STUDIES ON *BACILLUS* BACTERIOPHAGE POPULATIONS IN COMPOST

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by

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To my wife Viv

and our sons Joshua and Luke

ABSTRACT

Studies on *Bacillus* bacteriophage populations in compost.

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Members of the indigenous *Bacillus* and bacteriophage population of mushroom compost, recovered at 50°C were monitored throughout the composting process and used as the basis of a study into the interaction of bacteriophages and hosts in natural environments. Forty-eight *Bacillus* isolates were assembled into ten groups on the basis of colonial morphology. Thirty-eight of the isolates were characterised using a range of biochemical and physiological tests and it was apparent that isolates within a particular group were similar to one another, but distinct from isolates in other groups. Group one strains were identified with *B. licheniformis* and Groups four and eight were identified with *B. thermodenitrificans*. The recovery of bacteriophages from mushroom compost was demonstrated and those isolated at 50°C were all found to be lytic and relatively host specific to isolates within a particular morphological group. An apparently thermoinducible bacteriophage was isolated and this was used as the basis for experiments to construct a trackable bacteriophage, that could be monitored in release studies. Two *Bacillus* transformation systems were studied with the intention of developing an optimised transfection system. Transfection was demonstrated, although at a low frequency of 20 transfectants per μg of DNA. Attempts to optimise this system were unsuccessful. A tagging sequence was constructed using PCR and inserted into the bacteriophage genome using shotgun cloning techniques. However, it was not possible to demonstrate the presence of viable tagged bacteriophages after transfection experiments. Mutants were isolated and used for monitoring a previously demonstrated transduction system in *B. caldotenax*. However, the poor survival of this strain and reversion of the mutants meant that it was not possible to adapt this system to monitoring in mushroom compost. The interaction of bacteriophages and hosts in mushroom compost was investigated by releasing *B. subtilis* 168 carrying a tetracycline resistance plasmid and bacteriophage ø105 carrying the *lacZ* gene. The use of these markers allowed interactions to be monitored in the absence and presence of the indigenous compost population. Results indicated that phage infections occurred after release into microcosms and the persistence of free bacteriophages was greater in the presence of host cells.

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ABBREVIATIONS

cAMP	- adenosine cyclic monophosphate
mA	- milliamp(s)
ATCC	- American Type Culture Collection
bp	- base pairs
BSA	- bovine serum albumin
CAMR	- Centre for Applied Microbiology and Research
ccc	- covalently closed circular plasmid DNA
°C	- degree (s) Centigrade
CDTA	- diaminocyclohexanetetraacetic acid
cfu	- colony forming unit
DNA	- deoxyribonucleic acid
DSM	- Deutsche Sammlung von Mikroorganismen
EDTA	- ethylenediaminetetraacetic acid
g	- gram (s)
mg	- milligram (s) (10^{-3}g)
µg	- microgram (s) (10^{-6}g)
x g	- gravitational force
kb	- kilobase (s)
GMO	- genetically engineered microorganism
h	- hour (s)
l	- litre (s)
ml	- millilitre (s) (10^{-3}l)

µl	- microlitre (s) (10^{-6}l)
M	- molar
mM	-millimolar (10^{-3}M)
µM	- micromolar (10^{-6}M)
pM	- picomolar (10^{-12}M)
cm	- centimetre (s) (10^{-2}m)
mm	- centimetre (s) (10^{-3}m)
µm	- micrometre (s) (10^{-6}m)
nm	- nanometre (s) (10^{-9}m)
min	- minute (s)
MOI	- multiplicity of infection
NCIB	- National Collection of Industrial Bacteria
OD	- optical density
PCR	- polymerase chain reaction
PEG	- polyethylene glycol
pfu	- plaque forming unit (s)
PNK	- polynucleotide kinase
rpm	- revolutions per minute
$Strep^R$	- streptomycin resistance
Tet^R	- tetracycline resistance
UV	- ultraviolet
v/v	- volume per volume
w/v	- weight per volume

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INTRODUCTION

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Introduction

1.1 Genetically Engineered Microorganisms

The adaptations of naturally occurring microorganisms to their environment are highly diverse and represent an invaluable genetic tool. Biotechnology can exploit this, by using genetic engineering techniques that allow the manipulation of strains for more effective or varied applications. Recombinant DNA technology allows the insertion of genes from one organism into a heterologous host. This results in the generation of so called genetically manipulated microorganisms (GMO's). The unique inserted DNA sequence(s) distinguishes the recombinant organism from its parental strain.

Recently there has been increased commercial interest in the environmental exploitation of GMO's of all types. Possible environmental applications of this technology are far reaching and include the control of plant diseases and insect pests (Payne, 1988) and the removal of xenobiotic compounds from the environment (Trevors et al., 1987; Pipke et al., 1992). Already, the use of recombinant DNA technology has resulted in increased efficiency in the excretion of extracellular proteins by members of the genus *Bacillus*. (Aiba et al., 1983; Imanaka et al.,1981; Kuriki et al., 1988; Vehmaanpera et al., 1987). The best known environmental use of recombinant DNA technology has been in the biological control of frost injury to crop plants. Ice nucleation bacteria contribute to frost damage by initiating the formation of ice in plants that would otherwise supercool and avoid ice formation. Control of frost damage has

been achieved by developing an "ice minus" strain of *Pseudomonas aeruginosa* to out-compete indigenous ice nucleating bacteria, so preventing frost damage (Dar et al., 1993).

The full scale exploitation of these possibilities has however been limited. These limitations arise because there is a lack of fundamental knowledge concerning the ecological impact of GMO's released into the environment, either accidentally or deliberately. These considerations have led to strict legislation regarding the deliberate environmental release of GMO's. By 1993 it was reported that 27 distinct releases of recombinant microorganisms in the open environment had occurred (Wilson and Lindow, 1993). The principal concerns associated with the construction and consequent release of GMO's have been described (Trevors et al., 1987) and the effect of a recombinant microorganism on the functioning of an ecosystem must be assessed before a release can be considered. Also, there is the potential for genetic transfer of the engineered sequence into the indigenous bacterial population. Such gene transfer may establish new genotypes that cause fluctuations in the equilibrium of the ecosystem and might conceivably result in the creation of a pathogen. In considering these issues, it is fundamental that studies of the survival and transfer of genes released into the environment are underpinned by the genetic analysis of indigenous microbial populations and the establishment of the extent to which gene transfer occurs within these populations.

There are several mechanisms by which gene transfer in the environment may occur, one of which is transduction, mediated by bacteriophages. Bacteriophages are ubiquitous in natural environments (Wommack et al., 1992) and it has been recently

shown that the numbers of phages in the marine environment, for example, have been underestimated such that bacteriophage numbers in the environment are sufficient to facilitate gene transfer within populations by transduction (Bergh et al.,1989). This raises the question of the potential for phage interaction leading to transduction in other natural environments where the indigenous bacteriophage populations have been insufficiently studied.

Initially an introduction to the genus *Bacillus* will be presented. Bacteriophages and their role as agents in gene transfer will then be examined. Methods for monitoring the interaction between bacteriophages and bacteria in the environment will also be discussed.

1.2 The genus *Bacillus*

The family *Bacillaceae* comprises Gram positive rod shaped bacteria that are characterised by the formation of endospores. The obligately anaerobic members of the family comprise the genus *Clostridium*; the genus *Bacillus* encompasses aerobes and facultative anaerobes. The genus was first described by Ferdinand Cohn in 1872 and the Cohn strain of *Bacillus subtilis* remains the type species of the genus. The genus is one of the most heterogeneous in the Eubacteria with 65 validly described species in *Bergey's Manual of Systemic Bacteriology* and new strains of new species are continually being described (Priest et al., 1993). Vegetative cells of *Bacillus* species are rod shaped with rounded or square ends in the size range from 0.5-2.5μm x 1.2-10μm (Claus and Berkeley, 1986). The cells may occur singly or in chains of variable length depending on the particular culture conditions or species. Most *Bacillus* species are

motile by means of peritrichous flagellae. The cell wall peptidoglycan of most members of the genus is of the directly cross linked meso-diaminopimelic acid type. Endospore formation is a multistage process allowing *Bacillus* species to survive conditions that are unfavourable to vegetative cells. The endospores are generally spherical, ellipsoidal or cylindrical. Their position in the mother cell and whether it swells the sporangium or not are characteristic for each species. The degree of resistance of endospores depends largely on the environmental conditions under which they were formed. The process of sporulation in *Bacillus* species has been an important model for studies into the complex mechanisms of prokaryotic differentiation (Errington, 1993).

Bacillus spp. are widely distributed in the environment, members of the genus being recovered from soils (Oguma et al., 1993), freshwater (Ogan and Nwiika, 1993), estuarine and freshwater sediments (Wuertz et al., 1991). *Bacillus* spp. were also isolated in a study of the bacterial flora in food packaging and board (Valsanen et al., 1991), although, as the *Bacillus* spores are passively distributed and persistent, occurrence is not necessarily a sign of activity.

There is a broad diversity of physiological abilities within the genus, especially in relation to growth temperature. The obligate thermophile *Bacillus stearothermophilus* has an optimum growth temperature of 65°C and a range of 40°C to 80°C. Mesophilic species include *Bacillus subtilis* with an optimum growth temperature of 37°C and a range of 25°C to 55°C. A psychrophilic *Bacillus* spp. has recently been isolated (Davail et al., 1994) and facultative thermophilic and caldoactive members of the genus have also been studied (Suzuki et al., 1992; Bealin-Kelly et al., 1990)

In regard to their pathogenicity for humans, the association of *Bacillus* species with infections, with the exception of *B. anthracis*, the causative agent of anthrax, is often not sufficiently rigid for them to be regarded unequivocally as the aetiological agent. It is becoming increasingly apparent that several other *Bacillus* species must be taken more seriously as pathogens, for example certain *B. cereus* strains produce toxins that cause food poisoning (Tuazon, 1995).

There is considerable commercial interest in the properties of *Bacillus* species, many enzymes and antibiotics being produced by *Bacillus* fermentations (Arbige et al., 1993). Other metabolites are produced commercially from *Bacillus* species for a wide range of medical, agricultural, pharmaceutical and other applications. Table 1.1 lists some of the commercial uses of *Bacillus* species. Another example is the use of an insecticide based on *B. thuringiensis* , which is particularly important for pest control on food crops. Biological insecticides do not pollute the environment and have further advantages over their chemical counterparts by being host specific towards insect larvae (Priest and Grigorva, 1991). However, concerns have been expressed recently that the introduction of new varieties and toxin mixtures such as those derived from recombinant technology should be carefully evaluated and not be assumed safe to the community on the basis of previous work with earlier insecticides (Drobniewski, 1994).

The commercial importance of *Bacillus* species has led to their increased use as hosts for gene cloning experiments. The concerns that arise over the use of this technology, especially with regard to gene transfer to indigenous populations, have

Table 1.1 Examples of some commercial uses of *Bacillus* species.

Commercial use	*Bacillus* species
Enzyme production	
∝-Amylases	*B. licheniformis*
Serine proteases	*B. stearothermophilus*
Glucose isomerase	*B. coagulans*
Recombinant protein production	
Pertussis toxin subunits	*B. subtilis*
ß-Lactamases	*B. subtilis*
Diphtheria toxin	*B. subtilis*
Pneumolysis	*B. subtilis*
Peptide antibiotic production	
Bacitracin	*B. licheniformis*
Bacilysin	*B. subtilis*
Brevistin	*B. brevis*
EM49	*B. circulans*
Octapeptins	*B. circulans*
Polymyxins	*B. polymyxa*

The information contained in this table was obtained from that published by Zuber et al., (1993) and Arbige et al., (1993).

been mentioned. The possible mechanisms by which the recombinant DNA may be transferred in the environment will now be reviewed.

1.3 Mechanisms of gene transfer in the environment

The three most common mechanisms by which genes may be transferred between bacteria in the environment are transformation, conjugation and transduction. These mechanisms have been studied using well characterised laboratory strains, which may not present a complete picture of the actual behaviour of indigenous populations of bacteria (Trevors et al.,1986).

The process of natural transformation involves the active uptake and heritable incorporation by a bacterial cell of free extracellular DNA (plasmid and chromosomal). Natural transformation occurs in certain genera of bacteria including *Haemophilus, Staphylococcus, Bacillus, Acinetobacter* and *Pseudomonas*, that are naturally competent during a stage in their growth cycle (Lorenz and Wackernagel, 1994). The free DNA for natural transformation may be released by decaying microbial cells or excreted by actively dividing cells (Ueda and Hara, 1981; Lorenz et al., 1991). The presence of free DNA in marine (Paul et al., 1988) and soil environments (Torsvik and Goksoyr, 1978) has been demonstrated. Romanowski et al., (1993), employed the polymerase chain reaction and electroporation to suggest that genetic material may persist in natural soils for months after its release from cells. It is thought that the binding of free DNA to particulates in the environment may protect it from the action of nucleases, allowing it to persist (Lorenz and Wackernagel, 1987). Natural transformation of *Bacillus subtilis* attached to sand grains has been demonstrated (Lorenz et al., 1988), as well as

introduced bacterial strains in soil (Graham and Istock, 1978; Lorenz and Wackernagel, 1990), and marine environments (Paul et al.,1991), although it appears to provide only limited opportunities for gene exchange. This is because in some environments DNA is rapidly degraded (Paul et al., 1987), and, particularly in marine environments, transformation is limited by the low cell densities present.

Transfer by conjugation is the most profoundly studied mechanism of gene transfer in soil (Top et al., 1990). Conjugative plasmids present within certain bacteria, can mediate their own transfer by a process that requires cell to cell contact through a sex pilus. These appendages on the donor cell surface interact with specific sites on the recipient cell. All the functions needed for conjugation are coded by the transfer (*tra*) operon carried by conjugative plasmids. Conjugative plasmids may mediate the transfer of other plasmids that may be present in the same donor cell. The mobilisation of a nonconjugative plasmid can occur either by the formation of a cointegrate between the two, or through the system coded by the conjugative plasmid. The latter case is dependent on the presence of a mobilisation (*mob*) site on the nonconjugative plasmid (Davis et al., 1980) and does not require physical contact between the two plasmids.

Transfer by conjugation or mobilisation has been demonstrated in lakes (O'Morchoe et al.,1988), seawater (Paul et al.,1991), rivers (Trevors and Oddie, 1986) and soil (Top et al.,1990; Wellington et al.,1990). Conjugative mobilisation of plasmid DNA has been demonstrated in the laboratory between *Bacillus* species (Oskam et al., 1991). The extent of conjugation, as a means of gene transfer in natural environments is limited by the low densities of microbes in some environments, reducing the probability of cell to cell contact. The frequency of conjugation may also be affected

by environmental parameters. For example, Trevors and Oddie (1986) showed that the transfer of antibiotic resistance plasmids to introduced *E. coli* occurred in nutrient amended soil but not in unsupplemented soil.

Kruse and Sorum (1994) showed that conjugation and transfer of R plasmids can occur between bacterial strains of human, animal and fish origin even in the absence of antibiotics. In human and veterinary medicine, plasmid mediated transfer of antibiotic resistance genes represents a major threat to the treatment of infectious diseases. Recently it was demonstrated that conjugation was occurring between an introduced *P. aeruginosa* strain and genetically dissimilar species within the indigenous soil biota (Glew et al., 1993).

1.4 Bacteriophages

Bacteriophages are viruses that specifically infect bacteria and were discovered independently by Twort in England and D'Herelle in France in 1917. The bacteriophage can be defined simply as an obligate intracellular parasite of bacteria that manifests no independent metabolic activity. A bacteriophage can propagate only in its bacterial host and uses the host's ribosomes and macromolecular synthetic machinery to do so. When free in the environment, phages are metabolically inert and cannot multiply (Kokjohn and Miller, 1989).

The role of bacteriophages as mediators of gene transfer was first discovered by Lederberg and co-workers (Lederberg et al.,1951). They showed that the genetic transfer between two strains of *Salmonella typhimurium* occurred when one strain was exposed to a cell free extract of the other. This transfer was shown to be mediated by

bacteriophage particles and termed transduction. Bacteriophage activity is indirectly displayed by means of lysis of the host bacterium. This is most easily demonstrated by using the soft agar overlay technique (Adams, 1959), where phage growth is indicated by areas of lysis which are termed plaques. A plaque is a colony of bacteriophages that arises from a single bacteriophage particle.

The lytic effect of a bacteriophage is not the only possibility upon infection, and certain bacteriophages can establish a long term association with their host termed lysogeny. Bacteriophages that are lysogenic to their host are said to be temperate, while those bacteriophages that can only display a lytic response are termed virulent.

1.4.1 The lytic cycle of infection

The stages in the infective process of a lytic bacteriophage may be considered as (1) adsorption of free bacteriophage; (2) penetration of bacteriophage nucleic acid; (3) replication of the viral nucleic acid; (4) packaging of the bacteriophage genome into a capsid and consequent release of new phage.

The cycle of infection begins with an initial contact between bacteriophage and bacterium. If the bacteriophage possesses an adsorption site that is complementary to a specific receptor site on the bacterial cell surface, structural changes occur in the receptor and bacteriophage particle and irreversible adsorption occurs. Using bacteriophage SP50 and its host *B. subtilis* 168, Archibald et al., (1989), showed that adsorption was irreversible at different temperatures, but that the rate of binding was dependent on the teichoic acid content of the bacterial cell wall . Three bacteriophages, PBS1 and SP3 of *B. subtilis* and PBP1 of *B. pumilis* have been classified in the

literature as being flagellotropic. Such bacteriophages adsorb to the host's flagellae and proceed to the bacterial cell wall (Shea and Seaman, 1984). The host cell does not play an active part in bacteriophage adsorption (Stent, 1963) and the receptor sites of the bacterial cell render it highly specific for a small range of phages. Adsorption is often facilitated by the presence of cations that are postulated to neutralise repulsive electrostatic forces between phage and bacterium (Stent, 1963). Letellier and Labedan (1984) showed that the depolarisation of the *E. coli* bacterial membrane induced after adsorption of bacteriophage T4 was calcium dependent.

Once the bacteriophage particle is adsorbed and irreversibly bound, the bacteriophage nucleic acid is injected into the bacterial cell. This is usually by insertion of the tail core into the bacterial cell wall and contraction of the tail sheath, acting in a similar manner to a syringe. The empty protein coat remains on the outside of the bacterial cell.

The presence of mature bacteriophage particles cannot be detected if a bacterial cell is disrupted soon after the injection of phage nucleic acid. During this time the synthesis of viral proteins occurs, resulting in the formation of subunits for the phage head and tail structures. This highly-coordinated period is called the eclipse phase (Doermann, 1952). The length of time of this phase is variable and dependent on the metabolic state of the host, the bacteriophage species and the environmental conditions. At the end of the bacteriophage maturation cycle the infected cells begin to lyse and the progeny bacteriophages are released into the surroundings. Bacteriophage lysis has been recently reviewed where it was indicated that lysozyme dependent and independent mechanisms of lysis exist (Young, 1992) It was suggested by Steiner et al., (1993) that

a conserved lysis pathway may exist for most bacteriophages whether their host bacterium is Gram positive or Gram negative. The presence of a unique phage-encoded lysozyme has been demonstrated for ø29 of *B. subtilis* (Saedi et al., 1987). Lysis does not occur in infected cells until mature bacteriophages are formed and the average number of bacteriophages released is referred to as the burst size which is a characteristic of the infecting bacteriophage (Ellis and Delbruck, 1939; Stent, 1963; Hayes, 1968).

1.4.2 The temperate life cycle

Temperate bacteriophages gain entry into the bacterial cell in the same manner as virulent phages, but thereafter do not usually produce lysis. This repression of the lytic function is believed to be due to a phage-encoded DNA binding protein which is further discussed in section 1.5.1 (Ptashne, 1986). The viral genome is carried symbiotically in a lysogenic association and termed a prophage. Infection by temperate bacteriophages confers resistance on the host bacterium to superinfection with the same, or related, bacteriophages (Stent and Calender, 1978). The prophage can occasionally undergo a spontaneous change that allows the production of virulent bacteriophage particles resulting in host cell lysis. High levels of prophage induction to lytic growth can be achieved by exposure to agents that directly damage cellular DNA or inhibit DNA replication e.g. ultra-violet light, acridine orange or mitomycin C (Lwoff, 1953; Walker, 1984). IG1, a *B. subtilis* temperate bacteriophage isolated from soil, was strongly induced from its lysogens by the action of an azopyrimidine that selectively inhibited the host DNA polymerase III (Fernandes et al., 1990). There are various states

of existence for these prophage within a host cell. For example, the DNA of ø105, a temperate bacteriophage of *B. subtilis,* inserts directly into the host chromosome (Hemphill and Whiteley 1975) while *Pseudomonas aeruginosa* bacteriophage F116 is maintained as a low copy number extra-chromosomal element (Miller et al., 1977).

1.4.3 Maintenance of lysogeny

Much of the research on the lysogenic state of prophage has been carried out on *E. coli* temperate bacteriophage lambda. It is beyond the scope of this introduction to discuss this bacteriophage in detail, however the basic principles of lambda prophages can be applied to temperate bacteriophages generally and these will be discussed. The establishment and maintenance of lysogeny of lambda is under the control of both bacteriophage and host genes (Herskowitz and Hagen, 1980). During lambda lysogeny, a bacteriophage-encoded repressor protein binds to the viral chromosome and inhibits the transcription of much of the bacteriophage's genome. The repressor protein for lambda is the product of the *cI* gene (Ptashne, 1967). Similar systems of repression have been shown to occur in other lysogenic associations, for example D3 of *P. aeruginosa* (Miller and Kokjohn, 1987).

If the production of the repressor protein ceases or the protein is inactivated then lysis of the host cell occurs. Lysis does in fact occur at low levels in a sub population of lysogenized cells. This is displayed by the production of turbid plaques by temperate bacteriophages, when plated out onto a susceptible host.

The mechanism of prophage induction appears to be a result of the breakdown of the repressor protein. In bacteriophage lambda, an activated host encoded RecA

protein is produced in response to the inducing agent. This protein cleaves the *c1* repressor protein, eliminating its DNA binding affinity (Roberts and Devoret, 1983) and the lytic response is achieved when enough repressor protein is cleaved (Bailone et al., 1979).

1.5 Transduction

Transduction is defined as the transfer of genetic information mediated by bacteriophage particles (Masters, 1985). The outcome of the developmental cycle of a virulent bacteriophage is the production of mature virions each carrying their own specific DNA. The transduction process is a diversion of this cycle, to one in which bacterial DNA is incorporated into the maturing bacteriophage particle. Generalised transduction allows any genetic element within the host cell including chromosomal or plasmid DNA, to be transduced with an equal probability (Zinder and Lederberg, 1952; Ozeki and Ikeda, 1968). Specialised transduction only allows the transfer of specific genetic elements (Morse, 1954). The molecular mechanisms of the two are fundamentally different.

1.5.1 Generalised transduction

During the later stages of the lytic cycle, the host DNA is broken down, so that the host cell contains many linear chromosomal fragments. In generalised transduction, the transducing particles contain a fragment of this bacterial DNA, packaged into a bacteriophage capsid in place of the viral genome. Transducing particles contain only bacterial, chromosomal or plasmid DNA (Ikeda and Tomizawa, 1965; Yamagishi and

Takahashi, 1968; Chakrabarty and Gunsalas, 1969; Ebel-Tsipis et al.,1972; de Lancastre and Archer, 1981). Transducing particles can be formed by a lytic bacteriophage or during induction of a host-associated temperate bacteriophage, and generalised transduction has been reported in both virulent and temperate bacteriophages under appropriate conditions (Susskind and Botstein, 1978; Kokjohn, 1989). The fragment of DNA carried by the bacteriophage corresponds to about 1-2% of the total bacterial DNA. During the formation of a transducing particle, the amount of host DNA that can be packaged is restricted by the size of the bacteriophage capsid as bacteriophage DNA is packaged by a headful mechanism; once the capacity of the head is reached, it is cut by a nuclease at a particular site termed *pac*. Transducing particles are believed to be a result of sites in the host DNA that mimic this *pac* site (Chelala and Margolin, 1974). Bravo et al., (1990) cloned the *pac* site from a virulent *B. subtilis* bacteriophage into the host chromosome and observed the packaging of chromosomal DNA initiated at the inserted *pac* site. The transductional frequency per bacteriophage particle, for a selected gene is usually in the region of 10^{-5}-10^{-8} (Masters, 1985; Saye et al., 1987).

1.5.2 Abortive transduction

Once inside the recipient, the transduced fragments can integrate into the host chromosome in order to be stably inherited. For this recombination event to occur, there must to be a degree of homology between the transduced fragment and the host chromosome (Sandri and Berger, 1980). Furthermore, it has been shown that this recombination must occur soon after the appearance of the transduced fragment (Hayes,

1968). It is possible for the introduced fragment of bacterial DNA to exist without recombination. This is referred to as abortive transduction. The transduced DNA persists in the bacterial cell without replication and is transmitted unilinearly, whereby only one of the progeny cells receives the transduced DNA. If an entire gene is located on the transduced fragment, it is possible for this to be expressed. The gene expression will only be from the cell possessing the transduced fragment, remembering that only one of the progeny cells receives the transduced fragment (Davis et al., 1980). Under appropriate conditions, abortive transductants can be viewed as microcolonies. To prevent the degradation of the transduced fragment and allow its inheritance, the transduced fragment is believed to be associated with a protective protein that prevents degradation of the fragment (Ikeda and Tomizawa, 1965).

1.5.3 Specialised transduction

Specialised transducing particles are produced during the induction of a temperate bacteriophage, probably when the prophage excises from the host genome. At this stage it is thought that an illegitimate recombination event takes place that results in the replacement of a fragment of the bacteriophage DNA with a fragment of the host DNA (Davis et al., 1980). A unique DNA sequence on the bacteriophage genome, the *cos* site, is required for packaging. As before, the bacteriophage DNA is packaged by a headful mechanism, however only the DNA in close proximity to the *cos* site and therefore the bacteriophage integration site can be packaged. Thus infectious transducing bacteriophages are produced if the host DNA replaces bacteriophage DNA that is not essential for vegetative growth of the bacteriophage. These bacteriophages

can proceed to infect a susceptible host, still carrying the original host DNA. If an essential bacteriophage gene is replaced by the bacterial DNA, then a defective bacteriophage is produced. It is possible for a defective bacteriophage to transduce it's host DNA fragment but this requires the coinfection and integration of wild type helper bacteriophage. Upon induction, this doubly-lysogenic cell produces a lysate that contains roughly equal proportions of wild type and transducing bacteriophage particles, referred to as a high frequency transducing lysate.

1.6 Bacteriophages of Bacillus subtilis

The vast majority of the work investigating *Bacillus* species and their associated bacteriophages has been carried out using *B. subtilis* as the host. *B. subtilis* has been shown to be susceptible to both virulent and temperate bacteriophages (Zahler, 1993). All of the temperate bacteriophages studied contain linear double stranded DNA that inserts into the *B. subtilis* chromosome at a particular attachment site (*att*). These temperate bacteriophages have been classified into 4 groups (I-IV) based on serology, immunity, host range and adsorption site.

Group V bacteriophages are the defective prophages including PBSX. Wood et al., (1990) speculated that the widespread distribution of defective prophages in *Bacillus* strains may be because they carry out some essential function to the host. When induced, these group V bacteriophages contain bacterial DNA in their heads rather than bacteriophage DNA. Group III bacteriophages have large genomes in the region of 110-129 kb and include IG1. Bacteriophages in group III are DNA and structurally related, but may show differences in other characteristics (Dean et al., 1978:

Wilson et al., 1974: Zahler, 1988). The group I bacteriophages have typically small genomes ranging from 38.5-40.1 kb (Dean et al., 1978). The best studied of these phages is ø105, with a DNA content of 39.2 kb. (Errington and Pughe, 1987.) The ø105 phage genome integrates between the *leu* and *phe* genes on the *B. subtilis* chromosome (Zahler, 1993). ø105 has been extensively used as a bacteriophage vector for analysis of the *B. subtilis* chromosome. In particular the bacteriophage vectors have been used for gene cloning directly into the *B. subtilis* chromosome. To enable the identification of the product of a cloned gene in ø105, a promoterless *LacZ* gene has been inserted into the cloning site. In the ø105 derivative ø105 MU201, the *LacZ* gene is expressed during lytic growth from a bacteriophage promoter (East and Errington, 1989). By incorporating the *cts*-52 mutation into ø105MU201, a thermo-inducible derivative ø105 MU205, has been constructed (East and Errington 1989,). In the context of this study, the presence of the *LacZ* gene would allow the bacteriophage to be monitored *in situ*. Group II bacteriophages include SPO2 which is physically similar to ø105 but differs in that it encodes its own DNA polymerase which has been sequenced (Raden and Rutberg, 1984). Group IV contains SP16 which is not capable of generalised transduction and may replicate as a plasmid (Zahler, 1993).

SPO1 and related bacteriophages constitute a family of large virulent bacteriophages of *B. subtilis* that are distinguished by the complete replacement of thymine by hydroxymethyluracil in their DNA (Stewart, 1993). Bacteriophage taxonomy is being regularly updated with new *Bacillus* bacteriophage species being described (Ackermann et al., 1994). Table 1.2 details some other transducing bacteriophages isolated for other *Bacillus* species.

1.6.1 Transduction in mutants of *Bacillus caldotenax*

Sharp (1982), described and optimised a transducing system in *Bacillus caldotenax*, whereby thymine auxotrophs could be transduced by the bacteriophage JS017. This bacteriophage was isolated from compost and appeared to act as a specialised transducing phage. Transductants maintained the bacteriophage for several generations indicating that JS017 lysogenized the host or retained a close association with the host cell in a manner similar to the *Pseudomonas aeruginosa* bacteriophage F116. The thymine auxotrophs of *Bacillus caldotenax* were isolated following selection with trimethoprim.

Table 1.2 Transducing bacteriophages isolated for *Bacillus* species

Bacillus species	Type of transduction
B. alesti	Generalised
B. amyloliquefaciens	Plasmid
B. anthracis	Plasmid
B. cereus	Generalised
B. licheniformis	Generalised
B. megaterium	Generalised
B. pumulis	Plasmid
*B. stearothermophilus**	Generalised
B. subtilis	Specialised
B. thuringiensis	Generalised

The information in this table is a modification of that published by Kokjohn, (1989)

* Reference; Welker, (1988).

1.7 Transduction in the environment

Until recently, the role of transduction as a means of gene transfer in the environment had been underestimated. This was presumably because of the relatively restricted host range shown by bacteriophages and the fact that transduction was mediated by an external factor, the bacteriophage. It has been shown by *in situ* studies on transduction (Saye et al., 1987) that genetic transfer in the environment is very much dependent on the concentration of the individual biological components that make up the gene transfer system. The frequency of gene transfer is significantly influenced by the numbers of donor bacteria and their concentration relative to the numbers of recipients. Numbers of bacteria in soils range from 10^4 to 10^6 per gram in desert soils (Skujins, 1984), to as high as10^7 per gram of clay soil (Stotzky, 1989). There is considerable variation in bacterial concentrations in aquatic environments. Culturable cells in freshwater lakes have been estimated to range from 10^3 to 10^4 cfu/ml (O'Morchoe et al., 1988), while bacterial counts in waste waters and wet sludges have been estimated to be as high as 10^9 cfu/ml (Gealt, 1992). Bacteriophage titres in the environment have been shown to range from 1 - 10^3 pfu/ml in freshwater habitats to 10^8 pfu/ml in wastewater and sludge (Saye and Miller, 1989). Suttle and Chan (1994), showed that cyanophages that infect *Synechococcus* cells are present at levels of 4 x 10^5pfu/ml near the sea surface.

Studies on transduction in the environment have tended to be carried out using laboratory manipulated, well characterised bacteriophages and hosts. The available data on numbers of bacteria and their associated bacteriophages, that exist naturally within

particular ecosystems, appear to suggest that sufficient potential exists for the individual components of the transducing system to interact. However, the precise numbers and the interactions of phages and bacteria from indigenous populations, within natural environments have been insufficiently studied.

The transmission of DNA by transduction has been demonstrated in a Gram negative bacterial species in both sterile and non sterile soils. Much of this work has been carried out in soil using the *E. coli* generalised transducing phage P1. Transduction by P1 in sterile soil samples was shown to be greater than that demonstrated in non-sterile soil samples (Zeph et al., 1988; Stotzky, 1989). The frequency of transduction was not affected by amending the nutrient status of the soil samples. Examination of the indigenous bacteria for the transduced DNA fragment showed that transduction had not occurred between P1 and the indigenous population.

The potential for transduction in freshwater environments has been addressed using F116 or its variants. Morrison et al., (1978), showed transduction of streptomycin resistance during a 10-day incubation in a flow-through chamber suspended in a freshwater reservoir. Saye et al., (1987) showed transfer of a *Pseudomonas aeruginosa* plasmid by the generalised transducing bacteriophage DS1, both in the absence and presence of the natural microbial community. Here again, the presence of the natural flora resulted in a dramatic decrease in the numbers of donors and recipients and a concomitant decrease in the number of transductants recovered.

Although transduction in the environment has mainly been demonstrated in soils and freshwater, other environments have also been considered. Recently, Kidambi et al. (1994) investigated the potential of plant leaves to act as surfaces on which

transduction between bacteria could take place. Using *Pseudomonas aeruginosa* and its associated transducing phage F116, they demonstrated qualitatively and quantitatively that transduction occurred between donor and recipient on both the same leaf surface and when the strains were inoculated onto densely planted adjacent plants.

1.7.1 Implications of transduction in the environment

Bacteriophage mediated gene exchange has the potential to facilitate evolution by the transmission of genetic elements, thus expanding the diversity of the available gene pool. Bacteriophages may also serve as reservoirs of bacterial DNA in natural habitats (Stotzky, 1989). In terms of releasing genetically engineered microorganisms, transduction may have a highly significant impact on the transfer and establishment of novel DNA sequences within indigenous populations. Even if the original GMO did not persist, the recombinant DNA could persist in a bacteriophage capsid and reappear upon infection of a susceptible bacterial host.

1.8 Environmental factors that may influence the interaction of bacteriophage with bacteria

A number of environmental conditions offer potential barriers to the infection of a bacterial cell by a bacteriophage. These will ultimately influence the potential for gene transfer by transduction. Many bacteriophages show a temperature optimum for adsorption and replication (Seeley and Primrose, 1980: Primrose et al., 1982). This temperature is more often a reflection of the ecological origin of the bacteriophage rather than the growth temperature optimum of the host bacterium. Therefore

bacteriophage infections that are highly efficient under laboratory conditions may not necessarily occur as efficiently under environmental conditions.

The ionic composition of the environment is important because for example, some bacteriophages, have a specific requirement for the presence of divalent cations in order for adsorption and consequent DNA replication to occur. The solid surfaces within a particular environment will also influence the interaction of bacteriophages and bacteria. Soil can be regarded as a structured environment (Stotzky, 1989) in which the distribution of bacteria is generally limited to the liquid phase. The solid surfaces within soil may separate different microhabitats which may lead to a reduced interaction between bacteriophages and bacteria. The binding of bacteriophage to clays has been demonstrated to protect them from inactivation (Bystricky et al., 1975), but the adhesion of bacteria to soils may reduce the availability of phage attachment sites (Stotzky, 1989). Roper and Marshall (1978), showed that sorption of *E. coli* to clay particles less than 0.6μm in size inhibited bacteriophage-bacterium interaction.

As previously discussed, temperate bacteriophages can be induced to lytic growth by exposure to DNA damaging agents. One such agent is solar UV radiation, to which most free living microorganisms are exposed on a daily basis. Solar UV radiation wavelengths are in the region 290 nm to 400 nm, consisting of UV-B wavelengths (290-320 nm) and UV-A (320-400 nm); solar radiation contains light, mainly in the UV-A region that can induce strand breaks, alkali labile sites and DNA to protein cross links. The ability of UV-A to induce prophages present in natural populations is unknown (Kokjohn and Miller, 1992). Lwoff (1953), suggested that after exposure to DNA damaging stress, prophage induction in *B. megaterium* was dependent

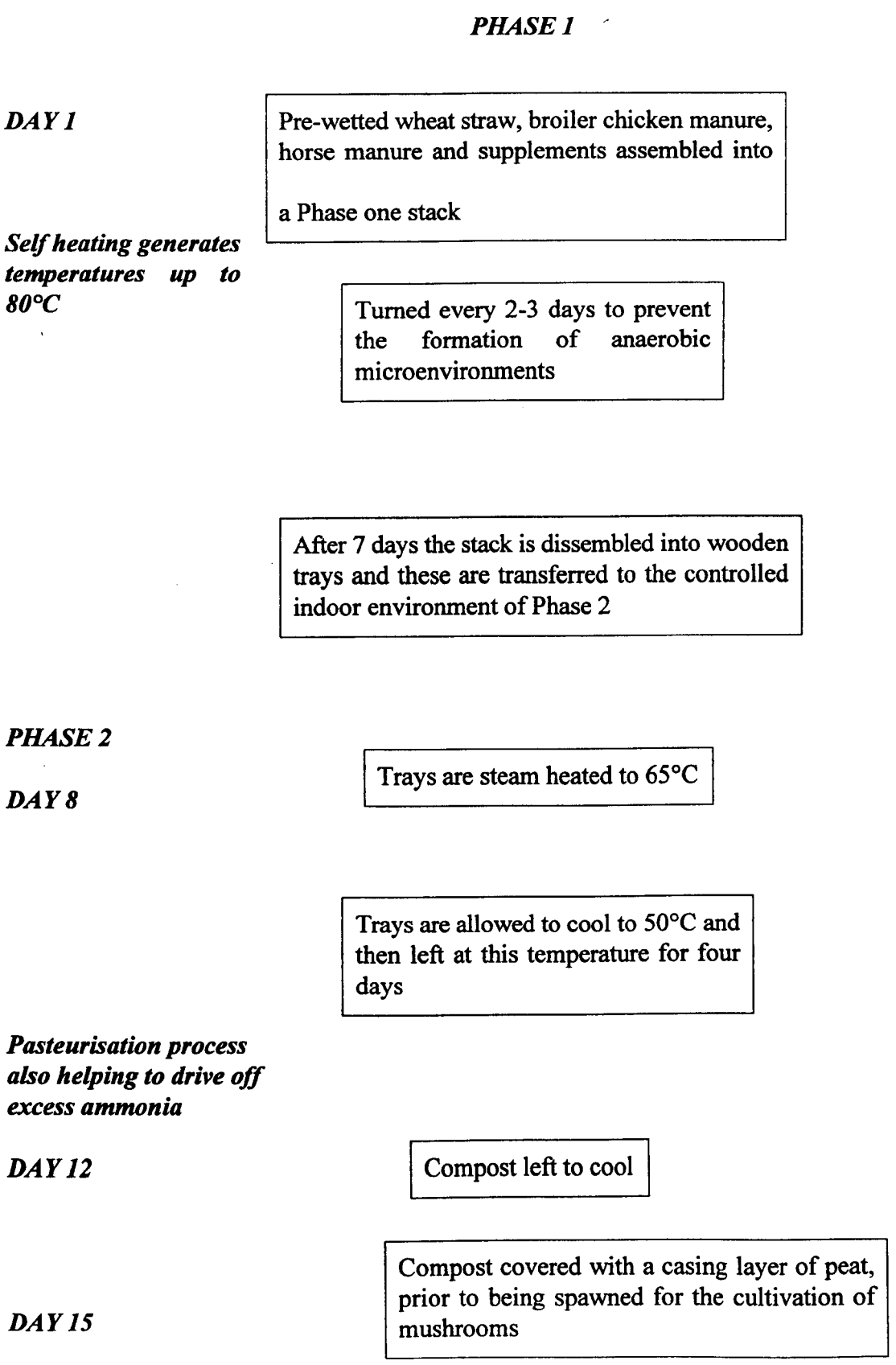
on the physiological state of the host. Cells on minimal media did not induce prophage whereas cells growing in a rich medium supported prophage induction. This is further evidence to indicate that within natural environments, prophage induction is linked to the nutritional status of the host cell.

1.9 Mushroom compost as a biological substrate

Compost is produced commercially as a substrate for the production of the edible mushroom *Agaricus bisporus* and the process is represented in Figure 1.1 . Mushroom compost has been selected as a model environment for this study because it contains a diverse and highly active microflora with *Bacillus* spp. present in large numbers (Amner et al., 1988). This substrate has been previously used in the development of risk assessment studies to investigate the consequences of the release of GMO's into the environment (section 1.9.1). The mushroom compost preparation process involves the mainly aerobic breakdown of solid organic matter by microbial activity, with the presence of anaerobic microenvironments reportedly contributing approximately 3.5% of the breakdown process. (Derikx et al., 1990).

Mushroom compost is prepared from a mixture of pre-wetted wheat straw, horse manure, broiler chicken manure and supplemented with gypsum (Beardsell, pers comm). The pre-wetting of the straw takes 14 days. The constituents are mixed and held outdoors in long stacks (windrows), approximately 1.5 metres high. This outdoor part of the composting process is referred to as Phase 1. As a result of the high biological activity, the insulating character of the composting material and the absence of forced air movements causes the temperature of the stack to rise as high as 80°C

Figure 1.1; Outline of the commercial preparation of mushroom compost



(Finstein and Morris, 1975). To prevent the increased development of anaerobic microenvironments, the Phase 1 compost stack is mechanically churned twice during the phase. Turning also contributes a degree of homology to the stack, removing any other environmental gradients that may have developed. Phase 1 normally lasts for seven days and apart from the turning there are no temperature or aeration controls during this time. Following Phase 1, the stack is treated indoors (Phase 2), with the temperature and aeration under close control. Phase 2 is the pasteurisation stage of the process in which the compost is split into trays and rapidly heated by steam to 60°C. The temperature is then maintained at 50°C for a further four days. The conditions of Phase 2 prepare the compost for the growth of mushroom spawn by driving off excess ammonia and removing any competitors or pathogens.

The composting is carried out by a succession of microorganisms from mesophilic to thermophilic species. These microorganisms take part in a decomposition process that results in the production of a nutrient rich substrate for the cultivation of *A. bisporus*. Stanek (1972), described the successive colonisation beginning with an increase in the number of mesophilic and thermo-tolerant bacteria and fungi e.g. phycomycetes. As the composting progresses towards the end of Phase 2, the main components of the microflora are the thermophilic actinomycetes (*Thermomonospora* spp, *Thermoactinomyces* spp, *Saccharomonospora* spp and *Streptomyces* spp) and *Bacillus* spp. (Amner et al., 1988). Mushroom compost samples collected from the end of phase 2 show a high biological activity and provide a relatively reproducible substrate that can be used in model microcosms. (Amner et al., 1988).

1.9.1 The application of mushroom compost to microcosm studies

Microcosms have been defined as physical models of the natural environment in which experimental conditions in the laboratory are intended to mimic a field setting (Pritchard and Bourquin, 1984). They allow experiments with GMO's to be performed under conditions in which environmental components necessary for expression of ecosystem processes are present while the GMO's are still maintained in contained situations (Nublein et al., 1992). Microcosms for soil (Bentjen et al., 1989), aquatic (Awong et al., 1990) and sediment (Wagner-Dobler et al., 1992), have been used to predict the fate of GMO's introduced into these environments. These contained laboratory systems can be as simple as adding sterile compost to test tubes (Amner et al., 1993). Providing that there is an awareness of the limitations of the particular microcosm under study, it can be regarded as an analytical tool for risk assessment studies, to monitor the impact of releasing GMO's into a particular model ecosystem. The advantage of using mushroom compost in microcosm studies lies in the fact that it is a readily available, reproducible substrate, that contains a relatively predictable microflora that is active and therefore provides a good test of gene survival under competitive conditions, and provides the opportunity for gene transfer to the indigenous population. The presence of mesophilic and thermophilic species within the indigenous population also facilitates studies of gene transfer between these two groups.

To date there have been limited release studies into mushroom compost microcosms involving *Bacillus* species (Amner et al., 1993; McDonald, 1992). The indigenous microbial population of mushroom compost can provide an ideal environment for risk assessment studies prior to releasing GMO's into the environment.

1.10 Methods for detecting DNA in the environment

To assess the potential role of bacteriophages as agents in gene transfer requires knowledge of both the bacteriophage replication and microbial growth, survival, activity and dispersal within the environment. It is important that the dynamics of bacteriophage activity in the environment are understood as a prerequisite to investigating their role in gene transfer. The development of practical monitoring techniques has allowed the detection of DNA in the environment such that phage DNA could be labelled with a marker unlikely to be widely distributed in the indigenous bacterial and bacteriophage population under investigation. Bacteriophages tagged in this way would require monitoring techniques that are specific, sensitive and reliable. There are techniques available that allow the analysis of DNA sequences in the environment and these could be used to study the interactions of bacteriophages and their hosts in their natural environment.

Molecular detection techniques can be categorized into three main groups: immunological methods; nucleic acid probing; molecular markers. The immunological detection methods employ either monoclonal or polyclonal antibodies raised against specific marker gene products or intact microorganisms that express the particular antigen. Monoclonal antibodies have been used successfully to track *P. putida* released in unsterile lake water samples (Ramose-Gonzalez et al., 1992). This section will concentrate on nucleic acid probing and the use of molecular markers as a means of monitoring bacteriophages in the environment.

1.10.1 Nucleic acid probing

Nucleic acid probes can provide a specific detection and monitoring tool to identify complementary DNA base sequences, in the organism of interest. The use of a probe depends on the re-annealing of two complementary strands of denatured DNA. The presence of a specific DNA sequence within a target organism can thus be demonstrated by hybridising a complementary labelled probe to it. A probe can be labelled either radioactively (e.g., with ^{32}P nucleotides) or non-isotopically (e.g., biotin labelling). The majority of current radioactive labelling procedures rely upon the enzymatic incorporation of the labelled moiety into the probe DNA, to produce either uniformly labelled or end-labelled probes. Uniformly labelled probes can be produced by nick translation, however single stranded probes cannot be labelled in this manner (Rigby et al., 1977). The enzyme T4 polynucleotide kinase is used to end label the 5' end of single stranded probes by the transfer of the ∝-phosphorous group from [γ-^{32}P]ATP to the 5' end of the sequence.

The hybridisation of nucleic acid probes allows the detection of specific nucleic acid sequences ranging from functional genes to oligonucleotides. The presence of these target sequences can, depending on the strategy used, be determined directly from environmental samples. Colony hybridisation techniques involve the probing of bacterial colonies immobilised onto filters and have been applied to the detection of a range of microorganisms including *Salmonella* spp. (Fitts et al., 1983), *Yersinia* spp. (Hill et al., 1983) and *P. putida* (Sayler et al., 1985). Other methods depend on the ability to extract DNA from environmental samples that can be used for subsequent analysis by probing or PCR for a particular sequence.

1.10.2 Extraction of utilisable DNA from environmental samples

There are two approaches to extracting DNA from environmental samples. A cell extraction procedure isolates intracellular nucleic acids by first separating the bacterial cells from the environmental matrix using differential centrifugation. The cells are then lysed and the nucleic acid recovered (Holben et al., 1988). Alternatively, DNA is extracted by direct disruption of the cells without prior separation from the environmental matrix (Steffan and Atlas, 1990). Larger amounts of DNA have been recovered by direct disruption procedures and this may indicate the presence of extracellular DNA (Steffan et al., 1988). Phenolic compounds such as humic acids, which are present in soils and sediments, interfere with enzymatic reactions and the DNA extracted from environmental samples must be purified before it can be utilised. The removal of humic acids can be achieved by adsorption to polyvinylpolypyrollidone (PVPP) during the extraction procedures (Steffan et al., 1988). In aquatic studies, sufficiently pure microbial nucleic acids to allow enzymatic analysis have been isolated (Ward et al., 1990; Weller and Ward, 1989). Other purification procedures that have been used include CsCl ultracentrifugation (Holben et al., 1988; Paul et al., 1990), phenol extractions and ethanol precipitations (Fuhrman et al., 1988).

1.10.3 Molecular marker systems

The incorporation of a genetic marker that is unlikely to be widely distributed in the environment can facilitate the identification of an organism or group of organisms within a sample. Such a marker can be considered in this study, as a tag to identify the bacteriophage, allowing them to be identified and monitored in release studies. Prosser,

(1994) further defined this tagging as the intentional introduction of genes conferring distinctive phenoytpic properties which enable the marked organism to be 'tracked' after its introduction into the environment.

If a molecular marker is to be used as a tag, it must not already be present within the indigenous bacterial or phage population. Furthermore the marker must be stably maintained within the host, with a level of expression sufficient to allow detection. For this purpose, marker genes are often introduced into the host genome rather than on a plasmid. Hwang and Farrand (1994) introduced the ability to utilise agropine (AGR) as a sole source of carbon and energy into a chromosomal site on *Pseudomonas fluorescens*. Catabolism of AGR was used to recover selectively the marked strain from mixed populations of closely related bacteria. Among the marker genes that have been used for detection in the environment are antibiotic resistance determinants, bioluminescence (*lux)* and *lac*ZY genes.

1.10.4 Antibiotic resistance markers

The basis of the use of antibiotic resistance markers is the selection of a viable cell population of marked organisms by plating out onto selective medium containing an appropriate antibiotic. There are limitations to the use of these markers. There are often high numbers of naturally occurring antibiotic resistant bacteria within an indigenous population (Amner et al., 1988; McDonald, 1992), reducing the sensitivity of this technique. Although not relevant to microcosm studies, there is also concern that releasing antibiotic resistance determinants may promote resistance among bacteria and ultimately render some antibiotic therapy ineffective. In spite of these limitations and

concerns, antibiotic resistance marked strains have been used in release studies (Van Elsas et al., 1986; Graham and Istock, 1978; Devanas et al., 1986) and have formed the basis for the development of many of the markers used in environmental release. Chao and Feng (1990), found that the effect of various plasmids on the survival of *E. coli* in the environment were highly inconsistent but postulated that if positive selection for plasmid-encoded markers were present increased host survival might result

1.10.5 Luminescence based detection

The introduction of the bacterial luciferase (*lux*) genes originally cloned from *Vibrio fischeri* or *Vibrio harveyi* into a microorganism, allows *in situ* monitoring of the gene system based on the detection of light. Luminescent strains of *E. coli* have been constructed and monitored in soil (Rattray et al., 1990) and the construction and characterisation of bioluminescent strains of a *Bacillus* spp. designed specifically for environmental detection has been recently reported (Cook et al., 1993). Disadvantages of *lux*-marked strains have been reported if the genes are located on a plasmid, since plasmid instability is a problem (Prosser, 1994). Also, successful detection of the *lux* tag depends on the cell being metabolically active.

1.10.6 The *lac*ZY genes system

The *lac*ZY genes used originate from *Escherichia coli* and code for β-galactosidase and lactose permease respectively. The detection of this gene system is based on the incorporation of the chromogenic substrate X-Gal (5-chloro-4-bromo-3-indolyl β-D-galactopyranoside) into the agar isolation medium. Growth of the Lac^+

genotype bacteria results in a cleavage of the X-Gal and formation of a blue-green colony. This distinguishes the *Lac*$^+$ colonies from the other Lac$^-$ cells. A more sensitive means of detection can be achieved using the fluorogenic substrate, 4-methylumbelliferyl-β-D-galactopyranoside (MUG).

The *lac*ZY gene system has been used as a marker for *P. fluorescens* (Hofte et al., 1990). Normally *P. fluorescens* is not able to utilise lactose as a sole carbon or energy source. Drahos et al.,(1986), introduced the *LacZY* genes, carried on a plasmid, into their strain creating a Lac$^+$ phenotype that could be monitored *in situ*. A loss of selectivity results if indigenous lactose catabolizing microorganisms exist in the ecosystem.

1.11 Isolation of bacteriophages and bacteria using microbiological techniques

In order to assess the potential for bacteriophage mediated gene transfer in natural environments, it is necessary to identify the bacteriophage and bacterial species composition of the particular ecosystem under study. The isolation of bacteriophage and bacteria from environmental samples can be carried out using standard microbiological techniques. These methods can provide an estimate of the culturable bacterial population, i.e. a viable count, and determine whether a particular isolate has an associated phage or phages. A viable count requires sample collection and cultivation in the laboratory on a suitable medium containing a carbon and/or other energy sources (Roszak and Colwell, 1987). The collection regimen needs to be arranged to ensure a representative sample of the population. During laboratory processing, the choice of

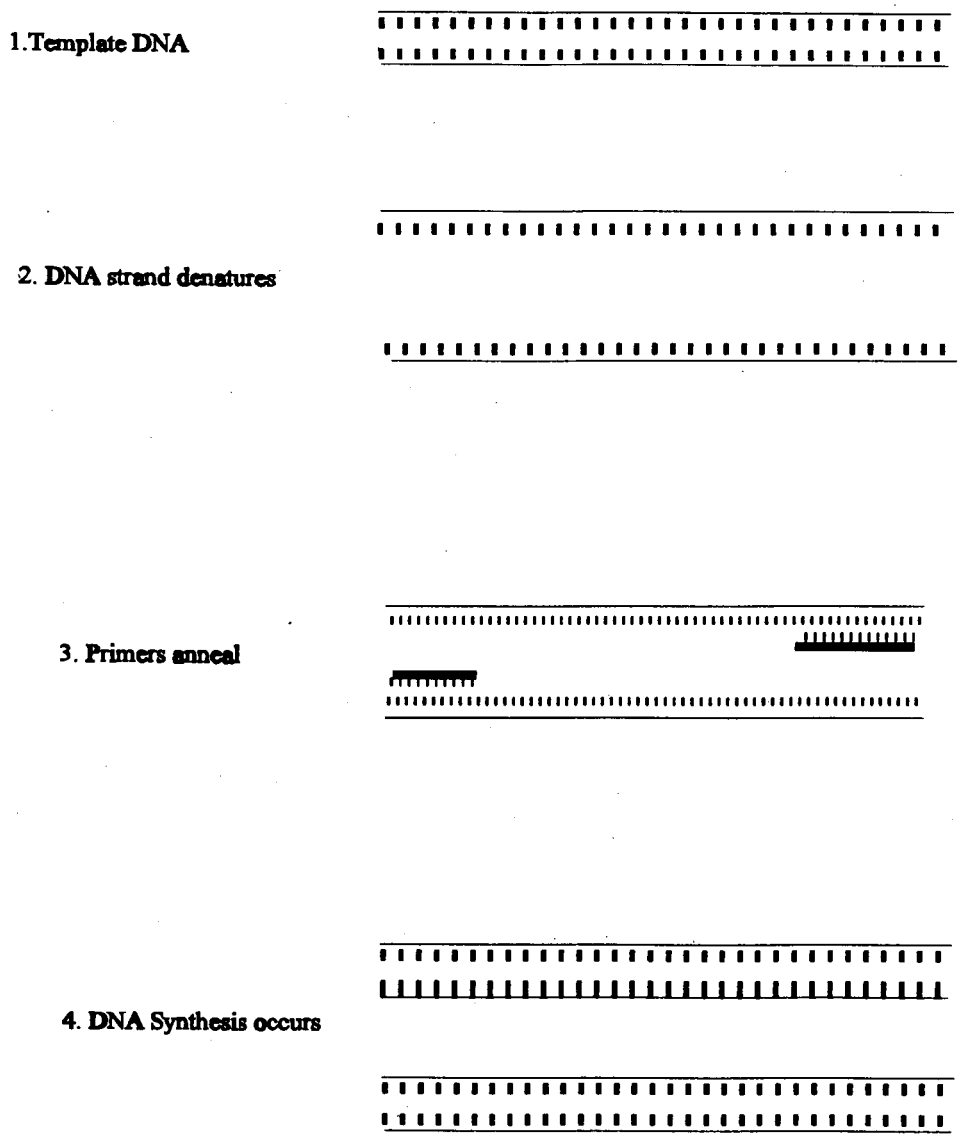
growth conditions and culture media can affect the recovery of bacteria from environmental samples. For example, it was only possible to recover around 2-4% of the total microscopical count observed in different soil samples (Olsen and Bakken, 1987). This was due to various factors including the observation of non viable organisms, inadequacies in culture technique and also that some bacteria enter a non culturable but viable (NCBV) state. The NCBV state has been shown to exist mainly for pathogenic bacteria e.g. *Salmonella typhimurium* and *Vibrio* spp. (Colwell et al., 1985; Roszak and Colwell, 1987). The limitations of the recovery procedure and the application of any conclusions made concerning the population as a whole must be acknowledged.

1.12 The polymerase chain reaction (PCR)

The PCR allows the sequential replication of a targeted sequence of DNA *in vitro,* resulting in an amplification of this sequence. In the context of this study, PCR will be used to construct and amplify a short DNA sequence that could be used as a tag to monitor indigenous bacteriophages in their natural environment. For PCR analysis of environmental DNA it is essential that the DNA is purified. The PCR reaction mixture consists of target sequence DNA, oligonucleotide primers, free dNTP's, *Taq* DNA polymerase and its reaction buffer. The oligonucleotide primers are designed to flank the particular sequence of interest. The PCR cycle is shown in Figure 1.2 and the whole process can be fully automated using a thermal cycler.

The PCR amplification permits the detection of as few as 100 cells per 100g

Figure 1.2; Schematic representation of the PCR.



The primer annealing and DNA synthesis occurs during a number of cycles. This results in the amplification of the template DNA.

sample and the PCR product can be quantified permitting estimates of the number of organisms in environmental samples (Steffan and Atlas, 1991). PCR based detection strategies can be targeted to sequences specific to the particular microbe, for example the *lacZ* genes of coliform bacteria (Bej et al., 1990) and the exotoxin A gene of pathogenic *Pseudomonas* strains (Khan and Cerniglia, 1994). However, a problem arises if the sequence is contained in other members of the indigenous population, where overestimates or false positive results could be obtained. Other PCR based strategies detect sequences that have been introduced into the host genome. Flemming et al., (1994) introduced the *luxA* gene into the chromosome of *P. aeruginosa* and detected it using PCR. This was used along with other detection strategies to provide sensitive detection and enumeration in soil. The gene system used to mark organisms must obviously be stably maintained within the host and this requirement is best met by inserting the gene into the chromosome. Solid phase PCR in which the amplification occurs directly on a membrane filter has been shown to have an increased detection sensitivity compared to culture methods when detecting airborne microorganisms (Alvarez et al., 1994). Another important use of the PCR is in cloning reactions where similar sequences in different organisms can be cloned, and PCR has also been used to construct new gene sequences (Yon and Fried, 1989).

1.13 Aims

This project presents studies designed to investigate the potential for bacteriophages to act as vectors in the transfer of recombinant DNA in the environment. The *Bacillus* population and the previously unstudied indigenous bacteriophage

population, present within the mushroom compost ecosystem were characterised and used as the basis for this study. It was intended to construct a trackable phage vector from an indigenous bacteriophage. In order to achieve this, transformation systems of *B. subtilis* 168 were attempted in order to develop a transfection system. Transduction in mutants of a thermophilic *Bacillus* species had been previously demonstrated (Sharp, 1982) and this system was investigated to see if it could be monitored in mushroom compost microcosms. The dynamics of bacteriophage and host interactions were further investigated by monitoring one of the ø105 derivatives along with *B. subtilis* after release into mushroom compost microcosms.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2

Materials and Methods

2.1 Bacterial strains and Bacteriophages

Bacterial strains and bacteriophages used during this study are listed in Tables 2.1 and 2.2. Strains were stored as cell suspensions in 20% (v/v) glycerol at -70^{0}C. Bacteriophages were stored as high titre suspensions in 2 x L broth at 4^{0}C.

2.2 Mushroom compost

The compost used in this study was a commercial preparation produced as detailed in Section 1.10. Samples were collected daily throughout phases 1 and 2 and were analysed immediately in the laboratory, with the remainder stored at 4^{0}C. The microbial population did not vary significantly during storage for up to 3 months.

2.3 Chemicals, reagents and buffers

All chemicals were supplied by BDH Limited or Sigma Chemical Company unless otherwise stated and were Analar or comparable grade. Restriction endonucleases, digest buffers, T4 DNA ligase, and alkaline phosphatase were supplied by Boehringer Mannheim, or Bethesda Research laboratories.

TE buffer: 10 mM Tris; 1 mM EDTA pH 8.0.

TES buffer: 50 mM NaCl; 30 mM Tris; 5 mM EDTA pH 8.0

Table 2.1 **Bacterial strains and sources.**

Strain	Reference/source
Bacillus caldotenax BT1 (DSM 406)	Heinen and Heinen (1972)/CAMR Culture Collection.
Bacillus coagulans ATCC 80388, ATCC 12245	CAMR Culture Collection
Bacillus thermodenitrificans DSM 465	CAMR culture collection
Bacillus licheniformis ATCC 14593	Gordon et al., (1973)/CAMR Culture Collection
Bacillus megaterium ATCC 4531	Gordon et al., (1973)/CAMR Culture Collection
Bacillus pumilus ATCC 7061	Gordon et al., (1973)/CAMR Culture Collection
Bacillus stearothermophilus ATCC 8005, ATCC 10149, ATCC 12016, EP 136, EP240, EP 262, LUDA 7210, NCA 1503, NCIB 8923,	CAMR Culture Collection
Bacillus subtilis 168	CAMR Culture Collection
Bacillus subtilis CU 267	J. Errington, Oxford
Bacillus spp. RS 8 RS 13	R. Sharp (pers comm.) CAMR Culture Collection

Table 2.2 **Bacteriophages and Plasmid DNA**

Bacteriophage	Host	Source
JS 017	*Bacillus caldotenax* BT1	R Sharp CAMR
ø105 MU201	*Bacillus subtilis* CU 267	J. Errington, Oxford
ø105 MU205	*Bacillus subtilis* CU 267	J. Errington, Oxford

Plasmid	Host	Resistance markers	Reference
pTB90	*B. subtilis* 168	Tetracycline. Kanamycin.	Imanaka et al. 1982

TBE electrophoresis buffer: 90 mM Tris; 90 mM Boric acid; 3 mM EDTA.

100 x Denhardts solution: 2% (w/v) Bovine serum albumin; 2% (w/v) Ficoll; 2% (w/v) Polyvinylpyrolidine.

20 x SSC: 3 M NaCl; 0.3 M Tri-sodium citrate.

Phosphate buffered saline (PBS):

138mM NaCl; 2.7mM KCl; pH 7.4

PMN buffer: 0.01 M KH_2PO_4; 0.05 M NaCl; 0.001 M $MgCl_2$; pH 7.0.

2.4 General growth media

The media listed below were used for the routine cultivation of bacteria and propagation of bacteriophages. The media were sterilised by autoclaving at 121^{0}C for 15 min and their pH adjusted with 2M HCl or 2M NaOH.

Tryptone soya broth agar (TSBA) (Oxoid) contained in g/l:

Pancreatic digest of casein, 17.0; Papaic digest of soybean meal, 3.0; Sodium chloride, 5.0; Potassium dihydrogen phosphate, 2.5; Glucose, 2.5; Agar 15.0; pH 7.3.

2 x L broth contained in g/l:

Tryptone (Difco), 30; Yeast extract (Difco), 10; NaCl, 10; pH 7.2.

Trypticase soft agar (TSA) contained:

500 ml 1.2% (w/v) Agar (Oxoid), pH 7.5; 500 ml of a solution containing: trypticase peptone (BBL), 20 g; yeast extract 5.0 g; $FeCl_3.6H_20$, 0.007 g; $MgSO_4.7H_20$, 0.015 g; $MnCl_2.4H_20$, 0.001 g. The two solutions were heated to 80^0C in a water bath and combined aseptically.

Penassay broth contained.

Beef extract, 1.5 g; yeast extract, 4.0 g; casitone, 5 g; bacto-peptone, 5.0 g; dextrose, 1.0 g; NaCl, 3.5 g; K_2HPO_4, 3.68 g; KH_2PO_4, 1.32 g; β glycerophosphate, 2.5 g; pH 7.0; made up to 1 l with distilled water. Niacin (4 mg) and Thymine-HCl (4 mg) were added after autoclaving, as filter-sterilised solutions (0.2 μm, Millipore).

Protoplast transformation media and solutions.

SMM buffer: 0.5 M sucrose; 0.02M malic acid; 0.02 M $MgCl_2$; pH 6.5. SMMP medium was prepared by mixing equal volumes of 4 x strength Penassay broth and 2 x strength SMM. PEG solution: 40 g PEG 6000 and 50 ml of 2 x SMM, in 100 ml. DM3 regeneration media consisted of the following sterile solutions, per litre: 200 ml 4% agar; 500 ml 1 M sodium succinate, pH 7.3; 100 ml 5% casamino acids (Difco); 50 ml 10% yeast extract (Difco); 100 ml 3.5% K_2HPO_4

and 1.5% KH_2PO_4; 25 ml 20% glucose; 20 ml 1 M $MgCl_2$; The sterile solutions were heated to 80°C and combined. 5 ml of filter sterilised (0.2 μm, Millipore) 2% bovine serum albumin was added post sterilisation. The agar medium was dispensed into Petri dishes. An alternative regeneration media (DM3T) described by Dunn (1988) was also used and consisted of the following sterile solutions; 500 ml, 35% sucrose pH 7.3; 400 ml, 5% agar, 1.25% casamino acids, 1.25% TES; 50 ml, 10% yeast extract; 12.5ml, 40% glucose; 10ml, 2M $MgCl_2.6H_2O$; 10 ml, 2M $CaCl_2.6H_2O$. Selective DM3 and DM3T contained 25μg/ml tetracycline unless otherwise stated. DM3TSA was DM3T containing 0.6% agar.

Kuhns minimal broth media contained in g/l.

MES, 9.52; NH_4Cl, 2.14; KH_2PO_4, 0.3; $MgCl_2$, 0.03; $CaCl_2$, 0.0051; $FeSO_4.7H_20$, 0.04; Kuhns salts solution 1.5 ml/l; The following additions of filter sterilised (0.2 μm, Millipore) solutions were made post sterilisation per 200 ml of broth: 1 ml 50% glucose; 330 μl 10% methionine; 30 μl 1% Biotin. Kuhns salts solution contained in g/l; $MnCl_2.4H_2O$, 15.13; $ZnSO_4.7H_2O$, 0.25; H_3BO_3, 2.5; $CuSO_4.5H_2O$, 0.125; $Na_2MoO_4.2H_2O$, 0.125; $CoNO_3.6H_2O$, 0.23; 2.5 ml 95.97% (v/v) H_2SO_4.

For solidified medium, Kuhns minimal broth was supplemented with 1.5% (w/v) agar.

2.5 Isolation of *Bacillus* strains from compost

Compost was sampled according to the technique developed by Amner et al., (1988). Compost samples (3 g fresh weight) in boiling tubes were suspended in 30 ml PBS and vortexed for 2 min. A 10-fold dilution series was prepared in PBS and 0.1 ml samples were spread onto the surface of dried Nutrient agar plates amended with 50 μg/ml cycloheximide. In order to determine spore counts, the dilution series was heated to 98°C for 15 min and plated out as before. Isolation plates were incubated at 50°C overnight.

Isolate selection was based on variation in colony morphology on the Nutrient agar plates. Isolates were purified by a minimum of three successive single colony transfers onto nutrient agar plates, assigned a number and stored as previously described (section 2.1).

2.6 Biochemical and physiological characterisation of the *Bacillus* isolates

The media and procedures listed below were used for the characterisation of *Bacillus* strains isolated from compost and reported in Chapter 3. All media were sterilised by autoclaving at 121°C for 15 min and their pH adjusted with 2M HCL or 2M NaOH unless otherwise indicated. Tests employing solid media in the form of plates, stabs or slopes were inoculated from overnight cultures grown on TSBA. Liquid media were inoculated (three drops from a Pasteur pipette) with a culture grown in 5 ml peptone broth for 5 hr. All incubations were at 50°C unless otherwise indicated.

Starch agar (Cowan and Steel, 1974) contained.

10g Potato starch (BDH); 50 ml distilled water and TSBA to 1000 ml.

The starch was mixed to a smooth cream with the distilled water and added to the molten TSBA at 65°C. After mixing, the medium was autoclaved at 115°C for 10 min and then distributed to Petri dishes.

Starch agar plates were inoculated and examined for the presence of hydrolysis after 1 day and 3 days incubation. In order to detect areas of starch hydrolysis, some of the colonies were removed and the plates flooded with 1-2 ml of Lugol's iodine. Hydrolysis was indicated by clear colourless zones while unhydrolysed areas remained blue/black. Controls: Positive hydrolysis, *B. stearothermophilus* NCA 1503; Negative Hydrolysis, *B. stearothermophilus* EP 136.

Casein agar (modified from Cowan and Steel, 1974) contained.

100 ml Skimmed milk powder (Oxoid); 100 ml 3% Agar (w/v).

The skimmed milk was sterilised at 115°C for 10 min and the agar at 121°C for 15 min. After allowing both solutions to cool to approximately 50°C they were gently mixed. The medium was used as an overlayer (6 ml) on TSBA plates.

Casein agar plates were inoculated and examined for areas of casein hydrolysis after 1 day and 3 days incubation. Hydrolysis was indicated by clear zones in the white agar overlay. Controls: Positive hydrolysis, *B. stearothermophilus* NCA 150; Negative hydrolysis, *B. stearothermophilus* EP 136.

Gelatin stabs contained in g/l.

Beef extract (Difco), 3.0; Peptone (Difco), 5.0; Gelatin (Gibco), 120.

The gelatin was dissolved by heating the medium to 50°C and the pH was adjusted to 7.0. The medium was distributed as 12 ml aliquots in 1 oz bottles and autoclaved at 121°C for 15 min.

Gelatin stabs were examined for liquefaction after 1 day and 3 days incubation. Before recording the results, the tubes were held at 20°C for 1 hr to allow unhydrolysed gelatin to solidify. An uninoculated control was included with each batch of tests. Controls: Positive control, *B. stearothermophilus* NCA 1503; Negative control, *B. stearothermophilus* EP 136.

Tyrosine stabs (modified from Gordon et al., 1973) contained:

L-tyrosine, 0.5 g; distilled water, 10 ml; Nutrient agar, 100 ml.

Tyrosine crystals were suspended in 10 ml of distilled water, autoclaved and mixed thoroughly with 100 ml of sterile nutrient agar. The medium was distributed as 12 ml aliquots in sterile universal bottles.

Tyrosine stabs were examined after 1, 3, 5 and 10 days incubation for the decomposition of the tyrosine crystals. Decomposition began at the top of the stab and progressed down through the agar. Observations were also made for the appearance of a brown pigment which appeared initially on the surface and progressed through the agar. Controls: Positive control, *B. stearothermophilus* EP 262; Negative control, *B. stearothermophilus* NCA 1503.

Phenylalanine slopes (Gordon et al., 1973) contained in g/l:

Yeast extract (Difco) 3 g/l; dlphenylalanine 2 g/l; Na_2HPO_4 (anhydrous) 1 g/l; NaCl 5 g/l; Agar (Oxoid No. 1) 12 g/l.

The medium was distributed as 12 ml aliquots into universal bottles to prepare slopes.

Phenylalanine slopes were inoculated and examined after 1 day and 3 days incubation. A few drops of 10% (w/v) $FeCl_3$ was allowed to run over the surface of the agar. The production of a green pigment in the agar immediately below the area of growth indicated the production of phenylpyruvic acid. Controls: Positive control, *B. megaterium* ATCC 4531; Negative control, *B. stearothermophilus* NCA 1503.

Hippurate broth (Gordon et al 1973), contained in g/l:

Tryptone (Difco), 10; beef extract (Difco), 3; yeast extract (Difco), 1; glucose, 1; Na_2HPO_4 (anhydrous), 5; sodium hippurate, 10.

The broth was dispensed as 10 ml aliquots in universal bottles.

Hippurate broth was inoculated and examined after 5 days and 10 days incubation. 1 ml of culture was added to 1.5ml of 50% (v/v) H_2SO_4. The appearance of crystals in the acid mixture was evidence of the formation of benzoic acid from the hippurate (Baird-Parker, 1963). Where dense growth in the broth obscured the formation of crystals, 1ml of culture was membrane filtered (0.45µm Millipore). Controls: Positive control, *B. pumilis* ATCC 7061; Negative control, *B. stearothermophilus* NCA 1503.

Nutrient broth with 3% (w/v) or 5% (w/v) NaCl. (Gordon et al., 1973);

The medium was dispensed as 10 ml aliquots in universal bottles

The supplemented nutrient broths were inoculated and examined for growth after 1 day and 3 days incubation. Controls: Positive control, 5% (w/v) NaCl, *B. stearothermophilus* EP 136; Positive control, 3% (w/v) NaCl, *B. thermodenitrificans* DSM 465; Negative control, *B. stearothermophilus* ATCC 8005.

Nutrient Broth at pH 7.0, 6.5, 6.0 and 5.5 (Sharp, 1982).

The medium was adjusted to the appropriate pH and dispensed as 10 ml aliquots in universal bottles.

The nutrient broths were inoculated and examined for growth after 1 day and 3 days incubation. If growth was not present at pH 7.0 then the tests were repeated. Controls: Growth at pH 6.5, but not 6.0, *B. stearothermophilus* ATCC 12016; Growth at pH 6.5, but not 5.5, *B. stearothermophilus* ATCC 8005; Growth at pH 5.5, *B. coagulans* ATCC 8038; Growth at pH 7.0 but not 6.5, RS 13.

Catalase production (Skerman, 1967).

The production of catalase was detected by adding a few drops of H_2O_2 to a plate culture. A positive reaction was indicated by the rapid evolution of bubbles. Controls: Positive control, *B. stearothermophilus* ATCC 12016; Negative control, *B. stearothermophilus* NCA 1503.

Oxidase activity (Kovacs, 1956).

A few drops of 1% (w/v) tetramethyl-p-phenylenediamine dihydrochloride were placed onto Whatman No 1 filter paper in a Petri dish. Colonies from a fresh overnight TSBA plate were streaked across the surface of the filter paper. A positive reaction was indicated by the production of a deep purple colour within 10 s. Controls: Positive control, *B. stearothermophilus* ATCC 12016; Negative control, *B. stearothermophilus* NCA 1503.

Vogues-Proskauer Test media (MRVP) contained in g/l.

Protease peptone (Difco), 7; glucose, 5; NaCl, 5; pH 7.2.

The broth was dispensed as 10 ml aliquots in universal bottles.

Glucose peptone broth was inoculated and examined for the production of acid and acetylmethylcarbinol. Acid production was detected by the production of a red colour in the broth following the addition of two drops of methyl red solution. The broth was examined for the production of acetylmethylcarbinol (Barrit< 1936) by adding 0.6 ml of 5% (w/v) a-napthol and 0.2 ml 40% (w/v) NaOH. The tube observed after 1 hr. A positive reaction was indicated by the production of a strong red colour. Controls: Positive control, Methyl red, *B. stearothermophilus* ATCC 10149; Negative control; Methyl red, *B. stearothermophilus* ATCC 8005; Positive control, VP test, *B. coagulans* ATCC 8038; Negative control, VP test, *B. stearothermophilus* ATCC 12016.

Citrate agar contained in g/l.

Sodium citrate, 2; NaCl, 1; $MgSO_4.7H_2O$, 0.2; $(NH_4)_2HPO_4$, 0.5 ; agar, 15; 20 ml 0.04%(w/v) phenol red; pH 6.8.

Slopes were prepared in universal bottles.

Propionate agar contained in g/l.

Sodium propionate, 2; NaCl, 1; $MgSO_4.7H_2O$, 0.2; $(NH_4)_2HPO_4$, 0.5; agar 15; 20 ml 0.04%(w/v) phenol red; pH 6.8.

Slopes were prepared in universal bottles.

Citrate and propionate agar slopes were inoculated and examined after 1 day, 3 days and 5 days incubation. The utilisation was demonstrated by the production of a red colour in the agar due to the alkaline reaction of the phenol red indicator. Controls: Positive control, Citrate, *B. stearothermophilus* ATCC 8005; Negative control, Citrate, *B. stearothermophilus* NCA 1503; Positive control, Propionate, *B. licheniformis* ATCC 14593; Negative control, Propionate, *B. pumilis* ATCC 7061.

Nitrate broth contained in g/l.

Peptone (Oxoid), 5; Beef extract (Oxoid), 3; $KN0_3$, 1; pH 7.2

The broth was dispensed as 10 ml aliquots in universal bottles containing Durham tubes.

The broths were inoculated and examined after 1 day and 3 days incubation. The presence of nitrite was indicated by the production of a deep red colour after adding 1 ml sulfanilic acid reagent and 1 ml dimethyl a-napthylamine reagent. The presence of

residual nitrate was indicated by a red colour after the addition of a small quantity of powdered zinc. Controls: Reduction of NO_3 to NO_2, *B. stearothermophilus* NCA 1503; Reduction of NO_3 to gas. *B. stearothermophilus* ATCC 12016; No reduction of NO_3, *B. stearothermophilus* EP 136.

Carbohydrate media contained in g/l unless otherwise stated.

$(NH_4)_2HPO_4$, 1; KCl, 0.2; $MgSO_4.7H_2O$, 0.2; yeast extract (Difco), 0.2; agar, 15; The pH was adjusted to 7.0 before the addition of 15 ml 0.04% (w/v) bromo cresol purple. A 10% solution of test carbohydrate was sterilised by filtration (0.2µm Millipore) and added give a final concentration of 0.5%.

The medium was dispensed as agar slopes in universal bottles.

Plates were inoculated and examined after 1 day and 3 days incubation.

Temperature growth tests.

Growth at 25°C, 37°C, 45°C, 50°C, 55°C, 60°C and 70°C was recorded after 3 days incubation on TSBA plates. Temperatures above 55°C were maintained in a thermostatically controlled water bath.

2.7 Morphological characterisation

Colony morphology was recorded after 16 h incubation on TSBA plates. Plates were previously dried at 40°C for 48 h before use. Morphological descriptions were restricted to the elevation, margin, surface and degree and form of spreading. The colony surface was described as either smooth and glossy, smooth and matt or rough.

The degrees of spreading after incubation were noted.

Elevation controls: Flat, *B. stearothermophilus* EP 136; Raised, *Bacillus* spp. RS8; Convex, *B. stearothermophilus* NCA 1503.

Form controls: Circular, *B. stearothermophilus* NCA 1503; Irregular, *B. thermodenitrificans* DSM 465.

Margin controls: Entire, *B. stearothermophilus* NCA 1503; Undulate, *B. stearothermophilus* LUDA T22. Erose, *B. thermodenitrificans* DSM 465.

Surface controls: Smooth/glossy, *B. stearothermophilus* NCA 1503; Smooth/matt, *B. coagulans* ATCC 12245; Rough, *B. thermodenitrificans* DSM 465.

Examination of spores.

Colonies were examined for the presence of spores after 2 days incubation using the staining method of Schaeffer and Fulton (1933)

2.8 Isolation of Bacteriophages from compost

Compost samples (1g in 50 ml conical flasks) were suspended in 50 ml TSB (Lab M) and incubated for 1 h at 50°C with shaking (200 rpm). The flasks were then allowed to stand at 22°C for 10 min, to allow compost debris to settle. Aliquots (10 ml) of the supernatant were centrifuged (SS34 rotor Sorvall RC5B) at 3500 rpm for 15 min, the supernatant was membrane filtered (0.45 μm Millipore) and stored at 4°C.

Bacteriophages were isolated by spotting 10 μl of the supernatant onto a TSBA plate seeded with a potential host. Plates were incubated overnight at 50°C. Any area of lysis was excised into 1 ml of PMN buffer and left to stand at 22°C for 2 h. The

suspension was membrane filtered (0.45 μm Millipore) and a 10-fold dilution series prepared in PMN buffer. Phages were detected using the soft agar overlay technique (Adams 1959), on 0.6% Trypticase soft agar (TSA) supplemented with 0.015M $CaCl_2$, as follows; phage dilutions (100 μl) were added to a series of 5 ml capped Pyrex tubes containing 3.5 ml of molten TSA, held at 50°C in a heating block. The host strain (100 μl of an overnight culture of the appropriate host) was added to each tube. After mixing, the contents of the tube were poured over the surface of an undried TSBA plate and incubated overnight at 50°C. Single plaques were picked off with sterile toothpicks and transferred to 1 ml of PMN buffer for re-plating. Three sucessive re-selection steps were made for each plaque type isolated, to ensure the purity of the phage stock. Phages were stored at 4°C as high titre suspensions in nutrient broth or 2 x L-broth.

The enumeration of bacteriophages was carried out by preparing a 10 fold dilution series of a bacteriophage suspension and using the soft agar overlay technique (Adams 1959).

2.9 Bacteriophage maintenance

Bacteriophages were stored as high titre lysates produced using either 1 l or 50 ml shake flasks, or universal tubes (Nunc). The host strain was grown in 2 x L-broth at 50°C with shaking at 150 r.p.m. At mid log phase (OD 0.55 - 0.60 at 420 nm) the phage suspension was added at a multiplicity of infection (MOI) not exceeding 0.1. $CaCl_2$ to a final concentration of 0.015M and 50% (w/v) glucose to a final concentration of 0.4% were also added. Incubation was continued for a further 2-3 h. Incubation was terminated if the bacterial suspension lysed before this time. The culture supernatant

was membrane filtered (0.45 μm Millipore) and stored at 4°C. Phages were titred as previously detailed (section 2.) Titres in the region of 10^8 to 10^9 pfu/ml were routinely obtained.

2.10 Bacteriophage characterisation

Bacteriophages were characterised according to their host range and plaque morphology. High titre bacteriophage suspensions, prepared as detailed earlier, were used. The host range of bacteriophages was determined by preparing a 10-fold dilution series of the bacteriophage suspension and plating out against the different bacterial hosts. The soft agar overlay technique was used and plaques were counted after overnight incubation at 50°C unless otherwise stated. Plaque morphology was examined after a single plaque had been purified 3 times (see section 2.9) and described according to plaque appearance.

2.11 Isolation of bacteriophage DNA

The rapid small-scale procedure of Jones and Errington (1987), was used to isolate bacteriophage DNA from 1 ml amounts of high titre lysates. The method was modified to allow a scaling up of the procedure to isolate bacteriophage DNA from 1 l lysates. The method of Jones and Errington is described below with the modifications that allow scaling up.

DNAase (1 μl of a stock solution, 1 mg/ml, stored in 150 mM-NaCl, 10% (v/v) glycerol at -20°C) and RNAase (5 μl of a stock solution, 10 mg/ml, stored in 150 mM-Tris/HCl, 5 mM EDTA, 100 mM NaCl at -20°C) were added to a 5 ml phage lysate .

The lysate was incubated at 37°C for 1 h and a 1 ml sample then centrifuged (Beckman microfuge 5 min) and the supernatant recovered. 500 µl of solution 1 (30%, (w/v) polyethylene glycol and 1.2 M NaCl) was added. After at least 1 h on ice, the precipitated phage was recovered by centrifugation (Beckman microfuge, 2 min), taking care to remove as much of the supernatant as possible. The phage pellet was resuspended in 50 µl of solution 2 (10 mM Tris/HCl pH 7.5, 5 mM CDTA, 15 mM mercaptoethanol and 0.25% (w/v) SDS). The mixture was vortex-mixed briefly, heated at 70°C for 10 min then cooled to 22°C. 12.5 µl solution 3 (3M potassium acetate) was added and the tube placed on ice for 30 min. Insoluble material was removed by centrifugation (Beckman microfuge, 5 min and the phage DNA in the supernatant was precipitated by adding 2 vols ice cold ethanol and incubating at -70°C for 1 hr. The precipitate was recovered by centrifugation (as above) and redissolved in 50 µl solution 4 (10 mM Tris/HCl, pH 7.5; 1 mM-CDTA; 15 mM 2-mercaptoethanol). The DNA was extracted with 1 vol phenol, and then 3 times with 1 vol ether. The DNA was reprecipitated with ice cold ethanol as before, washed with 80% (v/v) ethanol then dried *in vaccuo* and redissolved in 50 µl solution 5 (10 mM Tris/HCL, pH 7.5; 1 mM CDTA).

The isolation of bacteriophage DNA from a 1 l lysate involved scaling up the previous procedure accordingly, with the following modifications. The lysate was incubated at 4°C with solution 1 overnight. Bacteriophage were harvested by centrifugation (15000 x g) for 15 min at 4°C. After the first ethanol precipitation the bacteriophage DNA was redissolved in 2 ml of solution 4.

2.12 Isolation of plasmid DNA

The following method was used to isolate pTB90 plasmid DNA from *Bacillus subtilis* pTB90.

Bacillus subtilis cultures were grown overnight in 1 litre of 2 x L-broth at 37°C with shaking at 150 rpm. Cells were harvested by centrifugation (15,000 x g) for 15 min at 4°C and washed in 100 ml TES buffer and resuspended in 40 ml lysozyme solution (2% glucose, 10 mM Tris pH 8.0, 10 mM EDTA, 2 mg/ml lysozyme). After incubation at 37°C for 15 min, 80 ml of lysis buffer (1% SDS, 10 μg/ml RNAase) was added. After vigorous shaking of the lysis solution, the pH was adjusted to between 12.2 and 12.8 by the addition of 0.2 M NaOH, and incubated for 15 min with gentle shaking at 4°C. 60 ml 3 M sodium acetate (pH 4.) was added and the lysate incubated at 4°C for 1h. After centrifugation (12,000 x g, 30 min) at 4°C, the DNA was precipitated by the addition of 1 vol of isopropanol, and the suspension incubated for 30 min at 22oC. The sample was centrifuged (as above) at 22°C and the pellet washed in 70% (v/v) ethanol, dried *in vaccuo* and resuspended in a small volume of TE. Caesium chloride was added to the solution to a concentration of 1 g/ml. Ethidium bromide (10 mg/ml in distilled water) was also added (max 0.1 ml per ml DNA solution). The sample was ultracentrifuged 152,000 x g, for 40 h,) at 12°C. The band of plasmid DNA was detected under UV light, removed with a syringe, extracted with isoamyl alcohol to remove the ethidium bromide and dialysed against TE buffer to remove the caesium chloride.

2.13 Rapid mini-preparation of plasmid DNA

A modification of the rapid alkaline extraction procedure of Birnboim and Doly (1979) was used for small scale preparations of plasmid DNA. The STET plasmid preparation procedure of Holmes and Quigley (1981) was also attempted.

Rapid alkaline extraction.

Bacterial cultures were grown in 2.5 ml of 2 x LB overnight at 30°C with shaking at 150 rpm. 0.5 ml of culture was centrifuged (Beckman microfuge 2 min.) and the pellet resuspended in 100 µl of solution A (2 mg/ml lysozyme, 50 mM glucose 10 mM CDTA, 25 mM Tris/HCl pH 8.0). The tube was incubated at 37°C for 30 min and then 200 µl of solution B (0.2 N NaOH, 1% SDS), was added. The mixture was vortexed gently and kept on ice for 5 min, after which 150 µl of solution C (3 M sodium acetate pH 4.8) was added. The tube was mixed by gentle inversion, kept on ice for 1 h and centrifuged (Beckman microfuge 5 min). The plasmid DNA in the supernatant was precipitated by the addition of two volumes of ice cold ethanol and held at -70°C for 1 h. The precipitate was collected by centrifugation (as above), and dissolved in 100 µl of solution D (0.1 M sodium acetate, 0.05 M Tris/HCl pH 8.0). The DNA was reprecipitated as above, dried *in vacuo* and redissolved in 50 µl TE buffer.

STET plasmid preparation.

The culture was grown and centrifuged as detailed in the alkaline extraction method above. The pellet was resuspended in 120 µl STET buffer (8% (w/v) sucrose, 5% (v/v) Triton X-100, 5 mM EDTA, 50 mM Tris/HCl) and 8 µl lysozyme (10 mg/ml)

was added. The mixture was left on ice for 5 min then heated at 98°C for 40 sec followed by rapid cooling on ice. A further 50 µl of STET buffer was added and the mixture centrifuged (Beckman microfuge, 5 min). The supernatant was removed and the DNA precipitated and purified in the same way as used in the rapid alkaline extraction procedure.

2.14 Transformation protocols

Two transformation protocols were attempted during this study.

Protoplast transformation (Chang and Cohen 1979).

A modification of this protocol was used to prepare the protoplast and is as follows. Bacterial cultures were grown in 50 ml Penassay broth at either 37°C or 50°C, with shaking at 150 rpm. Cells were harvested at mid log phase (2,600 x g, 15 min),and resuspended in 5 ml SMMP solution. Lysozyme was added to a final concentration of 2 mg/ml and the suspension incubated at 37°C until 70% of the cells had protoplasted, as viewed under the light microscope (30-45 min). Cells were then harvested as before, washed by gently resuspending in SMMP and re-pelleted. The washed pellet was resuspended in 5 ml SMMP solution

Once prepared the protoplasts were used immediately in the transformation protocol as follows. Approximately 1 µg of DNA in 50 µl of TE buffer was mixed with an equal volume of 2 x SMM solution, followed by 1.5 ml 40% (w/v) PEG 6000. After 2 min, 5 ml of SMMP medium was added and the protoplasts recovered by centrifugation (2,600 x g, 10 min). The treated protoplasts were resuspended in 1 ml SMMP and either plated out immediately or incubated for 1.5 h at the appropriate

temperature and then plated out. The different regeneration media used are detailed in section 2.

KCl mediated transformation (Hiraoka *et al* 1992).

This method was used to successfully transform *B. subtilis* 168 and is detailed as follows. *Bacillus subtilis* 168 was grown in 2 x L-broth until mid to late exponential phase. A 0.33 ml aliquot of culture was pelleted (Beckman microfuge 5 min), and resuspended in 1 ml of 410 mM KCl. This was incubated statically at 30°C for 30 min. 50 µl of cell suspension was mixed with 5 µl TE buffer containing 0.5 µg plasmid DNA. 50 µl of PEG 6000 was then added and the suspension mixed by inversion several times. 1 ml of LC medium (L medium supplemented with 100 mM $CaCl_2$) was added and the cells pelleted as before. The pellet was resuspended in 0.5 ml 2 x L-broth and maintained statically at 37°C for 2 hr. 100 µl aliquots were plated out onto selective TSBA containing 25µg/ml tetracycline.

2.15 Isolation of DNA from mushroom compost

A 50 g sample of end of phase 2 mushroom compost was mixed with 100 ml PBS in a stomacher bag (Seward UK Ltd.). The material was homogenised in a Seward 400 stomacher (medium setting) for 1 min. The liquid phase was collected and the stomaching procedure repeated twice. The pooled liquid phases were centrifuged (12000 x g, 45 min, RT), the pellet resuspended in 30 ml PBS and the suspension centrifuged to remove large particulate matter (2500 x g, 5 min, RT). This was repeated three times. The pooled supernatants were centrifuged (12000 x g, 45 min, RT), and the

pelleted cells resuspended in 10 ml sucrose solution (1.3 g/ml). 15 ml sucrose solution was carefully layered underneath and the mixture centrifuged (5200 x g, 5 min, RT). The upper layer containing the cells was removed and centrifuged (12000 x g, 45 min, RT) to pellet the cells. This was repeated three times. The pellet was washed twice in 10 ml PBS and resuspended in 3 ml PBS.

An X 5 X-Press (A.B. Biox, Gothenburg, Sweden) was chilled at -70°C for 2 h. The 3 ml cell suspension was transferred to the top chamber and allowed to freeze. The plunger was inserted into the upper chamber followed by the piston. The apparatus was placed in a Beckman hydraulic press and a force of 4 tons applied until the frozen cell mixture was forced into the lower chamber of the X-press. The frozen cell lysate was removed from the lower chamber, allowed to thaw and aliquoted (100 µl) into sterile eppendorf tubes. The lysates were extracted once with one volume of phenol and then with one volume of chloroform twice. The DNA was then precipitated by the addition of 2 volumes ice cold ethanol and the tubes held at -70°C for 1 hr. The precipitate was collected by centrifugation (Beckman microfuge 5 min), and dissolved in 100 µl 0.1 M sodium acetate, 0.05 M Tris/HCl pH 8.0. The DNA was reprecipitated as above, dried *in vacuo* and redissolved in 100 µl TE buffer.

2.16 Enzyme analysis and agarose gel electrophoresis of DNA

All enzymatic manipulation of DNA samples was as described by Maniatis et al., (1982). Following the addition of 0.1 vol loading buffer (100mM EDTA, 50% (v/v) glycerol, 1% (w/v) SDS and 0.1% (w/v) bromophenol blue), DNA samples were loaded onto 0.8-3.0% (w/v) agarose gels. Gels were electrophoresed in TBE buffer, containing

0.5 μg/ml ethidium bromide at 30 mA overnight or 55 mA for 60 min. A 230 nm UV transilluminator was used to visualise the DNA bands.

2.17 DNA amplification using the Polymerase Chain reaction (PCR)

DNA amplification by PCR was performed using a MJR thermal cycler (GRI). The reaction mixture contained: 72.5 μl, sterile distilled H_2O; 10 μl of dNTP stock solution (2 mM solution of each of dATP, dCTP, dGTP, dTTP), 10 μl 10 x Taq polymerase buffer (BCL), 2 μl forward primer (50 pM), 2 μl reverse primer (50 pM), 1 μl template DNA (50-100 ng) and 2.5 μl Taq DNA polymerase (2.5U BCL). The mixture was overlaid with 75 μl light mineral oil. Melting, annealing and extension temperatures were 95°C, 55°C and 72°C respectively. The cycle parameters were as follows; 95°C for 40 sec; 55°C for 40 sec; ramp to 72°C at 1°C/10 sec; 72°C for 2 min; cycle five times, then; 95°C for 40 sec; 55°C for 40 sec; 72°C for 2 min; cycle thirty five times. Temperature ramps were at rates of 1°C/sec unless otherwise stated.

2.18 DNA blotting and Hybridisation

The method for plaque lifts was as detailed in Maniatis et al., (1982). To prepare dot blots, nitrocellulose membranes were pre-wetted in 2 x SSC solution for 30 min. Dot blots were performed using a vacuum manifold (Bio-Rad). The nitrocellulose membrane was placed on top of the dot blot manifold gasket and the wells secured above it. The DNA solution (250 μl) was denatured by the addition of 170 μl 1 M NaOH and 5 μl 1 M EDTA, and heating to 100°C in a boiling water bath for 10 min. Aliquots of denatured DNA were added to wells of the dot blot apparatus. An equal

volume of 2 x SSC was added to unused wells. A vacuum of 50-100 mbar was applied to the apparatus and the liquid drawn through the filter. a wash solution of 2 x SSC was added to all wells and drawn through the filter. The filter was removed, washed in 2 x SSC and dried on Whatman 3MM paper. The nitrocellulose filters were baked under vacuum at 80°C for 2 hr to fix the DNA. The membranes were then hybridised as described in Section 2.

The method for gel blots employed a Milliblot vacuum transfer apparatus to transfer DNA from an agarose gel onto a nitrocellulose membrane. The wells were removed from an agarose gel using a scalpel. The gel was soaked in 0.25 M HCL for 15 min before being vacuum blotted for 40-50 min. The filter was removed, washed in 2 x SSC and dried on Whatman 3MM paper. The nitrocellulose filters were baked under vacuum at 80°C for 2 hr to fix the DNA. The membranes were then hybridised as described in Section 2.

To prepare radiolabelled probes 10-30 pM of oligonucleotide probe was added to the labelling reaction mixture (3 µl 10 x PNK buffer solution, 1 µl Dithiothreitol (100 mM) 23.5 µl dH_2O, 1 µl $\gamma^{32}P$ ATP (50 µci aq.soln. ICN-Flow) and 10U PNK). The reaction mixture containing the probe was incubated at 37°C for 30 min and then 60°C for 10 min to denature the enzyme. The labelled probe was immediately used in the hybridisation as follows.

The membrane filter containing the DNA was prehybridised in 5 ml hybridisation solution (5 x SSC, 5 x Denhardts solution, 0.3% SDS) 1 ml denatured salmon sperm (10 mg/ml)) for 30 min at 55°C in a rotating hybridisation oven (Techne, HB1). The radiolabelled probe was then added and allowed to hybridise overnight at

55°C. The filter was then washed twice in hybridisation buffer at 55°C. The membrane was removed wrapped in Saran wrap and autoradiographed as follows.

Hybridised filters wrapped in Saran wrap were put up for autoradiography for 18 hr using X-ray sensitive film with intensifying screens at -70°C.

2.19 Induction of ø105 MU205

5 ml of 2 x L-broth was inoculated with a single colony from a fresh plate of the host. Incubation was at 37°C until the OD_{600} reached 1.0. The bacteriophage was induced by incubation at 48°C for 5 min, and then incubation continued at 37°C until lysis had occurred, usually after 2-3 hr. The lysate was sterilised by membrane filtration (0.45 μm, Millipore), and stored at 4°C.

2.19.1 Mitomycin C induction of bacteriophages

5 ml of 2 x L-broth was inoculated with a single colony from a fresh plate of the host. Incubation was at 37°C until the OD_{600} reached 0.5. 15 μl of mitomycin C (200 μg/ml in H_2O, stored at 4°C) was then added and the incubation continued for a further 25 min. The cells were harvested by centrifugation (3000 x g, 5 min, RT) and resuspended in 5 ml of fresh pre-warmed 2 x L-broth. Incubation was continued at the appropriate temperature until lysis had occurred, usually 2-3 h. The lysate was sterilised by membrane filtration (0.45 μm, Millipore), and stored at 4°C.

2.20 Isolation of thymine requiring mutants of *Bacillus caldotenax*

Mutants deficient in thymidylate metabolism were isolated using trimethoprim

as a selective agent. Kuhns minimal agar plates were prepared supplemented with trimethoprim (25 μg/ml) and thymine (50 μg/ml. A suspension of *B. caldotenax* BT1 was prepared in 0.09% (w/v) saline from an overnight plate, to give a cell density of 10^8-10^9 cell /ml. 0.5 ml was then spread onto the surface of the supplemented Kuhns plate and incubated at 55°C for 3 days. Large surviving colonies against a faint background of growth covering the plate were then replica plated onto a fresh plate an incubated for a further 2 days. The isolates were confirmed as thymine requiring strains by replica plating onto supplemented and unsupplemented Kuhns agar plates and incubating at 55°C for 2 days. Confirmed thymine requiring isolates were stored at 4°C.

2.20.1 Isolation of streptomycin resistant mutants of *B. caldotenax*

Mutants that were resistant to streptomycin were isolated in a similar manner to thymine requiring strains. The supplemented Kuhns agar plates contained streptomycin (25 μg/ml). Confirmed streptomycin resistant isolates were stored at 4°C.

2.21 Transduction in *Bacillus caldotenax* mutants

Phage JS017 titre was adjusted to 10^8 pfu/ml. The mutant strains isolated as detailed above, were cultured in 50 ml of supplemented Kuhns broth in a baffled flask (55°C 150 rpm). The growth was monitored by OD at 420 nm to an OD of 1.2 (corresponding to 1 x 10^8 cells/ml. 1ml volumes of cell suspension were transferred to capped 5 ml pyre tubes held at 55°C. Bacteriophage suspensions were added to give an MOI of 0.5. After 30 min the suspension were centrifuged (3000 rpm Beckmann microfuge) and washed in Kuhns broth to remove free phage particles. The cells were

re-suspended in 0.2 ml Kuhns broth and spread over the surface of Kuhns minimal agar plates. The plates were incubated at 55°C for 2 days. Controls were included for mutant reversion by using 0.5 ml PMN buffer in place of the bacteriophage suspension. Controls for phage contamination replaced 1 ml of cells with 1 ml of Kuhns broth.

Thy$^+$ transductant colonies were replicated onto TSBA plates and examined for the presence of lysogenic bacteriophages by overlaying with 3.5 ml of TSA seeded with *B. caldotenax* BT1. Pates were incubated overnight at 55°C. The presence of lysogenic bacteriophages was indicated by a hazy zone of lysis in the TSA around the transduced colony.

2.22 Survival of bacteria and bacteriophages in compost microcosms

Bacteria for release experiments were grown to mid-log phase in 50 ml 2 x L-broth at 50°C with shaking at 150 rpm unless otherwise stated. The cells were harvested and resuspended in PBS for use in release experiments. High titre lysates of bacteriophages prepared as previously described were used in release experiments. End of phase 2 compost samples (3g of untreated compost in boiling tubes with loose fitting caps) were inoculated with 1ml of bacterial suspension or 1ml of bacteriophage suspension or 1ml of both. Sterile distilled water (1ml or 2ml) was added to uninoculated control tubes. Where appropriate compost was sterilised by autoclaving at 121°C for 15 min on three successive days (unless otherwise stated). Recoveries onto selective and non-selective media were made as described in sections 2.5 for bacteria and 2.8 for bacteriophages, immediately after inoculation and at intervals thereafter.

CHAPTER 3

ISOLATION AND CHARACTERISATION OF *BACILLUS* SPECIES AND ASSOCIATED BACTERIOPHAGES THROUGHOUT THE MUSHROOM COMPOSTING PROCESS

Chapter 3

Isolation and Characterisation of *Bacillus* species and associated bacteriophages throughout the mushroom composting process.

3.1 Introduction

Mushroom compost has been used previously in microcosm studies to investigate the survival of genetically recombinant microorganisms. (Amner et al., 1988; McDonald, 1992). The active indigenous *Bacillus* population presents a good test of gene survival under competitive conditions and provides opportunity for gene transfer to the indigenous population to be studied. An important prerequisite for all release studies should be that the indigenous population is thoroughly characterised. This was attempted here to allow greater understanding in assessing the effect of a released GMO on the indigenous population dynamics. It may be that transfer of a recombinant DNA sequence from a released GMO to the indigenous population does not occur or occurs at frequencies below the limit of detection. This does not preclude the equilibrium of the indigenous population being altered due to the presence of the GMO.

The isolation of *Bacillus* species from mushroom compost has shown that the indigenous population comprises both mesophilic and thermophilic species (Amner et al., 1988; Amner et al., 1993). Previous studies have concentrated on mushroom compost obtained from the end of Phase two, as these samples have been shown to be relatively reproducible (McDermott, unpublished data). The association of the

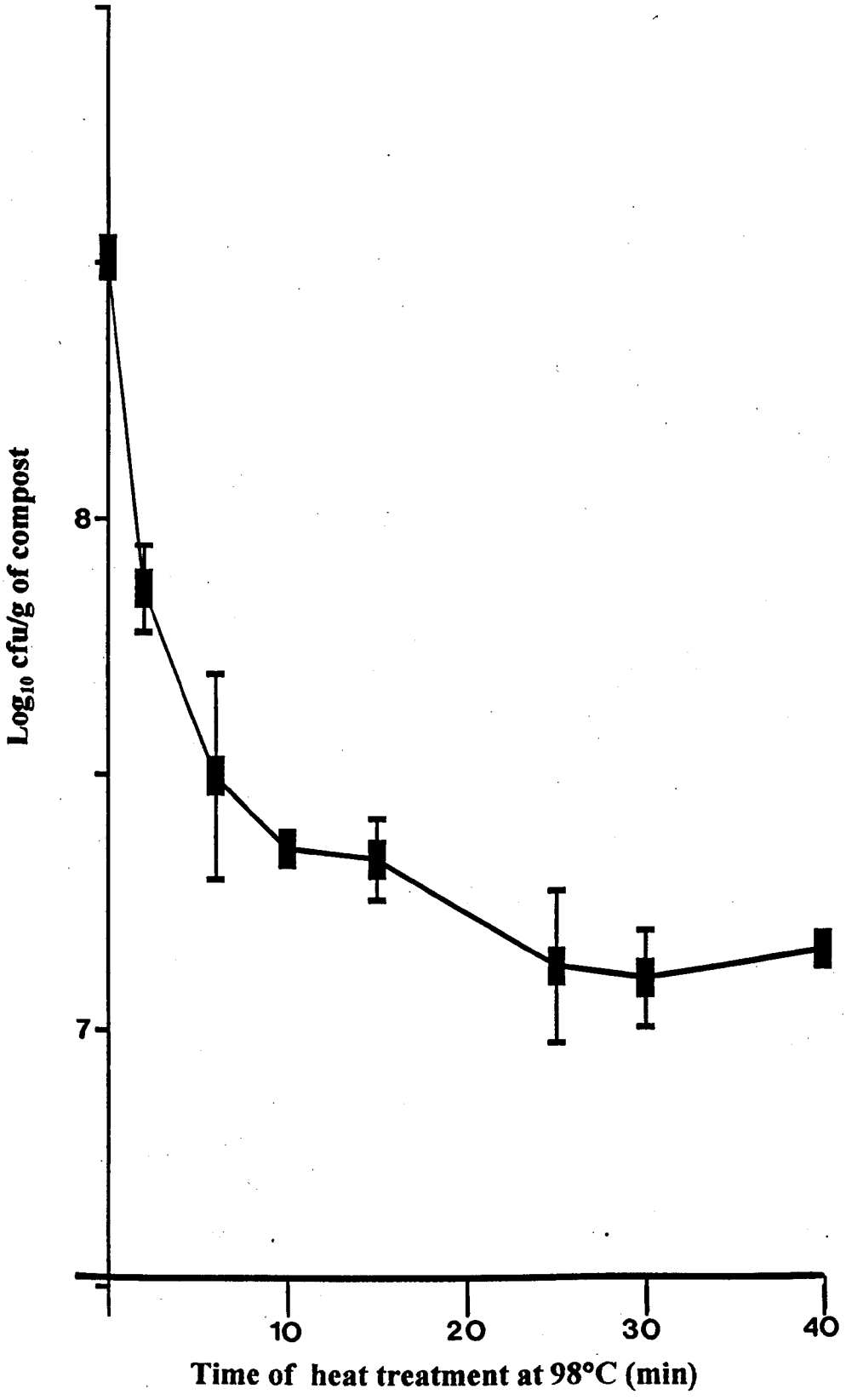
indigenous *Bacillus* population of mushroom compost with bacteriophages has not been studied previously.

It was the intention of this part of the study to monitor and identify the *Bacillus* species that could be isolated as vegetative cells or spores, through Phases one and two of the mushroom composting process. This involved daily sampling of the compost stack from the beginning of phase one to the end of phase two. In order to examine the thermotolerant and thermophilic species present, all incubations were carried out at 50^{0}C. It has also been previously reported that at this incubation temperature virtually all of the bacteria recovered, exhibiting non hyphal growth, are members of the genus *Bacillus* (Amner et al., 1988).

The initial requirements to fulfill these objectives were firstly, to formulate a sampling regimen that would allow representative samples to be collected from the compost stack and analysed in the laboratory. Secondly, to assemble a culture collection of *Bacillus* strains that represented the population present in Phases one and two. The collection of isolates was essential in order to monitor the occurrence of different *Bacillus* species throughout the composting process. The dilution plating procedure used in these studies (Methods 2.5) had previously been used to isolate *Bacillus* spp. in high numbers, from mushroom compost and solid waste compost samples (Amner et al., 1988; Strom, 1985).

Prior to the sampling experiments, the procedure for determining spore counts (Methods 2.5) was studied in order to determine if there was an adverse effect of the heat treatment on the spore cell count from end of Phase 2 mushroom compost. The results are presented in Figure 3.1 and show that the viable count decreased during the

Figure 3.1; Effect of heat treatment on the recovery of *Bacillus* spp. from mushroom compost; ■, Colony count determined at 50°C after heat treatment, on TSBA. All values are means of triplicate determinations. Error bars represent standard deviations of the mean


Log10 cfu/g of compost
8
7
10
20
30
40
Time of heat treatment at 98°C (min)

first ten minutes at 98^{0}C, presumably as the vegetative cells were killed. There was less of a decrease over the next 30 min, a result of the spores surviving the heat treatment. At the end of the experiment a sample of colonies were confirmed as *Bacillus* spp. by Gram staining. These results indicated that the spore population was not adversely affected by the heating procedure during this time and that a treatment of 15 min at 98°C was appropriate for determination of spore counts throughout these studies. The preliminary analysis of mushroom compost samples for this study, indicated that a variety of morphologically distinct *Bacillus* isolates could be obtained from compost samples using dilution plating procedures. It was concluded that the dilution plating procedures, although only giving relative data, would nevertheless provide a diversity of *Bacillus* isolates that could be used in further studies. The dilution plating on non-selective media did not appear to favour the selection of any one particular isolate.

3.2 Formulation of a sampling regimen.

The compost material collected at the end of Phase two has been viewed relatively predictable regarding its indigenous biological community (Amner et al., 1993). However the compost stack at the beginning of phase one, when the raw materials are brought together, represents a more heterogeneous and dynamic environment, with rapid activity resulting in rapid changes in population structure. It is important that any samples taken from this stage represent this. It was decided to sample the Phase one stack from a variety of locations and determine the vegetative and spore cell counts of *Bacillus* species for each location. This would give an indication of any variation in the collection sites. Five replicate samples were analysed for each

sampling site (Methods 2.5). The sampling sites and corresponding colony counts are shown in Table 3.1.

It was observed on some of the isolation plates in the above experiment, that the *Bacillus* colonies could not be enumerated because of the spreading of certain colony types. This spreading appeared as a confluent lawn of growth, even at the highest dilution, preventing any colony count being obtained. It was particularly evident when determining spore counts. The spreading was reduced by drying the isolation plates in a laminar hood for two hours before plating out samples.

Results in Table 3.1 indicate the variation in numbers of *Bacillus* isolates recovered at different sampling sites. This showed the difficulties of reproducibility when sampling environmental bacterial populations, because the compost material is heterogeneous and contains within it, different microenvironments with varying biological activity. It was decided that a representative sample of the compost stack could be attained by taking handful samples, from several different sites within the stack and combining these thoroughly. This was to be the sampling procedure for the daily monitoring of the indigenous *Bacillus* population from the beginning of Phase one to the end of Phase two.

3.3 Isolation of *Bacillus* species from mushroom compost.

Mushroom compost samples were collected from the beginning of Phase one and the end of Phase two according to the sampling regimen. Samples of spawned compost and spent mushroom compost were also obtained for analysis. The viable cell

Table 3.1 **Colony counts from different sampling sites of a Phase 1 compost stack.**

Sampling site*.	Total viable count[a] (cfu/g of compost)	Spore count[a] (cfu/g of compost)
End of stack (A)♯	7.7×10^7 (1.4×10^7)	1.5×10^7 (0.1×10^7)
End of stack (B)♯	3.0×10^8 (0.8×10^8)	1.2×10^7
Centre of stack	1.5×10^8 (0.5×10^8)	2.0×10^7 (0.4×10^7)
Side of stack	1.4×10^8 (0.5×10^8)	6.9×10^7
Top of stack	1.3×10^8 (0.3×10^8)	3.6×10^7

Values in parentheses indicate the standard error of the mean from duplicate counts of five replicate samples. Spore counts determined by enumeration of colonies that survived heat treatment of 98°C for 15 min/

* Handful samples were collected from the appropriate location at a depth between 10 and 40 cm

♯ End of stack samples (A and B) were at opposite ends of the same composting stack (ca 15m long).

a Total viable count and spore count of the isolated *Bacillus* population.

count for the indigenous *Bacillus* population was enumerated for each of the above stages from several different batches of compost. Table 3.2. details colony counts obtained from one set of samples. The main purpose of these experiments was to gain familiarisation with the indigenous *Bacillus* microflora in terms of its numbers and colony appearance under the isolation conditions.

Further fresh mushroom compost samples from Phases one and two were obtained over a period of two months, from different compost batches and analysed as above. The isolation plates were examined for morphologically distinct isolates which could be subcultured. Such isolates were subcultured three times on TSBA plates. It was beyond the scope of the study to subculture every single isolate observed, so a subjective view was taken in order to keep the number of isolates to a representative yet manageable level. Further consideration was given to the selection of isolates that were morphologically distinct to minimise the duplication of isolates within the collection. Each isolate was assigned an identification number. On examining the colonial morphologies of the isolates obtained, it was possible to divide them into ten groups. Members within a particular group showed a similar colonial morphology and were morphologically distinct from the isolates in the other groups. Groups 7,8,9 and 10 contained only one or two isolates to reduce duplication because there appeared to be little variations in the colony types, in comparison to the other groups. Colonial morphology was not considered at this stage to represent different *Bacillus* species. The groups of isolates are detailed in Table 3.3 together with a description of the typical colonial morphology observed.

The analysis of different batches of mushroom compost presented further

Table 3.2 Colony counts from different stages of the mushroom composting process.

Stage of composting process	Total cell count* (cfu/g compost)	Spore count* (cfu/g compost)
Phase one compost	2.1×10^7 (0.2×10^7)	1.1×10^7 (0.13×10^7)
Phase two compost	1.7×10^8 (0.1×10^8)	1.45×10^7 (0.1×10^7)
Spawned compost	1.4×10^8 (0.4×10^8)	Not determined
Spent compost	1.7×10^7	Not determined

Values in parentheses indicate the standard error of the mean from triplicate determinations.

* Colonies were determined after overnight incubation on TSBA at 50°C

Table 3.3 ***Bacillus*** **isolates from mushroom compost grouped on the basis of colony morphology.**

Group Number.	Isolate Reference No.	Typical colonial morphology.
1	MG001-MG009	Undulate edge; Irregular colony; Pulvinate; Glossy appearance.
2	MG010-MG018	Entire edge; Circular colony; Flat; Opaque; Spreading; Dry appearance.
3	MG019-MG022	Entire edge; Irregular colony; Flat; Opaque; Dry appearance.
4	MG023-MG029	Crenated edge. Irregular colony. Flat. Opaque. Rough appearance.
5	MG030-MG037	Entire edge. Circular colony. Raised. Glossy appearance.
6	MG038-MG043	Entire raised edge; Circular colony; Flat; Glossy appearance.
7	MG044-MG045	Entire edge; Circular colony; Flat; Glossy and translucent appearance.
8	MG046	Entire edge; Circular white colony Flat; Rough appearance.
9	MG047	Crenated edge; Circular colony; Flat; Glossy and translucent appearance.
10	MG048	Entire edge; Circular colony; Pulvinate; Glossy and translucent.

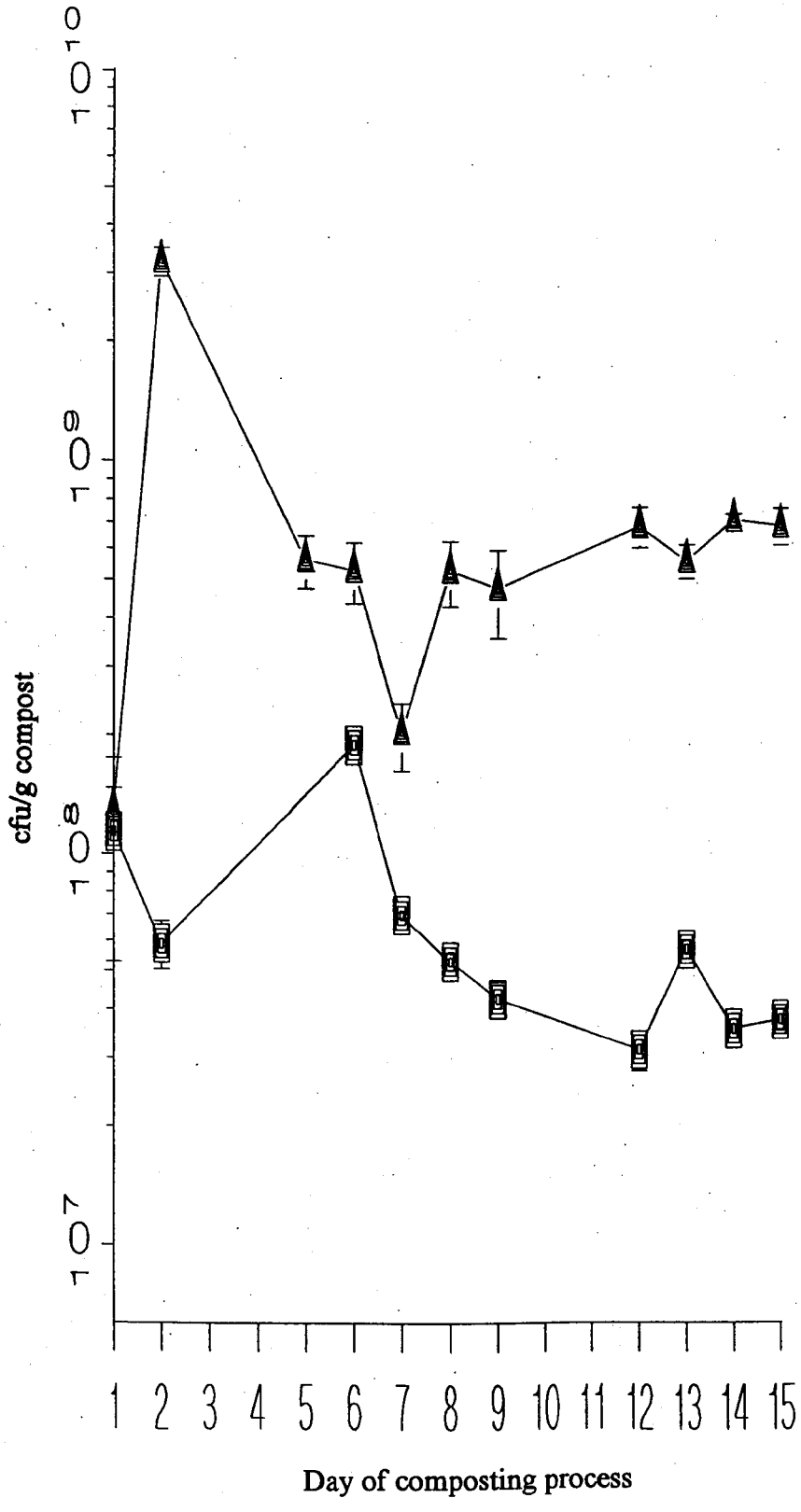
opportunities to maximise the number of morphologically distinct groups that could be isolated. Results showed that each compost batch appeared to have all of the groups present either in Phase one or Phase two or both. No further morphologically distinct *Bacillus* isolate groups were obtained. On a number of occasions when Phase one compost samples were analysed, an isolate was obtained that did not resemble any of the *Bacillus* isolates. When Gram stained and examined under the light microscope the isolate was found to be a Gram negative rod. The isolate was provisionally identified as a pseudomonad of the fluorescens group after it was shown to fluoresce under UV light and was also shown to produce a reddish pigment on TSBA plates. The isolation of pseudomonads capable of growth at 50°C from mushroom compost has been previously reported (Stanek, 1972), although in this laboratory they are recovered from mushroom compost samples only sporadically.

From these experiments, it had been possible to investigate the indigenous *Bacillus* population that was culturable under the conditions detailed. In total, a collection of 48 isolates was obtained that could be divided into ten groups based on differences in colonial morphology. The next stage in the investigations was to monitor the mushroom compost from day zero of Phase one to the end of Phase two. This monitoring would take the form of enumerating vegetative cells and spores daily under the isolation conditions detailed. The occurrence of the defined groups of isolates would also be monitored

3.4 Monitoring changes in the *Bacillus* population during phases one and two of the mushroom composting process.

The sampling regimen was used to collect daily samples from a mushroom compost stack. Day one was defined as the first sampling day after the raw materials were assembled into a Phase one stack. Five replicate samples were analysed for each sampling day (Methods 2.5). The results are presented in Figure 3.2. Vegetative cell counts were obtained by subtracting the spore cell count from the total cell count. At the beginning of Phase one the spore count is greater than the vegetative cell count. This may be a result of sporulation prior to the assembly of the stack. *Bacillus* spores require heat shock to germinate plus a lag time and this might explain why the majority of cells appeared to be present as spores. The compost stack was turned on Day two before sampling and again before sampling on Day five. The effect of turning is represented by an increase in the number of vegetative cells isolated on Day two. This is probably a result of germination of spores as there is a reduction in the number of spores isolated between these days. Unfortunately, it was never possible to sample the stack on either of days three and four. An increase in the spore population was evident on Day 6, presumably as a result of sporulation of vegetative cells. The compost was moved to Phase two on Day eight and was in this phase for the remainder of the sampling time. The relative biological stability of Phase two compost in terms of the indigenous *Bacillus* population is represented by less variation between the daily counts as Phase two composting progresses. During this time the compost is in a temperature controlled environment and many of the simple nutrients have been utilised adding stability to the remaining population. At the end of Phase two, the compost material

Figure 3.2; Analysis of the *Bacillus* population of mushroom compost during Phases one and two of the composting process. ▲ , Total cell population; ▤ , spore population; Colony counts determined at 50°C. All values are means of duplicate counts from five replicate samples. Error bars represent standard deviations of the mean


cfu/g compost
10^10
10^9
10^8
10^7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Day of composting process

had been broken down by the actions of the indigenous bacterial population resulting in a more uniform material physically, than was present in Phase one. This uniformity probably contributes further to the biological stability of different samples. Whereas in Phase one the sampled material was bulky and visibly different, ie bits of straw and manure, Phase two samples appeared physically similar to each other.

The occurrence of particular groups of isolates during the sampling is represented in Table 3.4. A particular group of isolates was scored as present, if there was an isolate of similar colonial morphology present on an isolation plate for that sampling day. Throughout the experiment, the isolation plates were again examined for the presence of other colonial morphologies not already recorded, however there were no additions to the number of groups. The results indicate that groups 1,2 5,7, and 8 could be isolated as members of the vegetative cell population and the spore population on each of the sampling days. These groups together, were observed to form the majority of the indigenous population that could be isolated. Analysis of the spore population showed that throughout the sampling days the majority of the isolates were from Group 2. The predominance of these spreading isolates was also noted during the experiments to formulate a sampling regimen. Group 10 isolates could not be detected on spore isolation plates on Days four and five. It may be that this group of isolates were present in lower numbers than the other groups, but it was not possible to identify them on the lower dilution spore isolation plates because of the confluent growth of the other groups. The isolation of only vegetative cells could be a result of the germination and outgrowth of spores after the compost had been turned. Group 9 isolates could not be isolated during Phase 2. The group was present in the vegetative

Table 3.4 Occurrence of the different groups of *Bacillus* isolates during the composting process

DAY	Group number									
	1	2	3	4	5	6	7	8	9	10
1	+◊	+◊	+◊	+	+◊	+◊	+◊	+◊	+◊	-
2	+◊	+◊	+◊	+	+◊	+◊	+◊	+◊	+◊	-
5	+◊	+◊	+	+◊	+◊	+◊	+◊	+◊	-	+
6	+◊	+◊	+	+◊	+◊	+	+◊	+◊	◊	+
7	+◊	+◊	-	+◊	+◊	+	+◊	+◊	◊	-
8	+◊	+◊	-	+	+◊	-	+◊	+◊	-	-
9	+◊	+◊	-	-	+◊	-	+◊	+◊	-	-
12	+◊	+◊	-	-	+◊	-	+◊	+◊	-	-
13	+◊	+◊	-	-	+◊	-	+◊	+◊	-	-
14	+◊	+◊	-	-	+◊	-	+◊	+◊	-	-

Key to symbols:

+ Present on total cell isolation plate
◊ Present on spore isolation plate
+◊ Present on total cell isolation plate and spore isolation plate

and spore cell form on Days one and two of Phase 1. The group was not isolated on Day five but was isolated in the spore form on Days six and seven. Again it appears that the presence of this group of isolates reaches a maximum during the early stages of Phase one. The isolates from Groups 3 and 6 were also only isolated during Phase one. Although these isolates were present in the vegetative and spore form it became apparent that neither group could be stored in the laboratory. Attempts were made to store the isolates on sealed plates at 4°C and in glycerol suspensions at -70°C. Both of these procedures were found to reduce the viability of the isolates such that they could not be recovered. It was not practical to sub culture the isolates daily, so Groups 3 and 6 were excluded from any further study. It may be that the isolates could not sporulate *in vitro* although no attempts were made to confirm this. The *Bacillus* population that could be isolated during phase two appeared to remain stable in terms of the morphological groups present. However, this does not mean that the other groups of isolates were not present in phase two, only that they could not be detected.

3.5 Characterisation of *Bacillus* isolates.

The results of biochemical, physiological and morphological characterisation of the *Bacillus* isolates (Methods 2.6) are presented in Table 3.5. A summary of the data is presented in Table 3.6. From these results it appeared that isolates within the original groups based on colonial morphologies were similar to each other based on the tests described. It may be that for these particular isolates colony morphology was a valid method of grouping isolates. All isolates were shown to be Gram positive rods that were catalase positive. Throughout the experiments, a particular characteristic was

Table 3.5 Results of phenotypic characterisation tests for *Bacillus* isolates.

Test	Isolate Number (MG No.)											
	001	002	003	004	005	006	007	008	009	010	011	012
Gram	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	+	-	-	-	+	-	-	-	-
Acid (MR)	+	+	+	+	+	+	+	-	+	+	+	+
Acetoin (VP)	+	+	+	+	+	+	+	+	+	-	-	-
Growth												
5% NaCl	+	+	+	+	+	+	+	+	+	+	+	+
pH 5.5	+	+	+	+	+	+	+	+	+	-	-	-
Hydrolysis of												
Hippurate	-	-	-	-	-	-	-	-	-	+	+	+
Tyrosine	-	-	-	-	-	-	-	-	-	-	-	-
Casein	+	+	+	+	+	+	+	-	+	-	-	-
Starch	+	+	+	+	+	+	+	-	+	-	-	-
Gelatin	+	+	+	+	+	+	+	+	+	-	-	-
Utilisation of												
Citrate	+	+	+	-	-	+	+	-	+	-	-	-
Propionate	+	+	-	-	+	+	+	-	-	-	-	-
Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-
Reduction												
NO_3-NO_2	+	+	+	+	+	+	+	+	+	-	-	-
NO_3-gas	-	-	-	-	-	-	-	-	-	-	-	-
Acid Prodn.												
Cellobiose	+	+	+	+	+	+	+	+	+	-	-	-
Ribose	+	+	+	+	+	+	+	+	+	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-	-
Growth-Temperature												
25°C	+	+	+	+	+	+	+	+	+	-	-	-
37°C	+	+	+	+	+	+	+	+	+	-	+	+
45°C	+	+	+	+	+	+	+	+	+	+	+	+
50°C	+	+	+	+	+	+	+	+	+	+	+	+
55°C	+	+	+	+	+	+	+	+	+	+	+	+
60°C	±	±	-	-	±	±	-	±	±	+	+	+
70°C	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.5 cont.

Test	Isolate Number (MG No.)											
	013	014	015	016	017	018	023	024	025	026	027	028
Gram	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	+	+	+	+	+	+
Acid (MR)	+	+	+	+	+	+	-	-	-	-	-	-
Acetoin (VP)	-	-	-	-	-	-	-	-	-	-	-	-
Growth												
5% NaCl	+	+	+	+	+	+	-	-	-	-	-	-
pH 5.5	-	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis of												
Hippurate	+	+	+	+	+	+	-	-	-	-	-	-
Tyrosine	-	-	-	-	-	-	-	-	-	-	-	-
Casein	-	-	-	-	-	-	-	-	-	-	-	-
Starch	+	-	-	+	-	-	+	+	+	+	+	+
Gelatin	-	-	-	-	-	-	+	+	+	+	+	+
Utilisation of												
Citrate	-	-	-	-	-	-	-	-	-	+	-	-
Propionate	-	-	-	-	-	-	-	-	-	-	-	-
Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-
Reduction	-	-	-	-	-	-	-	-	-	-	-	-
NO_3-NO_2	-	-	-	-	-	-	+	+	+	+	+	+
NO_3-gas												
Acid Prodn.	-	-	-	-	-	-	+	+	+	+	+	+
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-
Ribose	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-
Salicin												
Growth-Temperature												
25°C	-	-	-	-	-	-	-	-	-	-	-	-
37°C	+	+	+	+	+	+	-	-	-	-	-	-
45°C	+	+	+	+	+	+	+	+	+	+	+	+
50°C	+	+	+	+	+	+	+	+	+	+	+	+
55°C	+	+	+	+	+	+	+	+	+	+	+	+
60°C	+	+	+	+	+	+	+	+	+	+	+	+
70°C	-	-	-	-	-	-	+	+	+	+	+	+

Table 3.5 cont.

Test	Isolate Number											
	029	030	031	032	033	034	035	036	037	044	045	046
Gram	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+
Acid (MR)	-	+	+	+	+	+	+	+	+	-	-	-
Acetoin (VP)	-	+	+	+	+	+	+	+	+	-	-	-
Growth												
5% NaCl	-	+	+	+	+	+	+	+	+	+	-	-
pH 5.5	-	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis of												
Hippurate	-	-	-	-	-	-	-	-	-	-	+	-
Tyrosine	-	+	+	+	+	+	+	+	+	-	-	-
Casein	-	-	-	-	-	-	-	-	-	-	-	-
Starch	+	-	-	-	-	-	-	-	-	-	-	+
Gelatin	+	-	-	-	-	-	-	-	-	-	-	+
Utilisation of												
Citrate	-	-	-	-	-	-	-	-	-	-	-	-
Propionate	-	-	-	-	-	-	-	-	-	-	-	-
Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-
Reduction												
NO_3-NO_2	-	+	+	+	+	+	+	+	-	-	-	-
NO_3-gas	+	-	-	-	-	-	-	-	-	-	-	+
Acid Prodn.												
Cellobiose	+	-	-	-	-	-	-	-	-	-	-	+
Ribose	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-	-
Growth-Temperature												
25°C	-	-	-	-	-	-	-	-	-	-	-	-
37°C	-	+	+	+	+	+	+	+	+	+	+	-
45°C	+	+	+	+	+	+	+	+	+	+	+	+
50°C	+	+	+	+	+	+	+	+	+	+	+	+
55°C	+	+	+	+	+	+	+	+	+	+	+	+
60°C	+	+	+	+	+	+	+	+	+	+	+	+
70°C	+	-	-	-	±	-	-	-	-	-	-	+

Table 3.5 cont.

Test	Isolate number	
	047	048
Gram	+	+
Catalase	+	+
Oxidase	+	-
Acid (MR)	+	+
Acetoin (VP)	-	-
Growth		
5% NaCl	+	+
pH 5.5	-	+
Hydrolysis of		
Hippurate	+	+
Tyrosine	+	+
Casein	-	-
Starch	-	-
Gelatin	-	-
Utilisation of		
Citrate	-	-
Propionate	-	-
Phenylalanine	-	-
Reduction		
NO_3-NO_2	-	-
NO_3-gas	-	-
Acid Prodn.		
Cellobiose	-	+
Ribose	-	-
Melibiose	-	-
Salicin	-	-
Growth-Temperature		
25°C	-	-
37°C	-	-
45°C	+	+
50°C	+	+
55°C	+	+
60°C	+	-
70°C	-	-

+, Positive reaction; -, Negative reaction

Table 3.6 **Summary of taxonomic tests for the morphologically distinct groups.**

Test	Group Number								
	1	2	4	5	7a	7b	8	9	10
Gram	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	v	-	+	+	+	+	+	-	-
Acid (MR)	v	+	-	+	-	-	-	+	+
Acetoin (VP)	+	-	-	+	-	-	-	-	-
Growth									
5% NaCl	+	+	-	+	+	-	-	+	+
pH 5.5	+	-	-	-	-	+	-	-	+
Hydrolysis of									
Hippurate	-	+	-	-	-	+	-	+	+
Tyrosine	-	-	-	+	-	-	-	+	+
Casein	v	-	-	-	-	-	-	-	-
Starch	v	v	+	-	-	-	+	-	-
Gelatin	+	-	+	-	-	-	+	-	-
Utilisation of									
Citrate	v	-	v	-	-	-	-	-	-
Propionate	v	-	-	-	-	-	-	-	-
Phenylalanine	-	-	-	-	-	-	-	-	-
Reduction									
NO_3-NO_2	+	-	-	+	-	-	-	-	-
NO_3-gas	-	-	+	-	-	-	+	-	-
Acid Prodn.									
Cellobiose	+	-	+	-	-	-	+	-	+
Ribose	+	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-
Temperature									
25°C	+	-	-	-	-	-	-	-	-
37°C	+	v	-	+	-	-	-	-	-
45°C	+	+	+	+	+	+	+	+	+
50°C	+	+	+	+	+	+	+	+	+
55°C	+	+	+	+	+	+	+	+	+
60°C	v	+	+	+	+	+	+	+	-
70°C	-	-	+	v	-	-	+	-	-

+, Positive reaction; -, Negative reaction; v,Variable characteristic within the group. For colony morphologies of groups see Table 3.3

only assigned to an isolate if the result was supported by positive and negative controls. If this was found not to be the case, the experiment was repeated.

There follows a brief description of the main characteristics for each group of isolates. Where possible an attempt was made to identify an isolate at the species level, using the studies by Gordon et al., (1973) and White, (1991) as a reference. However, all of the isolates could not be readily assigned to a recognised species. This was not unexpected as *Bacillus* is a very heterogeneous genus where classification at the species level is incomplete. The number of tests used in this study are relatively small and further characterisations must be carried out before assigning the isolate to a particular *Bacillus* species. Although identification at the species level was not possible for all isolates, the results indicate that the distinction of colonial morphology that was originally made to group the isolates, does appear to be supported by the identification tests in the majority of cases.

Members of Group one were the most visibly distinct of all groups. All isolates grew at 25^{0}C and were the only isolates to do so in the study. Common to all isolates was the ability to hydrolyse gelatin, growth in 5% NaCl and at low pH. All isolates reduced nitrate to nitrite, gave a positive Vogues Proskauer reaction and produced acid from cellobiose and ribose. Starch and casein hydrolysis were demonstrated by all isolates except MG008. Phenylalanine and tyrosine decomposition results were negative for all isolates as were results for the hydrolysis of hippurate. There was some variation in the results of the tests for utilisation of citrate and propionate. Two isolates were oxidase positive. From this study it is evident that the original nine isolates can be grouped into five taxa. Overall, members of this group resembled *Bacillus*

licheniformis. The isolation of *B. licheniformis* from spent mushroom compost samples has been previously reported (Kleyn and Wetzler, 1981).

Isolates that were in Group 2 were originally characterised by their spreading colonial morphology. It was possible to distinguish the isolates MG016 and MG013 on the basis that they hydrolysed starch while the other members of the group did not. The other positive reactions for that were common for all members of this group were methyl red, growth in 5% NaCl and decomposition of tyrosine. The members of this group did not produce acid from any of the sugars tested.

Positive reactions for Group 4 isolates were the hydrolysis of starch, the hydrolysis of gelatin and the production of acid from cellobiose. Denitrification was also a characteristic of this group with gas being produced from nitrate. MG026 could be distinguished by the utilisation of citrate. This group was truly thermophilic since all of the isolates grew at 70^{0}C and the minimum growth temperature was generally 45^{0}C. On the basis of these results this group was provisionally identified as *Bacillus thermodenitrificans*.

The isolates in Group 5 were shown to be oxidase positive, capable of growth in 5% NaCl and reduced of nitrate to nitrite. All of the isolates were positive for the MRVP tests. The other reactions gave negative results. with members of this group not producing acid from any of the sugars tested. Growth of isolates was in the temperature range from 37^{0}C to 60^{0}C apart from MG033, which exhibited some evidence of growth at 70^{0}C. Apart from this difference, all of the isolates within the group gave similar results to one and other. It was not possible to determine whether the isolates represented the same or more than one strain.

The two isolates within Group 7 differed in some of the tests detailed and could be distinguished on the basis that MG044 was capable of growth in 5% NaCl and did not hydrolyse hippurate. MG045 was not capable of growth in 5% NaCl and did hydrolyse hippurate. It is probable that these two isolates represent different species but further characterisation is necessary to attempt identification at the species level.

The single isolate in Group eight gave the same results as six of the isolates in group four including the reduction of nitrate to gas. Accordingly, the isolate was provisionally identified as *Bacillus thermodenitrificans*. Originally this isolate was assigned to a different group because it did not posses the typical crenated edge colony appearance as those isolates in Group four.

The isolate in Group 9 was capable of growth in pH 5.5 broth and gave a positive result with the oxidase test and methyl red reaction. Hydrolysis of hippurate and tyrosine was also displayed. Growth temperature data showed that the isolate only grew between 45°C and 60°C. The remaining tests gave negative reactions. Identification of the isolate at the species level was not possible.

The Group 10 isolate gave positive reactions for the pH 5.5 and 5%NaCl growth methyl red and hydrolysis of hippurate and tyrosine tests. The isolate appeared similar to the group nine isolate in the test results and there was a restricted range in growth temperatures, with growth occurring between 45°C and 55°C only. It was distinguished from the group 9 isolate because it grew in 5% NaCl and produced acid from cellobiose. Again, identification of the isolate at the species level was not possible. A summary of the data for isolation, occurrence and identification for the colony morphology groups is presented in Table 3.7.

Table 3.7 Summary of *Bacillus* isolates recovered from compost

Colony morphology Group	No. of isolates	Recovery from compost	Preliminary identification
1	9	Phases 1 and 2	*B. licheniformis*
2	9	Phases 1 and 2	*Bacillus* spp.
4	7	Phase 1	*B. thermodenitrificans*
5	8	Phases 1 and 2	*Bacillus* spp.
7	2	Phases 1 and 2	***Bacillus* spp.**
8	1	Phases 1 and 2	***B. thermodenitrificans***
9	1	Phase 1	***Bacillus* spp.**
10	1	Phase 1	***Bacillus* spp.**

3.6 Isolation of bacteriophages from mushroom compost.

In order to assess the feasibility of the isolation procedure (Methods 2.8), initial experiments were based on a preparation of a compost supernatant from early in phase one of the mushroom composting process. This was assayed against some of the available isolates. The results showed evidence of zones of lysis indicating bacteriophage activity. Single plaques were isolated from the zones of lysis after the third successive plating, using the soft agar overlay technique. The plaques appeared clear indicating lytic bacteriophage activity, but it could not be determined whether the phage activity was the result of a single phage or several phages, as there were no observable differences in plaque morphologies on the isolation plates. These preliminary experiments suggested that bacteriophages could be isolated directly from compost without prior enrichment in the laboratory.

Further compost supernatants were obtained from samples collected on various days of the mushroom composting process. These were assayed for bacteriophage activity against forty of the *Bacillus* isolates and against *B. subtilis* 168. Bacteriophage activity was represented by a zone of lysis on a lawn of the host. The lysis was recorded only after three successive transfers, to eliminate lysis that may have been produced by bacteriocins or some other factors. As has been previously mentioned, it was not possible to detect whether the zones were caused by more than one bacteriophage. The results are presented in Table 3.8 and indicate that bacteriophage activity was recorded for 19 of the 34 isolates tested. At least one isolate within each of the original groups had a bacteriophage that infected it, present in the compost supernatants. Except for MG016, this bacteriophage activity was shown to be present

Table 3.8 Evidence of bacteriophage activity in compost extracts

		Compost supernatant					
Isolate	**Group**	**Day 1**	**Day 5**	**Day 7**	**Day 8**	**Day 9**	**Day 12**
001	1	+	+	+	+	+	+
002	1	+	+	+	+	+	+
003	1	-	-	-	-	-	-
004	1	-	-	-	-	-	-
005	1	-	-	-	-	-	-
006	1	+	+	+	+	+	+
007	1	-	-	-	-	-	-
008	1	+	+	+	+	+	+
010	2	+	+	+	+	+	+
012	2	-	-	-	-	-	-
013	2	+	+	+	+	+	+
015	2	+	+	+	+	+	+
016	2	+	+	-	+	+	+
017	2	-	-	-	-	-	-
018	2	-	-	-	-	-	-
023	4	+	+	+	+	+	+
024	4	+	+	+	+	+	+
026	4	-	-	-	-	-	-
027	4	+	+	+	+	+	+
028	4	+	+	+	+	+	+
029	4	-	-	-	-	-	-
030	5	+	+	+	+	+	+
031	5	-	-	-	-	-	-
032	5	-	-	-	-	-	-
033	5	-	-	-	-	-	-
034	5	-	-	-	-	-	-
035	5	-	-	-	-	-	-
036	5	+	+	+	+	+	+
037	5	+	+	+	+	+	+
044	7	+	+	+	+	+	+
045	7	-	-	-	-	-	-
046	8	+	+	+	+	+	+
047	9	+	+	+	+	+	+
048	10	+	+	+	+	+	+

Key; +, lysis; -, No lysis

from the beginning of Phase one to the end of Phase two. This is of interest especially with bacteriophages infecting those hosts not detected during Phase 2 as it suggests that the bacteriophages were still able to survive in the absence of a host population at a detectable level. A possible explanation of this is that the hosts were still present during Phase two but at too low a level to be detected. It may also be possible that the compost environment favours the survival of bacteriophage in the absence of a host. This was investigated and the conclusions are presented in Chapter 5. There was no bacteriophage activity detected against *Bacillus subtilis* 168 in any of the compost supernatants.

For these results, bacteriophage activity was indicated by a zone of lysis after three successive platings from the original compost supernatant. The zone of lysis was regarded as a confluent area of bacteriophage plaques. Individual bacteriophage plaques were obtained using the agar overlay method from the zones of lysis for each host. The overall intention of the experiments was to isolate a lysogenic bacteriophage that could be developed as a trackable bacteriophage. Agar overlays from the zones of lysis appeared to indicate that the majority of resulting plaques were clear indicating lytic bacteriophage activity. The presence of turbid plaques was displayed for MG028 from Group 4. However, the phage purification involving three successive single plaque isolations resulted in the turbid plaques appearing clear indicating the presence of a lytic bacteriophage. Therefore, isolation of a lysogenic bacteriophage from compost could not be shown for any isolate at this stage. Kokjohn and Miller (1992) suggested that the lysogenic state was common for most bacterial isolates in nature, however this could not be demonstrated in these experiments with the indigenous *Bacillus* population of

mushroom compost.

From the experiments detailed, it was not known how many different bacteriophages were actually present for each host. Plaque morphologies were similar for individual hosts and gave no indication of whether more than one bacteriophage was present. To maintain the numbers of individual bacteriophages to a manageable level, it was decided to only propagate and store bacteriophages from a single plaque for each host. Table 3.9 contains descriptions of the host and plaque morphology for each bacteriophage selected. Another reason for limiting the bacteriophage collection was that it was not considered worthwhile at this stage to construct a trackable phage vector, to monitor bacteriophage and host interactions, if the bacteriophage was lytic.

3.7 Bacteriophage host range

The bacteriophage host ranges were determined by infecting host cells in an agar overlay (Methods 2.10) and the results illustrated the specificity of bacteriophage-host interactions. There was no evidence to suggest that a bacteriophage would infect an isolate from a different morphological group. Indeed, within any particular group the host strain specificity was represented as not all isolates were infected (Table 3.9). The isolates that were not infected may be resistant or immune to infection. It was thought that in these experiments the isolates were probably resistant to infection because the presence of a bacteriophage within the host, conferring immunity, could not be demonstrated (see section below).

Table 3.9 Plaque morphology and host specificity of bacteriophages isolated from mushroom compost.

Host (group)	Plaque morphology	Number of isolates susceptible to infection within the group
MG008 (grp 1)	Clear, circular, 1-2mm diameter .	1
MG010 (grp 2)	Clear, circular, 1-3mm diameter, hazy edge.	3
MG028 (grp 4)	Clear, circular, 1-2mm diameter.	1
MG030 (grp 5)	Clear, circular, 3-4mm diameter.	1
MG044 (grp 7)	Clear, circular, 1-3 mm diameter.	1
MG046 (grp 8)	Clear, circular, 1-2mm diameter, hazy edge.	1
MG047 (grp 9)	Clear, circular, 4-5mm diameter, hazy edge.	1
MG048 (grp 10)	Clear, circular, 1-3mm diameter.	1

3.8 Mitomycin C induction of *Bacillus* isolates.

Lysogenic bacteriophage can be induced to lytic growth by the action of mitomycin C (Methods 2.19.1). The induction procedure was carried out for all the *Bacillus* isolates. Lysis of the culture within 1-2 h was only observed for isolates MG001, MG015 and MG036. Bacteriophage activity could not be detected when the resulting supernatants were spotted onto the original host or other isolates in the collection. A possible explanation is that a lysogenic bacteriophage was induced but its presence could not be detected using the original host, because the bacteriophage was already stably maintained in the isolate. This could confer immunity to infection on the host. A more likely explanation is that the presence of mitomycin C induced a defective prophage in each of the isolates, similar to induction of defective bacteriophages in *B. subtilis* (Zahler, 1993).

3.9 Isolation of a lysogenic bacteriophage.

Infections of bacteriophages previously discussed were carried out at 37°C. The reason for this was that the lower incubation temperature could have produced a lysogenic association under the control of a temperature regulator. At increased temperatures, this control may not be in operation causing the bacteriophages to be lytic,

It was found that øMG030, infecting a Group 5 isolate, produced turbid plaques at 37°C. Bacteriophages from a single plaque isolated at 37°C were purified and used to infect the same host at 50°C and 37°C. The resulting plaques were clear at 50°C and turbid at 37°C. Further experiments showed that bacteriophages isolated at 50°C from

the clear plaques, produced turbid plaques at 37°C. Flask infection studies at 37°C, where the bacteriophage was added at an MOI of 0.1 did not produce lysis of the culture. The same experiment repeated at 50°C did produce lysis. Cells infected at 37°C were washed to remove exogenous phage, streaked out and overlayed with phage sensitive cells. After incubation at 50°C, a zone of lysis in the overlay, around the streak was evident. It appeared that øMG030 may have been lysogenic at 37°C and lytic at 50°C. Further studies to construct a trackable phage vector from a bacteriophage indigenous to mushroom compost were therefore concentrated on øMG030, and this bacteriophage was designated øMGPR.

3.10 Discussion

The main objective of these studies was to isolate, enumerate and characterise the indigenous *Bacillus* population of mushroom compost and their bacteriophages. It was also the intention to isolate a lysogenic bacteriophage that could be used for further studies into phage host interactions. Nutritionally rich media were used in these isolations and it has been suggested that the use of such media recovers only 1-2% of the soil microflora (Priest and Grigorova, 1990), so it is probable that in this study only a very small proportion of the indigenous *Bacillus* population of mushroom compost has been studied. The classification of *Bacillus* spp. using numerical taxonomy based on a wide range of characterisation tests has been widely reported (White et al., 1993; Gil et al., 1986, Priest et al., 1988) In this study some of those characterisation tests were used to attempt identification of the *Bacillus* isolates obtained from mushroom compost.

The *Bacillus* isolates that formed the culture collection appeared to be representative of the indigenous population, that could be isolated from mushroom compost, using the procedures detailed. This is because the isolates were originally identified from different batches of compost, but were still shown to be present when a single batch of mushroom compost was monitored from Phase one to the end of Phase two. Analysis of the composting process indicated a reduction in the number of morphological groups observed as the compost progressed from Phase one to Phase two. The isolates were originally assembled into 10 groups, based on similar colonial morphologies. All of these groups were shown to be present at some stage during Phase one. During Phase two, five of the groups were present throughout. The groups present during Phase two also formed the majority of the isolates viewed during Phase one. There was less variation in the counts during Phase two than had been seen during Phase one. This observation and the reduction in the number of isolate groups during Phase two, indicates the homogeneity and reproducibility of end of Phase two mushroom compost, that has been previously reported (Amner et al., 1993).

The intention of the taxonomic studies was primarily to distinguish between the *Bacillus* isolates and it was also hoped to assign each isolate to a particular species. However identification of all isolates was not possible, with only two groups of isolates being identified at the species level. Group one was identified as *B. licheniformis* which has been previously been reported as the most common isolate in spent mushroom compost (Kleyn and Weltzer, 1981). Groups four and eight resembled the *B. thermodenitrificans*, that White et al., (1993) suggested be accepted as validly described species. It was apparent from the taxonomic data that the isolates within each

group gave similar results to each other and different groups could be distinguished by the characterisation tests, which was also one of the objectives of the study. Group 7 was an exception to this and it was unfortunate that groups 7,8,9 and 10 had only one or two members on which to base these conclusions. The lack of identification at the species level from the taxonomic data may have been obtained because these isolates might only be active in the compost ecosystem and physiologically inert in laboratory studies. This is particularly relevant for groups 3 and 6 which could not be stored in the laboratory. Despite this, it seems possible that the original groups may represent different species, however, this would need to be confirmed by a more extensive taxonomic study. Priest and Alexander, (1988) suggested that at least 30 tests should be done to be reasonably accurate in identification.

Bacteriophage studies demonstrated that bacteriophages specific to individual isolates were isolated without any need for prior enrichment. The bacteriophages were all shown to be lytic . A possible lysogenic bacteriophage was obtained after the infection temperature was lowered and was apparently thermoinducible.

Bacillus isolates and bacteriophages obtained here, represent a small proportion of the respective indigenous populations. In the context of this study the isolated indigenous *Bacillus* population has been monitored throughout the composting process and characterised to some extent. The presence of bacteriophages infecting *Bacillus* isolates has also been established.

CHAPTER 4

CONSTRUCTION OF A TRACKABLE BACTERIOPHAGE AND STUDIES WITH OTHER *BACILLUS* HOST AND BACTERIOPHAGE SYSTEMS

Chapter 4

Construction of a trackable bacteriophage and studies with other *Bacillus* host and bacteriophage systems

4.1 Introduction

The objectives of the studies reported in this chapter were three-fold. Firstly, to construct a tagged bacteriophage by introducing a marker sequence into the DNA of øMGPR. Secondly, to develop the transduction system reported by Sharp (1982), in order that transduction could be monitored in situ. Mutants of *B. caldotenax* were isolated with a view to their release into mushroom compost microcosms to monitor bacteriophage mediated gene transfer under environmental conditions. Thirdly, to investigate the detection of the *lacZ* gene carried by the *B. subtilis* bacteriophage ø105 derivatives, in order that they could be efficiently followed during release studies into mushroom compost.

The initial objective in the construction of a tagged bacteriophage was to develop an efficient and reproducible transfection system. As a starting point, *B. subtilis* transformation systems were studied and attempts made to optimise these for transformation with pTB90 plasmid DNA. This was deemed necessary since it has not been always possible to reproduce reported transformation results in this laboratory with the same efficiency as reported elsewhere (I. McDonald pers comm). Only when a reported transformation system for *B. subtilis* could be shown to be effective was, transfection attempted. The development of a successful transfection system was essential in order to transfect the tagged bacteriophage DNA into strain MG030. This

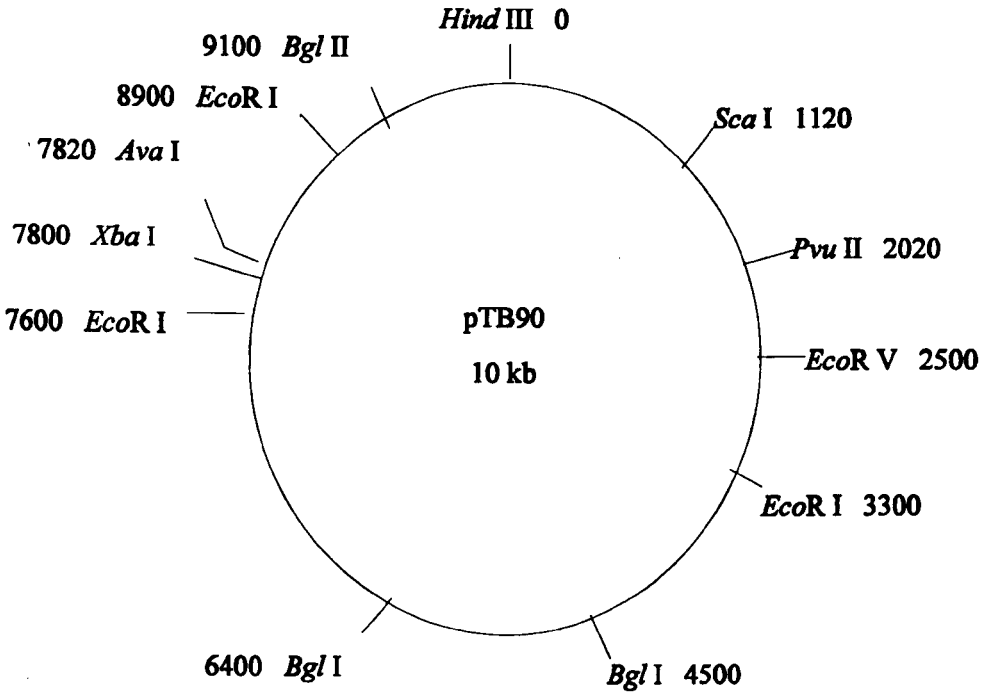
would allow replication, resulting in the release of tagged bacteriophage. As well as producing a trackable bacteriophage for release studies, the development of a tagged bacteriophage would facilitate further studies into the association of øMGPR with strain MG030.

Competent cell transformation (Young and Spizizen, 1961) as a method of transforming plasmid DNA was not attempted. Neither was electroporation attempted because of previous difficulties with reproducibility in this laboratory (P. Riley pers comm). Protoplast transformation (Chang and Cohen 1979) and alkali cation transformation (Hiraoka et al., 1992) were attempted. The following sections detail the results of the transformation and transfection experiments. Unless otherwise stated, prior to all reactions, transformations and transfections, DNA samples were purified usng the commercially available Geneclean kit (Bio 101 Inc. Vista, USA).

4.2 Protoplast mediated transformation of *B. subtilis* 168 with pTB90 plasmid DNA

The method detailed by Chang and Cohen (1979) was used to attempt transformation of *B. subtilis* 168 with the plasmid pTB90. A restriction map of pTB90 is presented in Figure 4.1. Plasmid encoded tetracycline resistance was used as the basis for selection of cells in which the plasmid was expressed. The preparation of pTB90 (Methods 2.12) yielded in the region of 900 μg of plasmid DNA from one litre of overnight culture and importantly for transformation experiments, did not contain any bacterial cells. Chang and Cohen (1979) reported that 80% of host cells could be transformed with 5 μg of pC194 plasmid DNA. This procedure (Methods 2.14) was

Figure 4.1; Restriction map of plasmid pTB90 (McDonald, 1992)



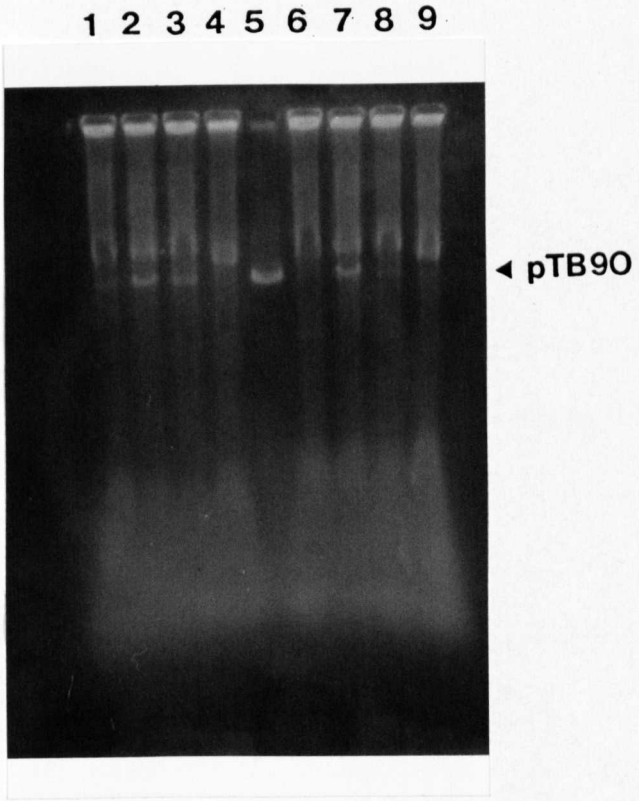
repeated with 5 μg of pTB90 as the transforming plasmid. Selection of transformants was based on the tetracycline resistance of transformed cells, but transformation could not be demonstrated in these experiments. Confluent growth of mucoid colonies was evident on the non selective DM3 agar plates (Methods 2.4) and this confluence was assumed to be caused by cell wall deficient L-forms of *B. subtilis* that could not regenerate their cell walls, although this was not confirmed. Growth of mucoid forms of *B. subtilis* after protoplast transformation has previously been reported (Matsuno et al., 1990). The procedure was modified by stopping the lysozyme treatment when approximately 70% of the cells were present as protoplasts, as determined by microscopy. This was normally after 25-30 min incubation in the presence of lysozyme. Differences of 5-10 min in this time were observed with repeated experiments. For this reason, protoplasting was always monitored microscopically under phase contrast, at 5 min intervals. Following transformation under these conditions, the mucoid form was absent. Growth was evident on the non selective plates after overnight incubation and assumed to be due to non protoplasted cells. After 2-3 days incubation, single colonies were visible on the selective DM3 plates containing 25μg/ml tetracycline. No tetracycline resistant colonies were obtained in control experiments in the absence of pTB90, or when *B. subtilis* was plated onto the selective media. Overall, an average of 31 tetracycline resistant colonies were obtained in repeated experiments. Plasmid extraction procedures were used to test for the presence of pTB90 plasmid DNA in a sample of transformants (Methods 2.13). Two rapid methods of plasmid extraction were attempted, alkaline lysis (Birnboim and Doly, 1979) and the STET plasmid preparation (Holmes and Quigley, 1981). Plasmid extraction was only demonstrated with the rapid

alkaline lysis procedure (Birnboim and Doly, 1979) and this was used throughout the study. The results of plasmid analysis of the transformants are presented in Figure 4.2 and confirm the presence of pTB90 DNA in the transformants. The transformation frequency of 6 transformants per μg plasmid DNA was significantly lower than that reported by Chang and Cohen (1979). Despite this, the results were encouraging since previous workers in this laboratory had failed to demonstrate any level of transformation using this procedure. It was decided that although transformation had been achieved, an alternative method would be sought to achieve an improved transformation frequency.

4.3 Alkali cation transformation of *B. subtilis* 168 with pTB90 plasmid DNA

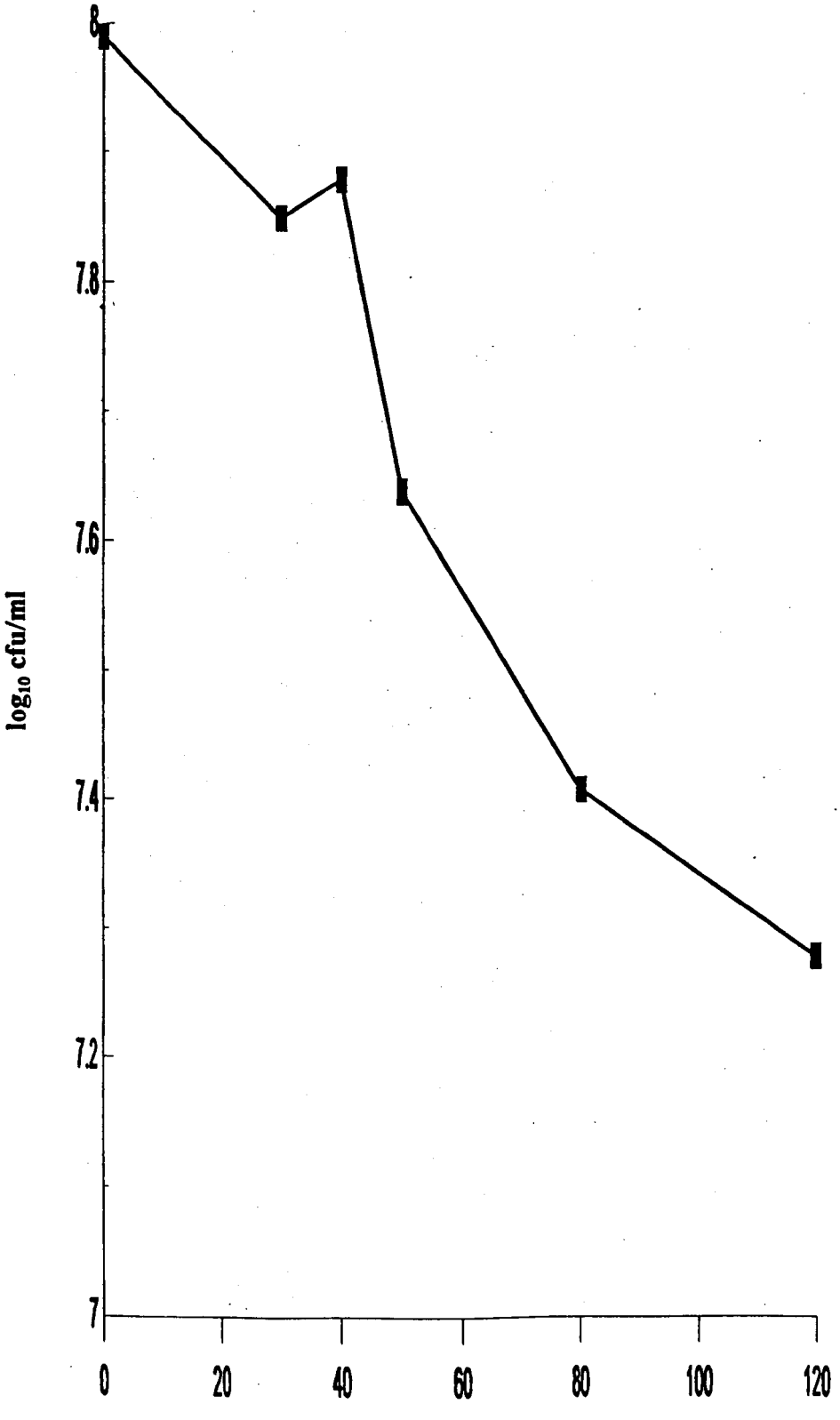
Hiraoka et al., (1992) proposed a rapid alkali cation transformation method applicable to strains of *B. subtilis*. They reported that the optimised procedure yielded several thousand transformants per μg of plasmid DNA. The basis of the technique is that potassium ions activate autolysins within the bacterial cell, inducing competency. Due to the apparent variations in attainable and published transformation frequencies, associated with previous procedures, the first experiment set out to demonstrate KCl mediated autolysis of *B. subtilis* 168, used in the transformation experiments. The results are presented in Figure 4.3. Autolysis appears to be evident as there is a reduction in cell number over the monitoring period. Although no actual values were presented by Hiraoka et al., (1992), the trend of the graph in Figure 4.3 compares favourably with their results. Following these results, attempts were made at

Figure 4.2; Agarose gel electrophoresis of plasmid pTB90 DNA isolated from randomly selected *B. subtilis* colonies recovered after protoplast transformation. Colonies were recovered on regeneration media (DM3) containing 25µg/ml tetracycline.



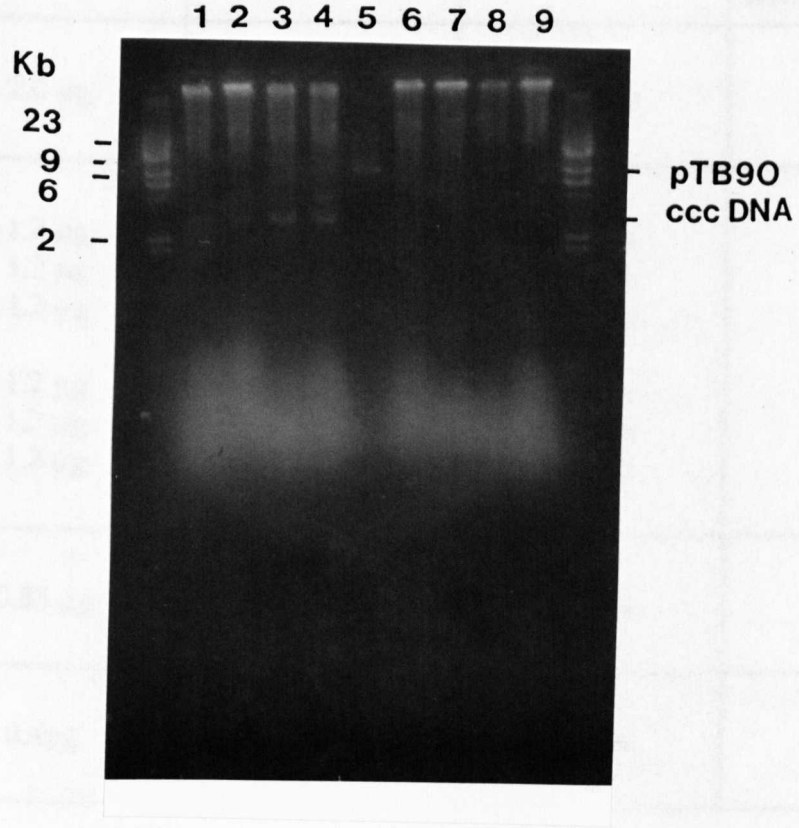
Lanes 1,2,3,4,6,7,8 and 9 contain *Pvu* II digests of pTB90 DNA isolated from Tc^R transformants. Lane 5 contains *Pvu* II digested pTB90 DNA used in the transformations.

Figure 4.3; Effect of incubation at 37°C, in 410mM KCl on *B. subtilis* 168 **viability.** Colonies were determined after overnight incubation at 37°C on TSBA. Values **are the** means of duplicate counts


8
7.8
7.6
7.4
7.2
7
log10 cfu/ml
0
20
40
60
80
100
120
Time (min)

transformation after 40 mins incubation in KCl with 0.85 μg of pTB90 DNA. After overnight incubation, selective plates contained on average only 2 tetracycline resistant colonies per plate. Plasmid extraction procedures demonstrated the presence of pTB90 plasmid DNA in a sample of these transformants. The results are shown in Figure 4.4. The plasmid was believed to be isolated as covalently closed circular (ccc) DNA which is represented in Figure 4.4. These forms of pTB90 ran alongside the marker pTB90 after restriction with *Pvu* II (results not shown) indicating the presence of pTB90 DNA in the transformants. This transformation method was quicker and more convenient than protoplast transformation therefore attempts were made to optimise the procedure for a more efficient transformation of *B. subtilis* with pTB90. The effect of variations in the DNA concentration, PEG concentration and incubation times in KCl on transformation were evaluated and the results are presented in Table 4.1. Plasmid DNA isolation from those transformants tested indicated the presence of pTB90 (results not shown). The best transformation frequency attained was only 12 transformants per μg pTB90 DNA. The absence of transformation with quantities of plasmid DNA higher than 1μg, may have resulted from a gene dosage effect, overburdening the host cells capacity to replicate and translate the introduced DNA. Protoplast transformation was demonstrated with higher amounts of plasmid DNA (Chang and Cohen., 1979; Bakhiet and Stahly., 1985) and it may be that the induction of autolysis reduces the cells replicative capacity, however this would need further investigation. Preparations of pTB90 DNA prior to transformation were thought to be mainly in the ccc form. It was decided to linearise the plasmid DNA by a Hind III digest, to determine if this was transformable. The DNA was reprecipitated and used to transform *B. subtilis* 168 under

Figure 4.4; Agarose gel electrophoresis of plasmid pTB90 DNA isolated from randomly selected *B. subtilis* colonies, recovered after KCl mediated transformation experiments. Colonies were recovered on TSBA plates containing 25µg/ml tetracycline.



Lanes 1,2,3,4,6,7,8 and 9 pTB90 DNA isolated from Tc^R transformants.

Table 4.1; KCl mediated transformation of *B. subtilis* with pTB90 plasmid DNA

Amount of pTB90	PEG concentration	Incubation time in KCl	Transformants per µg plasmid DNA
2.0 µg	70%	40 min	0
1.2 µg	70%	30 min	14
1.2 µg	70%	40 min	2
1.2 µg	70%	50 min	11
1.2 µg	40%	30 min	0
1.2 µg	40%	40 min	1
1.2 µg	40%	50 min	1
0.85 µg	70%	40 min	0
0.4µg	70%	40 min	0

the conditions that gave the best transformation frequency previously. No transformants were produced using linearised DNA. This suggested that the procedure needed further investigation for the intended use of transfecting ligated bacteriophage DNA mixtures. Overall, the results demonstrated that alkali cation transformation of *B. subtilis* 168 with pTB90 plasmid DNA was possible. Again however, the transformation frequencies were much lower than had been previously reported. Before any transfection attempts were made with this system and øMGPR DNA, it was essential to demonstrate the induction of autolysis in strain MG030. The results of this experiment are presented in Figure 4.5. As can be seen, strain MG030 did not demonstrate an autolytic effect in comparison to *B. subtilis*. Further experiments on developing a transfection system based on these methods were not attempted because autolysis was an essential part of the procedure. Transfection experiments were continued using the PEG mediated protoplast transfer system.

4.4 Isolation and restriction enzyme analysis of øMGPR DNA

The small scale isolation of DNA from øMGPR (Methods 2.11) yielded approximately 30 μg of DNA per reaction tube which was sufficient DNA for six restriction digests. The scale up of this procedure yielded approximately 1 mg of DNA in total from 1 litre of overnight culture. The bacteriophage DNA was studied by cleavage with various restriction enzymes (Methods 2.16). Negative images of the gels were recorded and analysed against known size markers using a laser densitometer. Graphical analysis of the data obtained enabled an estimation of the sizes of the various restriction fragments that were generated. These results are presented in Table 4.2. The

Figure 4.5; Effect of incubation at 37°C in 410 mM KCl on MG030 viability. Colonies were determined after overnight incubation at 37°C on TSBA. Values are the means of duplicate counts

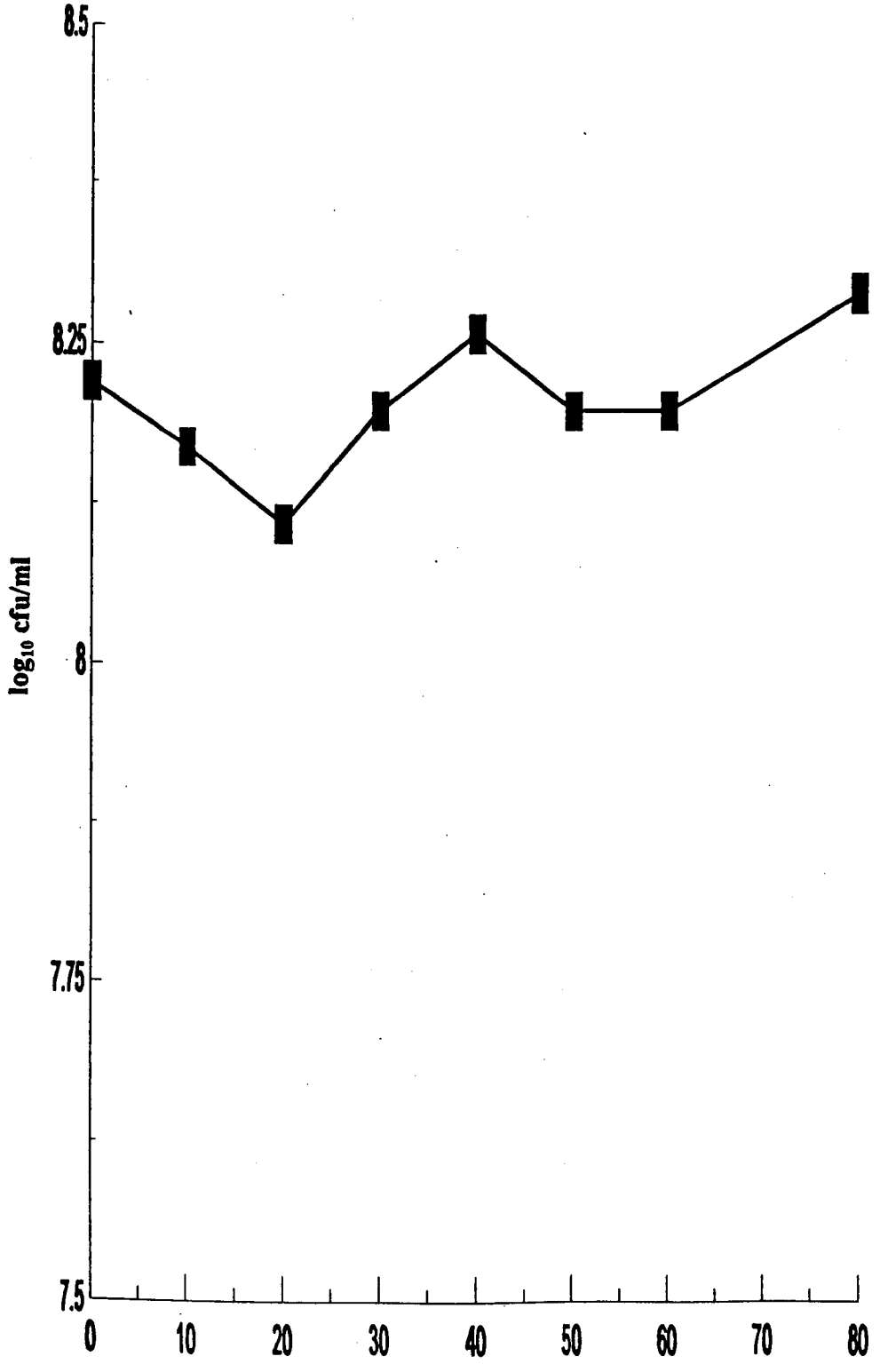

8.5
8.25
8
7.75
7.5
log10 cfu/ml
0
10
20
30
40
50
60
70
80
Time (min)

Table 4.2; Restriction enzyme analysis of øMGPR DNA

Restriction Endonuclease	No. of fragments	Size of fragments (kb)	Calculated size of genome (kb)
Pvu II	5	14.8; 10; 8.4; 4.5; 1.6.	39.3
*Eco*R I	9	11.8; 5.8; 3.9; 3.5; 2.6; 2.5; 2.4; 2.2; 2.1.	36.8
Sal I	2	~28; 10.2.	~38.2
Pst I	6	13.2; 9.1; 6.4; 5.6; 4.9; 2.1.	41.3
Hind III	2	~23; 17.8.	~40.8
Bgl II	5	12.7; 11.7; 7.6; 3.7; 3.2.	38.9

No sites for *Kpn*I or *Sst*I were detected.

sizes of these fragments were consistently obtained in separate experiments. It can be seen that the phage DNA size appears to be in the range 36.75kb to 41.3kb. These discrepancies may be due to some restriction fragments not being resolved because of their small size or that two fragments with similar sizes may have resolved as one. This could not be confirmed because it was not possible to increase the size of the gel to gain a better resolution. Double and triple digests of the DNA using different combinations of the enzymes that cut the DNA were attempted to investigate the genome size discrepancies and produce a restriction map. However a map could not be generated because of similar discrepancies in the total genome size for the different combinations of digests.

4.5 PEG mediated protoplast transfection of MGO30 using øMGPR DNA

Although protoplast mediated transformation could not be optimised in the earlier experiments, a modification of the procedure was attempted for the transfections detailed here. Bacteriophage DNA recovered using the scaled-up isolation procedure was used in these transfection experiments. Transfection of MG030 was attempted using essentially the same procedure described above for the transformation of *B. subtilis* 168 with pTB90. The modifications were similar to those reported by Errington (1984) for transfection with ø105 derivatives. After PEG treatment, the protoplasts were plated directly into a DM3TSA overlay seeded with host MG030. The transfection procedure assumed that the initial infectious event in a protoplast would lead to a lytic

cycle of bacteriophage replication. The progeny bacteriophage would then be released and infect the surrounding cells in the overlay media, eventually giving rise to a plaque. Transfection experiments were carried out on MG030 grown at 50°C. The protoplasting effects of lysozyme were observed at this temperature. Results indicated 70% protoplasting was attained after approximately 45 min incubation, and there was no need for any extra addition of lysozyme. Again, there were slight variations in this time with different transfection experiments, so the extent of protoplasting was monitored microscopically as before. Initial experiments used cells of strain MG030 grown at 50°C and 5µg of øMGPR DNA in transfections. No plaques were evident after incubation at 50°C for up to three days. Transfection was demonstrated when 1µg of øMGPR DNA was used under the same conditions, with a total of 21 plaques. The presence of øMGPR was demonstrated by isolating bacteriophages from the plaques and using these to infect MG030 at 50°C and 37°C. Clear plaques were produced at 50°C and turbid plaques were produced at 37°C. DNA isolation and restriction, as a method of identification was not attempted because of the time taken to propagate the phage from a single plaque to a high enough titre to isolate DNA. Exhaustive attempts were made to improve the transfection frequency. The effect of varying the regeneration medium was attempted by using DM3T which supports regeneration of thermophilic *Bacillus* protoplasts (Dunn et al., 1987). This medium did not readily support the growth of strain MG030 and was not investigated further. The effect of incubating the transfected protoplasts at 50°C, prior to addition to the overlay media, in order to induce lytic bacteriophage activity was also investigated. The resulting suspension was mixed with host cells in a TSBA overlay. Single plaques were evident after overnight

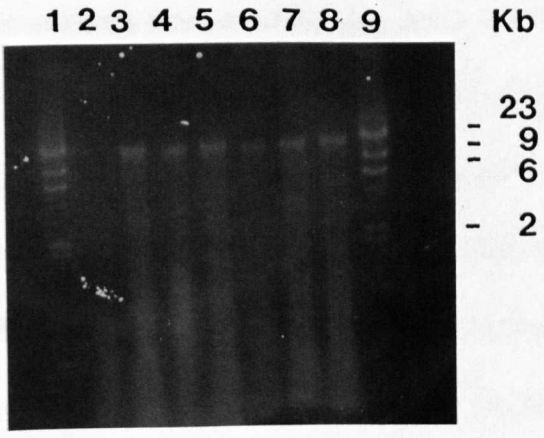
incubation at 50°C and were confirmed as øMGPR as previously detailed. However the transfection frequency was even lower than previously determined. It was assumed in this experiment that the plaques arose from bacteriophages released from transfected protoplasts, as the protoplasts were not regenerated, although no attempts were made to confirm this.

Although low, similar transfection frequencies were obtained with repeated experiments using the modified procedure detailed. Optimisation for efficient transfection was not achieved, however, the procedure was still used for transfections, once a suitable tagging system for øMGPR had been identified and cloned into the bacteriophage genome.

4.6 Isolation of DNA from compost

It was necessary to isolate DNA from mushroom compost in order to show that any tagging system used could be demonstrated to be absent from the indigenous compost population and therefore exclusive to the bacteriophage. The isolation of DNA from end of Phase two compost samples (Methods 2.15) yielded approximately 200 µg of DNA per 30 g of compost. The results of agarose gel electrophoresis of this DNA are shown in Figure 4.6. Upon precipitating the DNA, the pellet appeared yellow in colour compared to the white pellet normally seen in the earlier plasmid and bacteriophage DNA preparations. Mushroom compost has a high humic acid content and these were thought to be responsible for the colour difference observed. The isolated DNA was assumed to have been released from the indigenous compost microflora during the freezing and X-press treatment. The isolated compost DNA was

Figure 4.6; Agarose gel electrophoresis of DNA isolated from mushroom compost



Lanes 1 and 9 contain λDNA cut with *Hind* III; Lanes 3,4,5,6,7 and 8 contain DNA isolated from compost; Lane 2 contains sonicated compost DNA.

used in the following PCR and gene probing experiments indicating that it was isolated in a form that could be used in further experiments.

4.7 Selection of a marker for tagging øMGPR DNA

Various markers were available for use in tagging øMGPR DNA. A 500 bp sequence within a methanogen gene (P. Riley, pers comm) was originally proposed. However, PCR with methanogen primers (Methods 2.17) showed a similar sized amplified product from compost DNA samples (results not shown). This was thought to indicate that the methanogen sequence was already present in the compost ecosystem. A degenerate primer with the following sequence was used in this PCR; 5' GGTGGTGTA/CGGATTCACACAA/GTAC/TGCA/TACAGC 3'. The fact that PCR was possible in the presence of humic acids was surprising since they have been shown to inhibit the reaction. Sonicating of the compost DNA prior to PCR may have facilitated this by altering the association of the humic acids with the DNA. Also the extensive dilution of the compost DNA prior to the PCR, may have removed the inhibitory effect of the humic acids.

The *lac*ZY gene system has been thought to be probably in compost given the diversity of the indigenous bacterial population (McCarthy pers comm) and so it was discounted for use as a marker sequence at this stage. It was decided to synthesise a short oligonucleotide sequence that could act as a phage tagging sequence. A nucleotide sequence from the green fluorescent protein of the cnidarian *Aequorea victoria* (Prasher et al. 1992) was chosen, as this was thought to be unlikely to occur in the compost ecosystem. A 30 bp sequence from within the published sequence was to be used as the

basis of a tagging insert. The sequence was analysed using GENBANK 74 (C. Duggleby pers comm) and did not show homology to any other DNA sequence. The sequence was constructed and its purity confirmed using TLC, by Dr. J. Miller at PHLS CAMR, and is shown in Figure 4.7. The 30mer sequence was end labelled and used to probe the compost DNA sample as detailed in methods reference 2. . Compost DNA was spiked with methanogen DNA and probed with a methanogen sequence as a positive control in the experiments. DNA was diluted ten fold from 5.75µg down to 57.5pg. The results presented in Figure 4.8 show the synthesised tag probe did not bind to the compost DNA, indicating the sequence is not apparent in the sample of mushroom compost DNA. Binding of the methanogen probe to the spiked compost DNA, as the positive control is evident. Binding of the methanogen probe to unspiked compost DNA was not attempted. The next stage of constructing a tagged bacteriophage particle was to obtain a double stranded form of the tagging sequence that could be inserted into the øMGPR genome. PCR and shotgun cloning techniques were used to attempt this.

A cloning strategy was devised whereby *BamH* I linkers could be incorporated onto either end of the 30mer sequence. This involved synthesising versions of the linkers that could be used as primers in the PCR. A representation of this is displayed in Figure 4.9. The sequences of the primers were constructed and their purity confirmed using TLC, again by Dr. J. Miller at PHLS CAMR. *Bam*H I sites were chosen as the linker sequence because the cohesive ends produced after cleavage with this enzyme are compatible with several other restriction endonucleases. This allowed further possibilities of potentially introducing this marker sequence into other

Figure 4.7; Synthesised 30mer oligonucleotide sequence derived from the green fluorescent protein of *A. victoria* and used as the basis of a marker sequence for øMGPR

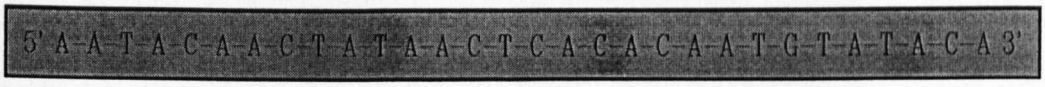
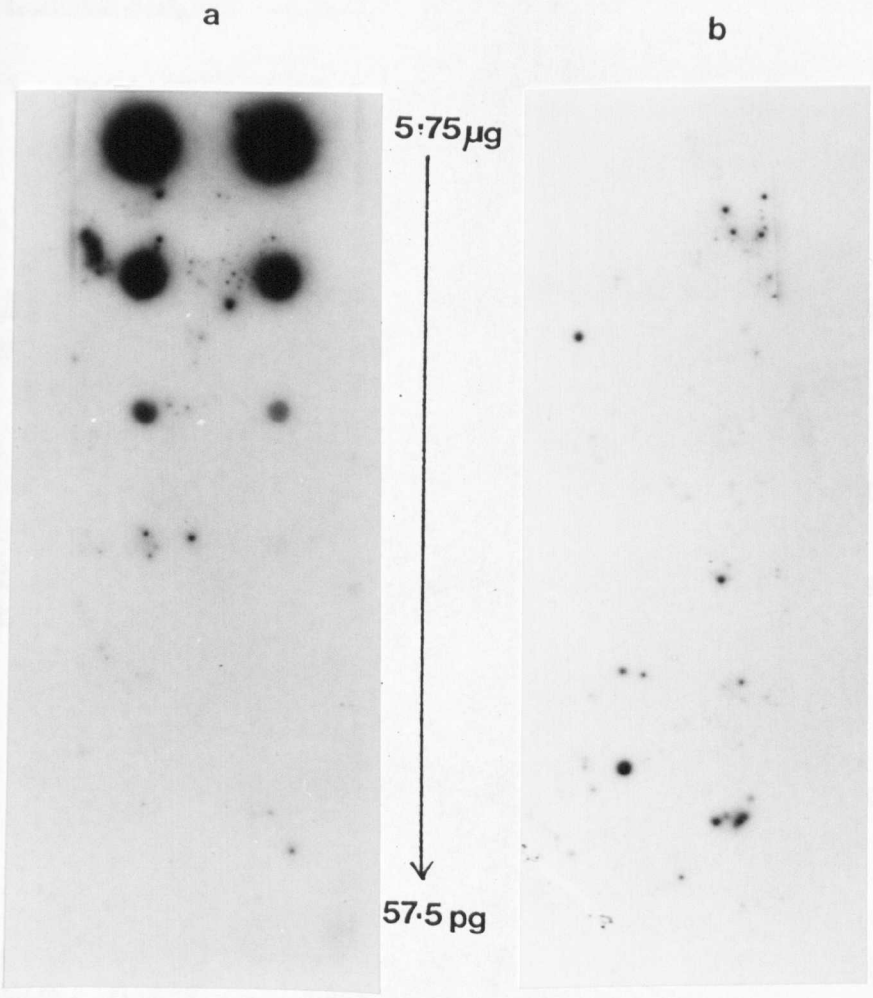


Figure 4.8; Radiolabelled probing of DNA isolated from compost



Autoradiograph a; Compost DNA spiked with methanogen DNA and probed with radiolabelled methanogen DNA probe.

Autoradioraph b; Compost DNA probed with radiolabelled 30mer sequence.

Figure 4.9; Schematic diagram of the construction of a ds DNA tagging sequence with *Bam*H I linker sites at either end, from the 30mer sequence using the PCR

1. 30mer and primer sequences in the PCR:

5' AATACAACTATAACTCACACAATGTATACA 3' 30mer

3' **TGTTACATATGTGCCTAGGC** 5' Primer 1

5' **CGGATCCGAATACAACTATA** 3' Primer 2

2. Primer 1 anneals during the first PCR reaction and synthesis of the 2nd strand occurs

5' AATACAACTATAACTCACACAATGTATACA 3'
3' **TGTTACATATGTGCCTAGGC** 5'

3. Primer 2 anneals to newly synthesised 2nd strand and DNA synthesis occurs resulting in synthesis of 46mer.

5' **CGGATCCGAATACAACTATA** 3'
3' TTATGTTGATATTGAGTGTGTTACATATGTGCCTAGGC 5'

4. The PCR of 46mer proceeds as above resulting in amplification of the 46bp dsDNA sequence shown below:

5' CGGATCCGAATACAACTATAACTCACACAATGTATACACGGATCCG 3'
3' GCCTAGGCTTATGTTGATATTGAGTGTGTTACATATGTGCCTAGGC 5'

5. Restriction of the PCR product with BamH I leaves the tagging insert with cohesive ends

5' GATCCGAATACAACTATAACTCACACAATGTATACACG 3'
3' GCTTATGTTGATATTGAGTGTGTTACATATGTGCCTAG 5'

bacteriophage genomes. As previously detailed, the *Bam*H I linkers were incorporated into two sequences that were used as primers in the PCR of the 30mer sequence. Careful design of the primers was essential in order that primer annealing did not lead to DNA synthesis.

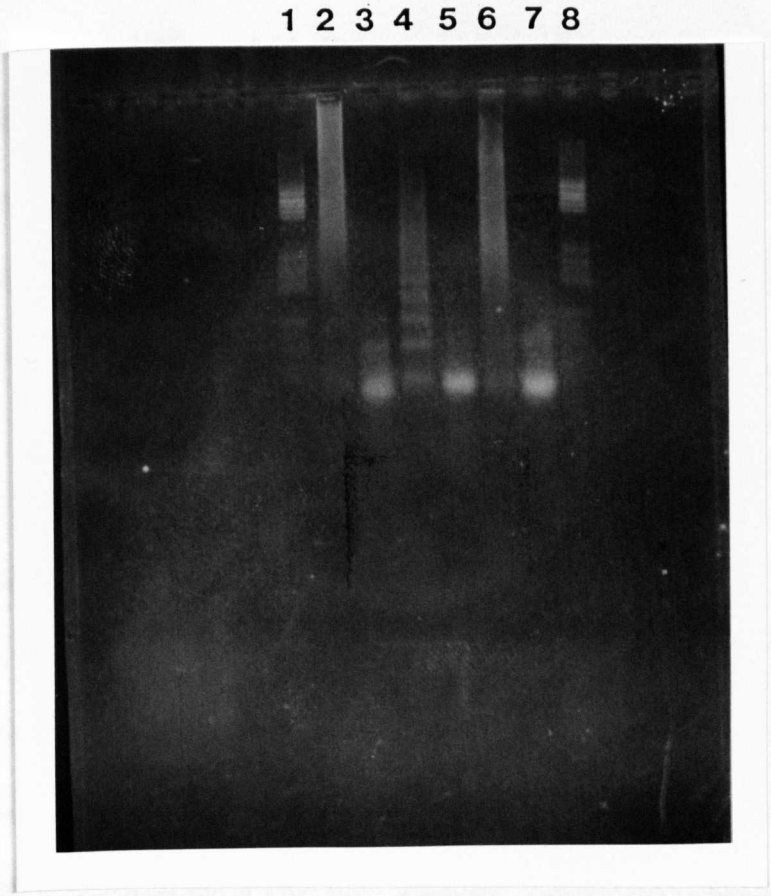
Restriction of the PCR product with *Bam*H I would leave the marker sequence with cohesive ends, which would facilitate its insertion into the bacteriophage genome. Annealing temperatures of 45^{0}C, 50^{0}C and 55^{0}C were used for the PCR. Analysis of the PCR products were carried out by agarose gel electrophoresis. In order to visualise the product it was necessary to run 3% agarose gels. Further problems were encountered in identifying the product under these conditions, since visualisation of molecular weight markers around the size of the PCR product was not always possible.

The presence of a PCR product was only demonstrated after annealing reactions at 55^{0}C and on no occasion was a single band produced under these conditions. Further PCR reactions were carried out under these conditions with different concentrations of 30mer oligonucleotide. The PCR products that resulted are presented in Figure 4.10a. It can be seen that the PCR product at two of the dilutions was viewed as a smear on the gel. A 1:1000 dilution of the oligonucleotide gave less smearing but still did not give a single PCR product. However, when the products were digested with *Bam*H I a single band was evident for all three samples. These results are presented in figure 4.10b. It appears that the smears, evident in the PCR products, are a form of associated 48mer products. This may be caused by the apparent suboptimal conditions for the PCR reactions. The results of PCR with different dilutions of 30mer oligonucleotide indicated that none of these concentrations were optimal because a

Figure 4.10a; Agarose gel electrophoresis analysis of PCR products after the PCR of the 30mer sequence with the modified *Bam*H I linkers as primers.

Lanes 1and 10 contain molecular weight marker V; Lanes 2 and 9 contain molecular weight marker VI; Lane 3 contains 30mer DNA sequence; Lanes 4 and 5 contain primer DNA sequence; Lane 6 contains PCR product with 1:10,000 diluted 30mer; Lane7 contains PCR product with 1:1000 diluted 30mer; Lane 8 contains PCR product with 1:100 diluted 30mer.

Figure 4.10b; Agarose gel electrophoresis analysis of *Bam*H I digested PCR products after the PCR of the 30mer sequence with the modified *Bam*H I linkers as primers.



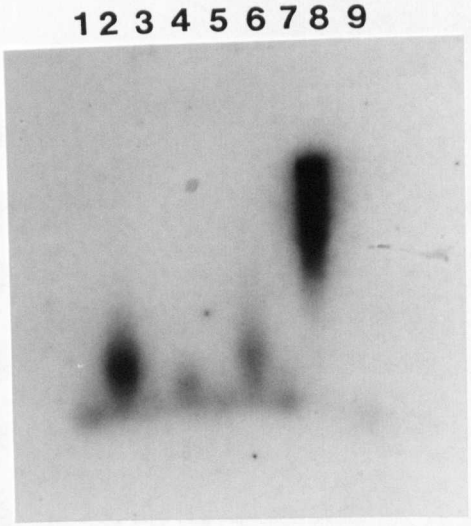
Lanes 1 and 8 contain molecular weight marker V; Lane 2 contains PCR product with 1:10,000 diluted 30mer; Lane 3 contains *Bam*H I digest of PCR product with 1:10,000 diluted 30mer; Lane4 contains PCR product with 1:1000 diluted 30mer; Lane 5 contains *Bam*H I digest of PCR product with 1:1000 diluted 30mer; Lane 6 contains PCR product with 1:100 diluted 30mer; Lane 7 contains *Bam*H I digest of PCR product with 1:100 diluted 30mer;

single PCR product was not obtained. The fact that a single product was evident after digestion with *Bam*H I suggested that the required product could be obtained and further optimisation experiments were not necessary. The presence of PCR products as smears was consistently observed. Probing of the digested and undigested PCR products with radiolabelled primer oligonucleotide DNA was performed. The results are presented in Figure 4.11. Only the PCR product in lane 2 was evident as a smear. In all cases it appeared that the single product obtained after digestion of the PCR product with *Bam*H I, contained the 30mer DNA. This single product appeared larger in size than the primer DNA and was assumed to be the required PCR product which could be cloned into the øMGPR genome.

4.8 Shotgun cloning of the marker sequence into the bacteriophage genome

Conditions for the partial digestion of øMGPR DNA with *Sau*3A were determined. The optimal conditions were found to be 0.075U Sau3A and 5µg bacteriophage DNA in a 15 min digest at 37°C. *Sau*3A was chosen because it cut the bacteriophage genome at nine sites allowing insertion of the tagging sequence with its compatible cohesive ends. Alkaline phosphatase treatment of partially digested DNA was necessary to prevent self-ligation of the bacteriophage DNA during the reaction. Partially digested bacteriophage DNA was purified, however it was not possible to purify the marker fragment with the Geneclean kit, prior to ligation because of its small size, so ethanol precipitation at -70°C was used. Different ratios of phage to DNA insert were used in the ligation reactions, ranging from 1:1 to 10:1, (phage:insert).

Figure 4.11; Probing of PCR products and *Bam*H I digested PCR products with radiolabelled primer DNA



Lane 1 contains 30mer oligonucleotide; Lane 2,4,6 and 8 contain PCR product; Lanes 3,5,7 and 9 contain *Bam*H I restriction digests of the adjacent PCR product.

After ligation, the purified reaction mixture was used in transfection experiments under the conditions previously detailed. Prior to transfections, ligated DNA was probed with the radiolabelled oligonucleotide as detailed in methods 2.18. Despite the poor quality of autoradiographs obtained, results indicated the binding of probe DNA to bacteriophage DNA, but no obvious binding to the low molecular weight marker sequence. This was interpreted as meaning the marker sequence had been incorporated into the bacteriophage genome.

The transfection procedure used was the same as the optimised procedure devised earlier, except that 1μg bacteriophage DNA containing the marker sequence replaced øMGPR DNA. Despite exhaustive attempts, the transfection procedures did not demonstrate viable tagged bacteriophage progeny. Control transfections using untreated øMGPR DNA were successful with similar frequencies to those previously detailed (section 4.5). Each of the repeated transfections used bacteriophage DNA that had newly synthesised marker sequence ligated to it. Viable bacteriophages were evident as 7 plaques were produced during one transfection, however, the tagging sequence was not present as determined by a plaque lift assay (autoradiograph not shown). It was assumed in this case that the transfecting DNA contained intact, undigested bacteriophage DNA. These results demonstrated that it was not possible to recover tagged bacteriophage progeny.

The implications of these results are discussed at the end of this chapter. Despite many attempts, it had not been possible to construct a trackable bacteriophage. Studies into the interaction of hosts and bacteriophages therefore had to be continued with a

previously described and optimised transducing system in *B. caldotenax* BT1(Sharp 1982) and also with a derivative of the *B. subtilis* bacteriophage ø105.

4.9 Isolation of streptomycin resistant and thymine requiring mutants of *B. caldotenax* BT1

In order to monitor transduction in this system it was decided to construct thymine requiring mutants and double streptomycin resistant and thymine requiring mutants of strain BT1. Transduction of *thy* mediated by the bacteriophage JS017 could then be monitored sequentially from wild type BT1 to the single mutant and thence to the double mutant. Isolation of mutants was originally carried out at 50°C in order that the transduction system could eventually be monitored in mushroom compost where the *Bacillus* population isolated at this temperature had been studied and partially characterised. As well as monitoring transduction, any effects on the indigenous population could also then be monitored.

Thymine requiring mutants of *Bacillus caldotenax* BT1 grown at 50°C, were isolated following selection on Kuhns minimal agar supplemented with thymine and trimethoprim. Of 52 colonies selected from these plates, 7 appeared as stable thymine requiring mutants. These auxotrophs were incubated in Kuhns minimal broth supplemented as before and with streptomycin, to see if the required double mutant could be selected. After five days incubation at 50°C there was no evidence of any growth in streptomycin containing media. These experiments were repeated using similarly supplemented solid media. In both sets of experiments, it was not possible to isolate the required double mutant from a thymine requiring mutant of BT1. Further

repetition of the experiments with the thymine auxotrophs at 65°C, again did not produce any growth of a streptomycin resistant mutant. As a result of these investigations, selection of a streptomycin resistant mutant was attempted prior to selection of a thymine requiring mutant.

Strain BT1 grown to late exponential phase growth (OD_{540} 0.9-1.0) in Kuhns minimal broth was used to inoculate Kuhns minimal agar plates with and without streptomycin (Methods 2.20.1). After three days incubation at 65°C, there was confluent growth on the unsupplemented Kuhns minimal agar plates. Single colonies were also evident on the streptomycin supplemented plates. These colonies were subcultured and confirmed as streptomycin resistant mutants by growing on Kuhns minimal media without streptomycin, demonstrating that the mutants did not have a requirement for streptomycin. Streptomycin can also select for streptomycin dependent mutants which require streptomycin for growth and so it was important to show that this had not occurred. A total of six streptomycin resistant mutants were obtained. Each of the streptomycin resistant mutants was infected with JS017 using the soft agar overlay method (Adams, 1959). The results are presented in Table 4.3 . It can be seen that all of the mutants were infected by JS017. Each mutant also had a higher efficiency of plating than the BT1 host. It may be that the mutants have a different membrane configuration that prevents the streptomycin entering the cell and binding to the ribosome. This difference in the outer membrane may facilitate the adsorption of JS017 giving an increase in the EOP compared to BT1.

Attempts were then made to isolate thymine requiring isolates from these streptomycin resistant mutants by their culture in Kuhns minimal broth supplemented

Table 4.3; JS017 infection of BT1 and streptomycin resistant mutants of BT1

Host	Titre of JS017 (cfu/ml)	EOP
BT1	3.7×10^8	1.0
str^R1	5.4×10^8	1.5
str^R2	5.6×10^8	1.5
str^R3	4.8×10^8	1.3
$str^{R4}4$	6.8×10^8	1.8
str^R5	1.0×10^9	2.7
str^R6	1.0×10^9	2.7

with streptomycin, trimethoprim and thymine. There was no evidence of any growth after five days incubation at 65°C. When the streptomycin resistant mutants were plated onto similarly supplemented Kuhns minimal agar plates and incubated as above, single colonies were evident after two days incubation at 65°C.. These colonies were assumed to be double mutants and were further replica plated onto Kuhns minimal agar and Kuhns minimal agar supplemented with streptomycin, trimethoprim, and thymine. Results confirmed that 6 out of 41 isolates were streptomycin resistant and had a requirement for thymine. These mutants were used in transduction experiments with JS017 and are detailed in Chapter 5.

4.10 Propagation and detection of bacteriophages ø105 MU201 and ø105 MU205

The induction of the ø105 derivatives was as detailed in the methods (2.19 for MU205 and 2.19.1 for MU201). Phage titres in the region of 10^7 to 10^8 pfu/ml were routinely obtained, but because of the less hazardous temperature induction of MU205, this bacteriophage was used in the following experiments. Two systems were available to detect the *LacZ* gene The chromogenic substrate X-Gal and MUG, a fluorogenic substrate. MUG was used initially because it was more sensitive (Errington 1986) and less expensive than X-Gal. *Bacillus* 168 containing ø105 MU205, *B. subtilis* 168, *Escherichia coli* and *B. subtilis* CU267 were streaked separately onto MUG agar and monitored for fluorescence at 37°C using a hand held UV illuminator. After three hours, fluorescence was evident for the ø105 lysogen and *E. coli*. No fluorescence of CU267, or 168 was evident, even after overnight incubation. There was no evidence of

fluorescence when TSBA replaced MUG agar in the above experiment. Infection studies of ø105 with *B. subtilis* CU267 and 168, in MUG overlays showed a diffuse fluorescence after overnight incubation. It was not possible to monitor the development of single plaques by looking for areas of fluorescence on the overlay plates. As a result of these investigations, MUG was not used as a means of detecting the *LacZ* gene in the ø105 derivatives.

Investigations using X-Gal as a means of detecting the *LacZ* gene showed that it was necessary to use the substrate at a higher concentration than that reported by Errington (1986). After overnight incubation, the ø105 lysogen appeared as a blue-green colony on media containing 80µg/ml X-Gal. When X-Gal was used in infection studies, blue-green plaques were evident after overnight incubation under similar conditions.

This detection system was used to monitor the survival and interactions of ø105 MU205 and *B.subtilis* 168 in sterile and fresh mushroom compost microcosms. The results are presented in Chapter 5.

4.11 Discussion

The main purpose of the investigations reported here was to construct a trackable bacteriophage based on øMGPR. An essential part of this process was to have an efficient transfection system to allow the replication of tagged bacteriophage DNA inside the host bacterial cell. In order to achieve this, two transformation systems for *B. subtilis* were investigated. Initial protoplast transformation experiments with pTB90 plasmid DNA were unsuccessful. This led to the lysozyme treatment being modified

in order to prevent complete protoplasting of the bacterial cells. Although transformation of stable L-forms of *B. subtilis* has been recently demonstrated, a high level of plasmid instability was also detected (Waterhouse et al., 1994). Furthermore, it has been suggested that reduced transformation frequencies were a result of problems associated with regeneration of protoplasts (Van Waasbergen et al., 1993). Protoplast transformation of *B. subtilis* with pTB90 plasmid DNA was eventually achieved, although with a low transformation frequency of 6 transformants per microgram of plasmid DNA. Transformation frequencies vary greatly depending on the *Bacillus* spp. used as hosts with reported values ranging from 10^2 per microgram of DNA in *Bacillus sphaericus*, through to 4×10^7 per microgram of DNA in *B. subtilis* (Qiao et al., 1992; Chang and Cohen., 1979). Such high values for protoplast transformation of *B. subtilis* are not always attainable. Matsuno et al., (1990) reported a low efficiency of protoplast transformation in *B. subtilis* NB22 and this study reported similarly disappointing results.

Transformation of *B. subtilis* using KCl treatment has been previously demonstrated (Hiraoka et al., 1992 and Matsuno et al., 1990). Although it was demonstrated in this study, transformation efficiencies did not match those reported despite attempts to improve efficiency. Furthermore, the induction of autolysis using KCl could not be demonstrated in MG030, so the method was not used in any transfection experiments.

Despite extensive experiments, a protoplast transfection frequency as efficient as those reported for other *Bacillus* spp. (Errington, 1984; Bakhiet and Stahly, 1985) could not be achieved for transfection of øMGPR DNA into MG030. The fact that

MG030 was not a well characterised laboratory strain and did not behave in a similar manner to *B. subtilis* was not unexpected. Further work is necessary to optimise the transfection procedure and investigate other forms of gene transfer. Difficulties were also experienced because the clarity of DNA bands on agarose gels could not be reproduced to a high quality on photographs.

Attempts to insert a marker sequence into the genome of øMGPR were based on the techniques of PCR and shotgun gene cloning. As a result of these procedures the sequence was thought to be present in the bacteriophage genome. Transfection of this DNA was not possible despite numerous attempts. The most likely explanation for these results, is that the marker sequence was always inserted into an essential bacteriophage gene. Consequently, although transfection may have occurred, viable bacteriophage would not have been produced. Defective bacteriophage may have been produced but there was no means of detecting these in the experiments. *Sau*3A was found to cut the bacteriophage DNA into nine fragments. The generation of more restriction fragments would allow a greater chance of inserting the sequence into a non essential section of DNA. Another possible explanation for the lack of transfection is that a restriction/modification system exists in MG030, that recognises a sequence in the marker sequence. Several restriction/modification systems have been reported for *B. subtilis* and related bacteria (Trautner and Noyer-Weidner 1993). Another alternative is that part of the marker sequence had ligated to itself during the cloning reaction. When the sequence was inserted into the bacteriophage DNA, the size of the genome exceeded the packaging capacity of the bacteriophage capsid and so no viable bacteriophage were produced. Also, the low frequency of transfection obtained in the

positive controls meant that the system was not optimised, further hindering the transfection of any DNA which may have been produced containing the marker sequence. The failure to construct a tagged bacteriophage meant that further studies on the interaction of the bacteriophage with its host and in particular, whether a lysogenic association was present, were not attempted due to a lack of time. More importantly release studies with a tagged bacteriophage could not be performed.

The remainder of the work reported in this chapter concerned developing alternative strategies for monitoring release studies into mushroom compost. Experiments with *B. caldotenax* BT1 generated single and double mutants that were to be used in transduction experiments with the bacteriophage JS017. Previous work had optimised the transduction of the *thy* gene from BT1 to thy$^-$ mutants of BT1 under laboratory conditions (Sharp 1982) but the production of double mutants had not previously been achieved and it was hoped that these could be used to monitor this transduction system in mushroom compost.

A means of detecting a ø105 derivative in future release experiments was also established, by the incorporation of the chromogenic substrate X-Gal into the agar medium. This allowed visualisation of colonies of lysogens and bacteriophage plaques, by the production of a blue-green colour.

CHAPTER 5

RELEASE EXPERIMENTS IN MUSHROOM COMPOST MICROCOSMS

Chapter 5

Release experiments in mushroom compost microcosms

5.1 Introduction

Commercially produced mushroom compost provides an environment with a high biological activity, where *Bacillus* spp. are present in high numbers (Amner et al., 1988). The indigenous *Bacillus* population of mushroom compost isolated at 50°C had been partially characterised (Chapter 3) to underpin any investigations into the interactions of bacteriophages and their hosts at this temperature, as well as providing an opportunity to monitor gene transfer to the indigenous population. Originally it was hoped to release a bacteriophage that had been isolated from the indigenous population and labelled with a marking sequence. However, despite extensive attempts (Chapter 4), construction of a marked bacteriophage was not possible. Two other bacteriophages and their hosts were available for release studies, these were ø105 infecting *B. subtilis* CU267 and JS017 infecting *B. caldotenax* BT1. As a result of preliminary investigations reported in this chapter, only ø105 was actually released into mushroom compost microcosms. Infection studies with ø105 showed that it did not infect *B. subtilis* 168 or *B. subtilis* CU267 at 50°C. This meant that release studies were carried out at 37°C without any prior characterisation of the indigenous *Bacillus* population that could be isolated at that temperature. The purpose of the release studies was to investigate the survival and interaction of ø105 with *B. subtilis* in sterile and untreated mushroom compost. Such studies are a prerequisite to investigating phage mediated

gene transfer in natural environments, by giving an indication of bacteriophage behaviour.

Sterile and fresh mushroom compost microcosms have been previously used in release studies (Amner et al., 1991; McDonald 1992), to investigate the survival of plasmid-bearing *B. subtilis* strains. Mushroom compost was sterilised in these cases by autoclaving on three successive days, to ensure that the indigenous spore population is removed. The effects of autoclaving have been reasoned to cause the reduced survival of vegetative cells of *B. subtilis* in sterile compost by possibly releasing toxic compounds and reducing nutrient availability (Amner et al., 1991; McDonald 1992). For this reason, steaming of the microcosm was considered as an alternative to autoclaving, for sterilisation of compost samples.

5.2 Comparison of steaming and autoclaving as methods to sterilise mushroom compost microcosms.

Microcosms were held in a steam oven for 1h to kill the vegetative cell population and then immediately incubated at 37^{0}C for 3h to allow germination of the indigenous spore population. This procedure was repeated again on three successive days. Autoclaved compost microcosms and steamed compost microcosms were compared in terms of sterility and mass after these procedures. The results showed that autoclaving resulted in an average mass loss of 16% compared to steaming which resulted in an average mass loss of 3%. This mass loss was thought to be due mainly to dehydration of the compost samples during the high temperatures of steaming and autoclaving. Analysis of the indigenous population after these processes showed that

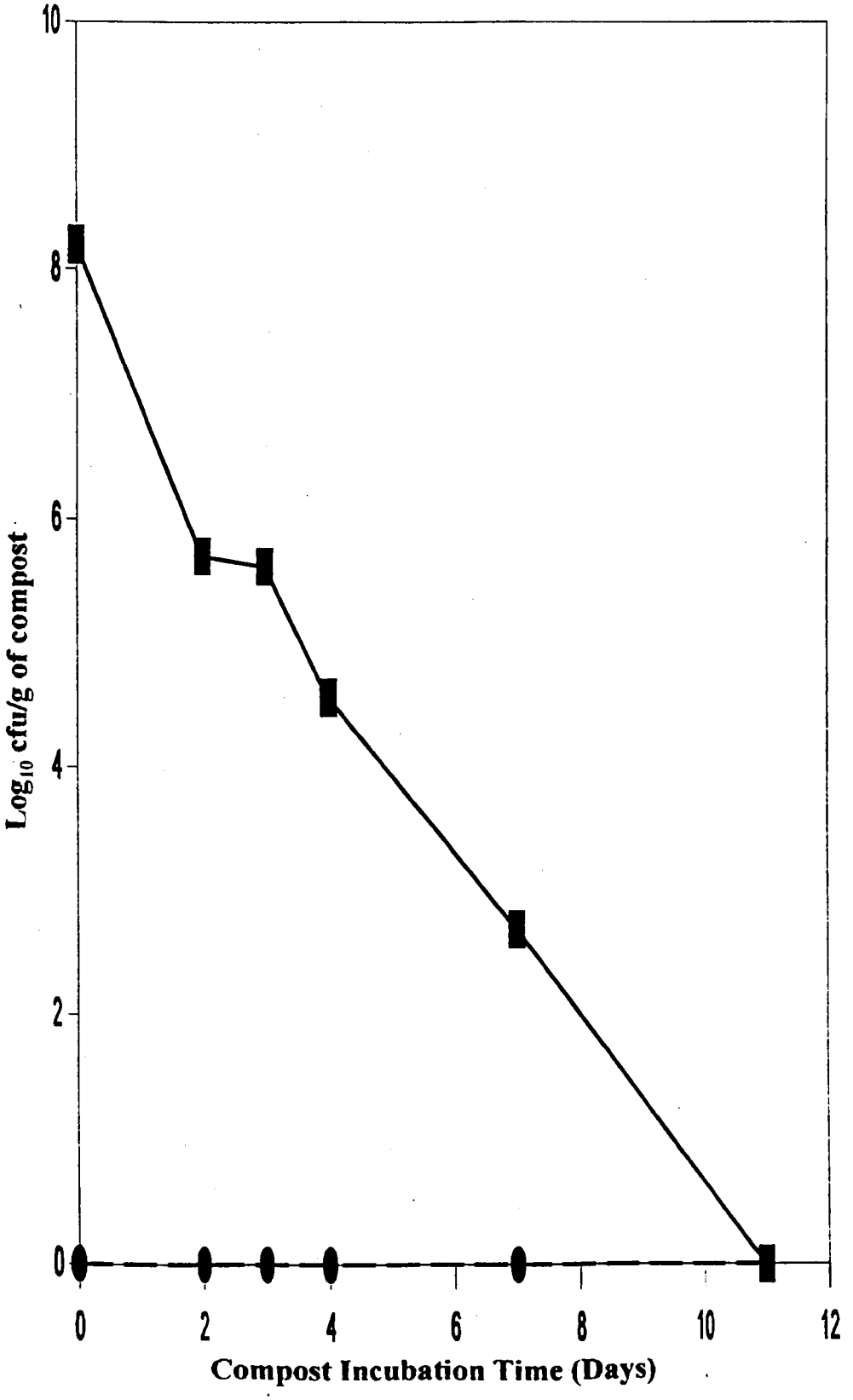
no bacteria could be isolated from the autoclaved compost microcosms. Varying colony counts were obtained from the steamed compost samples ranging from 1.7 x 10^3 cfu/g to 7.4 x 10^3 cfu/g, from an original count of approximately 1 x 10^8 cfu/g. The predominant isolates viewed here, appeared to resemble those in Group one of this study and it was concluded that compost microcosms could not be sterilised by steaming on three successive days. It was decided that although autoclaving produced an increased loss in mass, it would be used because it did appear to sterilise the compost. As the mass loss was thought to be due to dehydration an addition of sterile water was made to the compost, the volume of which was equal to the average weight loss of all microcosms sterilised. This addition was made after autoclaving on the third day and at least one day before the release experiments. The release of toxic compounds and the breakdown of nutrients during autoclaving could not be assessed. Initial experiments in sterile mushroom compost microcosms involved investigating the survival of *B. caldotenax* BT1 before any release studies investigating the described JS017 transducing system were attempted

5.3 Survival of *B. caldotenax* BT1 in sterile mushroom compost microcosms

The main objective of these experiments was to see if it was possible to release *B. caldotenax* BT1 and the mutant strains reported in Chapter 4, along with JS017 propagated on a thymine requiring strain into compost and demonstrate transduction.. Transduction would be demonstrated by the presence of streptomycin resistant mutants that did not require thymine, carrying JS017. As a prerequisite to these studies the

survival of *B. caldotenax* BT1 was monitored in sterile compost microcosms. The reason for using sterile compost was that *B. caldotenax* BT1 could not be distinguished from the indigenous compost population in untreated compost even with isolation onto Kuhns minimal media. Compost microcosms were incubated at 65^{0}C and the surviving counts are presented in Figure 5.1. It can be seen from the results that *B. caldotenax* BT1 did not appear to sporulate and did not colonise sterile compost. No survivors were detected after eleven days incubation. It may be because *B. caldotenax* BT1 did not sporulate that it could not survive and colonise sterile compost. Previous work with sterile compost has demonstrated that sporulation plays an important role in survival, because there was an increase in the number of spores present the end of similar releases in sterile compost with vegetative cells of *B. subtilis,* at this temperature (McDonald, 1992). Attempts were made to increase the survival of *B. caldotenax* BT1 by adapting it to compost, by growing it in nutrient broth supplemented with a sterilised (50%(v/v)) compost supernatant. Growth of *B. caldotenax* BT1 could not be demonstrated under these conditions, possibly because of the presence of growth inhibitors in the compost supernatant, although this was not investigated. It was concluded that BT1 could not be adapted to colonise sterile compost using these procedures. Background resistance of the indigenous compost population isolated at this temperature, to 25μg/ml streptomycin was shown to be 2.2 x 10^3 cfu/g compost. This indicates that it may be possible in the future, to select for transductants in untreated compost using streptomycin, if the survival of *B. caldotenax* BT1 could be achieved.

Figure 5.1; Recovery of *B. caldotenax* BT1 from sterile compost microcosms incubated at 65°C. Recovery at 65°C on TSBA: ■, vegetative cell count; ●, spore counts. All values are the means of triplicate determinations from single microcosms.


10
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2
0
Log10 cfu/g of compost
0
2
4
6
8
10
12
Compost Incubation Time (Days)

5.3.1 Transduction in *B. caldotenax* BT1 mutants

The previously isolated streptomycin resistant and thymine requiring double mutants reported in Chapter 4 were used in the optimised transduction experiments described by Sharp (1983) and detailed in the methods section 2.21. The results are presented in Table 5.1 and show that it was not possible to demonstrate the presence of transductants, as JS017 was not shown to be present in any of the *thy*$^+$ colonies. The apparent reversion of the thymine auxotrophs to prototrophy indicates that the mutants were not as stable as was previously thought even though streptomycin resistance was maintained. The colonies were considered to be true revertants to wild type BT1, after it was demonstrated that 48 replica plated mutants showed growth on Kuhns minimal media and did not show any evidence of the presence of JS017. Further studies of the transduction system were not attempted because insufficient time was available to try to re-isolate stable streptomycin resistant thymine auxotrophs. Furthermore, these results coupled with the failure of *B. caldotenax* BT1 to survive in compost, indicated that extensive work was necessary in order to monitor the transduction system in mushroom compost microcosms. As a result of these investigations the interaction of bacteriophages and their hosts in model compost systems was monitored using bacteriophage ø105 and its host *B. subtilis.*

5.4 Analysis of the indigenous compost population for the presence of the *lacZ* gene and tetracycline resistance

As previously detailed, two derivatives of bacteriophage ø105 were available, each carrying the *lacZ* gene. The heat inducible derivative MU205 was chosen for

Table 5.1 Transduction experiments with *B. caldotenax* BT1 and JS017

Strep^R mutant	Thy$^+$ phenotype (cfu/ml)	Transductants (cfu/ml)
1	8.4×10^5	0
2	5.7×10^5	0
3	3.5×10^5	0
4	2.3×10^4	0
5	7.4×10^5	0
6	6.2×10^4	0

release experiments because preparation of high titre bacteriophage stocks was less hazardous and more convenient than using mitomycin C, to induce the other derivative, MU201. Previous work with mushroom compost microcosms had shown that *B. subtilis* 168 was morphologically distinct from the indigenous bacterial population (McDonald, 1992). Furthermore, it was also demonstrated that plasmid DNA was stably maintained within *B. subtilis* 168 in mushroom compost microcosms (Amner et al., 1991; McDonald, 1992). As a result, it was decided to use *B. subtilis* 168 carrying pTB90 plasmid DNA as a host to MU205 in release experiments. Selection of the host was based on selecting for plasmid encoded tetracycline resistance. Selection of ø105 MU205 was based on the presence of the *lacZ* gene which previous studies (Chapter 4) showed was conveniently demonstrated using the chromogenic substrate X-Gal. Prior to release experiments the indigenous bacterial population isolated at 37°C, was examined for tetracycline resistance and the presence of β galactosidase and the results are presented in Table 5.2. Random isolates were confirmed as *Bacillus* by Gram staining and it can be seen that the distribution of tetracycline resistance in the indigenous *Bacillus* population was low and the distribution of tetracycline resistant, Lac^+ colonies was even lower, with all of the colonies being morphologically distinct from *B. subtilis.* Analysis of the compost supernatant indicated that no indigenous bacteriophages could be detected, that infected *B. subtilis* 168 or CU267. This indicated that a suitable detection system was available to monitor the interaction of bacteriophages and their host in mushroom compost. Although as it was previously thought β galactosidase would be common in the indigenous bacterial population of mushroom compost, it is clear that the combination of tetracycline resistance of the host

Table 5.2 **Distribution of tetracycline resistance and β galactosidase activity in the indigenous *Bacillus* population of mushroom compost**

Additions to isolation media	cfu/g compost*
Unamended	2.2×10^8
80μg/ml X-Gal	3.3×10^5 blue-green colonies
25μg/ml tetracycline	2.6×10^3
25μg/ml tetracycline + 80μg/ml X-Gal	8.0×10^2 blue- green colonies

* Values are the means of triplicate determinations from a microcosm containing untreated End of Phase 2 mushroom compost

and the *lacZ* gene carried by MU205, could be used as selectable traits, for studies on the interaction of bacteriophages and hosts in compost.

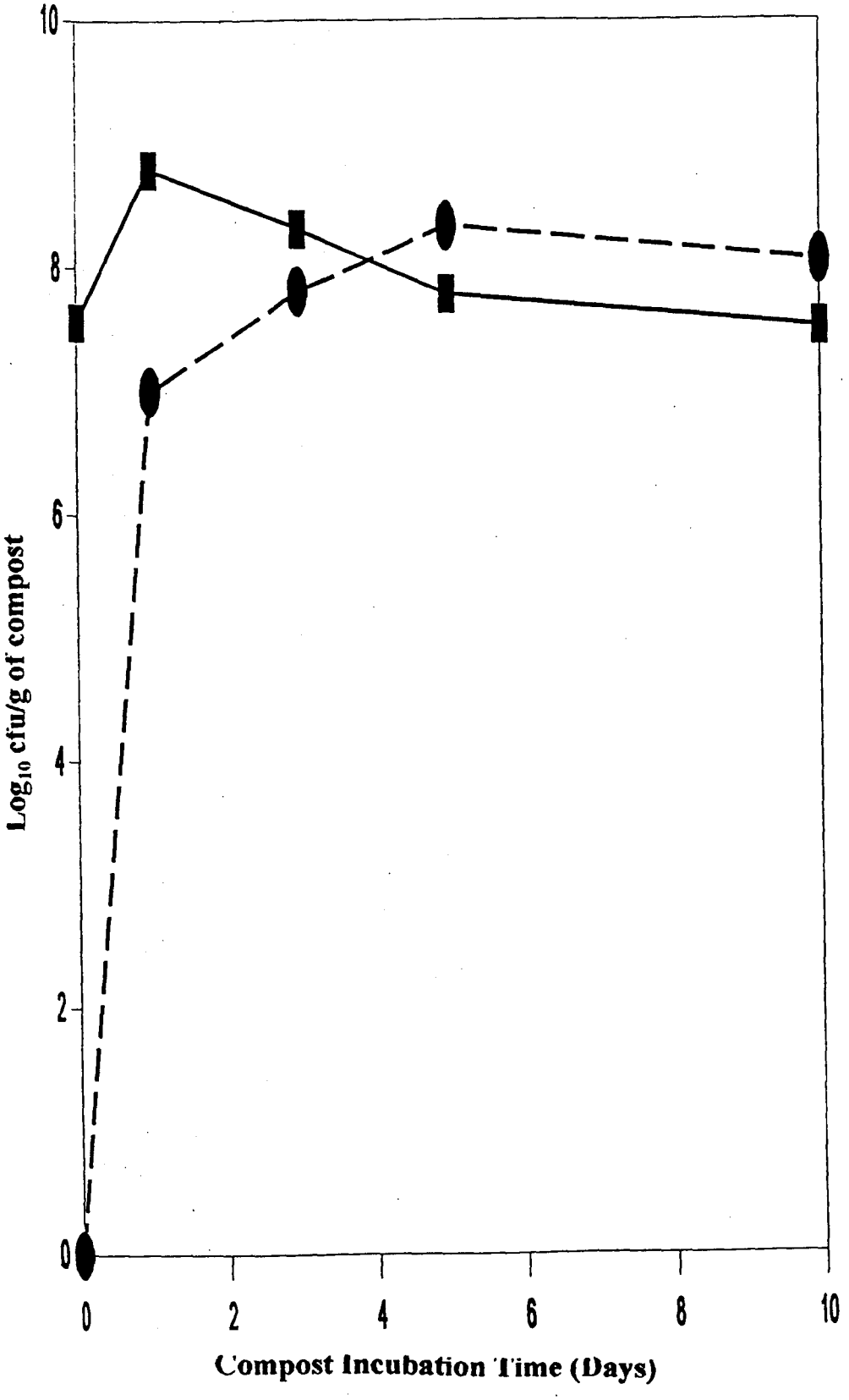
It was confirmed that MU205 infected *B. subtilis* carrying pTB90 plasmid DNA, with the production of blue-green plaques in an agar overlay containing 40µg/ml X-Gal. The maintenance of MU205 by *B. subtilis* pTB90 was demonstrated by 10 successive replica platings of 20 lysogens onto TSBA, TSBA amended with 25µg/ml tetracycline and TSBA amended with 40µg/ml X-Gal. Results indicated that tetracycline resistance and the *lacZ* gene were maintained for each replicate. After each incubation, the plates were overlaid with TSA containing *B. subtilis* 168 and results showed the presence of areas of lysis above each replicate further indicating the presence of MU205.

All release studies reported here were carried out at 37°C. Control sterile microcosms (as detailed in Methods 2.22) were analysed during these experiments and did not show any evidence of bacterial growth or bacteriophage activity. The limit of detection in these experiments was 10^2 *Bacillus* cfu/g compost.

5.5 Release of *B. subtilis* carrying pTB90 plasmid DNA in compost

The results of release experiments into sterile and untreated compost with *B. subtilis* containing pTB90 plasmid DNA are presented in Figures 5.2 and 5.3. Colonies were isolated onto TSBA amended with 25µg/ml tetracycline. Colonies were also isolated onto TSBA on the final days sampling and approximately thirty were tested for resistance to tetracycline. In each case, all of the colonies selected were tetracycline resistant and this was assumed to be due to the presence of pTB90 plasmid DNA.

Figure 5.2; Recovery of *B. subtilis* 168 containing plasmid pTB90 from sterile compost incubated at 37°C. Recovery at 37°C on TSBA amended with 25μg/ml tetracycline. ■, vegetative cell counts;●, spore counts; All values are means of triplicate determinations from single microcosms. The standard deviation of the mean values did not exceed 15%.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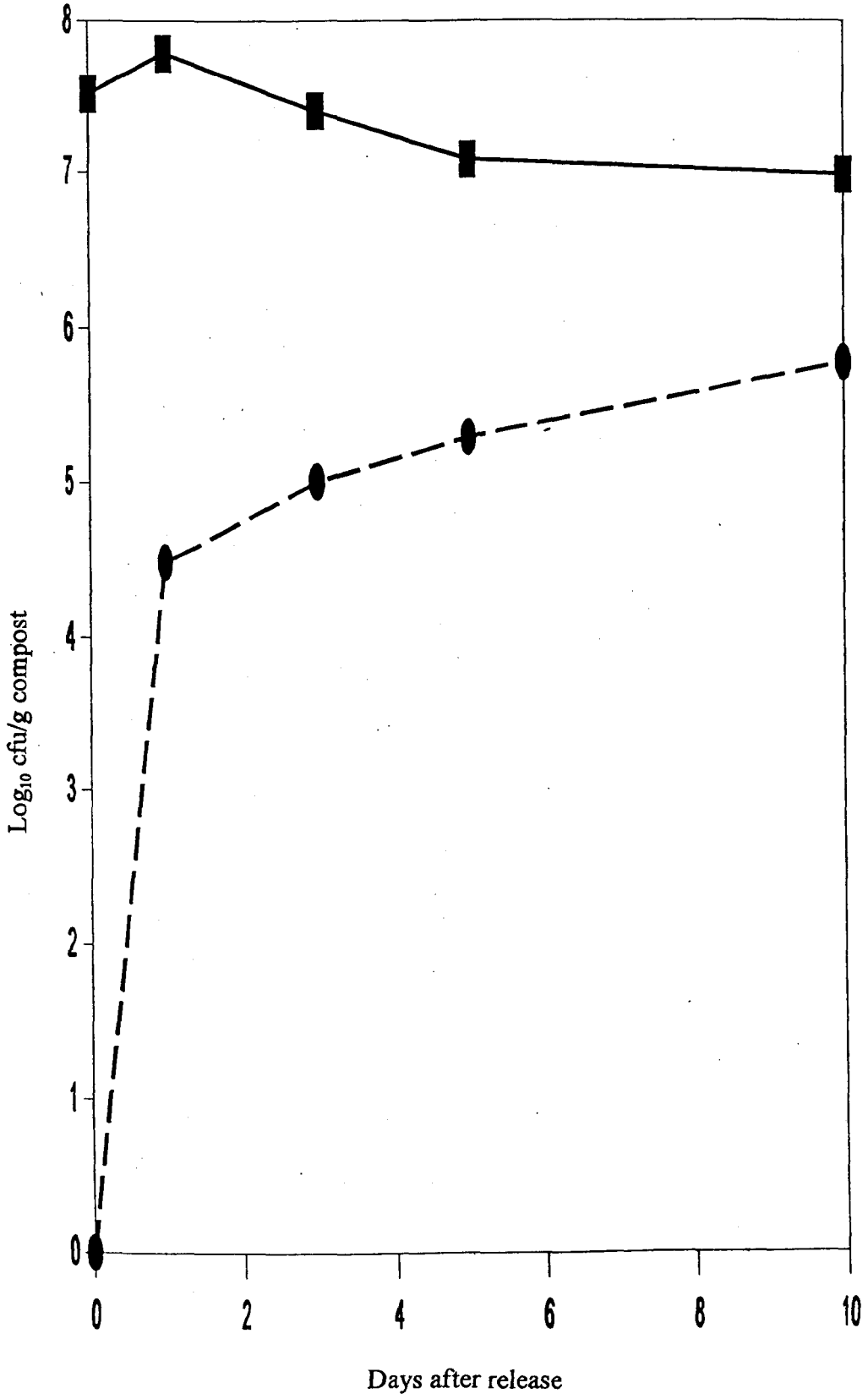
Log10 cfu/g of compost
Compost Incubation Time (Days)

Figure 5.3; Recovery of *B. subtilis* 168 containing plasmid pTB90 from untreated compost incubated at 37°C. Recovery at 37°C on TSBA amended with 25μg/ml tetracycline; ■, vegetative cell counts;●, spore counts; All values are means of triplicate determinations from single microcosms. The standard deviation of the mean values did not exceed 15%.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10
Log10 cfu/g compost
Days after release

Experiments involving sterile compost on Day 0 revealed that approximately 45% of the initial inoculum was recovered and this consisted of vegetative cells as no spores were detected. Sampling on Day 1 indicated that the spore population had increased, presumably due to sporulation of the released cells. Some vegetative cell growth was indicated by an increase in the number of vegetative cells isolated. This may have been due to the availability of nutrients and also because of the lack of competition from the indigenous microflora, in sterile compost. Between Days 1 and 5 it appeared that the sporulation of the released cells increased with a decrease in the vegetative cell population. By Day 5 the majority of the released population were observed to be present as spores and this continued until the final sampling on Day 10.

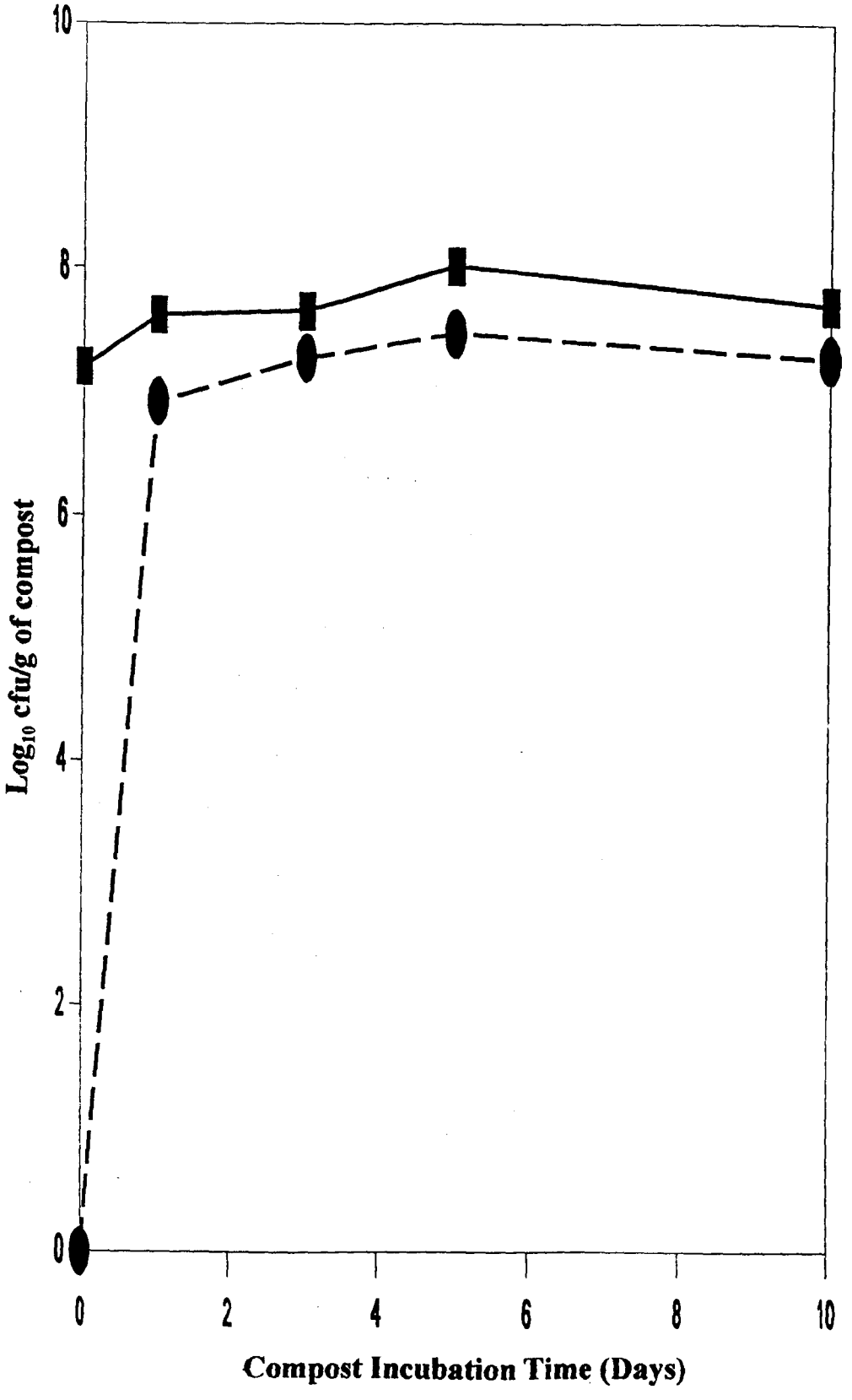
The recovery of *B. subtilis* containing pTB90 plasmid DNA from untreated compost is presented in Figure 5.3. Approximately 65% of the initial inoculum was recovered from the untreated compost, which was greater than that observed from sterile compost. This increase may have been because of an increased competition for space with the indigenous population. Again, no spores were detected in the initial inoculum, but as the monitoring progressed there was an overall increase in the spore population present however the extent of sporulation was not as great as that observed in sterile compost. Also in contrast to sterile compost the majority of the released bacteria were present as vegetative cells at the end of the sampling period. These results demonstrated the survival of *B. subtilis* containing pTB90 plasmid DNA in untreated and sterile compost over the sampling period. Further release experiments were carried out with a MU205 lysogen of *B. subtilis* containing pTB90 plasmid DNA.

5.6 Release of MU205 lysogen in compost

Figures 5.4 and 5.5 present the results of release experiments with the MU205 *B. subtilis* lysogen containing pTB90 plasmid DNA into sterile and untreated compost. Colonies were isolated onto TSBA amended with 25μg/ml tetracycline and 80μg ml X-Gal. Colonies were also isolated onto TSBA on the final day of sampling and approximately thirty were tested for resistance to tetracycline and the presence of the *lacZ* gene. In each case all of the colonies were tetracycline resistant and were blue-green in colour. This was assumed to be due to the presence of pTB90 plasmid DNA and MU205. Throughout the sampling experiments the colonies appeared blue-green on media containing X-gal, indicating the presence of the *lacZ* gene and no white colonies lacking the *lacZ* gene were recovered.

In sterile compost approximately 50% of the initial lysogen inoculum was recovered and no spores were detected on Day 0. Similar changes in the released population of lysogens were observed to those with the host population, with spores being isolated onwards from Day 1. However the majority of the population appeared to remain as vegetative cells during the sampling period. It was not possible to isolate bacteriophages from the microcosms on any of the sampling days, (results not shown) indicating that the numbers of free bacteriophages remained below the detection limit during the monitoring. The effect of the heat treatment to determine the spore count on MU205 needs to be considered. MU205 is induced by elevated temperature and it maybe that lysogens present as spores show induction of MU205 during the heat treatment. This would result in a decrease in the spore population due to bacteriophage lysis after the spore had germinated. However, no bacteriophages were isolated after

Figure 5.4; Recovery of a MU205 lysogen of *B. subtilis* 168 containing plasmid **pTB90** from sterile compost incubated at 37°C. Recovery at 37°C on TSBA amended with 25μg/ml tetracycline and 80μg/ml X-Gal; ■, vegetative cell counts;●, spore counts; **All** values are means of triplicate determinations from single microcosms.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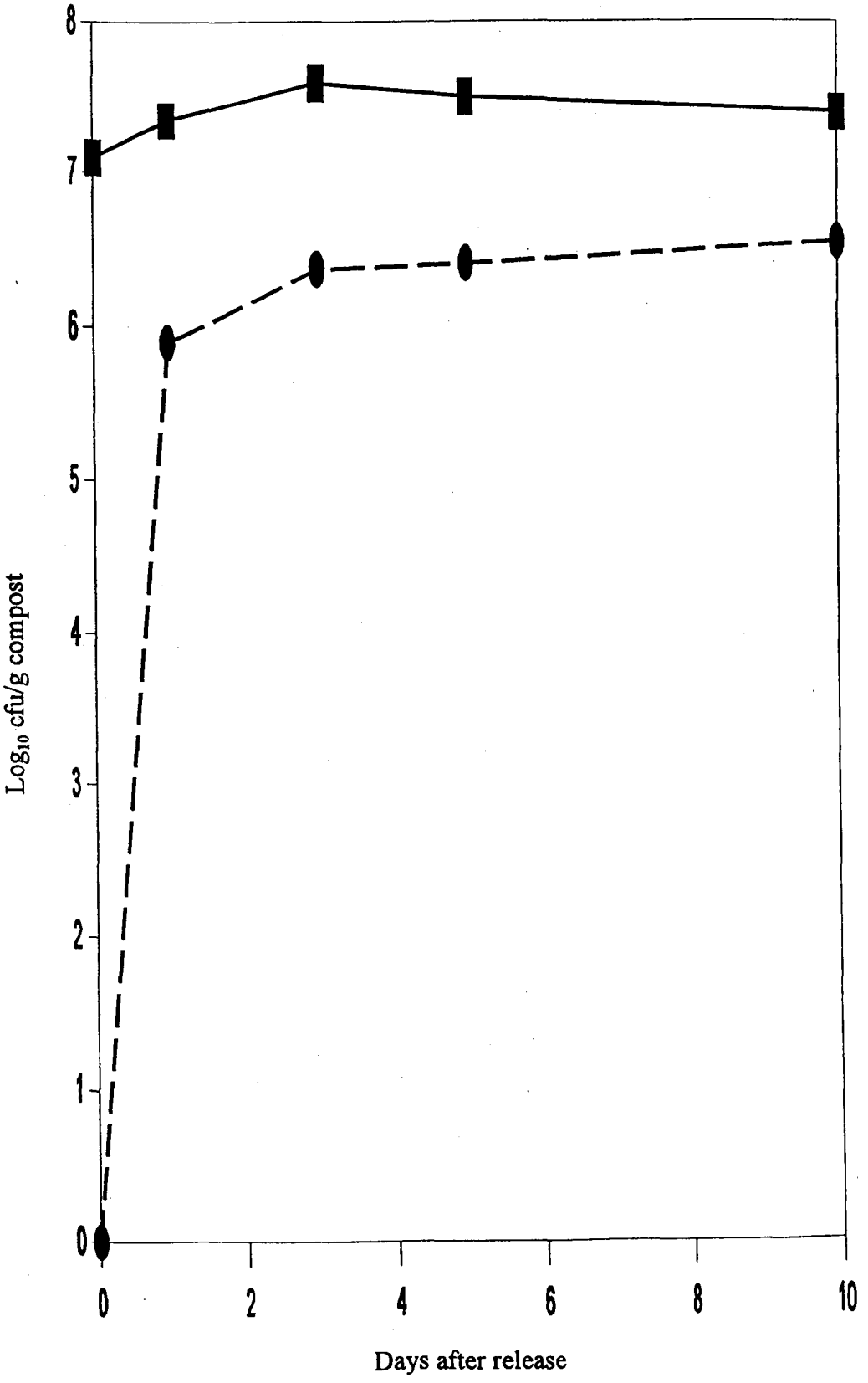
Log10 cfu/g of compost
Compost Incubation Time (Days)

Figure 5.5; Recovery of a MU205 lysogen of *B. subtilis* 168 containing plasmid pTB90 from untreated compost incubated at 37°C. Recovery at 37°C on TSBA amended with 25μg/ml tetracycline and 80μg/ml X-Gal; ■, vegetative cell counts;●, spore counts; All values are means of triplicate determinations from single microcosms.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Log10 cfu/g compost
Days after release

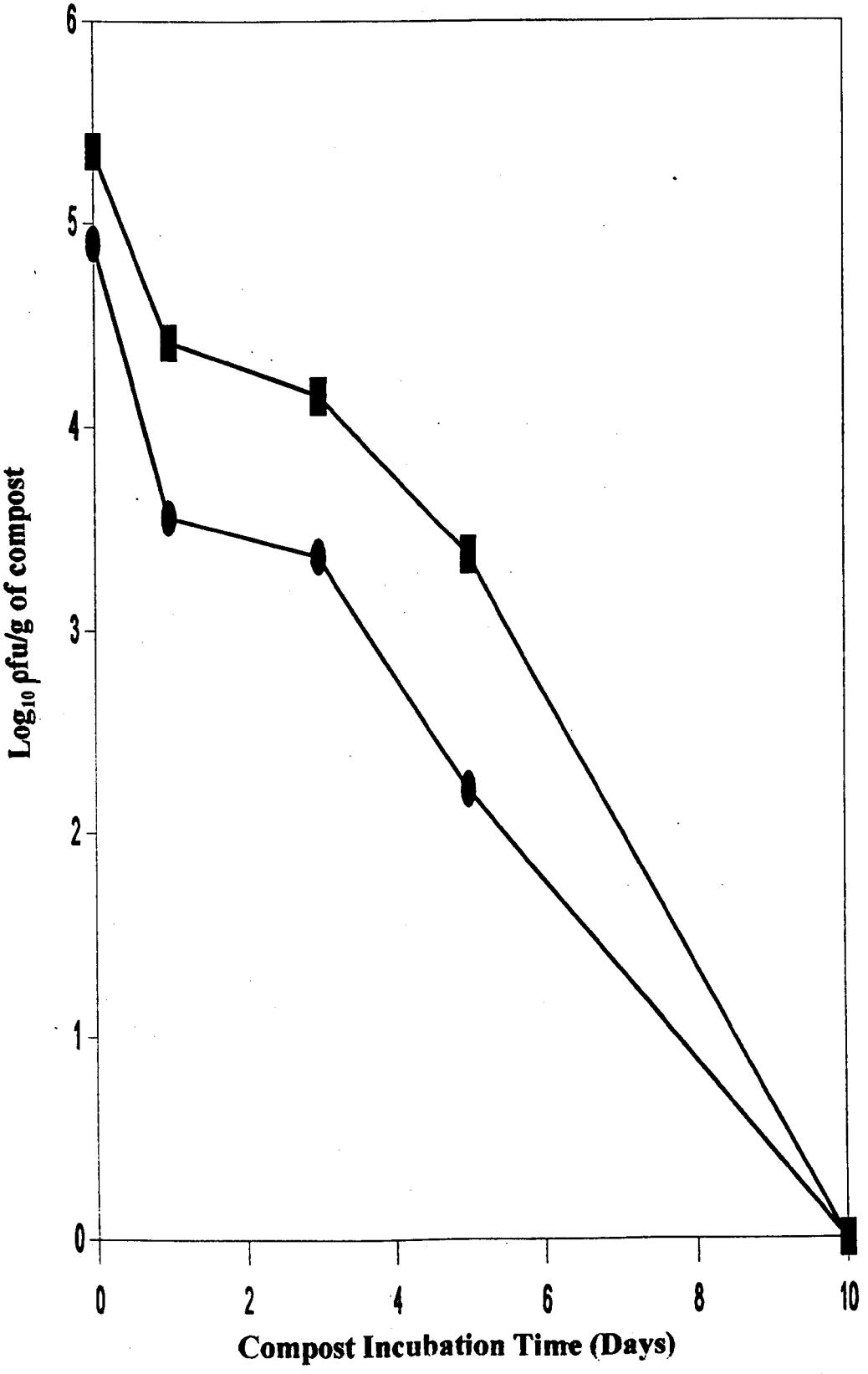
the heat treatment so it was assumed that the heat treatment to determine the spore count did not induce lytic bacteriophage activity and germination of the spore occurred with MU205 remaining as a lysogenic bacteriophage, although this does need further investigation.

Recoveries of lysogen released into fresh compost were again higher than those in sterile compost with 60% of the initial inoculum being recovered. Overall, similar results to release in sterile compost were obtained, with an increasing spore population as the sampling proceeded and the level of bacteriophage particles remaining below the detection limit. As with the release of host in untreated compost the extent of sporulation by the lysogen population was not as great as that observed in sterile compost. From these results it was concluded that the MU205 lysogen survived in untreated and sterile compost without detectable induction of the bacteriophage and maintaining the tetracycline resistance.

5.7 Release of MU205 in compost

After the survival of host and lysogen in sterile compost had been demonstrated, the survival of MU205 was monitored under the same conditions and the results are presented in Figures 5.6 and 5.7. Bacteriophages at two densities were released into sterile compost and it can be seen that in both cases the number of bacteriophages declined below the detection limit by Day 10, indicating that MU205 did not survive well in sterile compost. Recoveries of bacteriophages were less than 10% of the initial inoculum in both untreated and sterile compost and this may have been caused by adsorption of the bacteriophages to compost material. Also, the membrane filtration

Figure 5.6; Recovery of MU205 from sterile compost incubated at 37°C. Recovery at 37°C in TSA overlay containing 80µg/ml X-Gal and seeded with *B. subtilis* 168 containing pTB90 plasmid DNA;■, plaque counts from initial inoculum of 10^7 pfu/ml;●, plaque counts from initial inoculum of 10^6 pfu/ml; All values are means of duplicate determinations from single microcosms.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Log10 pfu/g of compost
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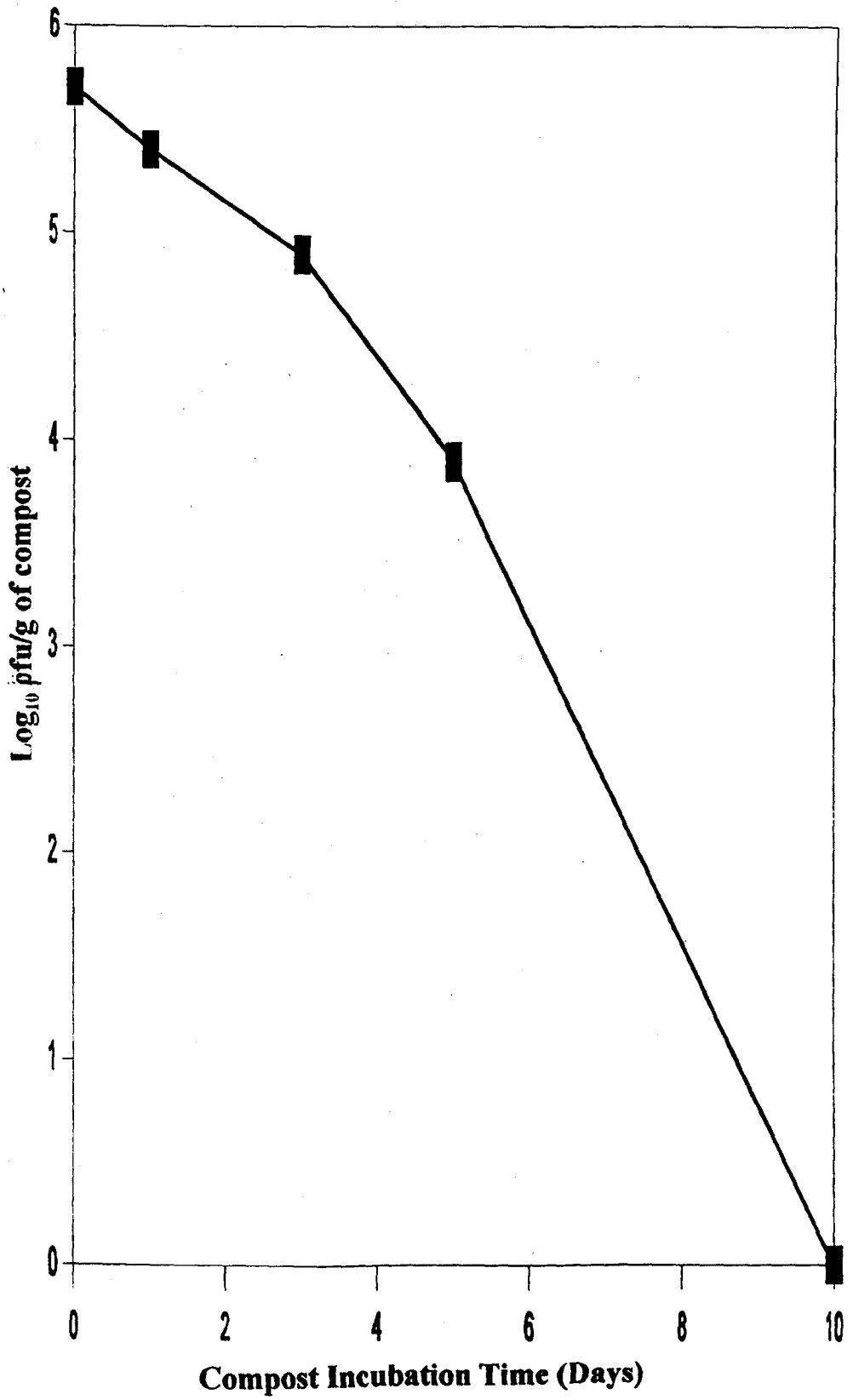
10
Compost Incubation Time (Days)

Figure 5.7; Recovery of MU205 from untreated compost incubated at 37°C. Recovery at 37°C in TSA overlay containing 80μg/ml X-Gal and seeded with *B. subtilis* 168 containing pTB90 plasmid DNA;■, plaque counts from initial inoculum of 10^7 pfu/ml; All values are means of duplicate determinations from single microcosms.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Log10 pfu/g of compost
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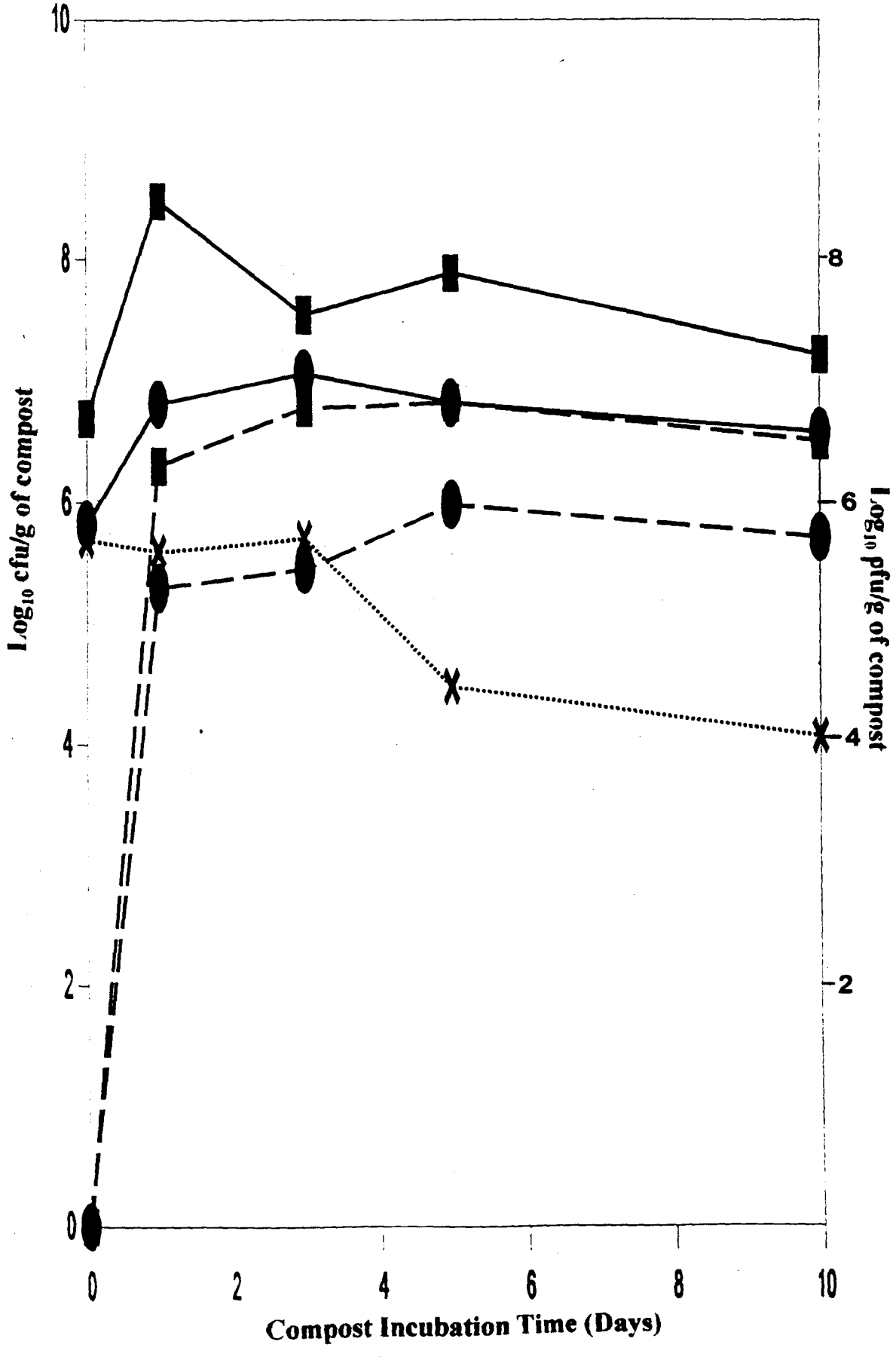
Compost Incubation Time (Days)

procedure may have removed bacteriophages from the compost supernatant, prior to plating out although this was not confirmed. In untreated compost, MU205 was only released at the higher density monitored in sterile compost. Again, in untreated compost at the end of the sampling period, the levels of bacteriophages were below the detection limit, indicating that MU205 did not survive in untreated compost.

5.8 Release of host and bacteriophage into compost

The final release experiments in untreated and sterile compost involved monitoring survival of bacteriophage and host released into the same microcosm and the results are presented in Figures 5.8 and 5.9. In order to confirm the presence of the *lacZ* gene and tetracycline resistance at the end of the releases, a sample of colonies from each recovery were analysed as previously detailed. Considering the survival of host in the sterile compost first, there was a lower initial recovery of 5% of the initial inoculum on Day 0, compared to 40% in sterile compost and 65% in untreated compost, inoculated with host only and reported earlier. There was also an increased recovery of bacteriophages on Day 0 compared to recoveries earlier. A possible interpretation of these results is that they may be due to infection and lysis of host vegetative cells by MU205, when the host and bacteriophage were inoculated into the microcosm. This would result in an increase in the numbers of bacteriophages present and a decrease in the number of hosts present. The microcosms were left for one hour prior to recovery on Day 0 and this may have been sufficient time for bacteriophage infection to occur. Lysogens were also recovered on Day 0 and because attempts were made to remove free bacteriophages from the compost supernatant prior to plating out,

Figure 5.8; Recovery of MU205, *B. subtilis* 168 containing pTB90 plasmid DNA (Host) and of MU205 lysogen of *B. subtilis* 168 containing pTB90 plasmid DNA (Lysogen) from sterile compost incubated at 37°C. Recovery of host at 37°C on TSBA amended with 25μg/ml tetracycline. ■——■, host vegetative cell counts;■- - -■, host spore counts; Recovery of lysogen at 37°C on TSBA amended with 25μg/ml tetracycline and 80μg/ml X-Gal; ●——●, lysogen vegetative cell counts;●- -●, lysogen spore counts; All values are means of triplicate determinations from single microcosms; Recovery of MU205 at 37°C in TSA overlay containing 80μg/ml X-Gal and seeded with *B. subtilis* 168 containing pTB90 plasmid DNA; X····X, plaque counts from initial inoculum of 10^7 pfu/ml; All values are means of duplicate determinations from single microcosms.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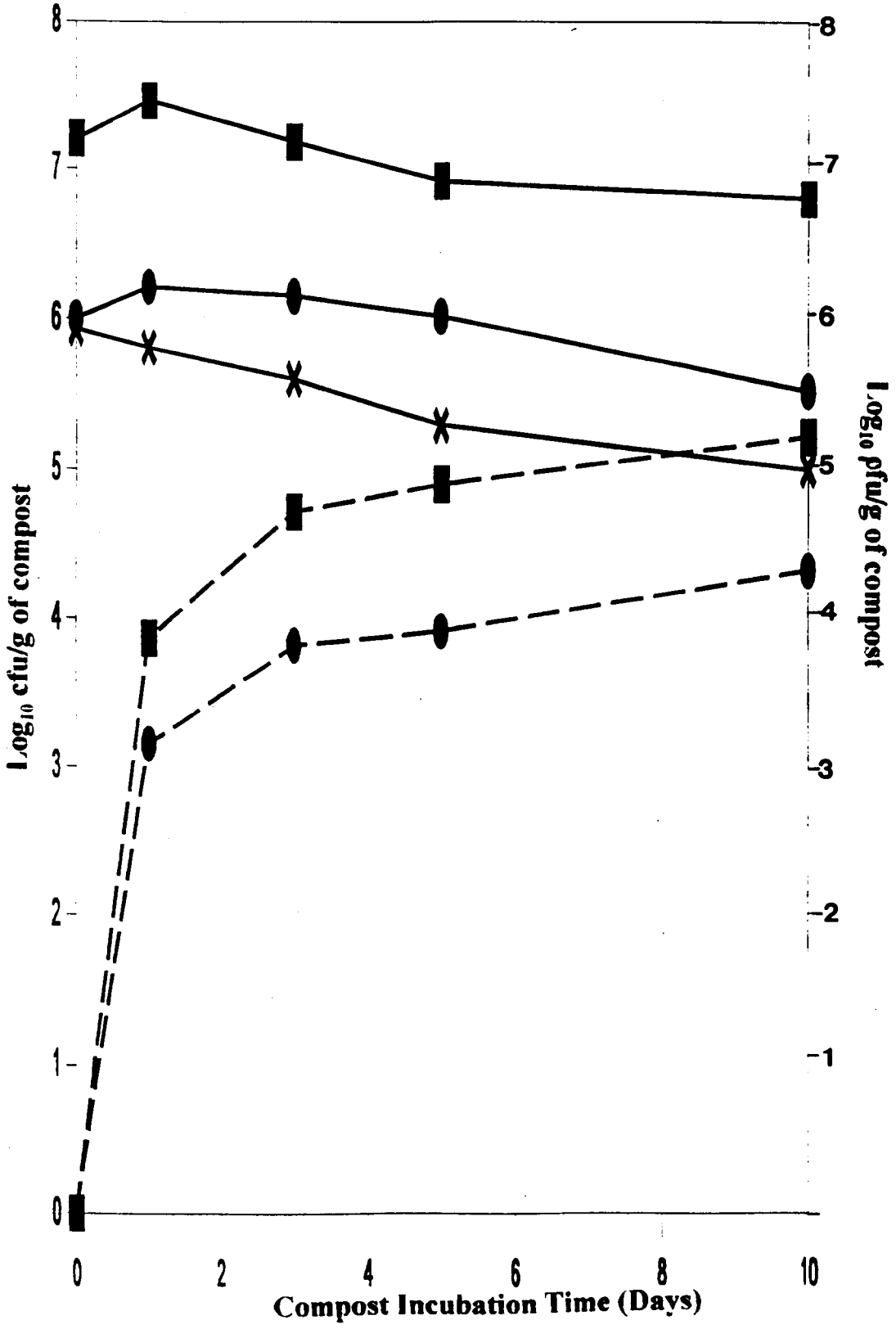
Log10 cfu/g of compost
Log10 pfu/g of compost
Compost Incubation Time (Days)

Figure 5.9; Recovery of MU205, *B. subtilis* 168 containing pTB90 plasmid DNA (Host) and of MU205 lysogen of *B. subtilis* 168 containing pTB90 plasmid DNA (Lysogen) from untreated compost incubated at 37°C. Recovery of host at 37°C on TSBA amended with 25µg/m l tetracycline. ■——■, host vegetative cell counts; ■-----■, host spore counts; Recovery of lysogen at 37°C on TSBA amended with 25µg/ml tetracycline and 80µg/ml X-Gal; ●——●, lysogen vegetative cell counts; ●- - -●, lysogen spore counts; All values are means of triplicate determinations from single microcosms; Recovery of MU205 at 37°C in TSA overlay containing 80µg/ml X-Gal and seeded with *B. subtilis* 168 containing pTB90 plasmid DNA; X·····X, plaque counts from initial inoculum of 10^7 pfu/ml; All values are means of duplicate determinations from single microcosms.


Log10 cfu/g of compost
Log10 pfu/g of compost
Compost Incubation Time (Days)
0
1
2
3
4
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10

it was assumed that the formation of lysogens occurred immediately after release into the microcosm. Sporulation of host and lysogen occurred as previously in the separate microcosms, however in both cases the number of vegetative cells was always greater than the number of spores throughout the sampling. This may be because the bacteriophages were released in nutrient broth which would increase the availability of nutrients within the microcosm facilitating survival in the vegetative cell form. The presence of a host bacterial population appeared to increase the survival of MU205, as free bacteriophages were present on Day 10, in contrast to the earlier release of MU205 in the absence of host when MU205 could not be detected on Day 10.

The same release experiment in untreated compost gave similar results as above, with the increased survival of MU205 being demonstrated. It was also apparent that the overall reduction in free bacteriophage numbers was greater in sterile compost than in untreated compost. As with the release in sterile compost, the formation of lysogens occurred on Day 0 and there was a reduction in the recovery of the initial inoculum of the host on Day 0 presumably for the same reasons as discussed earlier. In both sterile and untreated compost the formation of lysogens indicated bacteriophage infection in situ. As with the separate releases of host and lysogen, the extent of sporulation of the host and lysogen in the same microcosm, was less in untreated compost when compared to sterile compost.

5.9 Discussion

Release and recovery experiments were carried out with a bacteriophage, a host and a lysogenic host, in sterile and untreated mushroom compost microcosms.

Unfortunately the *B. caldotenax* transducing system (Sharp, 1983) could not be demonstrated *in vitro* because of apparent mutant reversion and further monitoring of the system in mushroom compost was not attempted. *B. subtilis* containing a tetracycline resistance encoding plasmid which had previously been shown to be stably maintained (McDonald, 1992) and the bacteriophage ø105 derivative, MU205 carrying the *lacZ* gene, were used in release studies at 37°C. The combination of the distinct morphology of *B. subtilis* and selecting for tetracycline resistance and the *lacZ* gene, meant that bacteriophage interactions could be sufficiently detected and monitored in mushroom compost microcosms.

Initial release experiments demonstrated the survival of *B. subtilis* pTB90 in sterile and untreated compost. This survival was thought to be as a result of sporulation in sterile compost whereas in untreated compost, survival was mainly in the vegetative cell form. The effects of autoclaving, particularly on the nutrient status are not known and it may be that the excessive heat treatment releases toxic compounds or destroys available nutrients, causing the released bacteria to sporulate. Increases in the vegetative cell population after inoculation into sterile compost imply that nutrients are available for vegetative cell growth and these may have resulted from carry-over of growth media used to prepare the inoculum, or may not have been affected by autoclaving. The extent of sporulation of the lysogen was less than that of *B. subtilis* pTB90 in sterile compost. It was not possible to determine whether this was due to the presence of MU205 or some other factor. It appeared from the results that the lysogenic association was stably maintained in compost, as no bacteriophages or Lac$^-$ phenotype *B. subtilis* pTB90 were isolated during these release experiments.

When free bacteriophages were released into sterile and untreated compost microcosms in the absence of any host, their presence was not detected by Day 10. In untreated compost, the presence of MU205 lysogens was not detected indicating that MU205 had not infected the indigenous population. Bacteriophages have been shown previously to have a finite half life in a specific ecosystem (Wiggins and Alexander, 1985; Yates et al., 1985). The sorption of bacteriophages to particulate matter has also been shown to limit recovery (Williams et al., 1987) and this probably occurs within the mushroom compost microcosm. As previously mentioned the membrane filtration procedures might have reduced the free bacteriophages present and also the small amount of supernatant recovered for bacteriophage isolation may not be a true estimate of the bacteriophage population (Seely and Primrose, 1982).

The interaction of bacteriophages and their hosts was monitored by releasing MU205 and *B. subtilis* pTB90 together into sterile and untreated compost microcosms. The results indicated that bacteriophage survival was increased to such an extent that bacteriophage were recovered up to the final sampling day. This indicates that the presence of a host possibly facilitates bacteriophage survival. The importance of the presence of a metabolically active host population for bacteriophage survival has been reported for *P. aeruginosa* bacteriophages (Kokjohn and Miller, 1992). It is also possible that the bacteriophages are not surviving, but being replaced by bacteriophages released from infected cells. Previous experiments revealed that the production of free bacteriophages from lysogens was not detected so the bacteriophages would have to be produced from a lytic cycle of infection in a vegetative cell. It was also found that the formation of lysogens occurred immediately on inoculation into both sterile and

untreated compost with the lysogen surviving the remainder of the experiment predominantly in the vegetative cell form.

In conclusion, it appears that hosts and lysogens can survive in untreated and sterile compost, however, released bacteriophages do not survive unless a host or lysogen is present within the indigenous population. The studies reported here indicate that it was possible to monitor interactions between bacteriophages and hosts in the presence of an indigenous population.

CHAPTER 6

CLOSING DISCUSSION

Chapter 6

Closing Discussion

The aim of this project was to investigate the *Bacillus* and bacteriophage populations within mushroom compost. The role of bacteriophages as agents of gene transfer in the environment has been insufficiently studied. Given the concerns over the environmental release of GMO's, this study set out to investigate the behaviour of indigenous hosts and bacteriophages in natural environments. Studies on the survival and transfer of genes in the environment need to be underpinned by a genetic analysis of the natural population, to assess any changes in population equilibrium as a result of the presence of a GMO. The studies reported in Chapter 3 investigated that fraction of the *Bacillus* population in mushroom compost, that could be recovered at 50°C. Forty-eight isolates were obtained and divided into ten groups on the basis of similarities in colonial morphologies. The prevalence of these groups during the preparation of mushroom compost was monitored and results indicated that five groups were present throughout Phases one and two, in both vegetative cell and spore form. The stability of end of Phase two mushroom compost was also demonstrated with the isolates recovered being from the previously mentioned five groups.

Attempts were made to characterise the isolates using biochemical, morphological and physiological tests. Although it was only possible to identify two groups at the species level, it was apparent that isolates within a particular morphological group gave similar results to each other. This indicated that differences

in colonial morphology of compost isolates may represent different *Bacillus* species. However this would need to be confirmed by a more extensive taxonomic study of the isolates. The lack of identification at the species level may have also be caused because although the isolates are apparently active in the mushroom compost ecosystem, they may appear relatively inert under laboratory conditions. These studies allowed the isolates from the *Bacillus* population isolated at 50°C from mushroom compost, to be assigned to a particular morphological group. As well as being morphologically similar, isolates within each group gave similar results in taxonomic tests.

The presence of bacteriophages infecting the majority of the previously detailed isolates was reported in Chapter 3. These bacteriophages were isolated without any prior enrichment. Host range of the bacteriophages was shown to be restricted to isolates within a particular colonial morphology group of the original host. The original intention of the study was to isolate a lysogenic bacteriophage, however it was demonstrated that all detected bacteriophage infections at 50°C produced clear plaques, indicating lytic bacteriophage activity. Compost contains an active *Bacillus* population and such conditions may not be favourable to the establishment of lysogeny and it may be that lysogens are present, but at too low a level to be detected. It may be necessary in future studies to reduce the activity of the *Bacillus* population of mushroom compost in order to encourage the development of a lysogenic association.

A lysogenic bacteriophage that was apparently thermoinducible, was isolated after the infection temperature was reduced to 37°C. This bacteriophage formed the basis of the studies detailed in Chapter 4, to construct a trackable bacteriophage that could be monitored in release studies in mushroom compost. The perceived advantage

of this system was that because the bacteriophage was isolated from the indigenous population and would be returned, there would be a minimal disturbance to the population equilibrium. This would allow the interaction of bacteriophages and hosts to be monitored in their natural environments.

It must be remembered that this study has investigated a very small proportion of the indigenous *Bacillus* population of mushroom compost. Also, the dilution plating technique, although being convenient, excludes members of the population that are present in lower numbers than other indigenous bacteria.

Construction of a trackable bacteriophage was dependent on selecting a suitable marker sequence and the development of a successful transfection system. In order to achieve transfection, *Bacillus* transformation systems were studied in order to gain an optimised system that could be further developed for transfection. Two systems of transforming *B. subtilis* 168 with pTB90 plasmid DNA were demonstrated. Unfortunately, reported transformation frequencies could not be achieved and despite exhaustive attempts, an optimised system was not obtained. Transfection of protoplasts was achieved although again at a low frequency. This was not unexpected since the isolate MG030 was isolated from compost and was not a laboratory strain. However, it was still encouraging that transfection of indigenous *Bacillus* species was achieved, although further work is necessary to optimise the system.

A suitable marker sequence was constructed using PCR. Conditions for these reactions were not optimal as indicated by the presence of more than one PCR product. However, upon *Bam*HI restriction, a single fragment was obtained. This was assumed to be the double stranded marker sequence. Shotgun cloning techniques were used to

insert the sequence into the bacteriophage genome, but it was not possible to isolate tagged bacteriophages after transfection. Possible reasons for this failure have already been discussed. Future experiments here could use the PCR based cloning strategy to facilitate the construction of marker sequences. Shotgun cloning techniques could also be continued, hoping to insert the sequence into a non essential gene. Furthermore, if this is achieved, the PCR cloning strategy allows the insertion of a unique restriction enzyme site into the marker sequence. A marker gene could be incorporated into this restriction site in a similar manner to well characterised bacteriophages being used as cloning vectors.

Although it was not possible to construct viable tagged bacteriophages, further systems were developed that enabled host and bacteriophage interactions to be monitored in the environment. Transduction in *B. caldotenax* was investigated, however the survival of *B. caldotenax* BT1 in mushroom compost could not be demonstrated. Also, mutants isolated for use in transduction experiments were found to be unstable. Although disappointing, this system can be developed to monitor bacteriophage and host interactions. If *B. caldotenax* BT1 can be induced to sporulate this may aid survival in compost. The use of streptomycin resistance as a selection agent appears to be favourable if stable mutants can be isolated, since the background resistance of *Bacillus* spp. at 65°C was shown to be relatively low.

Release experiments in mushroom compost investigated the interaction of ø105 MU205 with *B. subtilis* in untreated and sterile mushroom compost microcosms. It may be possible in future experiments to increase recovery rates, using alternative sampling procedures such as stomaching. Results of release experiments indicated that presence

of a host appeared to favour the persistence of bacteriophages. It may be that in these cases the bacteriophages are not surviving but are being released from infected vegetative cells and the bacteriophage population is therefore constantly being replenished. This could be further investigated by releasing bacteriophages with different densities of host cells to determine if a threshold of host numbers exists, below which bacteriophage survival is reduced. No attempts were made to investigate bacteriophage mediated gene transfer, however this system allows the release of MU205 lysogen to be released alongside *B. subtilis* 168 carrying pTB90 plasmid DNA. The presence of tetracycline resistant MU205 lysogens would indicate that MU205 had been released from a lysogen and infected a different host within the microcosm, although in this study the, presence of free bacteriophages released by a lysogenic population was not detected. The *lacZ* gene and pTB90 transfer to other members of the indigenous population as a result of bacteriophage activity could also be investigated with appropriate probing techniques. Also, the effects of inducing the MU205 lysogen to lytic activity, inside the microcosm could also be assessed. As with the intended studies at 50°C, it is essential that the indigenous population at 37°C is characterised to underpin gene transfer studies.

Overall, this study has provided preliminary information in addressing the questions of bacteriophage and host interactions and the potential for bacteriophage mediated gene transfer in mushroom compost.

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