B) Population changes in KC301 released from *S. lividans* TK24 (KC301) and consequent lysogeny of *S. lividans* TK23.

Fig. 7.3

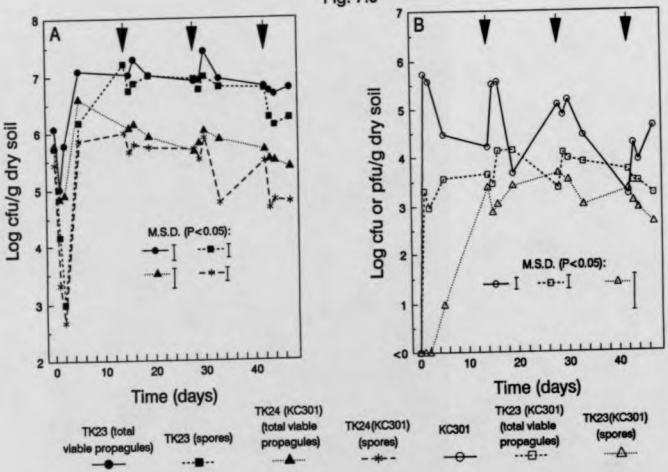
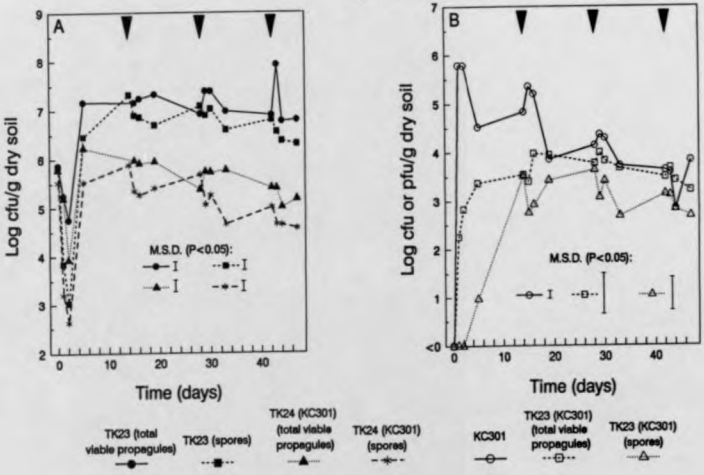

A
B
Log cfu/g dry soil
Log cfu or pfu/g dry soil
M.S.D. (P<0.05):
Time (days)
TK23 (total viable propagules)
TK23 (spores)
TK24 (KC301) (total viable propagules)
TK24(KC301) (spores)
KC301
TK23 (KC301) (total viable propagules)
TK23(KC301) (spores)

Fig. 7.4 **Long-term survival of *S. lividans* TK23 and *S. lividans* TK24 (KC301) in sterile unamended soil containing no thiostrepton**

(A) Population changes in *S. lividans* TK23 and *S. lividans* TK24 (KC301).

(B) Population changes in KC301 released from *S. lividans* TK24 (KC301) and consequent lysogeny of *S. lividans* TK23.

Fig. 7.4


A
B
Log cfu/g dry soil
Log cfu or pfu/g dry soil
Time (days)
M.S.D. (P<0.05):
TK23 (total viable propagules)
TK23 (spores)
TK24 (KC301) (total viable propagules)
TK24 (KC301) (spores)
KC301
TK23 (KC301) (total viable propagules)
TK23 (KC301) (spores)

S. lividans TK23 (KC301) lysogens arising from infections by KC301 released from *S. lividans* TK24 (KC301) were detected from day 1 onwards, and reached a maximum population density of about 10^4 c.f.u. g^{-1} (Figs. 7.3 and 7.4). Putative lysogens arising from infections in the microcosms (*i.e.* colonies that were both spectinomycin and thiostrepton resistant) were confirmed as being lysogenic using the methods described in sections 2.7.1 and 2.13.

The free phage population in the microcosms inoculated with *S. lividans* TK24 (KC301) alone stabilized at around 10^4 p.f.u. g^{-1} (Fig. 7.2), whereas in the microcosms containing *S. lividans* TK23 and *S. lividans* TK24 (KC301) and no thiostrepton, this population steadily declined (Fig. 7.4). A definite trend cannot be seen in the equivalent microcosm containing thiostrepton because of the erratic nature of the KC301 counts after day 19 (Fig. 7.3).

The extraction efficiency of thiostrepton from soil at day 0 was 71 %. Although the level rapidly declined up to day 15, thiostrepton in a recoverable active form was detected at a level which could effect *S. lividans* TK23 (Wellington *et al.*, 1993) up until the end of the incubation period (Fig. 7.1a). Thiostrepton is plotted on Fig. 7.1a as means of activities measured in the three microcosms containing the antibiotic.

7.4 Competition between lysogenic and uninfected populations of *S. lividans* in sterile unamended soil: discussion

KC301 was released from lysogenic populations following inoculation (microcosms C and D) and soil turnover. These inputs into the free phage pool were comparable to those observed previously. There was, however, no reduction in free phage density here, as opposed to when KC301 was introduced into soil as a lysate. This may have been due to the fact that the lysogen was introduced rather than formed in the soil, and was present at a far greater density (between 10^6 and 10^7 c.f.u. g^{-1}) than encountered before (Chapter 6; about 10^3 c.f.u. g^{-1}). None of the

inputs of free phage in microcosms C and D of this experiment would have been due to infections because of homoimmunity. Based on the phage and spore counts at days 0 and 1 in microcosms inoculated with *S. lividans* TK24 (KC301) alone, and assuming the mean burst size observed *in vitro* (55) was similar to the burst size in soil, it was calculated that about 1 % of the spores released KC301 on germination in soil, compared to 10 % *in vitro* (Chapter 3; Lomovskaya *et al.*, 1980). This suggested that the different conditions in the soil of this experiment compared to the peptone broth of the experiments described in Chapter 3 may have exerted different influences on the lysis-lysogeny choice for KC301. The poor nutrient conditions of the soil may have been responsible for causing a greater proportion of the prophage pool to remain in the lysogenic cycle rather than being released into the free phage pool.

S. lividans TK24 (KC301) achieved a population density one order of magnitude lower than that of *S. lividans* TK23 (either when coinoculated or in separate microcosms) which suggested that the fitness of the lysogen in soil was somehow compromised. This effect may have been due to lysis, although the presence of foreign genes which are being expressed in hosts is known to cause a metabolic burden on that host as has been observed with plasmids (Ogden *et al.*, 1992). The lower extraction efficiencies of *S. lividans* TK24 (KC301) compared to those of *S. lividans* TK23 may be a further indication of a difference in fitness. *S. lividans* TK23 may therefore out-compete *S. lividans* TK24 (KC301) in the long-term. This competitive advantage was not reversed by the presence of thiostrepton. Sporulation compensated for the killing effect of thiostrepton during the first mycelial phase of *S. lividans* TK23. Thiostrepton induces production of various proteins in *S. lividans* via the *tipA* promoter (Murakami *et al.*, 1989), which may degrade thiostrepton (C.J. Thompson, personal communication). This may explain the decreasing levels of antibiotic in soil throughout the experiment, although levels of thiostrepton in soil in the absence of *S. lividans* were not monitored. The level of thiostrepton used in this experiment was obviously not high enough to cause long-

term depletion of sensitive populations. Recent studies have shown that much higher concentrations (500 $\mu g. g^{-1}$) cause death of sensitive streptomycetes in soil, and result in selection for resistant strains introduced into nonsterile soil by causing depletion of indigenous streptomycetes (I.K. Toth, personal communication). 500 $\mu g. g^{-1}$ is however a very high concentration, unlikely to be encountered in natural soil, which is another reason why the level in this experiment was set at a lower concentration.

The maximum population densities of both *S. lividans* TK23 and *S. lividans* TK24 (KC301) were lower when growing together in soil than when growing separately, which was probably due to intraspecific competition for resources such as space and nutrients.

As in earlier experiments, the population levels of lysogens arising from *in situ* infections stabilized at densities much lower than those of the parent strains. This may be due to order of establishment of populations in soil, which is an important factor in the achievement of population levels. Richaume *et al.* (1992) noted in studies of transconjugant populations that order of establishment influenced final densities of pseudomonads inoculated into soil. Dynamics of transconjugant populations may however differ from those of lysogenic populations, especially when considering the effects different host and phage densities might have on infection efficiencies, and therefore loss of host numbers by lysis (Williams *et al.*, 1987).

CHAPTER 8

Competitive interactions between *S. lividans* TK23 and *S. coelicolor* J1501, and the influence of lysogeny of *S. coelicolor* J1501 by KC301 in sterile unamended soil

8.1 Introduction

Establishment, growth and survival of microbial hosts of genetically engineered material in natural habitats are dependent on the genetic composition of the host, as well as the physical, chemical and biological factors of a particular habitat (Stotsky and Babich, 1986). As shown in Chapter 7 and by Herron (1991), the growth of *S. lividans* TK24 (KC301) in soil was compromised compared to that of uninfected *S. lividans*, and thus the potential survival of *S. lividans* in soil was impaired by the presence of engineered DNA in the form of a prophage. This compromised survival ability may be aggravated in the presence of other fitter species in the natural habitat, and therefore result in a total population crash of a soil inoculant introduced, for example, into agricultural soil for a specific task. In terms of biological factors of a particular habitat, the survival of an introduced organism would depend on the outcome of positive or negative interactions with indigenous species. The result of such interactions would also affect the probability of gene transfer (Stotsky and Babich, 1986). Interspecific gene transfer between *S. violaceolatus* ISP 5438 (pIJ673) and *S. lividans* TK24 (mediated by a plasmid) was performed in both sterile and nonsterile soil by Wellington *et al.* (1990), but interspecific gene transfer between streptomycetes in soil mediated by phage (by either transduction or lysogenic conversion) has not been demonstrated. The aim of this experiment was to study a streptomycete widely used in genetic engineering (*S. coelicolor*) in terms of its survival (in lysogenic and uninfected forms) in soil in the presence of *S. lividans* TK23, and the effects of interspecific gene transfer. The

particular strain of *S. coelicolor* used was *S. coelicolor* J1501 (Chater *et al.*, 1982), chosen because it has no known plasmids. *S. coelicolor* J1501 is a histidine and uracil auxotroph, and therefore competitively compromised compared to *S. lividans* TK23. This property also facilitated the study of a genetically engineered streptomycete in competition with a fitter prototrophic strain.

8.2 Procedure for comparing survival of *S. lividans* TK23 and *S. coelicolor* J1501 (KC301) in sterile unamended soil

Renewable soil microcosms were prepared as described in section 2.15.2. These were inoculated as described in Table 8.1 and incubated at 22°C for nineteen days, and underwent soil turnover at day 14 as described in section 2.15.2. Spores, total viable propagules and actinophage were sampled and enumerated using the methods of section 2.16. As it could not grow on RASS, *S. coelicolor* J1501 was plated on R2.5 containing streptomycin, and *S. coelicolor* J1501 (KC301) was plated on R2.5 containing streptomycin and thiostrepton. *S. lividans* TK23 was plated on RASS containing spectinomycin, and *S. lividans* TK23 (KC301) arising from infections by KC301 released from *S. coelicolor* J1501 (KC301) was selected for on RASS containing spectinomycin and thiostrepton. The possibility of spectinomycin and streptomycin resistant colonies arising in microcosm B was checked by plating extracts on R2.5 containing these two antibiotics.

Table 8.1 **Microcosms used for determining interactions between *S. coelicolor* J1501 (KC301) and *S. lividans* TK23 in sterile unamended soil**

Microcosms	*S. coelicolor* J1501 (c.f.u. g^{-1} dry soil)	*S. coelicolor* J1501 (KC301) (c.f.u. g^{-1} dry soil)	*S. lividans* TK23 (c.f.u. g^{-1} dry soil)
A	2.4×10^5	-	-
B	2.4×10^5	-	3.4×10^5
C	-	-	3.4×10^5
D	-	2.4×10^5	-
E	-	2.4×10^5	3.4×10^5

8.3 Competition between *S. lividans* TK23 and *S. coelicolor* J1501 or *S. coelicolor* J1501 (KC301) in sterile unamended soil

The mean extraction efficiencies for the total viable propagules method were 359 %, 169 % and 134 % for *S. lividans* TK23, *S. coelicolor* J1501 and *S. coelicolor* J1501 (KC301) respectively. For the spore specific extraction method, efficiencies were 74 %, 42 % and 21 % for *S. lividans* TK23, *S. coelicolor* J1501 and *S. coelicolor* J1501 (KC301) respectively. No spectinomycin and streptomycin resistant colonies were isolated from microcosm B.

S. coelicolor J1501 germinated and resporulated in sterile unamended soil (Fig. 8.1a) following a similar pattern to that of *S. lividans* TK23 (Fig. 8.2a). *S. coelicolor* J1501 spores did not reach a level after germination comparable to the inoculum until day 7, whereas *S. lividans* TK23 spores had reached this level by day 5. *S. coelicolor* J1501 and *S. coelicolor* J1501 (KC301) both reached a maximum population level of 10^7 c.f.u. g^{-1} when growing alone (Fig. 8.1), whereas *S. lividans* TK23 reached a maximum population level of 10^8 c.f.u. g^{-1}. In the microcosm containing coinoculated populations of these two species, *S. lividans* TK23 reached 10^8 c.f.u. g^{-1} (Fig. 8.2b), similar to the maximum population when it grew alone in soil (Fig. 8.2a). *S. coelicolor* J1501 could only achieve 10^6 c.f.u. g^{-1} in the presence of *S. lividans* TK23 (Fig. 8.2b), whereas growing alone in soil

Fig. 8.1 **Population changes in *S. coelicolor* J1501 and *S. coelicolor* J1501 (KC301) in sterile unamended soil**

(A) Survival of *S. coelicolor* J1501 in soil.

(B) Survival of *S. coelicolor* J1501 (KC301) and release of KC301 in soil.

Fig. 8.1

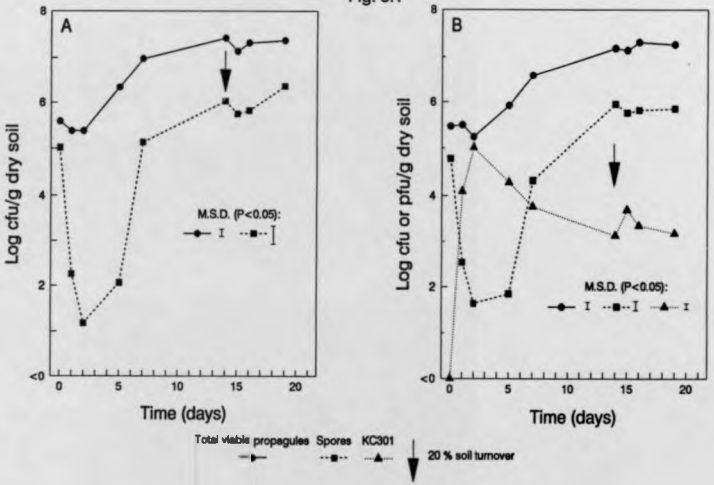
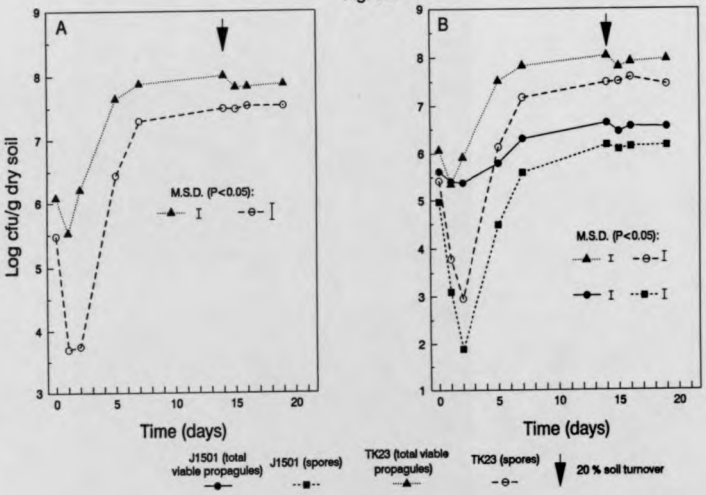

A
B
Log cfu/g dry soil
Log cfu or pfu/g dry soil
M.S.D. (P<0.05):
M.S.D. (P<0.05):
Time (days)
Time (days)
Total viable propagules
Spores
KC301
20 % soil turnover

Fig. 8.2 **Population changes in *S. lividans* TK23 in sterile unamended soil and competition with *S. coelicolor* J1501**

(A) Survival of *S. lividans* TK23 in soil.

(B) *S. lividans* TK23 and *S. coelicolor* J1501 in soil.

Fig. 8.2


A
B
Log cfu/g dry soil
Time (days)
M.S.D. (P<0.05):
J1501 (total viable propagules)
J1501 (spores)
TK23 (total viable propagules)
TK23 (spores)
20 % soil turnover

it reached 10^7 c.f.u. g^{-1} (Fig. 8.1a). Germinating *S. coelicolor* J1501 (KC301) lysed and released KC301 after inoculation and soil turnover (Fig. 8.1b).

S. lividans TK23 growing in the presence of *S. coelicolor* J1501 (KC301) reached 10^8 c.f.u. g^{-1} (Fig. 8.3a). *S. coelicolor* J1501 (KC301) however only reached 10^5 c.f.u. g^{-1}, less than the maximum level attained by *S. coelicolor* J1501 when growing in the presence of *S. lividans* TK23. When growing alone in soil, germinating *S. coelicolor* J1501 (KC301) released KC301 which had a peak level at 10^4 p.f.u. g^{-1}, which diminished to 10^3 p.f.u. g^{-1} after seven days (Fig. 8.1b). Growing in the presence of *S. lividans* TK23 in soil, germinating *S. coelicolor* J1501 (KC301) released KC301 to a peak density of 10^6 p.f.u. g^{-1}, which diminished to 10^4 p.f.u. g^{-1} after seven days (Fig. 8.3b). There was no significant increase in free phage density at soil turnover in the microcosm inoculated with both *S. lividans* TK23 and *S. coelicolor* J1501 (KC301) (Fig. 8.3b).

S. lividans TK23 (KC301) arising from infections by KC301 released from lysing *S. coelicolor* J1501 (KC301) were detected from day 1 onwards (Fig. 8.3b), and this population reached a maximum density of 10^5 p.f.u. g^{-1}.

S. lividans TK23 (KC301) which resulted from infections in the soil were confirmed as being lysogenic using the methods described in sections 2.7.1 and 2.13.

8.4 Competitive interactions between *S. lividans* TK23 and *S. coelicolor* J1501, and the influence of lysogeny of *S. coelicolor* J1501 by KC301 in sterile unamended soil: discussion

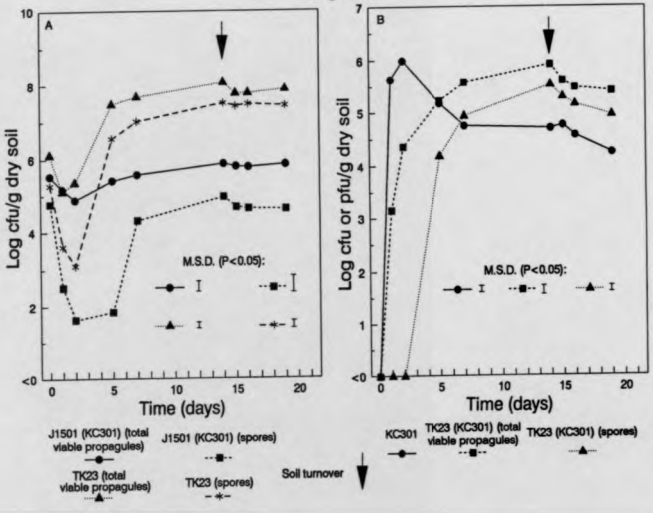
The auxotroph *S. coelicolor* J1501 survived less well than prototrophic *S. lividans* TK23 in sterile unamended soil, and this disadvantage was aggravated by

Fig. 8.3 **Competition between *S. lividans* TK23 and *S. coelicolor* J1501 (KC301) in sterile unamended soil, and formation of lysogenic *S. lividans* TK23**

(A) Population changes in *S. lividans* TK23 and *S. coelicolor* J1501 (KC301).

(B) Population changes in KC301 released from *S. coelicolor* J1501 (KC301) and consequent lysogeny of *S. lividans* TK23.

Fig. 8.3


A
B
Log cfu/g dry soil
Log cfu or pfu/g dry soil
Time (days)
M.S.D. (P<0.05):
J1501 (KC301) (total viable propagules)
J1501 (KC301) (spores)
TK23 (total viable propagules)
TK23 (spores)
Soil turnover
KC301
TK23 (KC301) (total viable propagules)
TK23 (KC301) (spores)

the presence of a prophage in *S. coelicolor* J1501 (KC301). Lysogenic conversion of *S. lividans* TK23 by KC301 released from *S. coelicolor* J1501 (KC301) resulted in a population of *S. lividans* TK23 (KC301) which could attain a similar population density to *S. coelicolor* J1501 (KC301). Hence a third population at least as fit as *S. coelicolor* J1501 (KC301) (probably even fitter as it was not auxotrophic) had resulted from *in situ* gene transfer. This was a model of what may occur in natural soil habitats, as both bacteria and bacteriophage concentrations found in natural ecosystems are high enough to allow transduction to take place (Kokjohn and Miller, 1992).

Based on the quoted mean burst size of øC31 *in vitro* (55; Chater *et al.*, 1986), 0.4 % of the germinating spores released KC301. In *S. coelicolor* A3(2) (øC31) cultures, 1 % of germinating spores spontaneously lyse *in vitro*. The difference seen with *S. coelicolor* J1501 (KC301) may have been due to starvation conditions in soil, although physiological differences between *S. coelicolor* A3(2) and *S. coelicolor* J1501, and between øC31 and KC301 cannot be ruled out as influencing differences such as these.

CHAPTER 9

Growth and survival of *S. coelicolor* J1501 (KC301) and KC301 in nonsterile amended soil

9.1 Introduction

The effects of release of KC301 into natural soil were investigated in Chapter 4. Although the survival of free phages in soil is reported to be good (Williams *et al.*, 1987), the results of Chapters 6, 7 and 8 suggest that the survival capacity of KC301 was enhanced by the presence of susceptible hosts and by its ability to exist in the prophage state. It could therefore be postulated that KC301, introduced into natural soil via inoculation of a lysogenic host, would survive longer than if it were introduced in the free state as in Chapter 4. This would in turn increase the probability of spreading phage-borne genes into natural populations. The aims of this experiment were to study the fate of *S. coelicolor* J1501 (KC301) inoculated into nonsterile amended soil, and determine whether KC301 was released and could infect indigenous hosts. The experiment reported in Chapter 8 may be viewed as a prototype to predict the outcome of this experiment. The prediction was that *S. coelicolor* J1501 (KC301) would be competitively inferior to indigenous streptomycetes (as it was to *S. lividans* TK23), but that prophage induction would result in a high soil titre of free KC301, which would be capable of establishing itself in indigenous hosts as in Chapter 4.

9.2 Procedure for monitoring S. coelicolor J1501 and *S. coelicolor* J1501 (KC301) in nonsterile amended soil

Three sets of batch microcosms (section 2.15.1) were amended with 2 % (w/w) chitin as described in section 2.15. It was decided to amend the soil to give

the auxotrophic (but chitinolytic) inoculant a chance to germinate in the presence of competition from indigenous microorganisms. Batch microcosms were used so that high volumes of soil could be sampled, and thus increase the probability of detecting indigenous lysogens in the spore-specific extracts. The microcosms were inoculated as shown in Table 9.1, and incubated at 22°C for fourteen days.

The microcosms were sampled for spores, total viable propagules and actinophage (section 2.16). Cycloheximide and nystatin (Table 2.2) were included in all bacterial plating media. *S. coelicolor* J1501 (KC301) was enumerated on R2.5 containing streptomycin and thiostrepton; *S. coelicolor* J1501 was enumerated on R2.5 containing streptomycin; indigenous streptomycetes were enumerated on RASS; indigenous thiostrepton resistant streptomycetes were enumerated on RASS containing thiostrepton (this medium was also intended to select for lysogens). Extracts from microcosm set C were plated out on all the media used in this experiment to ensure no naturally resistant streptomycetes grew on the various selective media. In addition, extracts from all microcosm sets were plated on RASS containing rifampicin and thiostrepton in an attempt to select for lysogens against background thiostrepton resistance in microcosm set A, the rationale being that selection for a double resistance would allow a high probability of detecting indigenous lysogens if present as in Chapter 4.

Table 9.1 **Microcosms used for studying survival of *S. coelicolor* J1501 and *S. coelicolor* J1501 (KC301) in nonsterile unamended soil**

Microcosm	*S. coelicolor* J1501 (KC301) (c.f.u. g^{-1} dry soil)	*S. coelicolor* J1501 (c.f.u. g^{-1} dry soil)
A	2.4×10^5	-
B	-	2.4×10^5
C	-	-

Microcosm set C was inoculated with SDW only.

9.3 Survival of *S. coelicolor* J1501, *S. coelicolor* J1501 (KC301) and KC301 in nonsterile amended soil

The efficiencies for the total viable propagules extractions were 121 % and 117 % for *S. coelicolor* J1501 (KC301) and *S. coelicolor* J1501 respectively. For the spore specific extractions, efficiencies were 5 % and 1 % for *S. coelicolor* J1501 (KC301) and *S. coelicolor* J1501 respectively. In microcosm set C, no streptomycetes grew on R2.5 containing either streptomycin or streptomycin plus thiostrepton.

The proportion of p.f.u. enumerated in microcosm set A that were KC301, and homology between KC301 and indigenous phages in microcosm sets B and C was checked as follows. Fifty plaques per sample day isolated from all microcosms were picked onto SNA overlays containing *S. lividans* TK24 on NA, and incubated at 30°C for twenty-four hours. Plaque DNA was fixed to nylon filters as described in section 2.12, and probed with DIG-labelled KC301 DNA (section 2.14). Only plaques isolated from microcosm set A hybridized positively to DIG-labelled KC301 DNA (Fig. 9.1). The proportion of KC301 diminished from 96 % at day 1, when lysis of hosts was first detected, to 28 % at day 14 (Fig. 9.2b).

The densities of clear p.f.u. was always lower than those of turbid p.f.u. (Figs. 9.2a, 9.3b, 9.4b and 9.5a).

Fig. 9.1 **Nylon filter containing plaque DNA hybridized with DIG-labelled KC301 DNA**

About 50 plaques per sample day were picked from phage assay plates from the microcosm sets. This filter represents isolates from microcosm sets A, B and C sampled on days 1 and 2. Plaques from sample set A can be seen as largely positive hybridizations. The number of positive hybridizations out of 50 (sample set A) represented the proportion of p.f.u. on assay plates that were KC301. Positive and negative controls were *S. lividans* TK24 (KC301) and *S. lividans* TK24 respectively.

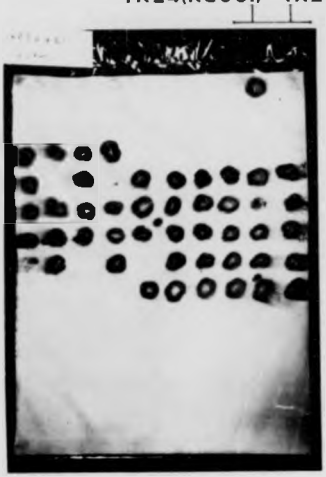


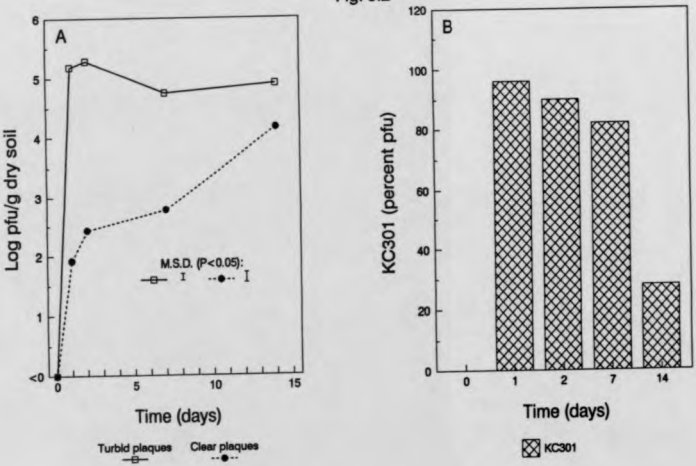
Fig.9.1

Fig. 9.2 **Survival of actinophages in nonsterile amended soil inoculated with *S. coelicolor* J1501 (KC301)**

(A) Population changes in actinophages. A proportion of phages represented by the turbid plaques was KC301 (B).

(B) Proportion of plaques which positively hybridized to DIG-labelled KC301 DNA (Fig. 9.1).

Fig. 9.2


A
Log pfu/g dry soil
6
5
4
3
2
1
<0
0
5
10
15
Time (days)
M.S.D. (P<0.05):
Turbid plaques
Clear plaques
B
KC301 (percent pfu)
120
100
80
60
40
20
0
0
1
2
7
14
Time (days)
KC301

S. coelicolor J1501 (KC301) germinated and lysed in nonsterile amended soil releasing KC301 (Fig. 9.3b), but its density gradually diminished over fourteen days, and there was no significant re-sporulation (Fig. 9.3a). The density of spores at day 14 was two orders of magnitude lower than that of *S. coelicolor* J1501 (Fig. 9.4a). *S. coelicolor* J1501 significantly re-sporulated between days 2 and 14 (Fig. 9.4a), the final spore density being 10^5 c.f.u. g^{-1} (this may have been as high as 10^6 c.f.u. g^{-1} when considering the low extraction efficiency of the spore-specific method for *S. coelicolor* J1501 in nonsterile soil). Indigenous streptomycete spores reached a maximum density of 10^7 c.f.u. g^{-1}, whether in the presence or absence of inoculated *S. coelicolor* J1501 or *S. coelicolor* J1501 (KC301) (Figs. 9.3a, 9.4a and 9.5a). In some cases spore counts were higher than total viable propagules counts, and this cannot be explained other than as an unpredictable factor in dealing which nonsterile soil.

Thiostrepton resistant indigenous streptomycetes were isolated from all microcosms (Figs. 9.3b, 9.4b and 9.5b). None of these spontaneously released phage when picked onto SNA overlays of *S. lividans* TK24 (section 2.7.1), nor did they positively hybridize to radio-labelled KC301 DNA (section 2.13). No thiostrepton and rifampicin resistant streptomycetes were isolated from any of the microcosms.

9.4 Release of KC301 from *S. coelicolor* J1501 (KC301) in nonsterile amended soil: discussion

Lysogeny of indigenous hosts by KC301 was not demonstrated in this experiment. This may have been due to several factors, the first being that the soil was amended with chitin (there were no amendments made to the soil in the experiment described in Chapter 4 where lysogeny of indigenous populations did arise). This would have created a relatively nutrient-rich environment, which may have caused reduced frequencies of lysogenic infections (Freifelder, 1987; section

Fig. 9.3 **Survival of *S. coelicolor* J1501 (KC301) and release of KC301 in nonsterile amended soil**

(A) Population changes in *S. coelicolor* J1501 (KC301) and indigenous streptomycetes.

(B) Population changes in indigenous actinophages and KC301 released from *S. coelicolor* J1501 (KC301). KC301 constituted a proportion of the turbid plaques (Figs. 9.1 and 9.2). Indigenous streptomycetes naturally resistant to thiostrepton are also shown.

Fig. 9.3

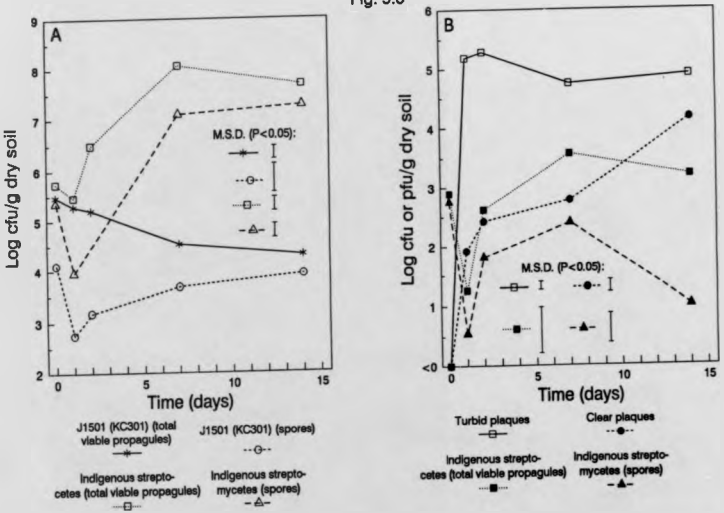

A
Log cfu/g dry soil
Time (days)
M.S.D. (P<0.05):
J1501 (KC301) (total viable propagules)
J1501 (KC301) (spores)
Indigenous streptocetes (total viable propagules)
Indigenous streptomycetes (spores)
B
Log cfu or pfu/g dry soil
Time (days)
M.S.D. (P<0.05):
Turbid plaques
Clear plaques
Indigenous streptocetes (total viable propagules)
Indigenous streptomycetes (spores)

Fig. 9.4 Survival of *S. coelicolor* J1501 in nonsterile amended soil

(A) Population changes of *S. coelicolor* J1501 and indigenous streptomycetes.

(B) Population changes of indigenous actinophages and naturally thiostrepton resistant indigenous streptomycetes.

Fig. 9.4

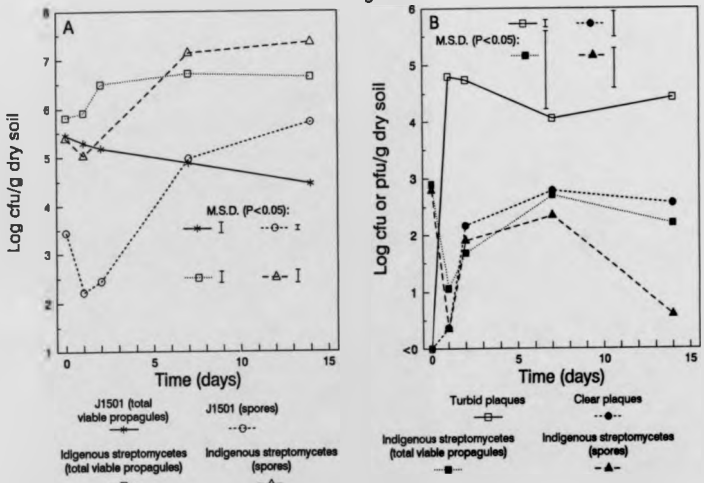
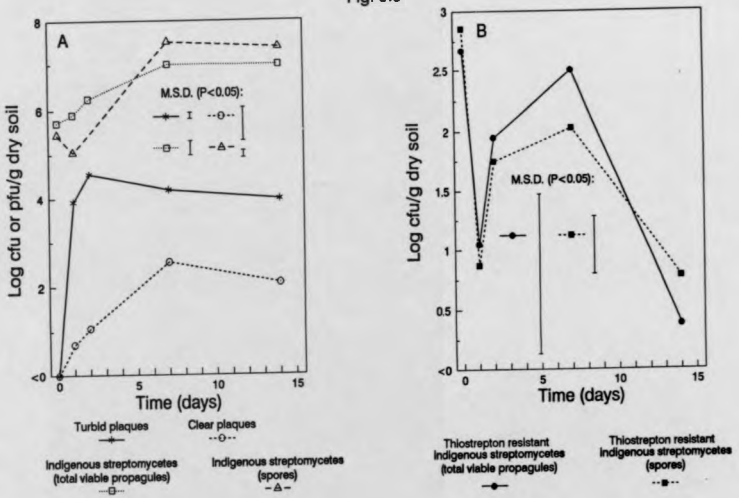

A
Log cfu/g dry soil
M.S.D. (P<0.05):
Time (days)
J1501 (total viable propagules)
J1501 (spores)
Idigenous streptomycetes (total viable propagules)
Indigenous streptomycetes (spores)
B
M.S.D. (P<0.05):
Log cfu or pfu/g dry soil
Time (days)
Turbid plaques
Clear plaques
Indigenous streptomycetes (total viable propagules)
Indigenous streptomycetes (spores)

Fig. 9.5 Population changes of indigenous streptomycetes and actinophages in nonsterile amended soil

(A) Population changes in indigenous streptomycetes and actinophages.

(B) Population changes in indigenous naturally thiostrepton resistant streptomycetes.

Fig. 9.5


A
M.S.D. (P<0.05):
Log cfu or pfu/g dry soil
8
6
4
2
<0
0
5
10
15
Time (days)
Turbid plaques
Clear plaques
Indigenous streptomycetes (total viable propagules)
Indigenous streptomycetes (spores)
B
M.S.D. (P<0.05):
Log cfu/g dry soil
3
2.5
2
1.5
1
0.5
<0
0
5
10
15
Time (days)
Thiostrepton resistant indigenous streptomycetes (total viable propagules)
Thiostrepton resistant indigenous streptomycetes (spores)

1.3.2), and therefore lysogens were not detectable. The localization of indigenous bacteria in soil aggregates relative to inoculated bacteria is important when considering genetic interactions between these two communities. Recorbet (personal communication) found that in soil fractionation studies, the majority of indigenous soil bacteria were located in large aggregates (> 100 μm), whereas inoculated *E. coli* were only detected at a low level in that fraction. If the indigenous streptomycetes in this experiment were similarly located in large aggregates, they may have been in locations inaccessible to inoculated spores, so that when KC301 was released on lysis of germinating spores, it was not in the immediate vicinity of the majority of indigenous streptomycetes. Conversely, in the experiment described in Chapter 4, KC301 was added in a lysate, and therefore may have been able to permeate soil aggregates to a degree not possible by far larger spores, and thus come into contact with indigenous spores as they germinated.

The enumerated clear p.f.u. may have represented virulent phages, although they may have originated from indigenous lysogens, and formed clear plaques due to the conditions used for enumeration. However, if these clear plaques did represent virulent phages, they existed at a far lower density than temperate phages (counted as turbid plaques, whether KC301 or indigenous). This balance of virulent/temperate population in favour of temperate phages may have been due to natural selection for temperate phages in soil, or because the conditions in which the soil had been kept prior to the experiment, (*i.e.* desiccated), selected for temperate phages.

The proportion of p.f.u. enumerated from the microcosm set inoculated with *S. coelicolor* J1501 (KC301) which were KC301 declined from day 1 onwards. In nonsterile soil inoculated with KC301 lysate as described in Chapter 4, the proportion of recovered p.f.u. remained the same (100 %) throughout the fifteen days of incubation. The cause of this difference was likely to be the different methods of inoculation, *i.e.* the KC301 particles inoculated via the lysate were not necessarily in contact with large populations of re-sporulating streptomycetes

following wetting of the soil. The KC301 particles inoculated via *S. coelicolor* J1501 (KC301) in this experiment would be in close proximity of their host, and probably became adsorbed to host material following induction and were therefore less extractable than encountered in Chapter 4.

CHAPTER 10

Relationships between growth, activity and infection of *S. lividans* TK24 by KC301 in sterile unamended soil

10.1 Introduction

Herron and Wellington (1990) indicated that decreases in spore density of *S. lividans* in soil corresponded to increases in phage density, and therefore indicated germination of the host spores. Direct measurements of host activity in soil were not made other than detecting increases in phage density and viable counts during apparent germination of the host. It was therefore necessary to investigate the metabolic activity of *S. lividans* TK24 and *S. lividans* TK24 (KC301) in sterile unamended soil, and to correlate measurements with changes in KC301 density. There are many methods of measuring microbial activity in soil such as measuring potential enzyme activity (Parkinson *et al.*, 1971; Burns, 1978) and soil respiration (Parkinson *et al.*, 1971; Anderson, 1982). Actual activity in soil can only be realistically estimated by taking measurements from undisturbed soil *in situ*. For measurements of particular soil enzymes such as chitinases and ureases, soil samples must be taken and treated under defined conditions, which results in estimations of potential activity which may relate to the actual activity (Parkinson *et al.*, 1971). Urease activity has been widely studied in soil because of its importance in breakdown of urea-based fertilizers (Bremner and Mulvaney, 1978). There is much extracellular urease present in soil, which can persist due to binding to soil colloids such as clay particles. This presence does not necessarily correspond to *in situ* microbial activity as it is an intracellular enzyme, its exogenous state in soil being largely due to release from dying cells. Hence assay methods such as that of McGarity and Myers (1967) rely on addition of substrate to soil samples in order to estimate the urease activity of soil microflora, and so is a less direct method of

activity measurement than, say, estimation of extracellular enzymes which are present in soil due to excretion from active cells. Chitinase is an example of extracellular soil enzymes, and is particularly important in the *Streptomyces* spp. life-cycle (Locci, 1989) for the biodegradation of fungal wall and arthropod exoskeleton material. Wirth and Wolf (1992) developed a method for estimating fungal chitinase in soil. This method was adapted for streptomycetes by using pH 8.0 buffer in the chitinase assay of this study (section 2.19.1). *S. lividans* TK24 produces extracellular chitinase in minimal media with chitin as the sole carbon source, and at low levels in the absence of chitin (Neugebauer *et al.*, 1991). Chitinase activity was monitored in sterile unamended soil inoculated with *S. lividans* TK24 spores in the presence and absence of KC301 (*i.e.* repeating a batch microcosm experiment described by Herron and Wellington (1990) with the additional monitoring of chitinase activity). Actual metabolic activity was also monitored by measuring CO_2 evolution from renewable microcosms inoculated with *S. lividans* TK24 or *S. lividans* TK24 (KC301), using the lysogen in this case to show release of KC301 corresponded to growth of germinated spores in soil.

10.2 Procedure for measuring chitinase activity in sterile unamended soil inoculated with *S. lividans* TK24 and KC301

Batch microcosms (section 2.15.1) of the 10 g, 20 g and 100 g type were inoculated with *S. lividans* TK24 and KC301 as shown in Table 10.1. Microcosm set A was inoculated with SDW only for obtaining background measurements of activity. Microcosms were incubated for fifteen days at 22°C and on days 0,1,2,7 and 15, *S. lividans* TK24 spores, total viable propagules and KC301 were extracted and enumerated as described in section 2.16. *S. lividans* TK24 was plated on RASS containing streptomycin.

Table 10.1 **Microcosms used for monitoring growth of *S. lividans* TK24 and KC301 and chitinase activity in sterile unamended soil**

Microcosm (MOI)	*S. lividans* TK24 (c.f.u. g^{-1} dry soil)	KC301 (p.f.u. g^{-1} dry soil)
A	-	-
B	2×10^5	-
C (1)	2×10^5	2×10^5

Chitinase activity in the soil from the 10 g microcosms was monitored (section 2.19.1) on the same days plate counts were made.

10.2.1 Correlation between the life cycles of *S. lividans* TK24 and KC301 and chitinase activity in sterile unamended soil

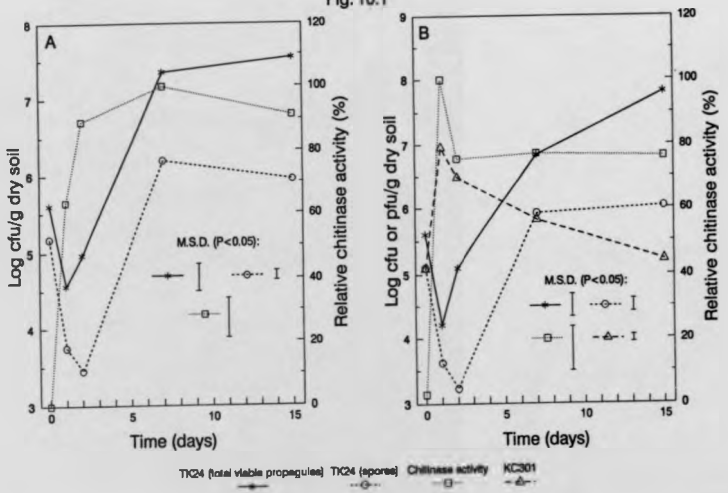
The mean efficiencies for the total viable propagules and spore specific extraction methods were 205 % and 70 % respectively. The phage extraction method was 61 % efficient. The spore counts dropped nearly two orders of magnitude between days 0 and 2, after which the spore density rose to stabilize at about 10^6 c.f.u. g^{-1} between days 7 and 15 (Fig. 10.1). The total count (total viable propagules) rose to 10^7 c.f.u. g^{-1}. There was a significant increase in KC301 density between days 0 and 1, after which the level of free phage gradually

Fig. 10.1 The relationship between chitinase activity and growth of *S. lividans* TK24 and KC301 in sterile unamended soil

(A) Population changes of *S. lividans* TK24 and corresponding chitinase activity.

(B) Population changes of *S. lividans* TK24 with KC301 and corresponding chitinase activity.

Fig. 10.1


A
B
Log cfu/g dry soil
Log cfu or pfu/g dry soil
Relative chitinase activity (%)
Time (days)
M.S.D. (P<0.05):
TK24 (total viable propagules)
TK24 (spores)
Chitinase activity
KC301

decreased with time (Fig. 10.1b). Significant increases in chitinase activity were detected between days 0 and 2 in soil inoculated with *S. lividans* TK24 spores (Fig. 10.1a), after which there were no significant changes in activity which levelled off at around 95 % of maximum activity, the maximum activity occurring at day 7. In soil inoculated with *S. lividans* TK24 and KC301, the chitinase activity was at its maximum at day 1, after which it significantly dropped and levelled out at about 77 % of maximum activity. This early chitinase peak correlated with the KC301 peak.

10.3 Procedure for measuring carbon dioxide evolution from sterile unamended soil inoculated with *S. lividans* TK24 or *S. lividans* TK24 (KC301)

Growth patterns of *S. lividans* TK24, *S. lividans* TK24 (KC301) and KC301 in sterile unamended soil have been well established (Chapters 5, 6 and 7), so it was therefore decided to monitor CO_2 evolution from soil using the system described in section 2.19.2, without taking samples for plate counts during incubation. This would eliminate aberrations to CO_2 flux caused by removal of soil samples. Renewable microcosms of 500 g of air-dried sterile unamended soil (section 2.15.2) were inoculated with *S. lividans* TK24 and *S. lividans* TK24 (KC301) as shown in Table 10.2. Microcosm A was inoculated with SDW only to act as the control (section 2.19.2) for the measurement of any CO_2 evolution from sterile soil not associated with microbial growth.

Table 10.2 **Microcosms used for monitoring carbon dioxide evolution from sterile unamended soil inoculated with *S. lividans* TK24 and *S. lividans* TK24 (KC301)**

Microcosm	*S. lividans* TK24 (c.f.u. g^{-1} dry soil)	*S. lividans* TK24 (KC301) (c.f.u. g^{-1} dry soil)
A	-	-
B	4.5×10^5	-
C	-	2.0×10^5

Samples were taken at day 0 and day 12 (*i.e.* immediately before and immediately after the incubation period throughout which CO_2 evolution was monitored). Total viable propagules and KC301 were extracted and enumerated from these samples as described in section 2.16. *S. lividans* TK24 was plated out on RASS containing streptomycin, and *S. lividans* TK24 (KC301) was plated out on RASS containing streptomycin and thiostrepton. CO_2 readings were taken on days 0, 1, 5, 9 and 12 as described in section 2.19.2.

10.3.1 Carbon dioxide evolution from sterile unamended soil inoculated with *S. lividans* TK24 or *S. lividans* TK24 (KC301)

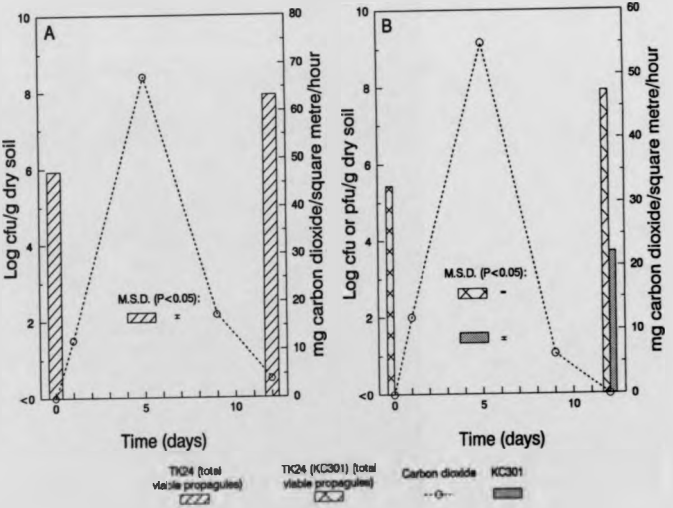
The total viable propagules extraction method for *S. lividans* TK24 and *S. lividans* TK24 (KC301) was 195 % and 175 % efficient respectively. Significant rises in *S. lividans* TK24 and *S. lividans* TK24 (KC301) density occurred between days 0 and 12 (Fig. 10.2). No free KC301 were detected on day 0, but free phage

Fig. 10.2 **Activity of *S. lividans* TK24 and *S. lividans* TK24 (KC301) in sterile unamended soil measured as respiration**

(A) Evolution of CO_2 from soil containing *S. lividans* TK24.

(B) Evolution of CO_2 from soil containing *S. lividans* TK24 (KC301), and release of KC301 over twelve days.

Fig. 10.2


A
B
Log cfu/g dry soil
Log cfu or pfu/g dry soil
mg carbon dioxide/square metre/hour
M.S.D. (P<0.05):
Time (days)
TK24 (total viable propagules)
TK24 (KC301) (total viable propagules)
Carbon dioxide
KC301

was detected at day 12 at 10^3 p.f.u. g^{-1} (Fig. 10.2b). The highest rate of CO_2 evolution occurred between days 1 and 5 (Fig. 10.2), after which time the rate fell to relatively low levels between days 9 and 12 (it was not possible to take replicate samples for CO_2 readings, so no statistical analysis has been performed on these results). A preliminary experiment (data not shown) confirmed that the pattern of CO_2 evolution shown in Fig. 10.2 was repeatable under the conditions cited.

10.4 Relationships between growth, activity and infection of *S. lividans* TK24 by KC301 in sterile unamended soil: discussion

The measurements of metabolic activity in soil inoculated with host and phage populations showed that cycles of infection and release of phage from lysogens in sterile soil microcosms were associated with host growth. Furthermore, these results justify the conclusions of Herron and Wellington (1990) in that the fall in spore counts observed after inoculation as detected using the spore specific extraction method corresponded to germination of spores and consequent lysis by KC301. Potential and actual host activity measurements (chitinase and CO_2 respectively) showed that phage-host interactions in soil occurred during times of host activity. The fall in chitinase relative activity to less than 80 % between days 1 and 2 in the presence of KC301 suggested there may have been an impact on the host population by the presence of phage. This may have been due not only to lysis of susceptible propagules by lytic infections, but also to release of lysozyme from lysing cells, which may build up on soil aggregates and affect growth of uninfected propagules.

CHAPTER 11

General discussion

11.1 General considerations of the project

Most experiments relied on extraction and enumeration of viable soil populations, and monitoring of various substances in soil such as thiostrepton and molecules arising from microbial activity. The advantages and limitations of these methods require discussion (section 11.2) to place them in perspective of the observations made and conclusions that were drawn.

The impact of phage populations due to lysis of host populations is a basic consideration in any study of viral ecology. The basic reproductive rate (R_0), the number of secondary infections arising from one infected individual introduced into a large population of uninfected individuals, is an important concept in the theory of infectious diseases (Nowak, 1991). For an infection to spread in a population of susceptible individuals, the condition $R_0 > 1$ must be met. This condition is limited by the host reproductive rate, and as discussed in section 11.3, sporulation limited the impact of KC301 on host numbers (probably reducing the condition $R_0 > 1$ to $R_0 < 1$), and therefore in soil, net KC301 infections only occurred early in experiments.

A major issue throughout the project concerned the question: which mode of cell-to-cell transmission predominates in a temperate phage population in soil? In simple terms the phage can firstly choose horizontal transmission, where infection is rapidly followed by release of multiple copies of the original particle. This is a fast way of multiplication but at a high risk, *i.e.* the destruction of the infected cell and the probability that the progeny particles may not find new hosts to infect. Secondly, the phage may choose vertical transmission, where the integrated prophage is multiplied with the host chromosome in a slow but more secure mode

of reproduction. Observations of the behaviour and effects of the KC301 prophage on hosts in soil (discussed in section 11.4) may answer the above question. It is notable that temperate phages are analogous to the latent viruses of eukaryotes (Nowak, 1991), and the sudden appearence of epidemics of viral diseases (*e.g.* AIDS) may be due to changes in environmental conditions, which affect reversion of the latent state to the virulent state. The interpretations of the experiments of this project may therefore be seen as basic models of viral epidemics.

11.2 Use and adaptation of existing extraction methods

Examination of extraction efficiencies for both total viable propagules and spore-specific extraction methods reveals a great deal of variation. The very high extraction efficiencies of the total viable propagules method (usually well over 100 %) were no doubt caused by vigorous shaking fragmenting spore chains and mycelium, thus increasing the number of colony forming units (Skinner, 1951). The spore-specific method (Herron and Wellington, 1990) was designed to overcome the problem of fragmentation by gentle chemical extraction of streptomycetes from soil samples, although as Herron (1991) found, the efficiencies for this method still showed much variation, usually at a range between 20 and 200 %. Despite these variations, the efficiencies of extraction from batch microcosms appeared to be lower than those from the renewable type. This was probably due to the different modes of inoculation, *i.e.* in batch microcosms, populations were poured (in SDW) onto the soil and allowed to percolate downwards, whereas in the renewable microcosms, populations were mixed into the soil by thorough stirring. This action may have broken up spore chains and mycelial fragments in the soil, thus increasing the number of colony forming units. The difference was reversed in the case of the phage extraction method, which was generally more efficient (between about 60 and 100 %) with batch microcosms than with the renewable type (about 30 %). Again this was possibly due to the different inoculation techniques, where stirring of the

renewable microcosm soil may have caused an increased rate of irreversible adsorption of phage to soil particles. In all cases, extraction efficiencies were lower in nonsterile soil, possibly due to irreversible binding to soil substrates (*e.g.* organic matter) whose binding properties were changed in sterile soil due to autoclaving.

The total viable count results for phage and hosts must therefore be regarded as estimates of relative abundance rather than actual measurements of density, especially with respect to the rather arbitrary practice of using colony forming units to describe filamentous organisms. The spore-specific method, especially when applied to counts after initial germination and re-sporulation of inoculants (*i.e.* after days 5 or 7) can however be regarded as actual measurements of propagule densities in the microcosms, as the host populations appeared to exist mainly as spores after this time. Other methods may be used for detection and enumeration of streptomycetes in soil. The difference in resilience of streptomycete cell walls and spore walls, to physical and chemical disruption, has been exploited to differentially extract plasmid DNA from soil inoculated with a *Streptomyces* species from defined stages of the growth cycle, and thus monitor the life cycle in soil (Cresswell *et al.*, 1991). There are numerous examples of the use of DNA extraction and gene probes to detect specific sequences in soil samples, although these are largely concerned with detection rather than population monitoring over time (Ogram *et al.*, 1987; Holben *et al.*, 1988; Tsai and Olsen, 1991; Pillai *et al.*, 1991; Tsai and Olsen, 1992a; Tsai and Olsen, 1992b; Bruce *et al.*, 1992). Many of these involved use of polymerase chain reaction (PCR) technology to amplify sequences in samples. Picard *et al.* (1992) used PCR to enumerate soil bacteria by comparing results to most probable number (MPN) tables, made possible by knowing the copy number of specific sequences in the populations being enumerated. This technique could be applied to the study of streptomycete populations in soil. Probes for RNA sequences could be used in a similar way (via inclusion of reverse transcriptase in reactions). Fluorescently labelled 16S rRNA probes could be used to detect actively growing mycelia in soil (Hahn *et al.*, 1990; Hahn *et al.* 1992), and quantification by U.V.

Hahn *et al.* 1992), and quantification by U.V. microscopy could be employed to quantify viable mycelium *in situ*. Monoclonal antibodies can be raised against *S. lividans* outer spore polypeptides (Wipat *et al.*, 1992), so these could be used in the same way if fluorescently labelled, although they would not be specific for metabolically active material.

The reduction in recovery of active thiostrepton over 50 days in sterile soil to about 16 % of the original concentration (Chapter 7) was comparable to the findings of Wellington *et al.* (1993), who noted the reduction of the antibiotic to 20 % after 70 days in sterile soil with no inoculants (12 % in nonsterile soil). The result in Chapter 7 therefore suggested reduction in antibiotic activity due to non-biological factors, such as binding to clay particles, although the presence of *S. lividans* which produces thiostrepton induced proteins which may degrade this antibiotic (C.J. Thompson, personal communication) cannot be ruled out as a factor influencing reduction of activity in soil.

The chitinase and CO_2 extractions (Chapter 9) supported the conclusion of Herron and Wellington (1990) that the dip in spore count between days 0 and 5 after inoculation of soil with *S. lividans* correlated with metabolic activity. This was also confirmed by the peaks in soil phage titre and lysogeny of hosts, which could only occur in the presence of actively growing hosts. The establishment of transconjugant populations of streptomycetes in soil (Wellington *et al.*, 1990; Cresswell *et al.*, 1992) during the first five days after inoculation also corresponds to this period of increasing chitinase and high CO_2 activity.

Although the batch microcosms could only be sampled once, and the inoculation method meant uneven distribution of populations (which would have been filtered out as SDW percolated down the soil, thus creating a density gradient), they did facilitate the detection of rare events such as lysogeny of indigenous hosts. This was because of the large volumes of soil used, and by use of the spore-specific extraction method in concentrating populations in extracts. The renewable microcosms allowed some interesting findings, supporting earlier conjecture that

streptomycetes exist mainly as spores in soil (Williams, 1978). The 20 % turnover method was expected to cause extensive re-germination of spores in sterile soil (manifested as large dips in the spore counts). However, once the apparent carrying capacity had been reached (about 10^8 c.f.u. g^{-1}), there was no net germination on subsequent turnovers. This may partly explain the findings of Cresswell *et al.* (1992), who noted the step-wise decline of inoculated *Streptomyces* spp. in similar nonsterile soil dynamic microcosms. That is, once initial net germination of inoculum was complete, no further net growth occurred on subsequent turnovers, so the inoculum was gradually diluted out (predation by indigenous microorganisms may also have been an important factor in this).

11.3 Interactions between KC301 and hosts in soil

Continually alternating oscillations of host and phage numbers might be expected in a steady state system in liquid, but this assumption is based on the presence of a continually growing host population (section 1.3.3). The results of the long-term phage and host survival experiments (Chapters 6 and 7) illustrated that dynamic interactions were not continually taking place, but only at times of soil disturbance when bursts in free phage occurred, with no apparent lysis of hosts (based on host densities). Williams *et al.* (1987) proposed a mathematical model to make trajectories of streptomycete and lytic actinophage systems in soil, based on parameters including phage infection efficiency (section 2.21) and burst size, the soil's carrying capacity (a density dependent function) and the proportion of hosts that exist as spores. At low infection efficiencies (10^{-8}), a steady state of coexistence was predicted as described above, whereas at high infection efficiencies (10^{-6}), the host population crashed, closely followed by the phage population. The lack of impact on the host populations in the long-term experiments may be explained in this way (*i.e.* infection efficiencies were too low to influence host numbers) in addition to KC301 being temperate. The sporulation of most propagules

following net initial germination would, however, protect the host population from further attack on a large scale. This last point was supported by one case where the infection efficiency of KC301 was 10^{-5}, where 10^3 c.f.u. g^{-1} of *S. lividans* TK24 and 10^4 p.f.u. g^{-1} of KC301 were inoculated into sterile soil in the density dependence experiment (Fig. 5.2a). Whereas normally the total viable count (spores plus mycelia) significantly ($P<0.05$) rose between days 1 and 2 after inoculation into sterile soil, here there was no rise, possibly caused by the high infection efficiency. But on sporulation between days 2 and 5, the host density reached the same level as normally expected at low infection efficiencies, or without the presence of phage. This observation directly concurs with the finding of Heddle (personal communication), who observed that when the combined infection efficiency of two heteroimmune actinophage populations (A4 and KC301 respectively) was 10^{-5}, it resulted in the same lack of increase in total viable count of *S. lividans* TK24 between days 1 and 2, which was compensated for by subsequent sporulation. This effect of sporulation compensating for death during hyphal growth was also evident with the presence of thiostrepton in soil (Chapter 7). The predictions of Williams *et al.* (1987) appear to be contradicted by these results, in that the differential life-style of streptomycetes protects them from phage-based extinction in soil. The net protracted sporulation would also result in low probabilities of formation of phage-resistant mutants (Pantastico-Caldas *et al.*, 1992) due to limited numbers of generations at a microsite at a given time. This would be beneficial to free infective actinophages where local germination occurred.

In all cases except *S. coelicolor* J1501, phage populations were established at densities lower than the susceptible host population. This phenomenon was also noted with *B. subtilis* and its phage, the equilibrium hypothesized to be maintained by the host (resource-limited) density being below the threshold for a phage epidemic (Pantasico-Caldas *et al.*, 1992). The equilibrium in this case may have been due to spore protection from phage attack, as well as the stationary state of the soil matrix restricting phage-host contact, as with *S. lividans* and KC301 in soil.

A trend was apparent of gradual reduction in height of the successive phage peaks at soil turnovers during the long-term survival experiments (Chapters 6 and 7). This may have been due to progressive utilization of resources by hosts during the repeated periods of growth, which resulted in eventual relative stasis (*i.e.* no net growth; only areas of isolated growth becoming less numerous with each turnover). A similar case was presented by Germida (1986), who noted that bursts in *Azospirillum brasilense* phage occurred on nutrient amendment of soil after seven days of incubation. No host or phage activity was detected following a repeated nutrient amendment after thirty-five days of incubation.

11.4 The role of lysogeny in the growth of KC301 and hosts in soil

Germination of *S. lividans* TK24 (KC301) spores in soil resulted in a 10^{-1}-fold reduction (from 10 to 1 %) of lysis compared to that *in vitro*. Conversely, there was little difference between the quoted percentage lysis of *S. coelicolor* lysogens *in vitro* (1 %; Lomovskaya *et al.*, 1980) and that of *S. coelicolor* J1501 (KC301) in soil. This value of 1 % may therefore represent the minimum frequency at which the øC31 prophage of *Streptomyces* spp. lysogens are induced into the lytic cycle regardless of environmental conditions. Furthermore, øC31 will mutate to the clear plaque phenotype (loss of *c* gene function) at a rate of 1×10^{-3} to 5×10^{-3} (Lomovskaya *et al.*, 1980). Therefore the phage bursts of about 10^5 p.f.u. g^{-1} in soil from *S. lividans* TK24 (KC301) (Chapter 7) may have included between 2×10^2 and 0.9×10^3 p.f.u. g^{-1} clear plaque mutants. Lysis of germinating lysogens due to such mutations may have accounted for a proportion of the phage bursts, although no clear plaques were detected on the NA plates (counting plaques at high extract dilutions may have eliminated the probability of detecting clear plaque mutants). Additionally, these mutation frequencies were based on infections using wild-type øC31 lysates (Lomovskaya *et al.*, 1972), *i.e.* mutations occurred during

the lytic cycle. Clear plaque mutations during cell division of lysogens may therefore occur at a far lower rate.

The 10^{-1}-fold reduction of KC301 induction from *S. lividans* TK24 (KC301) in soil (Chapters 3 and 7) was possibly due to resource limitation of the soil habitat compared to the abundant nutrients in *in vitro* experiments. The limited nutrients available in soil would make large scale induction disadvantageous to the prophage population because of temporal limitations in host growth, therefore 99 % of the prophage population (possibly via the influences discussed in section 1.3.2) remained uninduced. Kokjohn *et al.* (1991) similarly found that the *Pseudomonas aeruginosa* phage UT1, whilst being apparently lytic *in vitro*, was largely pseudolysogenic under induced starvation conditions in aquatic microcosms. Phage UT1 enters this carrier state in *P. aeruginosa* much sooner when incubated in nonsterile lake water microcosms than in sterile lake water microcosms, which may be indicative of environmental nutrient status influencing the lysis/lysogeny switch. Around 40 % of natural *P. aeruginosa* isolated from lake water were found to contain UT1 by Southern blot analysis (Ogunseitan *et al.*, 1990; Ogunseitan *et al.*, 1992). One major difference between results of these aquatic studies and those from soil microcosm studies was that lysogeny frequencies for inoculated *S. lividans* were high in sterile soil, and nonexistent in nonsterile soil (Herron and Wellington, 1990; Chapters 5, 6, 7 and 8). This difference may have been due to factors other than soil nutrient status, such as the low germination efficiency of *S. lividans* inoculum in nonsterile soil and the differentiated life-style of streptomycetes compared to the undifferentiated life-style of *P. aeruginosa*. Lysogeny frequency of indigenous streptomycetes in nonsterile soil (8.6×10^{-5}; Chapter 4) was comparable to that of *S. lividans* TK24 in sterile soil (1.6×10^{-5}) given the fact that different conditions and recipients were involved. This suggested that KC301 would lysogenize susceptible (due to positioning in soil and active growth) streptomycetes in soil regardless of nutrient status. Environmental factors did, however, appear to influence frequency of induction of *S. lividans* TK24 (KC301).

The presence of the KC301 prophage in mixed populations of *Streptomyces* spp. caused lysogenic populations to achieve lower maximum densities. This may be conceived as having the effect of allowing two populations (lysogenic and uninfected), in direct competition for local resources, to coexist at relatively high levels. The absence of prophage-induced debilitation (Cooper and MacCallum, 1984) would result in fiercer competition for resources thus negatively affecting community equilibrium. Unfortunately *S. lividans* TK24 and *S. lividans* TK23 were not incubated together in soil in the absence of KC301 to test this hypothesis. *S. coelicolor* J1501 and *S. lividans* TK23 were incubated together, resulting in the reduced population level of the former species, so lysogenic *S. lividans* in the same soil as the less fit *S. coelicolor* J1501 could have resulted in a reduced effect on *S. coelicolor* J1501, if resources were the limiting factors in this interaction. Similar balancing of mixed populations of *E. coli* by the presence of coliphages was demonstrated in continuous culture (Chao *et al.*, 1977). In another example of lysogeny altering host competitiveness, Abebe *et al.* (1992) demonstrated that lysogeny prevented *Bradyrhizobium japonicum* from nodulation on soybeans, even though the prophage was not inserted into the known nodulation genes of the host.

KC301 populations in the free state in soil gradually declined in infectivity with time, which was most likely due to physical soil factors including chemical damage to the adsorption apparatus and irreversible binding to soil particles such as clays. If left for a long time, free KC301 may therefore become undetectable *i.e.* extinct in a particular ecosystem. This temporal limitation to the virion population was nullified by the presence of KC301 as a prophage population, which not only maintained the population in host spores and thus facilitate long-term survival, but also enabled small inputs of free phage into the environment at times of intermittent lysogen germination. This would apportion some virions to the spread of the population to new microsites via water movement through soil fractures (McKay *et al.*, 1993), whilst most phages were retained as an inexhaustible supply of prophages, so long as the host population did not become extinct. A high density of

lysogens in soil (*ca.* 10^7 c.f.u. g^{-1}; Chapter 7) ensured the maintenance of the free phage pool at a defined level. Below this host density, the free phage density experienced the gradual decline as seen with phage and no susceptible hosts in soil. Pantastico-Caldas *et al.* (1992) found that a virulent mutant of *B. subtilis* temperate phage SP10C existed within viable spores of the host when incubated together in soil over a long period of time. This may partly explain the presence of clear and turbid plaques which appeared at day 1 but not day 0 when air-dried nonsterile soil was wetted with SDW (Chapters 4 and 9). That is, not only temperate phages, but also virulent phages may find refuge in dormant host stages during long periods of inactivity, to be released on resumption of host growth. Gradual desorption of infective phages from soil particles cannot be ruled out as another explanation of this observation.

11.5 General conclusions

Interactions between phages and hosts in soil relying on contact was density dependent, probably more so than in liquid ecosystems, because of the temporal and spatial limitations of the soil habitat. A derivative of a wild-type temperate actinophage survived in soil by lysogenizing streptomycetes which could persist as spores for long periods of time. Furthermore, this phage could lysogenize indigenous streptomycetes and thus persist as a prophage in nonsterile soil. Only a very small fraction of the prophage pool was released from lysogenic populations to supply the free phage pool, which was maintained at an infective density if susceptible hosts were present at a suitable density. Lysogens were competitively compromised in comparison to their uninfected counterparts in terms of population growth, although this disadvantage was negated by other phage-encoded benefits such as superinfection immunity and antibiotic resistance. The differentiated life-style of *Streptomyces* spp. counterbalanced killing effects of population challenges such as lysis by phage and mortality due to an antibiotic in the soil.

The prophage state is the long-term survival state of a high proportion of individual temperate phage populations, which while compromising host populations, facilitates renewal of free phage populations without further detriment to hosts.

11.6 Future work

The same environmental conditions were imposed in the microcosms in all experiments. A hypothesis arising from this thesis suggested that temperate phage populations exist in soil largely as prophages, thus facilitating their long-term survival. It is known that virulent phages may persist in soil for long periods (Williams *et al.*, 1987), but future experiments could usefully compare long-term survival of a virulent actinophage to a temperate one. This could be done by using a wild-type virulent actinophage heteroimmune to øC31, which would be able to propagate on øC31 lysogen populations. A virulent mutant of øC31 (as opposed to a clear plaque mutant, *i.e.* the binding sites of the *c* gene products are altered, resulting in øC31 *vl* mutants heteroimmune to wild-type øC31 (Lomovskaya *et al.*, 1980)) could be used in experiments to directly compare the difference between temperance and virulence in otherwise identical phage populations. These experiments would include leaving the phage in soil for long periods of time, without intermittent host growth (*i.e.* simulating protracted drought) until the soil had dried and all detectable free phage had disappeared. The soil could then be wetted, and assayed for free phage to deduce whether such adverse conditions were selective for temperate rather than virulent phages, by detecting the reappearance or not of free phage. It would also be useful to inoculate sterile soil containing no hosts with lysates, and allowing the soil to completely dry. After long periods of time, assaying soil for phage would indicate whether free phages (temperate or virulent) could persist infectively in inactive soil, for example by protective adsorption to soil

particles, or whether the presence of host populations (actively growing or dormant) was an absolute necessity for persistence.

A logical step following the studies in sterile soil described in this thesis would be to investigate similar phage-host interactions in natural (nonsterile) soil systems. KC301 can be detected in nonsterile soil by plating on specific host lawns on assay dishes (Chapters 4 and 9), but this requires the presence of that population in high densities. Molecular detection techniques, such as MPN-PCR (Picard *et al.*, 1992) could be used to monitor phages, with known primer sequences, at low levels in soil against a background of indigenous phages. Differential DNA extraction has already been used to monitor host streptomycete DNA in soil (Cresswell *et al.*, 1991; Cresswell *et al.*, 1992; Karagouni *et al.*, 1993), so this method could be employed in monitoring prophage DNA in soil, and compare measurements to host activity and free phage levels. Past studies of nonsterile soil (Herron and Wellington, 1990; Cresswell *et al.*, 1992) involved inoculating microcosms with laboratory strains. Although such an approach yields some information on interactions in the presence of competitive pressures of indigenous soil microflora, the situation is still artificial as large densities of foreign populations are added to soil containing previously established populations. It would therefore be of value to observe actual interactions of already present hosts and phages in nonsterile soil. Direct counting of phages using TEM, rather than relying on plaque formation on bacterial lawns, could be used to monitor fluctuations in phage populations in relation to host activity as in studies of marine systems (Bergh *et al.*, 1989; Bratbak *et al.*, 1990; Hara *et al.*, 1991; Miller and Sayler, 1992). Moreover, studies relating host activity to phage survival in soil (as initially attempted in Chapter 10) would help evaluate the frequency of net host activity required for the survival of phage populations. A detailed assessment of the numerically dominant streptomycetes in a particular soil, together with infection studies of representative phage isolates using soil extracts (obtained by the Lanning and Williams (1982) method C) would be a necessary prerequisite for such experiments. A constraint of

this approach is the requirement for host isolates to be culturable on laboratory media.

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