

GENETIC STUDIES OF STREPTOMYCES CATLEYA
AND STREPTOMYCES OLIVACEUS

by

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TABLE OF CONTENTS

	Page
Abstract	
Chapter 1 Introduction	1
Chapter 2 Isolation and characterization of an actinophage virulent amongst beta- lactam antibiotic producing <u>Streptomyces</u>	17
Chapter 3 Studies of DNA repair in <u>Streptomyces</u> <u>cattleya</u> using phage VC11	63
Chapter 4 Transfection of <u>Streptomyces cattleya</u> and <u>Streptomyces olivaceus</u> protoplasts with phage VC11	102
Chapter 5 Cloning strategies for the identification of the origin of replication of actinophage VC11	133

	Page
Chapter 6 Detection of the <u>tuf</u> gene in <u>Streptomyces</u> <u>cattleya</u> and <u>Streptomyces olivaceus</u>	192
Chapter 7 Discussion	229
Appendix General media and buffers	237
Literature Cited	243

ABSTRACT

Actinophage VC11 is able to virulently infect 11 of the 20 Streptomyces strains tested. Examination of VC11 infection of Streptomyces cattleya, Streptomyces olivaceus and Streptomyces lividans TC10 indicated the absence of restriction-modification systems which affect VC11 infectivity of these Streptomyces strains.

VC11 fits the B1 group of Bradley's morphological phage classification system, consisting of an icosahedral head (47 x 47 nm) and a long non-contractile tail (196 x 7.8 nm). The VC11 genome is comprised of a double-stranded DNA molecule which was estimated to be 45.2 kb in length and found to possess a G+C content of 50.4%. The presence of a cos site indicates that the phage genome replicates according to the rolling circle model. Although restriction enzyme analysis of VC11 DNA revealed the presence of numerous recognition sequences for 16 endonucleases and the absence of restriction sites for a further 21 restriction enzymes, partial-digestion of the phage DNA with BglII allowed the construction of a linear restriction map of the VC11 genome. Complete BglII digestion of VC11 DNA generated 5 fragments of 23.2, 11.1, 7.6, 2.6 and 0.65 kb in length.

Whereas survival of UV-irradiated VC11 followed simple one-hit

kinetics when propagated on untreated S. cattleya spores, UV-irradiation of the host spores resulted in an increase in the survival of UV-damaged phage by 100% or more. This observation indicates the induction of a DNA repair system in the UV-treated S. cattleya spores which is able to restore UV-irradiated phage. The e.o.p. of undamaged VC11 propagated on untreated S. cattleya spores was not affected by the presence of either caffeine or sodium arsenite. Thus, a caffeine-mediated decrease in the survival of UV-irradiated phage propagated on untreated S. cattleya spores identified a constitutive host-mediated UV repair system in S. cattleya, while a similar decrease in the survival of UV-damaged phage propagated on UV-irradiated S. cattleya spores revealed the presence of a caffeine inhibitable, inducible DNA repair mechanism in this Streptomyces strain. These results indicate that either two separate DNA repair systems exist in S. cattleya or caffeine inhibits a constitutive and an inducible component of the same DNA repair system. Sodium arsenite failed to influence the survival of UV-damaged VC11 propagated on both untreated and UV-irradiated S. cattleya spores. The possible interaction between DNA repair systems and genetic instability in S. cattleya are discussed.

S. cattleya and S. olivaceus were sensitive to lysozyme hydrolysis at 37°C, yielding protoplasts after 90 min incubation. S. olivaceus produced viable colonies after incubation at 30°C

for 48 h, while S. cattleya exhibited very small colonies following 6 days incubation. The regeneration frequency of S. olivaceus protoplasts was estimated to be 26.7%, while the S. cattleya protoplast regeneration frequency of 0.5% reflected the slow rate of colony-formation on R2 agar. Attempts to improve the regeneration frequency of S. cattleya protoplasts were unsuccessful. S. cattleya protoplasts were not transfected with VC11 DNA. However, 1 ug of VC11 DNA generated 5.3×10^2 S. olivaceus transfectants. Thus, VC11 DNA transfected S. olivaceus protoplasts at a frequency of 0.24×10^{-6} per phage DNA molecule and 1.3×10^{-5} per viable protoplast. Although transfection of S. cattleya protoplasts with VC11 DNA was unsuccessful, indirect 'transfection' of S. cattleya with phage DNA was accomplished by the regeneration of VC11 transfected S. olivaceus protoplasts in the presence of S. cattleya spores to yield 55 'transfectants' per microgram of VC11 DNA. This procedure should prove useful for the introduction of broad host range phage cloning vectors into recipient strains in which protoplast transfection systems have not been established.

An attempt to identify the ori region of the VC11 genome by 'shotgun' cloning BglII restricted phage DNA into the BamHI site of the Bacillus subtilis plasmid pHV60 was unsuccessful. Similarly, the phage ori region was not isolated following the destruction of the replicon of the Streptomyces plasmid pLJ702

and subsequent transformation of S. olivaceus protoplasts with BglII restricted VC11 DNA ligated to BclI digested plasmid DNA. However, transformation of S. olivaceus protoplasts with pIJ702 resulted in expression of the plasmid borne tyrosinase and thiostrepton- resistance genes in this organism indicating that pIJ702 may be utilized as a DNA cloning vector in S. olivaceus.

A single copy of the tuf gene which encodes the polypeptide chain elongation factor Tu was identified in S. cattleya and S. olivaceus by DNA hybridization. Although restriction enzyme digests indicate differences in the nucleotide sequence of the tuf gene region in these Streptomyces species, the Streptomyces tuf gene bears strong sequence homology to the carboxyl-terminal half of the Escherichia coli tufA gene. The detection of the tuf gene of S. cattleya and S. olivaceus has several potential applications for the study of the molecular genetics of these important beta-lactam antibiotic-producing Streptomyces species.

CHAPTER 1

INTRODUCTION

1.1. General

An antibiotic may be defined as a product of secondary metabolism with an incidental action in minimal concentration on growth processes (Zähner, 1978). Primary metabolism involves the synthesis of essential macromolecules such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), protein, lipids and polysaccharides via a highly specific network of enzymatic pathways. In most cases secondary metabolism occurs during the late exponential and stationary phases (idiophase), which follows the trophophase or growth phase, of a bacterial culture. The idiophase is characterized by a decrease in DNA synthesis and growth rate due to the depletion of nutritional growth-limiting components from the medium (Martin and Demain, 1980).

Secondary metabolites include a wide range of compounds belonging to different chemical groups. These include amino sugars, quinones, coumarins, epoxides, ergot alkaloids, glycosides, naphthalenes, indole derivatives, macrolides and tetracyclines. In addition, secondary metabolites often possess unusual chemical

configurations such as beta-lactam rings, cyclic peptides and large rings of macrolides (Martin and Demain, 1980). These various compounds may be synthesized by diverse organisms or a single strain. For example, chloramphenicol is produced by a number of actinomycete genera including Streptomyces, Kitasatoa, Nocardia and Streptosporangium, while Streptomyces lipmanii synthesizes penicillin N, cephalosporin C and a thienamycin (Wellington and Cross, 1983). The ability of single strains and separate taxa to produce various secondary metabolites is a reflection of the low specificity of the enzymes involved in secondary metabolism (Martin and Demain, 1980).

Secondary metabolites are not essential for cell growth and consequently, errors in the biosynthesis of these substances, due to the low specificity of the enzymes involved, are not lethal. Secondary metabolism is governed by specific regulatory mechanisms such as induction, catabolite regulation and end-product regulation, as well as general regulatory processes determined by growth rate (Martin and Demain, 1980). Although the function of secondary metabolites in the producing organism is often obscure, the fact that anti-microbial secondary metabolites are produced once the cell growth rate decreases below a specific level, has allowed the formulation of a number of hypotheses concerning the selective advantage these substances impart on their producing organisms.

One obvious function of antibiotics is to inhibit the growth of other micro-organisms. Nutrients are limited in most natural habitats; for example, soil and water. Thus antibiotic production bestows a competitive advantage on the manufacturing organism (Martin and Demain, 1980). However, it has been demonstrated that the level of microbial antibiotic production in unsterile soil is not the same as in synthetic nutrient media. Moreover, there seems to be no correlation between the ability of an organism to inhibit rival organisms in soil and its ability to synthesize inhibitory antibiotics to these organisms (Gottlieb, 1973).

As morphological differentiation occurs during the idiophase (for example, endospore formation in bacilli and sporulation in Streptomyces), antibiotics may function as differentiation regulators (Martin and Demain, 1980; Hopwood, 1981a). This implies that antibiotic production must occur specifically when its action is needed during the differentiation process. Indeed, antibiotic production occurs between stage 0 and I of the sporulation procedure in bacilli (Martin and Demain, 1980).

Mukherjee and Paulus (1977) isolated two mutants of Bacillus brevis ATCC 8185 which did not produce linear gramicidins, but continued to produce tyrocidine. These workers found that the addition of gramicidin at a critical point preceeding

sporulation, restored the heat resistance and dipicolinic acid content of the spores to that of the wild type. Ristow et al. (1979; 1982) observed that the addition of tyrothricin (a tyrocidine-gramicidin complex) to early vegetative colonies, grown in a nitrogen deficient medium induced sporulation. Modest et al. (1984) isolated two classes of B. brevis ATCC 8185 mutants. S 18 and S 19 were unable to produce tyrocidine or gramicidin and were asporogenous, while mutant BM 44 produced gramicidin only and was able to sporulate. The authors noticed that exogenous tyrocidine stimulated sporulation in S 18 and S 19 under nitrogen deprivation, while BM 44 synthesized gramicidin and sporulated in rich medium without the presence of tyrocidine. Earlier, Ristow and Paulus (1982) had demonstrated that tyrocidine stimulated gramicidin synthesis which resulted in sporulation. However, Modest et al. (1984) have shown that tyrocidine does not function solely as an inducer of gramicidin synthesis as it initiated sporulation, in the absence of detectable gramicidin synthesis. in mutants S 18 and S 19. Consequently, the authors suggested that the two peptide antibiotics regulate different aspects of sporulation and that their mutants had somehow surmounted the absence of one of the regulatory mechanisms. Ristow et al. (1975a; 1975b) showed interactions between the effects of tyrocidine and linear gramicidin on transcription in vitro and suggested that sporulation may be controlled by a balanced antagonism between the two antibiotics. It

is significant to note that no sporulating mutant of B. brevis ATCC 8185, lacking both peptide antibiotics, has been isolated to date (Modest, 1984).

Lazaridis et al. (1980) found that the addition of gramicidin S to late vegetative cultures of B. brevis Nagano gramicidin S negative mutants restored wild type spore outgrowth properties. Similarly, an antibiotic-like substance released by germinating Streptomyces viridochromogenes spores inhibited germination of other spores (Ensign, 1978; Hirsch and Ensign, 1978). Hence it would seem that secondary metabolites produced by B. brevis Nagano and S. viridochromogenes govern spore dormancy. This inhibition of sporulation may serve as a timing device in order to ensure that the entire spore population does not germinate during adverse environmental conditions.

While there is evidence supporting antibiotic participation during sporulation and spore germination in some antibiotic-producing strains, no general deductions can be reached as to the role of antibiotics in nature. However, when one considers that over 1% of the genome of some Streptomyces species is devoted to antibiotic production, it is clear that secondary metabolite synthesizing micro-organisms derive some selective advantage from these substances (Hopwood, 1981a; Hopwood et al., 1983a).

Secondary metabolite research has focussed mainly on antibiotics due to their commercial and medicinal value. Characteristic of secondary metabolites, the antibiotics are a group of diverse chemical compounds possessing a wide range of structures (Hopwood, 1981a). Chloramphenicol was originally isolated from Streptomyces venezuelae and possesses a relatively simple structure (Malik, 1983). The beta-lactam (penicillins and cephalosporins), macrolide (erythromycin) and tetracycline groups are polycyclic compounds (Hopwood, 1981a), while the peptide antibiotics include linear and cyclic forms (linear gramicidin and tyrocidine, respectively) and may possess up to 19 amino acids (alamethicin) (Kleinkamf and von Dohren, 1983). The aminocyclitol antibiotics (neomycin, kasugamycin, streptomycin, etc.), synthesized by Streptomyces, Micromonospora and Bacillus, are based on a rare secondary metabolite cyclitol 2-deoxystreptamine (Davies, 1982; Davies and Yagisawa, 1983). Moreover, antibiotics attack a broad range of biological targets: DNA replication, transcription, translation (ribosome function and aminoacyl-tRNA binding), and cell wall and membrane synthesis (Hopwood, 1981a). Consequently, antibiotics exhibit a wide range of selective toxicity, inhibiting bacteria, viruses, fungi, as well as possessing anticancer, antiparasitic and coccidiostatic properties (Martin and Gil, 1984).

Micro-organisms have been screened for the production of

clinically important antibiotics for the last 35 years. Of the more than 5,000 different antibiotics that have been isolated approximately 100 are used to treat human, animal and plant diseases (Martin and Gil, 1984). The actinomycetes are responsible for the production of more than 70% of the antibiotics described to date, while the fungi account for 20% and the remaining 10% is produced by unicellular bacteria (Bushell, 1983). More than 60% of the antibiotics synthesized by the actinomycetes are produced by the genus Streptomyces (Martin and Gil, 1984).

In 1929, Sir Alexander Flemming observed antibiosis between a Penicillium mould and neighbouring bacterial cultures. He named this antibacterial fungal metabolite penicillin, after the producing strain Penicillium notatum (Flemming, 1929). The beta-lactam ring structure of penicillin was elucidated by Abraham and co-workers in 1943. The second beta-lactam antibiotic, cephalosporin C, was found to be produced by Cephalosporium acremonium (Aoki and Okuhara, 1980). The isolation of the nuclei of penicillin and cephalosporin, 6-aminopenicillanic acid and 7-aminocephalosporanic acid respectively, allowed the construction of semisynthetic beta-lactam antibiotics.

Beta-lactam antibiotics selectively inhibit the final step of

peptidoglycan synthesis (Tomasz, 1979). Peptidoglycan imparts mechanical strength on procaryotic cell walls and thus is an essential component of these cells. The absence of peptidoglycan in mammalian cells renders beta-lactam antibiotics optimal chemotherapeutic agents. These antibiotics are excellent bacterial inhibitors and have no toxic effect on eucaryotic cells (Aoki and Okuhara, 1980).

For many years, semisynthetic beta-lactam antibiotics were the only source of new compounds effective against resistant (due to beta-lactamase production) or non-susceptible bacteria. However, the discovery of 7-methoxycephalosporin production in certain Streptomyces species, including Streptomyces clavuligerus, S. lipmanii and Streptomyces griseus, led to renewed efforts in the search for novel, natural beta-lactam antibiotics produced by procaryotes (Ogawara, 1981).

Novel screening procedures, such as the utilization of beta-lactam hypersensitive mutants (Kitano, 1983) and the target screening approach which involved the examination of large numbers of micro-organisms for the ability to perform a specific function (for example, beta-lactamase inhibition) (Bushell, 1983), led to the detection of new beta-lactam antibiotics produced by various types of micro-organisms. As a result of screening for beta-lactamase inhibitors, clavulanic acid was

isolated from S. clavuligerus and found to possess strong beta-lactamase inhibitory and low antibacterial properties (Ogawara, 1981; O'Sullivan and Ball, 1983). In addition, S. clavuligerus produces penicillin N, deacetoxycephalosporin C, cephamycin C and three novel clavam compounds (Kitano, 1983; O'Sullivan and Ball, 1983). Clavulanic acid, used together with penicillin, has a synergistic effect against many resistant bacteria (Ogawara, 1981).

The first of a novel group of beta-lactam antibiotics, known as the carbapenems, was discovered with the aid of a cell-free peptidoglycan synthesizing system (Bushell, 1983). Thienamycin, produced by Streptomyces cattleya together with penicillin N, cephamycin C, N-acetylthienamycin, N-acetyldehydrothienamycin (Kahan et al., 1979; Lilley et al., 1981) and a cyclopentenone antibiotic (Noble et al., 1978), is a beta-lactamase resistant broad spectrum antibiotic effective against, amongst others, Pseudomonas, Serratia and Bacteroides (Ogawara, 1981). Olivanic acids, closely related to thienamycin, were isolated from Streptomyces olivaceus (Brown et al., 1977; Butterworth et al., 1979; Hood et al., 1979). Other carbapenems include epithienamycins from Streptomyces flavogriseus, the PS series from Streptomyces cremeus subspecies auratilis, the asparenomycins produced by Streptomyces tokunonensis and Streptomyces argenteolus, and the carpetimycins (Kitano, 1983).

The nocardicins were the first monocyclic beta-lactam antibiotics isolated. These antibiotics were originally extracted from Nocardia uniformis subspecies tsuyamanensis using a beta-lactam hypersensitive mutant of Escherichia coli (Ogawara, 1981; O'Sullivan and Ball, 1983; Kitano, 1983).

A similar screening procedure led to the discovery of the first beta-lactam antibiotics produced by unicellular bacteria. Sulfazecin is synthesized by Pseudomonas acidophila, while isosulfazecin is produced by Pseudomonas mesoacidophila (Kitano, 1983). A similar group of antibiotics, known as the monobactams, have been isolated from Gluconobacter strains, Acetobacter strains, Chromobacterium violaceum and Agrobacterium radiobacter (Kitano, 1983; O'Sullivan and Ball, 1983).

The beta-lactam antibiotics can be classified into at least five groups: the penicillins, the cephalosporins, the clavams, the carbapenems and the monocyclic beta-lactams (O'Sullivan and Ball, 1983). The fungi are limited to the production of penicillins and cephalosporins, while the monobactams are produced solely by Gram-negative eubacterial species. Nocardicins have been isolated from both Nocardia and Streptomyces species. No beta-lactam antibiotic production has been observed in yeasts and Gram-positive eubacteria. Thus, the streptomycetes, the major producer of these antibiotics, are the most important source of

novel, natural beta-lactam antibiotics (Kitano, 1983).

The genus Streptomyces belongs to the family Streptomycetaceae. They are aerobic actinomycetes exhibiting extensive branching substrate and aerial mycelia. These soil-dwelling micro-organisms possess a type 1 cell wall composition and produce chains of spores on the aerial mycelium (Cross and Goodfellow, 1973). Besides being a major source of beta-lactam antibiotics, Streptomyces produce aminoglycosides, macrolides, tetracyclines, polyethers, ansamycins and peptide antibiotics (Chater et al., 1982a). Moreover, Streptomyces produce a greater assortment of extracellular hydrolytic enzymes than E. coli, including proteases, amylases, cellulases, lipases and nucleases (Chater et al., 1982a).

Novel antibiotics will always be required to combat natural and, more importantly, acquired antibiotic resistance in microbial pathogens. Although new antibiotics are still discovered with the use of current screening procedures, it is becoming increasingly difficult to isolate unusual antibiotics. Recently, great progress has been made in the application of recombinant DNA technology in the field of Streptomyces genetics (Hopwood et al., 1983a; Martin and Gil, 1984). Although the elucidation of the molecular genetics of antibiotic production in Streptomyces is presently at an early stage, revolutionary strategies may

already be deployed in the search for novel, natural antibiotics.

DNA isolated from an antibiotic-producing streptomycete, introduced into other distinct Streptomyces species, may well lead to the discovery of novel antibiotics. The creation of uncommon antibiotic-producing gene pools, together with the low specificity characteristic of enzymes involved in secondary metabolism, may result in a shift in the biosynthetic pathway of the organism to produce a different end-product (Hopwood et al., 1983a; Thompson and Davies, 1984; Martin and Gil, 1984). As no interspecific barriers, with regard to transcription and translation, have been found to occur in Streptomyces, it is likely that foreign DNA will be expressed in these hosts (Hopwood et al., 1983a). The introduction of genes coding for rate-limiting enzymes involved in antibiotic production could result in increased yields of industrially important antibiotics (Martin and Gil, 1984; Thompson and Davies, 1984), as well as the isolation of antibiotics normally produced in amounts too low to detect (Hopwood et al., 1983a; Thompson and Davies, 1984). Similar techniques may permit the production of semisynthetic antibiotics more economically; for example, the aminoglycoside antibiotic amikacin, chemically synthesized from kanamycin, could be synthesized in a kanamycin-producing streptomycete after the organism was transformed with the gene coding for the production of hydroxyaminobutyric acid (Thompson and Davies, 1984).

As antibiotics are produced by a large number of Streptomyces strains, a very broad host range vector or many host-vector systems will have to be developed to facilitate the manipulation of antibiotic production genes (Martin and Gil, 1984). A number of both low- and high-copy number plasmids, as well as phage ϕ C31-derived vectors, have been constructed and proved useful in gene cloning in Streptomyces (Hopwood et al., 1983a; Martin and Gil, 1984). These vectors may be deployed in the genetic manipulation of a number of Streptomyces species including Streptomyces lividans, Streptomyces coelicolor A3(2), Streptomyces acrimycini, Streptomyces albus, Streptomyces ambofaciens, Streptomyces aureofaciens, Streptomyces azureus, Streptomyces fradiae, S. clavuligerus, S. griseus, Streptomyces parvulus and Streptomyces rimosus (Martin and Gil, 1984). In addition, the majority of cloning studies have been performed in S. lividans 66 and S. coelicolor A3(2), while Streptomyces kasugaensis M518, S. ambofaciens and Streptomyces griseofuscus C581 are potentially useful as hosts for specific plasmids (Martin and Gil, 1984).

1.2. Aim of this study

S. cattleya NRRL 8057 produces cephamycin C, penicillin N, thienamycin, N-acetylthienamycin and N-acetyldehydrothienamycin (Kahan et al., 1979; Bushell and Fryday, 1983). S. olivaceus has

been shown to synthesize the olivanic acids MM-4550, MM-13902, MM-17880, as well as epithienamycin A (MM-22380), epithienamycin B (MM-22382), epithienamycin C (MM-22381) and epithienamycin D (MM-22383) (Aoki and Okuhara, 1980). The construction of a DNA cloning vector, able to transform these Streptomyces species, would allow the genetic study of the biosynthetic pathways involved in the production of the carbapenem antibiotics and the isolation of novel, natural antibiotics with the aid of gene cloning techniques. As no endogenous plasmids have currently been reported in S. cattleya and S. olivaceus, it would be useful to isolate a broad host range actinophage which could be utilized as a potential DNA cloning vector in these Streptomyces species. A DNA cloning vector, incorporating the replication region of the phage genome and a Streptomyces antibiotic-resistance gene, would have a number of advantages. The use of the replicon of a broad host range actinophage would enable replication of the DNA cloning vector in a variety of Streptomyces species which are susceptible to infection by the intact phage. If the replicon originated from a virulent actinophage, the DNA cloning vector would be replicated at a high copy number, allowing amplification of inserted foreign DNA. In addition, the absence of the phage lytic genes would ensure survival of the host organism, while the lack of a phage capsid would remove packaging constraints. Finally, ligation of the phage replicon to an antibiotic-resistant marker would permit direct selection of

transformants.

A fundamental requirement for the application of recombinant DNA technology to S. cattleya and S. olivaceus, is their ability to take up foreign DNA. Thus, protoplast formation, transfection and protoplast regeneration of these Streptomyces strains will be investigated.

An important academic and industrial problem is the characteristic genetic instability in S. cattleya with respect to antibiotic production, growth rate, aerial mycelium production and arginine biosynthesis (Coyne et al., 1984). The observation that genetic instability is induced by UV light in a number of Streptomyces species (Freeman et al., 1977; Coyne et al., 1984) implies that DNA repair systems may be involved in this phenomenon. As phages such as lambda are dependent on host-mediated DNA repair systems, the availability of a phage infectious to S. cattleya would enable investigation of DNA repair in this streptomycete by the examination of the survival of UV-damaged phage propagated on untreated and UV-irradiated S. cattleya spores. Moreover, the effect of chemical inhibitors of E. coli DNA repair systems on the survival of UV-treated phage could be studied. Thus, it may be possible to obtain an insight into the relationship between UV light and genetic instability in S. cattleya.

Portions of the E. coli tufA gene, which encodes the polypeptide chain elongation factor Tu, have been shown to be highly conserved amongst evolutionary distinct procaryotes (Filer and Furano, 1980; Filer and Furano, 1981). Utilization of the E. coli tufA gene as a probe in order to identify the tuf gene of S. cattleya and S. olivaceus will permit further investigation into the nature of Streptomyces gene structure and regulation. As the Bacillus subtilis and E. coli tuf genes are closely associated with the ribosomal protein gene cluster (Smith and Paress, 1978; Filer and Furano, 1980), the detection of the tuf gene in S. cattleya and S. olivaceus will facilitate the isolation and study of the ribosomal protein genes of these Streptomyces species. Moreover, as the E. coli tuf genes are highly expressed (Yokota et al., 1980; van der Meide et al., 1983), utilization of the Streptomyces tuf gene promoter in an expression vector would be advantageous for the elucidation of gene function in S. cattleya and S. olivaceus.

Thus, these four areas were investigated in order to establish a base from which the genetic and biochemical processes inherent in these important beta-lactam antibiotic-producing streptomycetes could be elucidated. As each of these four aspects of investigation cover complex topics, a comprehensive review of the literature has been included at the beginning of each chapter.

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF AN ACTINOPHAGE VIRULENT AMONGST BETA-LACTAM ANTIBIOTIC PRODUCING STREPTOMYCES

SUMMARY

Actinophage VC11 is able to virulently infect 11 of the 20 Streptomyces strains tested. Examination of VC11 infection of S. cattleya, S. olivaceus and S. lividans TC10 indicated the absence of restriction-modification systems which affect VC11 infectivity of these Streptomyces strains.

VC11 fits the B1 group of Bradley's morphological phage classification system, consisting of an icosahedral head (47 x 47 nm) and a long non-contractile tail (196 x 7.8 nm). The VC11 genome is comprised of a double-stranded DNA molecule which was estimated to be 45.2 kb in length and found to possess a G+C content of 50.4%. The presence of a cos site indicates that the phage genome replicates according to the rolling circle model. Although restriction enzyme analysis of VC11 DNA revealed the presence of numerous recognition sequences for 16 endonucleases and the absence of restriction sites for a further 21 restriction enzymes, partial-digestion of the phage DNA with BglII allowed

the construction of a linear restriction map of the VC11 genome. Complete BglIII digestion of VC11 DNA generated 5 fragments of 23.2, 11.1, 7.6, 2.6 and 0.65 kb in length.

2.1. INTRODUCTION

As is the case with other bacterial genera, Streptomyces are infected by a wide variety of bacteriophages known as actinophages. Lanning and Williams (1982) detected up to 2×10^4 plaque-forming units (p.f.u.) per gram of soil, indicating the widespread occurrence of actinophage in the natural habitat of Streptomyces. Indeed, Streptomyces phages can be isolated from most soils of pH greater than 5 (Lomovskaya et al., 1980).

The majority of the actinophages isolated belong to the B1 group of Bradley's morphological phage classification system (Bradley, 1967; Ackermann, 1978). Phages belonging to this classification group possess polyhedral heads and long non-contractile tails (VP5; VP7; ϕ C31). Three actinophages (MSP8, MSP2 and R2) possess elongated heads and long non-contractile tails, conforming to Bradley's group B2, while the phage ϕ 17 is a member of the C1 morphological group, exhibiting a polyhedral head and a very short tail (Berthiaume and Ackermann, 1977). No actinophages which lack tails, have contractile tails, or are filamentous have

been isolated (Lomovskaya et al., 1980). In addition, all actinophages contain double-stranded DNA and no icosahedral RNA phages or sex-phages have been isolated (Hopwood et al., 1973).

Both virulent and temperate phages infectious to Streptomyces species have been isolated. The broad or narrow host range of these phages is determined by adsorption specificity and host-specific restriction and modification systems (Lomovskaya et al., 1980). Okanishi et al. (1968) demonstrated that the removal of the cell walls of Streptomyces violaceoniger and Streptomyces acidomyceticus, naturally resistant to phage PK-66 infection, allowed infection by phage PK-66 DNA. The limitation of actinophage host range by host-specific restriction-modification systems is due to the occurrence of restriction enzymes which protect many Streptomyces species against invading foreign DNA (Roberts, 1983; Cox and Baltz, 1984). Host-specific restriction-modification systems will be discussed in greater detail below.

Dowding (1973) showed that the efficiency of plating (e.o.p.) of the virulent phage VP11 was affected by the age of the Streptomyces host. In the case of both S. coelicolor A3(2) and Streptomyces glaucescens, the highest e.o.p. of VP11 was obtained with spore suspensions of these phage hosts. Rosner and Gutstein (1981) examined the adsorption of actinophage Pal 6 to S. albus

mycelia of varying age. The authors found that phage adsorption to germinating spores increased with age, being maximal at approximately 4 h after the start of germination. Thus, the most efficient phage adsorption occurs in the presence of freshly germinated spores.

One-step growth experiments have been performed with a variety of Streptomyces phages (Lomovskaya et al., 1980). These experiments have indicated a 40 to 60 min latent period, followed by a 40 to 60 min rise period, and finally, a burst size of 10 to 50 p.f.u. for the majority of actinophages. However, some exceptions do exist. For example, the S. rimosus phage RP2 has a 360 min latent period, a rise period of 240 min and a subsequent burst size of up to 120 p.f.u. (Hranueli et al., 1979). One of the problems in performing quantitative studies with actinophages is the mycelial nature of their hosts. As germinating spores age, the young mycelia begin to grow around one another to form large mycelial clumps. Although each mycelial clump plates as one infective centre, they are able to attach large numbers of phage particles and consequently release many individual phage bursts, thus creating an artificially high burst size (Dowding, 1973; Rosner and Gutstein, 1982).

The temperate phage ϕ C31 is the most extensively studied actinophage. Temperate actinophages have been isolated directly

from soil enrichment cultures, for example VP5 (Dowding and Hopwood, 1973), or as a resident prophage. ϕ C31 was originally isolated by the latter method when S. coelicolor A3(2) liquid cultures were propagated on lawns of Streptomyces anthocyanicus 31 and S. lividans 66 and 130 (Lomovskaya et al., 1980). The phage consists of a polyhedral head 53 nm wide, a long non-contractile tail 100 x 5 nm, a basal plate 15 nm in diameter with at least one pin, and a prominent knot between the head and tail (Suarez et al., 1984). In addition, 17 polypeptides have been detected, four of which (51, 38.5, 29.5 and 28 kDa) comprise 84% of the total protein of the phage particle (Suarez et al., 1984).

ϕ C31 is thought to adsorb to (as yet unidentified) surface receptors on the cell wall of susceptible hosts, particularly to young mycelia of recently germinated spores (Lomovskaya et al., 1980). The ϕ C31 genome consists of a linear double-stranded DNA molecule of 41.2 kb in length (Chater et al., 1981c). The presence of cohesive ends at either end of the linear genome suggests that, following penetration into the host cell via an unknown mechanism, the cohesive ends ligate to generate a covalently closed circular molecule (Lomovskaya et al., 1980).

The phage establishes lysogeny by insertion into the S. coelicolor A3(2) chromosome at an attachment site located between

the pheA1 and uraA1 markers (Lomovskaya et al., 1980). The chromosomal attachment site of the heteroimmune temperate phage VP5 was mapped between hisE and argD in another region of the S. coelicolor A3(2) genome (Dowding and Hopwood, 1973). The isolation of clear-plaque ϕ C31 mutants which were unable to lysogenize their host, indicated the presence of a gene (c gene) which synthesizes a repressor protein for establishing and maintaining lysogeny (Lomovskaya et al., 1980). Heteroduplex analysis of ϕ C31 deletion mutants allowed the mapping of the c gene to a 1.7 kb DNA fragment located in the centre of the ϕ C31 genome (Sladkova et al., 1980a). In addition, the gene (att P site) at which insertion of the ϕ C31 prophage into the host chromosome occurs, was found to be situated at a distance of 2.4 kb from the right end of the linear DNA molecule (Sladkova et al., 1980b; Chater et al., 1982b). It is thought that the product(s) of this gene allows a recombination event by recognizing particular base sequences in the phage and host DNA (Lomovskaya et al., 1980). ϕ C31 lysogens produce phage spontaneously and are immune to superinfection. A reduction in the effective concentration of repressor protein results in the induction of the prophage in non-dormant cells. The action sites of the repressor protein are not known. ϕ C31, VP5 and R4 prophages are not induced by UV light, while a low level of UV induction of the temperate phages SH10 and SV2 has been detected (Lomovskaya et al., 1980; Stuttard, 1982).

Very little information concerning the events following induction of the prophage is available. As the h gene affects phage adsorption, Lomovskaya et al. (1980) suggested that this gene specifies a component of the end of the phage tail and that the closely-linked N and E genes may also encode tail components. Thus, the time of expression of the N and E genes (20 to 25 min) may indicate the time of expression of genes for tail components.

The homoimmune phages ϕ C43 and ϕ C62, detected as prophages in S. lividans 803 and Streptomyces caesins 55, were found to be serologically related, have identical specificity of immunity, and similar genomes to ϕ C31 (Sladkova et al., 1978). Heteroduplex analysis of these phages showed 93-96% homology between the DNA of these actinophages and that structural changes occurred exclusively in the right-hand portion of their genomes. Thus, it seems probable that the late functions may all be specified by the left half of the phage genome, with the genes for some early functions (B, L) more centrally located, closely linked to the c gene (Lomovskaya et al., 1980).

The presence of cohesive ends on either side of the ϕ C31 DNA molecule, indicates that the circular phage genome replicates by the rolling-circle model to produce concatameric DNA molecules which are subsequently cleaved during packaging by a site-specific endonuclease to generate linear DNA monomers.

Mature phage particles are released 40 to 50 min after the initiation of the lytic cycle (Lomovskaya et al., 1980).

An endemic problem in the application of recombinant DNA technology in Streptomyces, is the widespread occurrence of restriction-modification systems in these organisms which limit the interspecific transfer of DNA cloning vectors. DNA restriction is considered to be a strain-specific defence mechanism against foreign genetic material that penetrates a particular cell (Arber, 1974). Foreign genetic material may enter a cell during uptake of free DNA by transformation, by transfer of cellular DNA in sexual conjugation, and phage infection. Restriction endonucleases encoded by either chromosomal or plasmid genes, interact with double-stranded DNA at specific recognition sites resulting in cleavage of the DNA into a number of fragments. A bacterial strain can protect its own DNA from cleavage by its restriction endonucleases by the action of a DNA modification methylase. Both the restriction enzyme and methylase of a specific cell recognize the same base sequences on the substrate DNA.

Closely related bacteria often differ in their restriction-modification specificities (for example, the E. coli strains K12 and B) and two unrelated restriction-modification systems can be expressed in a single strain (Arber, 1974).

Indeed, Streptomyces are a rich source of restriction enzymes. Twenty-one well-characterized and eight less-studied restriction endonucleases have been isolated from Streptomyces species, while Streptomyces stanford has been shown to contain at least 4 restriction enzymes (Roberts, 1983).

The occurrence of a restriction system in S. coelicolor A3(2) was suggested by the greatly reduced frequency of conjugal transfer of the plasmid SCP1 from S. lividans to S. coelicolor as opposed to transfer in the opposite direction (Hopwood and Wright, 1973). In addition, plasmid or phage DNA propagated in E. coli can be introduced into S. lividans 66, but not directly into S. coelicolor A3(2) (Hopwood et al., 1983b).

Bacterial restriction-modification systems were originally discovered in research with bacteriophage (Arber, 1974). In each case, it was observed that the passage of a phage through one bacterial host greatly reduced its e.o.p. on a second bacterial host. Bacteriophages have subsequently proved to be extremely useful tools for the genetic study of restriction-modification systems both in vivo and in vitro.

A number of Streptomyces species have been found to possess restriction-modification systems which act on actinophage DNA. The virulent phage Pal 6 was shown to be restricted by S. albus G

2396 following growth on S. albus 1160 (Chater and Wilde, 1976). The e.o.p. of unmodified Pal 6 (last propagated on strain 1160) was reduced by 40-fold in comparison to modified Pal 6 (formerly grown on strain 2396). In addition, S. albus G has been shown to produce at least two restriction enzymes, one of which (SalI) recognizes the nucleotide sequence GTGAC (Roberts, 1983). Unmodified Pal 6 DNA was restricted by SalI, isolated from a cell-free extract of S. albus G 2396, at a single cleavage site, while modified Pal 6 DNA was almost completely protected from endonuclease digestion in vitro (Chater and Wilde, 1976). A restriction-deficient mutant of S. albus G 2396 was found to lack the SalI restriction enzyme and consequently was highly sensitive to infection with unmodified Pal 6 phage.

In addition, Voeykova et al. (1980) demonstrated that the restriction-modification system of S. albus G acts on the phage ϕ C31 following its propagation on the indicator strain S. lividans 66. However, passage of ϕ C31 from S. lividans 66 to a S. albus G $r^- m^-$ mutant resulted in an e.o.p. identical to that on S. lividans 66, indicating that the S. albus G mutant had become deficient in the production of the restriction enzyme SalI. ϕ C31 was again restricted by wild-type S. albus G after transfer from the restriction-modification deficient S. albus G mutant. Restriction enzyme analysis of ϕ C31 DNA demonstrated the presence of approximately 20 SalI restriction sites (Sladkova and

Rebentish, 1980; Chater et al., 1981c).

The presence of the second restriction-modification system, mediated by the restriction enzyme SalII, in S. albus G was detected with the temperate phage Pg2 (Voeykova et al., 1980). Transfer of the phage Pg2 from S. griseus Krl5 to S. albus G resulted in an e.o.p. of $< 10^{-9}$. However, the phage developed with an e.o.p. of 10^{-4} on the SalI-deficient S. albus G mutant, indicating that the second restriction-modification system of S. albus G was responsible for the reduced propagation efficiency of the phage.

Similarly, Chater and Carter (1978) demonstrated restriction and modification of a mutant of the wide host range temperate phage R4 by S. albus P which produces the restriction enzyme SalPI. Unmodified DNA of the R4 mutant was cleaved in the presence of a cell-free extract containing SalPI, while modified DNA of the mutant was relatively resistant.

Voeykova et al., (1979) identified a restriction-modification system in S. lividans 67 which acted on the temperate phage VP5. As restricted VP5 phage was subsequently able to propagate on S. coelicolor A3(2) (known to possess a restriction system acting on the plasmids SCP1 and SCP2; Lomovskaya et al., 1980) and S. lividans 67 at its original e.o.p., the authors speculated that

either the restriction-modification systems in these Streptomyces species are identical or the phage VP5 does not possess recognition sites for the restriction-modification system of S. coelicolor A3(2).

Although interspecific transfer of the plasmid SCP1 indicated that S. coelicolor A3(2) possesses a restriction-modification system (Hopwood and Wright, 1973), attempts to detect this system with the use of actinophages ϕ C31 and VP5 had been unsuccessful (Voeykova et al., 1979). However, a hybrid strain (Rcg 2), obtained by recombination between S. coelicolor A3(2) and S. griseus Kr 15, was able to restrict the narrow host range virulent phage Pg81 (Lomovskaya et al., 1977). The hybrid strain Rcg2 had inherited the restriction-modification system, streptomycin resistance, and the ability to adsorb phage VP5 from S. coelicolor A3(2), while several chromosomal markers and the ability to adsorb phage Pg81 were inherited from S. griseus Krl5.

Further analysis showed that Rcg2 restricted Pg81 following growth on S. griseus Krl5, while Pg81 modified by Rcg2 was subsequently restricted by S. griseus Krl5 (Voeykova et al., 1979). Pg81 modified by S. griseus Krl5 was restricted by S. griseus Kr20 and Rcg2, while phage obtained from the latter two strains was again restricted by S. griseus Krl5. Pg81 modified by S. griseus Kr20 was not restricted by Rcg2 and vice versa.

Passage of Pø81 from Streptomyces griseofovillus 43 to either S. griеus Krl5 or Rcg2, and subsequently to S. griseofovillus 43, abolished restriction of the phage by the latter strain. In addition, when Pø81 phage modified by S. griеus Krl5 was transferred from S. griseofovillus 43 to either S. griеus Krl5 or Rcg2, no host-mediated restriction by S. griеus Krl5 was observed, while the latter restricted the phage. In contrast, Rcg2 modified Pø81, transferred from S. griseofovillus 43 to either S. griеus Krl5 or Rcg2, was restricted by the former strain and partially restricted by the latter.

From these complex results, the authors suggested that the restriction-modification systems of Rcg2 and S. griеus Kr20 are identical, the restriction-modification systems of Rcg2 and S. griеus Krl5 are different, and the recognition sites of the restriction-modification system of S. griseofovillus 43 overlaps with those of the restriction-modification systems of S. griеus Krl5 and Rcg2 (Voeykova et al., 1979; Voeykova et al., 1980). These conclusions are supported by the observation that the restriction enzyme SgrII, isolated from both Rcg2 and S. griеus Kr20, restricted DNA from Pø81 modified by growth on S. griеus Krl5, while DNA obtained from phage grown on either S. griеus Kr20 or Rcg2 was not cleaved by the restriction endonuclease (Voeykova et al., 1980).

ϕ C31 propagated on the restriction-deficient S. albus G mutant sal43r^{-m}⁺ was restricted by S. coelicolor A3(2), but not by S. lividans 66 (Lomovskaya et al., 1982). The restriction enzyme SgrII, not detected in cell extracts of S. coelicolor A3(2), was isolated from Rcg2 (which supposedly had inherited the S. coelicolor A3(2) restriction-modification system) and found to restrict DNA of ϕ C31 propagated on the mutant sal43r^{-m}⁺. However, DNA prepared from ϕ C31 phage grown either on S. lividans 66 or S. coelicolor A3(2) was not cleaved by SgrII, suggesting that the recognition sites of this restriction enzyme undergo modification in these strains (Lomovskaya et al., 1982).

Stuttard (1982) detected four different restriction-modification systems in four closely-related strains of S. venezuelae which affect the temperate phages SV1 and SV2. Both phages propagated on S. venezuelae 13s were restricted in each of the other three strains, while S. venezuelae 13s restricted phage previously propagated in the other hosts, indicating that S. venezuelae 13s has a unique host specificity. The S. venezuelae strains PDO4828 and ATCC 10712 showed a second restriction-modification system since neither strain restricted phage propagated in the other strain, but both restricted phage isolated from S. venezuelae 13s. This restriction-modification system was also present in S. venezuelae ATCC 10595 which contained a third restriction-modification system acting on phage SV2. SV1 was not

affected by the unique restriction-modification system of S. venezuelae ATCC 10595 as the phage lacked target sites specifically recognized by this system. Restriction of SV2, previously propagated on S. venezuelae ATCC 10595, by the S. venezuelae strains PDO4828 and ATCC 10712, suggested the existence of a fourth restriction-modification system in the latter two strains. The author postulated that SV1 was not restricted due to the absence of specific nucleotide sequences recognized by this system.

Voeykova et al. (1980) utilized 15 actinophages to investigate the presence of restriction-modification systems in 120 strains representing 70 species of the genus Streptomyces. However, restriction-modification systems were observed with only 5 of the 15 phages. On the basis of the transfection of S. violaceoniger and S. acidomyceticus with DNA isolated from the phage PK-66 which is normally unable to infect these Streptomyces species (Okanishi et al., 1968), the authors attempted to detect restriction-modification systems by transfecting Streptomyces protoplasts with phage DNA. Thus, transfection of S. griseus Krl5 protoplasts with ϕ C31 DNA resulted in the detection of the restriction-modification system in this micro-organism which is not normally infected by the phage ϕ C31. The transfection frequency of S. coelicolor A3(2) and S. griseus Kr 15R⁻ (restriction-deficient mutant) was identical, while the frequency

of transfection of S. griseus Krl5 protoplasts was greatly reduced (Voeykova et al., 1980). Thus, it is possible that restriction-modification systems are widespread in Streptomyces and the use of protoplast transfection will facilitate further detection of these systems in other strains.

Cox and Baltz (1984) analyzed the relationship between the failure of phages to infect Streptomyces hosts and the susceptibility of the DNA of these phages to cleavage by the restriction enzymes produced by the corresponding hosts. The authors noted that each of the Streptomyces hosts that produced characterized restriction enzymes exhibited a unique pattern of plaque-formation by the 10 phages, while each phage differed from the others in plaque-formation patterns on the 8 hosts. In addition, Streptomyces species which contained a restriction enzyme did not allow infection by a phage containing more than one recognition site for that particular enzyme and phage lacking recognition sequences were able to infect Streptomyces species which produced the corresponding restriction enzyme. The overall frequency of plaque-formation by the 10 phages on the 8 hosts which produced restriction enzymes was 0.55. Similarly, the overall frequency of plaque-formation by the 10 phages on 21 species uncharacterized for restriction enzyme production was 0.44 indicating that although only approximately 50% of potential phage infections were prevented, restriction endonucleases in

Streptomyces function to restrict phage development and production of these restriction enzymes may be very widespread amongst members of this genus. Moreover, as the authors scored only very inefficient plaque-formation as negative, restriction may inhibit a greater percentage of potential phage infections (Cox and Baltz, 1984).

Restriction enzyme analysis of phage DNA allows the identification of physically-defined segments of the genome and an assessment of the potential of the molecule as a DNA cloning vector can be made. Restriction profiles of DNA isolated from various phages have shown an abnormal distribution of restriction sites. Thus, ϕ C31 DNA contains no BamHI, XhoI, BglII, SstI or PstI sites and more than 15 SalI, SacI, SmaI, AvaI, HgiAI, FspAI sites (Sladkova and Rebentish, 1980; Chater et al., 1981c; Harris et al., 1983), the DNA of the temperate phage SP10 possesses no EcoRI, BamHI, SmaI, HindIII, PstI, StuI or XbaI sites and more than 20 SalI, SacI, SacII, HaeIII, HhaI, HpaII sites (Isogai et al., 1982), R4 phage DNA includes no SmaI, SlaI (XhoI), BamHI, HindIII or XbaI sites and more than 30 SalI, SacI, SacII sites (Takahashi et al., 1982), and the DNA of the temperate phage SH10 has no BamHI, PstI, SmaI, HpaI, XhoI, HindIII, XbaI, SstI or SacII sites and more than 9 BglII, HindII, SalI, BalI recognition sites (Walter et al., 1981).

On average, restriction enzymes which recognize adenosine-thymine (A+T) rich sequences produce very few cuts in phage DNAs with a high guanine-cytosine (G+C) content, but digest A+T rich phage DNA to a greater degree than would normally be expected. However, actinophage DNA is not digested to any large extent by restriction enzymes which recognize G+C rich sequences, irrespective of base composition (Lomovskaya *et al.*, 1980). Thus, Pal6 DNA, which has a G+C content of 55%, includes more than 40 EcoRI and HindIII sites (recognize A+T rich sequences), while the G+C rich (67%) DNA of phage VP12 is not digested by these two restriction enzymes. In addition, neither phage DNA contains restriction sites for the endonucleases BamHI and PstI, both of which recognize G+C rich sequences (Lomovskaya *et al.*, 1980).

Analysis of the DNA of three actinophages for the presence of recognition sequences for restriction enzymes produced by Streptomyces species showed a significant bias for either the existence of many or no restriction sites for the 6 endonucleases examined (Cox and Baltz, 1984). The authors suggested that Streptomyces phages have evolved to totally bypass certain restriction systems by eliminating all restriction sites recognized by specific endonucleases produced by Streptomyces in accordance with the theory that restriction enzyme production is the major determinant for broad host range actinophage

specificity in Streptomyces species. Restriction sites not recognized by the endonucleases produced by the host strains are consequently accumulated.

The majority of actinophage DNAs possess large numbers of SalI recognition sites. Since this restriction enzyme is produced by S. albus G, it would be expected that phages would have attempted to eliminate the SalI recognition sequences, which occur at higher frequencies than expected in phage genomes with a G+C content greater than 60%, in order to resist restriction by this host (Lomovskaya et al., 1980). Indeed, S. albus G has been found to be resistant to infection by the virulent phages VP8, VP11, VP12, VP13 and f6, and the temperate phages VP5 and ϕ C31 (Chater and Wilde, 1976). Thus, the nucleotide sequence recognized by SalI restriction enzyme may have some important function in the DNA of these phages (Lomovskaya et al., 1980).

The virulent phage FP22, isolated by Cox and Baltz (1984), is the first actinophage reported to lack SalI restriction sites and therefore infects S. albus G at a high e.o.p. Moreover, the phage was able to virulently infect 21 of the 29 species tested by the authors, including 6 of the 8 species which produce well-characterized restriction enzymes. The genome of FP22 is unique amongst Streptomyces chromosomes, plasmids and phage DNAs as it has a relatively low G+C content of 49%, suggesting that

FP22 may have been genetically better suited to eliminate more potential restriction sites during the course of evolution than phages with high G+C contents (Cox and Baltz, 1984). Thus, restriction endonucleases that cleave G+C rich nucleotide sequences probably function to restrict actinophage infection of Streptomyces species in the natural soil environment and consequently, phage genomes have evolved to maximize the probability of bypassing many of these restriction systems (Cox and Baltz, 1984).

S. cattleya NRRL 8057 and S. olivaceus ATCC 31126 produce the commercially important antibiotics thienamycin and olivanic acid respectively (Kahan et al., 1979; Butterworth et al., 1979). Restriction-modification systems which act on the S. albus P phage FF4 were detected in S. cattleya, S. olivaceus and S. lipmanii (Flett et al., 1979). FF4, modified on any one of the three hosts, exhibited a high e.o.p. on the remaining Streptomyces species, indicating that all three strains possess restriction-modification systems of similar specificity.

In view of the successful application of molecular genetics to S. coelicolor A3(2) and S. lividans 66 (Hopwood et al., 1983a), the development of a DNA cloning vector for S. cattleya and S. olivaceus would provide a bridge for the extension of recombinant DNA technology to these Streptomyces species. An endogenous DNA

cloning system could facilitate the elucidation of the genetic and biochemical pathways for the production and regulation of antibiotic biosynthesis in these species, and subsequently, would lead to new possibilities for the development of novel antibiotics and for strain improvement (Suarez and Chater, 1981). Since no endogenous plasmids have currently been reported in these strains, it is necessary to construct a phage vector which would allow the transfer and amplification of DNA sequences in both S. cattleya and S. olivaceus. Such a cloning vector would rely on the isolation of a phage which is resistant to the restriction-modification systems present in these Streptomyces species.

The availability of a phage capable of infecting S. cattleya and S. olivaceus would provide several additional advantages. The incorporation of artificial target sites for the restriction systems in these micro-organisms would facilitate the isolation of restriction-deficient host mutants (Lomovskaya et al., 1980) which would consequently broaden the scope of genetic manipulation of these important antibiotic-producing streptomycetes. In addition, a phage could be utilized for the investigation of the genetics and biochemistry of differentiation, the elucidation of UV repair mechanisms, and the identification of nonsense suppressors (Dowding, 1973).

Thus, soil samples were screened for an actinophage which exhibited a high e.o.p. on S. cattleya and S. olivaceus. Subsequently, the phage was characterized with respect to morphology, host range and the nature of its genome.

2.2 METHODS

2.2.1 Bacterial strains

The Streptomyces strains utilized are indicated in Table 1.

2.2.2 Preparation of Streptomyces spore suspensions

Sterilized 20% (v/v) glycerol (5 ml) was introduced to each malt-3 agar slant of sporulated Streptomyces colonies. The spores were resuspended by whirly-mixing and subsequently filtered through non-absorbent cottonwool to remove the mycelia. The spore suspensions, containing approximately 10^7 colony-forming units (c.f.u.) per ml, were stored at -20°C with no resulting loss in viability.

2.2.3 General phage techniques

Phage assays were performed using the double-layer agar

TABLE 1. STREPTOMYCES STRAINS

Strain	Source	Antibiotic
<u>S. alboflavus</u>	U.C.T. Stock Collection	oxytetracycline
<u>S. ambofaciens</u> ATCC 15154	American Type Culture Collection	spiramycins I, II, III and congocidin
<u>S. argenteolus</u> ATCC 11009	American Type Culture Collection	vitamin B12
<u>S. aureofaciens</u> ATCC 10762	American Type Culture Collection	chlortetracycline
<u>S. cattleya</u> NRRL 8057	Glaxo Research Ltd.	thienamycin
<u>S. chartreusis</u> ATCC 14922	Squibb	cephamycin A and B
<u>S. cinnamomensis</u> ATCC 15413	American Type Culture Collection	monensin
<u>S. coelicolor</u> ATCC 19832	American Type Culture Collection	none
<u>S. fulvoviridis</u> ATCC 21954	American Type Culture Collection	beta-lactamase inhibitors
<u>S. griseus</u> ATCC 10137	American Type Culture Collection	streptomycin
<u>S. griseus</u> ATCC 11429	American Type Culture Collection	streptomycin
<u>S. griseus</u> ATCC 31031	American Type Culture Collection	deacetoxy- cephalosporin C
<u>S. griseus</u> W4	Squibb	streptomycin
<u>S. hygroscopicus</u> ATCC 31039	American Type Culture Collection	deacetoxy- cephalosporin C
<u>S. lipmanii</u> ATCC 27357	American Type Culture Collection	penicillin N
<u>S. lividans</u> TC10	John Innes Institute	none
<u>S. olivaceus</u> ATCC 21397	American Type Culture Collection	olivanic acid
<u>S. parvulus</u> ATCC 12434	American Type Culture Collection	dactinomycin
<u>S. sporophyticus</u>	Glaxo Research Ltd.	none
<u>S. tanashiensis</u> ATCC 23967	American Type Culture Collection	luteomycin

technique. Phage dilutions were prepared in soft nutrient agar prior to the addition of spores. A phage suspension (2 ul) and 125 ul of a Streptomyces spore suspension were mixed in 2 ml soft nutrient agar at 42°C. The mixed suspension was subsequently overlaid onto nutrient agar supplemented with 0.5% (w/v) glucose and 8 mM $\text{Ca}(\text{NO}_3)_2$. The plates were incubated at 30°C for 12 h or until a confluent lawn of growth was produced by the spores of the corresponding Streptomyces host in the absence of phage.

2.2.4 Phage isolation

Five soil samples (obtained from the slopes of Table Mountain, Cape Town) were assayed for actinophage infectious to S. lividans TC10 and S. olivaceus using the specific enrichment technique developed by Dowding (1973). Each soil sample (10 g) was placed in 20 ml nutrient broth containing 0.5% (w/v) glucose and 8 mM $\text{Ca}(\text{NO}_3)_2$. The suspension was subsequently inoculated with 1 ml of either a S. olivaceus or a S. lividans TC10 spore suspension and incubated at 30°C on an orbital shaker for 12 h. Each sample was filtered through Whatman No.3 filter paper and subsequently through a 0.22 um Millipore filter to ensure the removal of the soil and bacteria. Serial dilutions (100-fold) of each filtrate were prepared in 2 ml soft nutrient agar at 42°C and plated in the presence of a spore suspension of the original Streptomyces species utilized in the enrichment step by the method described

in section 2.2.3. Following overnight incubation at 30°C, a single large clear plaque was removed from each enrichment with a sterile toothpick, transferred to 1 ml of nutrient broth and further purified by a number of passages on the original Streptomyces host.

2.2.5 Confluent lysates

Purified phage strains were plated at 10^0 and 10^{-1} on the appropriate Streptomyces host and incubated overnight at 30°C. Plates exhibiting confluent lysis were flooded with 2 ml nutrient broth, the soft nutrient agar removed with a glass rod and incubated at room temperature for 2 h. The agar was extracted by centrifugation at 12,000 rpm for 15 min in a SS 35 rotor. The supernatant was sterilized by filtration through a 0.22 μ m Millipore filter and stored at 4°C.

2.2.6 Isolation of virulent phage

The high-titred phage isolates were tested for virulence on S. lividans TC10, S. olivaceus and S. cattleya by plating 100-fold serial dilutions of each isolate on each of the alternate Streptomyces hosts and screening for large, clear plaques. Single plaques were subsequently repropagated on their original hosts to test for their ability to reinfect these Streptomyces

strains.

2.2.7 Restriction-modification of VC11 phage

VC11 was routinely propagated on S. cattleya as high titre phage (2×10^{12} p.f.u. per ml) is readily obtained with this host. S. olivaceus and S. lividans TC10 were examined for the presence of restriction systems operating on VC11 by infecting these strains with phage previously grown on S. cattleya. Thus, the occurrence of host-mediated phage modification in these Streptomyces species was determined by the successive passage of VC11, initially grown on S. cattleya, through either S. olivaceus or S. lividans TC10. The ability of these streptomycetes to restrict or modify actinophage VC11 was subsequently established by the comparison of the e.o.p. of the phage on each of the hosts relative to that on S. cattleya. The e.o.p. of VC11 on each of the Streptomyces species was calculated by expressing the phage titre obtained on each host as a fraction of the titre of the phage on S. cattleya, where the phage titre was obtained in parallel with each successive passage. The e.o.p. of VC11 on S. cattleya is equal to 1.

2.2.8 Host range of VC11 phage

In addition to S. cattleya, S. olivaceus, and S. lividans TC10,

the host range of VC11 was determined by plating 100-fold serial dilutions of the phage, previously grown on S. cattleya, on 17 strains representing 14 Streptomyces species. The ability of VC11 to infect these various streptomycetes was determined by comparing the phage e.o.p. obtained on each strain with that on S. cattleya.

2.2.9 Electron microscopy

VC11 actinophage preparations were negatively stained with uranyl acetate and viewed on a Zeiss 109 electron microscope. The phage dimensions (nm) were calculated by dividing the average of 10 independent phage measurements (nm), obtained from 5 electron micrographs, by the print magnification.

2.2.10 Isolation of phage DNA

Seventeen disposable bioassay trays (22.5 x 22.5 cm) containing nutrient agar (0.9%, w/v) supplemented with 0.5% (w/v) glucose and 8 mM $\text{Ca}(\text{NO}_3)_2$, were each inoculated with 30 ml soft nutrient agar (0.3%, w/v) at 42°C containing 2 ml S. cattleya spores and 320 μl VC11 phage. The bioassay trays were incubated at 30°C for 24 h. The soft nutrient agar was soaked in 100 ml nutrient broth at room temperature for 2 h. The agar was removed by centrifugation at 11,000 rpm in a GSA rotor for 20 min at 4°C and

the pelleted agar was subsequently re-extracted. The pooled supernatant was centrifuged at 22,500 rpm for 90 min at 15°C in a Type 35 rotor. The sedimented phage was resuspended in 4 ml SM buffer (see Appendix). The phage suspension was centrifuged in a SS 34 rotor at 16,000 rpm for 20 min at 4°C (in order to remove contaminating organic debris) and filtered through a 0.22 µm Millipore filter. The protein coat of the phage was disrupted by three cycles of treatment with 10 mM Tris-EDTA, pH 8.0 (TE) buffer-saturated phenol and the phenol was subsequently removed by two cycles of ether extraction. Sodium acetate (to a final concentration of 0.3 M) and 2 volumes of cold 96% ethanol were added to the suspension which was subsequently incubated at -70°C for 30 min. The phage DNA was sedimented by centrifugation for 5 min at 12,000 rpm in a microfuge at 4°C and the DNA pellet was subsequently washed by the addition of a second aliquot of cold 96% ethanol (without disturbing the pelleted DNA) followed by centrifugation at 12,000 rpm in a microfuge for 1 min at 4°C. Finally, the phage DNA was resuspended in TE buffer to yield a concentration of 1.6 mg/ml.

2.2.11 Restriction endonuclease analysis of the VCL1 genome

Phage DNA (1 µg) was digested with 2 u of various restriction enzymes (Boehringer-Mannheim) for 2 h at 37°C. The digestion

buffers were supplemented with a final concentration of 5 mM spermidine in order to overcome agar impurities contaminating the phage DNA (due to the procedure utilized for preparation of VC11 DNA) which may inhibit the action of the restriction enzymes. Spermidine is thought to form inactive complexes with polyanions which are a component of agar (Bouché, 1981). Digested phage DNA was analyzed on 0.8% (w/v) 89 mM Tris-Borate-EDTA, pH 8.0 (TBE)-buffered agarose gels containing ethidium bromide at a final concentration of 0.5 ug/ml. Electrophoresis was performed in a horizontal direction at 40 V (constant voltage) for 12 h. The DNA was subsequently visualized by UV light at 254 nm and photographed with a Polaroid Land camera.

2.2.12 Detection of cohesive ends on the phage DNA molecule

The location of the cohesive ends (cos) on the VC11 genome was determined by digestion of 2 ug phage DNA with 2 u BglII restriction enzyme for 1 h at 37°C. Half the digested DNA was subsequently heated to 80°C for 10 min and rapidly cooled on ice. Both aliquots of DNA were analyzed by electrophoresis in a horizontal direction through a 1.4% (w/v) TBE-buffered mini-agarose gel, supplemented with ethidium bromide at a final concentration of 0.5 ug/ml, at 80 V (constant voltage) for 3 h. The DNA was subsequently visualized by UV light at 254 nm and

photographed with a Polaroid Land camera.

2.2.13 Construction of a restriction enzyme map of VC11 DNA

A restriction enzyme map of the VC11 genome was constructed using partial digestion of the phage DNA with BglII endonuclease. VC11 DNA (2 ug) was digested with 0.2 u BglII for 15 min at 37°C. The DNA was subsequently heated to 80°C for 10 min and rapidly cooled on ice in order to open the cos site. The DNA was analyzed by electrophoresis through 0.6% (w/v) TBE-buffered agarose gel containing a final ethidium bromide concentration of 0.5 ug/ml in a horizontal direction at 40 V (constant voltage) for 12 h. The number and sizes of the partially-digested phage DNA fragments were compared with DNA completely digested with BglII restriction enzyme. The sizes of the VC11-BglII DNA fragments were determined from a lambda DNA-HindIII standard.

2.2.14 Determination of the G+C content of VC11 DNA

VC11 DNA (500 ul) was dialyzed into 0.1 x SSC. The DNA was disrupted by sonication for 30 sec and subsequently de-aerated using a vacuum pump. The melting temperature (T_m) of the phage DNA was determined by monitoring the change in absorbance at 260 nm while slowly heating the DNA to 100°C in a Beckman Du8

spectrophotometer. The G+C content of the phage was subsequently calculated from the following equation:

$T_m = 16.6 \log(\text{Na}^+) + 0.41 (\%G+C) + 81.5$, where the Na^+ concentration in 0.1 x SSC is 0.0165 M.

2.3 RESULTS

2.3.1 Isolation of virulent actinophage

Phage infecting either S. olivaceus or S. lividans TC10 was obtained from 5 of the soil samples tested. Isolate 1 produced large colony-centred plaques, indicating that it is a S. lividans TC10 temperate phage. Isolate 2 exhibited small clear plaques on S. lividans TC10, while the remaining three phage isolates (3-5) resulted in large clear plaques on S. olivaceus.

The 5 high-titred phage isolates were tested for infectivity on alternate Streptomyces species: S. olivaceus, S. lividans TC10 and S. cattleya (Table 2). It was found that all the phage isolates were able to infect these Streptomyces hosts. Isolates 1-3 exhibited virulent infection of all three Streptomyces strains. However, phage isolates 1-4, propagated on S. cattleya and then transferred to their respective alternate hosts S. olivaceus and S. lividans TC10, were moderately infectious (Table

TABLE 2. HIGH TITRE PHAGE ISOLATES INFECTING ALTERNATE HOSTS

Host.	Actinophage isolates				
	1	2	3	4	5
<u>S. lividans</u>	1.0	1.0	1.7×10^{-2} _a	1.5×10^{-6}	7.5×10^{-5}
<u>S. olivaceus</u>	2.4×10^{-1}	1.4×10^{-1}	1.0	1.0	1.0
<u>S. cattleya</u>	4.8×10^4	1.4×10^5	1.7×10^{-1}	5.0×10^{-1}	5.0×10^{-5}

a: In order to express the relative infectivity of the phage isolates on alternate hosts, values were calculated by expressing the phage titre obtained on the alternate host as a fraction of that on the original host, where the titre is made to be equal to 1.0. Phage data were obtained from duplicate experiments.

TABLE 3. PHAGE ISOLATES PROPAGATED ON S. CATTLEYA AND THEN TRANSFERRED TO ALTERNATE HOSTS

Host	Phage isolate.				
	1	2	3	4	5
<u>S. lividans</u>	-	-	1.0×10^{-3} _a	1.0×10^{-8}	4.0×10^{-2}
<u>S. olivaceus</u>	2.1×10^{-5}	5.0×10^{-7}	-	-	-

a: Relative infectivity of phage by expressing the phage titre as a fraction of the titre obtained on S. cattleya, where the phage titre is equal to 1.0. Phage data were obtained from duplicate experiments.

TABLE 4. PROPAGATION OF PHAGE ISOLATES ON THEIR ORIGINAL HOSTS

Phage isolate.	Previous host.	Original actinophage host.	
		<u>S. lividans</u> TC10	<u>S. olivaceus</u>
1.	<u>S. olivaceus</u>	3.2×10^{-4} _a	
	<u>S. cattleya</u>	1.0×10^{-5}	
2.	<u>S. olivaceus</u>	1.5×10^{-1}	
	<u>S. cattleya</u>	5.0×10^{-2}	
3.	<u>S. lividans</u>		1.0×10^{-3}
	<u>S. cattleya</u>		6.0×10^{-2}
4.	<u>S. lividans</u>		no infection
	<u>S. cattleya</u>		9.0×10^{-6}
5.	<u>S. lividans</u>		4.3×10^{-2}
	<u>S. cattleya</u>		7.0×10^{-1}

a: Relative phage infection values obtained by calculating the fraction of the phage titre on the original host over that on the previous host. Phage data were obtained from duplicate experiments.

TABLE 5. RESTRICTION-MODIFICATION OF VC11 BY S. OLIVACEUS
AND S. LIVIDANS TC10

Second host ^a	Third host	Indicator strain	Efficiency of plating relative to <i>S. cattleya</i> ^b
-	-	OLIVACEUS	1.0
OLIVACEUS	-	OLIVACEUS	6.8×10^{-2}
OLIVACEUS	CATTLEYA	OLIVACEUS	1.2×10^{-1}
-	-	LIVIDANS TC10	5.0×10^{-2}
LIVIDANS TC10	-	LIVIDANS TC10	6.8×10^{-3}
LIVIDANS TC10	CATTLEYA	LIVIDANS TC10	6.4×10^{-2}

a: First host in all cases was *S. cattleya*.

b: Phage e.o.p. calculated by expressing the phage titre on each host as a fraction of the titre of the phage suspension, used to infect each host, on *S. cattleya*. The e.o.p. of VC11 phage on *S. cattleya* is equal to one. The phage data were obtained from duplicate experiments.

3). Isolate 5 was virulent on S. lividans TC10.

Actinophage isolate 2 remained virulent on S. lividans TC10 after infection of S. olivaceus and S. cattleya (Table 4). Isolate 1 was less virulent on its original streptomycete host. Phage isolates 3 and 5 remained highly infectious to S. olivaceus following infection of S. cattleya and S. lividans TC10, while the ability of isolate 4 to infect S. olivaceus was reduced after passage through S. cattleya.

2.3.2 Restriction-modification of VC11 (phage isolate 2)

As a result of the decreased plating efficiency of phage VC11 on S. olivaceus and S. lividans TC10 following propagation on S. cattleya (Tables 3 and 4), these Streptomyces strains were examined for the presence of restriction-modification systems affecting this actinophage. VC11 exhibited a decreased e.o.p. after passage through S. olivaceus and S. lividans TC10 (Table 5). However, the e.o.p. of the phage, initially propagated on S. cattleya and subsequently transferred to S. olivaceus and S. lividans TC10, remained relatively constant.

2.3.3 Host range of actinophage VC11

Including S. cattleya, S. olivaceus and S. lividans TC10, the

TABLE 6. EFFICIENCY OF PLATING OF PHAGE VC11 ON STREPTOMYCES STRAINS

Strain	e.o.p. of VC11 ^a
<u>S. albobflavus</u>	3.2×10^{-6}
<u>S. ambofaciens</u> ATCC 15154	3.1×10^{-1}
<u>S. argenteolus</u> ATCC 11009	$< 1 \times 10^{-12}$
<u>S. aureofaciens</u> ATCC 10762	9.4×10^{-6}
<u>S. cattleya</u> NRRL 8057	1.0
<u>S. chartreusis</u> ATCC 14922	4.5×10^{-1}
<u>S. cinnamomensis</u> ATCC 15413	$< 1 \times 10^{-12}$
<u>S. coelicolor</u> ATCC 19832	$< 1 \times 10^{-12}$
<u>S. fulvoviridis</u> ATCC 21954	$< 1 \times 10^{-12}$
<u>S. griseus</u> ATCC 10137	$< 1 \times 10^{-12}$
<u>S. griseus</u> ATCC 11429	4.6×10^{-2}
<u>S. griseus</u> ATCC 31031	1.8×10^{-1}
<u>S. griseus</u> W4	$< 1 \times 10^{-12}$
<u>S. hygroscopicus</u> ATCC 31039	$< 1 \times 10^{-12}$
<u>S. lipmanii</u> ATCC 27357	$< 1 \times 10^{-12}$
<u>S. lividans</u> TC10	5.0×10^{-2}
<u>S. olivaceus</u> ATCC 21397	1.0
<u>S. parvulus</u> ATCC 12434	1.5×10^{-2}
<u>S. sporophyticus</u>	1.0
<u>S. tanashiensis</u> ATCC 23967	$< 1 \times 10^{-12}$

a: The e.o.p. of VC11 on each Streptomyces strain was determined by expressing the phage titre on each host as a fraction of the number of p.f.u./ml on S. cattleya. The e.o.p. of VC11 propagated on S. cattleya was equal to one.



FIGURE 1. Electron micrograph of actinophage VC11.

Bar equals 75 nm.

**TABLE 7. DISTRIBUTION OF RESTRICTION ENDONUCLEASE
SITES IN VCL1 DNA**

Enzyme	Recognition sequence	No. of sites
Alu I	AG CT	>20
Apa I	GGGCC C	0
Ava I	G PyCGPuG	>20
Bam HI	G GATCC	0
Bcl I	T GATCA	10
Bgl I	GCCNNNN NGGC	23
Bgl II	A GATCT	4
BstE II	G GTNACC	18
Cla I	AT CGAT	0
Dra I	TTT AAA	0
Eco RI	G AATTC	0
Eco RV	GATAT C	0
Hha I	GCG C	>20
Hind III	A AGCTT	0
Hinf I	G ANTC	>20
Hpa I	GTT AAC	0
Hpa II	C CGG	>15
Kpn I	GGTAC C	0
Mbo I	GATC	>20
Mlu I	A CGCGT	0
Nde I	CA TATG	0
Nru I	TOG CGA	>20
Paer 7	C TOGAG	0
Pst I	CTCGA G	0
Pvu I	CG(^{Me} A) _A T CG	>20
Pvu II	CAG CTG	0
Rsa I	GT AC	0
Sac I	GAGCT C	>20
Sac II	COGC GG	0
Sal I	G TOGAC	>20
Sph I	GCAATG C	0
Sau 3a	G(^{Me} A) _A TC	>20
Sau 96	G GNCC	>20
Sma I	CCC GGG	0
Stu I	AGG CCT	0
Taq I	T CGA	>20
Xba I	T CTAGA	0
Xho I	C TOGAC	0

phage VC11 was found to infect 11 of the 20 strains tested (Table 6). The observation that VC11 phage was infectious to 2 of the 4 S. griseus strains is of particular interest.

2.3.4 Morphology of actinophage VC11

Electron microscopic examination of actinophage VC11 indicated that the phage consists of an icosahedral head and a long non-contractile tail (Fig. 1). Measurements taken from electron micrographs indicated that the phage possesses a head of 47 nm x 47 nm and a striated tail of 196 nm in length and 7.8 nm in width. No terminal structures on the phage tail were observed.

2.3.5 Nature of the actinophage VC11 genome

Restriction enzyme analysis of the DNA of VC11 revealed the presence of a large number of recognition sequences for 16 different restriction enzymes and the lack of restriction sites recognized by a further 21 endonucleases (Table 7). The absence of these restriction sites was confirmed by their ability to digest indicator DNA included in the VC11 DNA reaction mix (data not shown). Following restriction enzyme digestion of VC11 DNA with BglII (4 recognition sites) (Fig. 2), the genome size of VC11 was estimated from a lambda DNA-HindIII standard to be 45.2 kb. In addition, DNA fragment 2 (12 kb) of a Bgl II digest of phage DNA was shown to contain the cos site (Fig. 2). Disassociation of

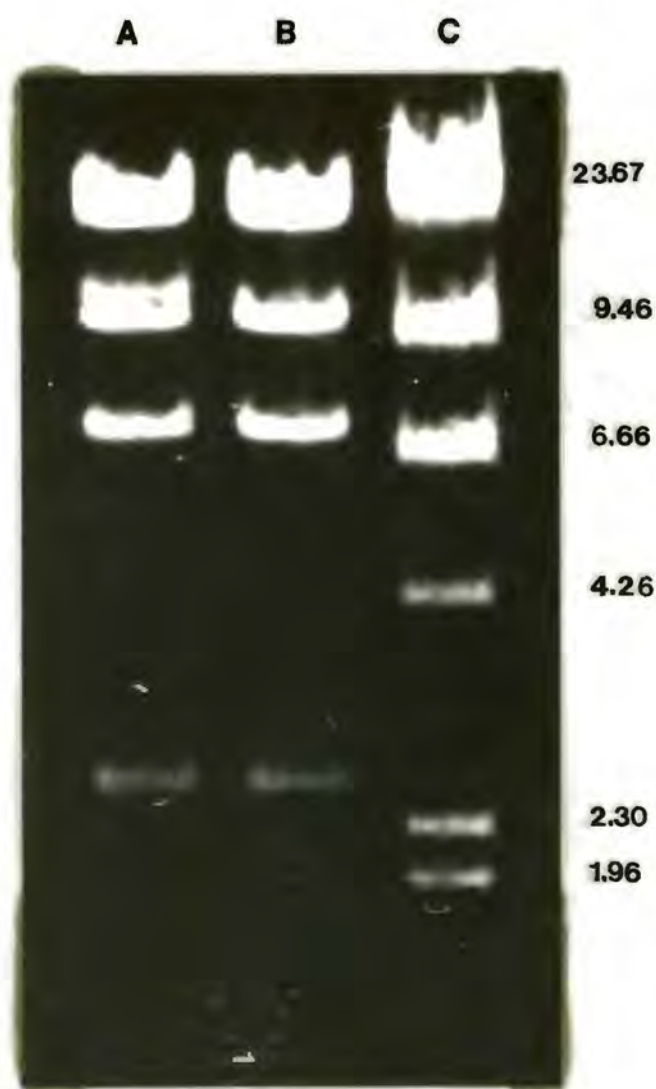


FIGURE 2. BglIII restriction analysis of VC11 DNA.

Lanes: A, VC11 DNA prior to heating to 80°C for 10 min; B, VC11 DNA heated for 10 min at 80°C; C, lambda DNA digested with HindIII. The sizes of the HindIII restriction fragments are measured in kilobases. Arrow indicates the position of the 0.65 kb VC11 DNA fragment.



FIGURE 3. Linear BglII restriction map of genome of actinophage VC11.
Restriction fragment sizes measured in kilobases.

the cos site by heating the digested DNA, produced two DNA fragments: 11.1 kb and 0.65 kb in length. The VC11 DNA melting point (T_m) of 72.4°C was used to calculate a G+C content of 50.4%.

The distribution of restriction enzyme sites in VC11 DNA precludes the construction of a complete restriction map of the phage genome. However, as only 4 BglII restriction sites are present in the DNA of VC11, a BglII restriction map was constructed of the phage genome by partial digestion of VC11 DNA with this endonuclease which generated 4 DNA fragments of 24, 17.5, 11.2 and 7.2 kb in length. The 11.1 and 0.65 kb DNA fragments, bearing the cohesive ends of the DNA molecule, were located at either end of the linear map. The partially-digested 17.5 kb fragment included the 11.1 and 7.6 kb DNA fragments obtained by complete BglII digestion, indicating that these two DNA fragments are linked. The juxtaposition of the BglII-generated 7.6 and 2.6 kb DNA fragments was inferred from the 11.2 kb DNA fragment following partial digestion with this endonuclease. Similarly, the occurrence of a 24 kb DNA fragment implied that the 23.2 and 0.65 kb BglII DNA fragments are connected. The linear map of VC11 DNA, indicating the positions of the BglII restriction sites and the order of the 5 VC11-BglII DNA fragments, is depicted in Fig. 3.

2.4 DISCUSSION

Actinophage VC11 was the most consistent in its ability to produce a high level of infection on a wide range of beta-lactam antibiotic-producing Streptomyces species. The reduced infectivity obtained on S. olivaceus, following propagation on S. cattleya, may be exaggerated by the high e.o.p. of VC11 on the latter strain (Tables 2 and 3). The results presented in Table 5 indicate that none of the Streptomyces species tested possess a restriction-modification system affecting VC11. The decreased e.o.p. of VC11, following passage through S. olivaceus and S. lividans TC10, reflected the different rates of sporulation and growth between S. cattleya and the other Streptomyces strains. S. olivaceus had a lower sporulation rate than S. cattleya, while their respective growth rates were similar. The growth rate of S. lividans TC10 was approximately 3-fold lower than that of S. cattleya. The sporulation efficiency of this strain was comparable to S. olivaceus. Dowding (1973) has shown that the degree of sporulation of a Streptomyces host, which would affect the titre of germinating spores, can influence the plating efficiency of actinophages. In addition, the rate of Streptomyces spore germination and growth determines the rate of phage adsorption and the subsequent number of p.f.u. (Rosner and Gutstein, 1981). In comparison with the other phage isolates, VC11 showed the least reduction in its ability to infect its original streptomycete host after passage through its alternate

hosts.

As with the majority of actinophages, VC11 fits the B1 group of the morphological phage classification system developed by Bradley (Ackermann, 1978). This virulent phage can be transferred between S. lividans TC10 and S. olivaceus, S. lividans TC10 and S. cattleya, and from S. lividans TC10 to S. cattleya to S. olivaceus. In addition, the phage is able to infect Streptomyces chartreusis, S. griseus ATCC 31031, S. griseus ATCC 11429, S. ambofaciens, S. parvulus, Streptomyces alboflavus, S. aureofaciens and Streptomyces sporophyticus.

The theoretical number of restriction sites for endonucleases which recognize six base pair sequences, calculated relative to the G+C content of VC11 DNA (1 site per 4,096 base pairs), is 12 sites per VC11 DNA molecule. Comparison of the number of restriction sites in lambda DNA and the theoretical number of six base pair recognition sequences (10 sites per lambda DNA molecule) indicates a close relationship between the two values: 6 BamHI sites, 6 EcoRI sites and 8 HindIII sites. It is evident that the restriction profile of VC11 DNA varies from the predicted restriction site distribution (Table 7). Enzyme-linked immunosorbent assay (ELISA) studies of the total cellular proteins of the Streptomyces strains infected by VC11 indicate that these species are very diverse (Kirby et al., 1984). Thus,

VC11 would seem to possess a taxonomically broad host range and this suggests an explanation for the restriction enzyme profile of the phage. Such taxonomically diverse Streptomyces species would have diverse restriction-modification systems and therefore, create a specific selection against the base sequences recognized by these systems. Indeed, Cox and Baltz (1984) suggested that host-mediated restriction systems influence the host range of Streptomyces phages. The authors observed a strong correlation between the inability of phage to infect Streptomyces species that produce restriction enzymes which recognize nucleotide sequences present in the phage genome. Moreover, phage lacking restriction sites recognized by restriction enzymes produced by specific Streptomyces species were able to infect these organisms at high frequency.

Thus, the inability of VC11 to infect 9 of the 20 strains (Table 6) may reflect the presence of restriction-modification systems in these streptomycetes which recognize sequences present in the phage genome. However, it is more likely that the very low phage e.o.p. is due to the failure of VC11 to adsorb to the cell walls of these Streptomyces strains. The observation that S. griseus ATCC 31031 and S. griseus ATCC 11429 are susceptible to VC11 infection, while the S. griseus strains ATCC 10137 and W4 are resistant, may indicate the presence of a common restriction-modification system in the latter two strains.

Alternatively, the inability of VC11 to infect the S. griseus strains ATCC 10137 and W4 may indicate a difference in adsorption specificity between these S. griseus strains and strains ATCC 31031 and ATCC 11429, in which case the S. griseus strains ATCC 31031 and ATCC 11429 may be taxonomically unrelated to strains ATCC 10137 and W4. If so, then actinophages should prove useful tools for the typing of Streptomyces strains to elucidate their taxonomic relatedness.

The inability of VC11 to infect S. lipmanii is of particular interest as a restriction-modification system, common to S. lipmanii, S. cattleya and S. olivaceus, was reported by Flett et al. (1979). The resistance of S. lipmanii to VC11 infection may be explained by two possibilities. Either the strain lacks specific surface receptors necessary for VC11 adsorption prior to infection, or the strain possesses a second restriction-modification system which is absent in S. cattleya and S. olivaceus and does not affect the actinophage FF4. The use of VC11 phage as a potential DNA cloning vector for the application of recombinant DNA technology to S. cattleya and S. olivaceus will be discussed in section 5.1.

CHAPTER 3

STUDIES OF DNA REPAIR IN STREPTOMYCES CATTLEYA
USING PHAGE VC11SUMMARY

Whereas survival of UV-irradiated VC11 followed simple one-hit kinetics when propagated on untreated S. cattleya spores, UV-irradiation of the host spores resulted in an increase in the survival of UV damaged phage by 100% or more. This observation indicates the induction of a DNA repair system in the UV-treated S. cattleya spores which is able to restore UV-irradiated phage. The e.o.p. of undamaged VC11 propagated on untreated S. cattleya spores was not affected by the presence of either caffeine or sodium arsenite. Thus, a caffeine-mediated decrease in the survival of UV-irradiated phage propagated on untreated S. cattleya spores identified a constitutive host-mediated UV repair system in S. cattleya, while a similar decrease in the survival of UV-damaged phage propagated on UV-irradiated S. cattleya spores revealed the presence of a caffeine inhibitable, inducible DNA repair mechanism in this Streptomyces strain. These results indicate that either two separate DNA repair systems exist in S. cattleya or caffeine inhibits a constitutive and an inducible component of the same DNA repair system. Sodium arsenite failed

to influence the survival of UV-damaged VC11 propagated on both untreated and UV-irradiated S. cattleya spores. The possible interaction between DNA repair systems and genetic instability in S. cattleya are discussed.

3.1 INTRODUCTION

Genetic instability has been observed in a number of Streptomyces species. This phenomenon is associated with antibiotic production and resistance, arginine biosynthesis, aerial mycelium formation and growth rate.

Freeman et al. (1977) noticed that chloramphenicol resistant (Cml^R) strains of S. coelicolor A3(2) and S. lividans 66 spontaneously produced chloramphenicol sensitive (Cml^S) variants at frequencies of 0.5 to 2%. The frequency of Cml^S variants increased to 5% following ultraviolet light (UV) irradiation. The authors reported that the Cml^S variants spontaneously reverted to Cml^R at frequencies one- to three-fold lower than the frequencies at which Cml^R colonies produced Cml^S variants. Cml^S variants were again obtained from the Cml^R revertants.

Similar phenomena have been observed in other Streptomyces strains. Spontaneous antibiotic sensitive variants have been

reported at frequencies between 0.1 and 1% in S. griseus and Streptomyces glaucescens (streptomycin sensitive), and S. acrimycini (erythromycin and chloramphenicol sensitive) (Freeman and Hopwood, 1978). UV irradiation of these Streptomyces strains resulted in a 10-fold increase in the occurrence of antibiotic sensitive variants. Moreover, the antibiotic sensitive variants were unstable; spontaneously reverting to antibiotic resistance.

Shaw and Piwowarski (1977) reported the loss of streptomycin production and resistance in Streptomyces bikiniensis following treatment with ethidium bromide or acriflavine. In addition, the variants (2 to 16% of the treated spores) had simultaneously lost the ability to produce aerial mycelia. Although the loss of antibiotic production and resistance appeared stable, transfer of the variants to fresh medium restored aerial mycelium production to these isolates.

However, Kirby and Lewis (1981) demonstrated that S. bikiniensis, having lost the unstable streptomycin resistance (Sm^R) phenotype, was able to revert to Sm^R at a low frequency. In addition, these revertants (Sm^R) were found to have simultaneously lost the ability to produce aerial mycelium (Amy^-). The Amy^-Sm^R clones were able to subsequently revert to Amy^+Sm^S at a high frequency. Moreover, selection of Sm^R revertants from Sm^S variants on malt-3 agar containing streptomycin resulted in the isolation of

Amy⁻Sm^r clones, of which 3.8% were auxotrophic. Of these, 6 were not identifiable, 2 were Arg⁻ and 1 Met⁻. Thus, other genes, besides those coding for antibiotic resistance and production, are affected by the instability phenomena.

Similarly, Redshaw et al. (1979) demonstrated the simultaneous loss of aerial mycelium formation (Amy⁻), arginine biosynthesis (Arg⁻), pigment production and earthy odour in Streptomyces alboniger, Streptomyces scabies and Streptomyces violaceusniger. The simultaneous loss of these differentiated functions occurred at a frequency of 2 to 20% in the presence of intercalating dyes such as acriflavine. Loss of arginine production was the result of a metabolic block in the arginine biosynthetic pathway involving a deficiency of the enzyme arginosuccinate synthetase.

In most cases, the genetic instability observed in a number of Streptomyces species is reversible, indicating that these cyclic phenotypic changes are not the result of an irreversible loss of genetic material. Kirby and Lewis (1981) proposed three hypotheses whereby the genetic instability in Streptomyces could be explained. The first hypothesis involved the insertion or deletion of a transposable element, bearing the genes coding for antibiotic resistance, which was able to exert an effect on the promoters of other genes. Likewise, the second explanation also entailed the insertion or deletion of a transposable element.

However, this insertion sequence would interfere with the operon specifying antibiotic biosynthesis and resistance and, in so doing, produce pleiotropic effects on the genes coding for aerial mycelium and arginine production. In the third case, the authors postulated the presence of a controlling element which does not bear genes expressing antibiotic resistance, nor directly interacts with these genes. However, the orientation of the controlling element would either switch on or off the antibiotic resistance and other genes. The controlling element could either operate in cis from a position close to these genes on the chromosome or in trans from a plasmid.

Unlike most transposition events in eubacteria, genetic instability in Streptomyces is induced by acriflavine treatment or UV irradiation (Freeman et al., 1977; Shaw and Piwowarski, 1977; Redshaw et al., 1979; Kirby and Lewis, 1981). The observation that UV light is able to induce genetic instability in Streptomyces implies that DNA repair systems may be involved in this phenomenon (Coyne et al., 1984).

Current understanding of DNA repair systems have originated mainly from studies involving E. coli. These studies have frequently served as models to explain similar phenomena in other procaryotes and eucaryotes, including mammals. A number of reviews dealing with aspects of DNA repair and mutagenesis have

been published. These include UV repair systems in E. coli (Witkin, 1976; Walker, 1984), bacteria and mammals (Hanawalt et al., 1979; Jones, 1979; Hall and Mount, 1981) and bacteriophages (Blanco and Devoret, 1973; Devoret et al., 1975; Bernstein, 1981).

Various carcinogenic chemicals, UV irradiation and other agents which damage DNA or interrupt DNA replication in bacterial and mammalian cells induce DNA repair mechanisms which produce mutations in these organisms. Elucidation of these DNA repair systems has shown that mechanisms for replicating damaged DNA are functionally related to cellular control of UV-induced mutagenesis. UV irradiation produces a variety of photoproducts in DNA. The most lethal, mutagenic and tumorigenic effects have been shown to be caused by intrastrand cyclobutane dimers of adjacent pyrimidines. Pyrimidine dimers may be removed from the DNA by two repair mechanisms: photoreactivation and excision repair. Dimers not removed by these repair systems hinder DNA replication. A third system, namely postreplication repair, allows DNA replication forks to proceed past these dimers and renews the interrupted DNA segments generated by the dimers.

Photoreactivation is mediated by a distinct enzyme which consists of a single polypeptide chain. Photoreactivating enzyme, found in both bacterial and mammalian cells, combines specifically with

cyclobutyl pyrimidine dimers in UV damaged DNA. This enzyme substrate complex is able to form in the absence of light. However, visible light (wavelength 320 to 410 nm) is necessary to activate the enzyme which then converts the dimers to monomeric pyrimidines without removing them from the DNA. As much as 80% of the lethal damage induced by UV irradiation may be overcome by photoreactivation.

A light-independent multienzymatic DNA repair system, excision repair, is able to remove pyrimidine dimers from UV irradiated DNA. An endonuclease recognizes the distortion of the double helix caused by the presence of a pyrimidine dimer. This endonuclease, the product of the uvr A and uvr B genes of E. coli, creates a single-strand nick on the 5' side of the pyrimidine dimer. The dimer, together with some bases on either side of it, is subsequently removed from the DNA by exonucleic excision. The excision gap is repaired by a polymerase which uses the second DNA strand as a template. The excision and resynthesis steps are carried out simultaneously by DNA polymerase I. Finally, polynucleotide ligase restores the phosphodiester bond. The product of the uvr C gene prevents the ligase from prematurely resealing the nick created by the endonuclease, thus allowing excision repair to occur.

Excision repair occurs via two pathways. These two pathways,

known as short patch and long patch excision repair, may be distinguished on the basis of the gene products involved and the physiological requirements. The system described above, short patch excision repair, is able to produce short patches of repair synthesis (up to 30 nucleotides), is independent of nutrients, and proceeds in the presence of chloramphenicol.

The second pathway for excision repair requires a complete growth medium and is inhibited by chloramphenicol. This pathway, known as long patch excision repair, generates long patches of repair synthesis (up to 1,500 nucleotides) and requires the expression of the rec A, rec B, rec C, lex A, pol C and leg genes. In addition, the products of the rec F, pol B and uvr D genes have been implicated in alternate branches of long patch excision repair. Both DNA polymerase II and III (pol B and pol C, respectively) may be involved in the repair synthesis step. Since these DNA polymerases are unable to bind at DNA nicks, gap expansion by exonuclease V (rec B, rec C) occurs prior to DNA polymerization. Finally, long patch excision repair is terminated by polynucleotide ligase.

Pyrimidine dimers, which have not been monomerized by photoreactivation or removed by excision repair mechanisms, produce gaps (up to 1,000 nucleotides) in the daughter strands following DNA replication. These gaps correspond to the position

and number of pyrimidine dimers in the parental strand which inhibit base pairing. The daughter strand gaps are filled via a postreplication repair pathway. Postreplication repair has been observed in cells capable of performing excision repair, implying that excision repair does not prevent the replication fork from encountering some pyrimidine dimers. The general mechanism whereby postreplication repair joins the discontinuous daughter strands involves a recombination process utilizing an intact strand of parental DNA. The gaps remaining in the parental strand would then be filled by repair synthesis.

Genes involved in postreplication repair include rec A, rec B, rec C, rec F, lex A, uvr D, pol A and pol C. The enzymes involved in postreplication repair processes are common to those utilized in some steps in recombination and are also similar to those which function in long patch excision repair. Polynucleotide ligase is required for completion of postreplication repair.

The fact that mutations in either the rec A or lex A genes abolish UV-induced mutations, suggests that these mutations arise as errors in DNA repair. Although visible light, necessary to activate photoreactivation enzyme, may be mutagenic, the process of photoreactivation is essentially non-mutagenic. Similarly, short patch excision repair is error-free as shown by studies

involving rec A⁻ and lex A⁻ strains (Witkin, 1976). Although low frequencies of UV-induced mutations have been detected in studies involving long patch excision repair and postreplication repair, the two repair pathways are not significantly error-prone.

Error-prone DNA repair is not a function of the constitutively expressed DNA repair systems in E. coli, but is induced as a result of UV irradiation and other mutagenic treatments (Witkin, 1976). The inducible error-prone DNA repair system, known as SOS repair, is dependent on the rec A and lex A gene products. The induction of the SOS system, in response to DNA damage or interrupted DNA synthesis, results in the expression of a number of diverse phenotypes which include prophage induction, Weigle reactivation and mutagenesis of bacteriophage, chromosomal mutagenesis, uvr A⁺ B⁺ C⁺-dependent repair, long patch excision repair and filamentation (Walker, 1984). In addition to the induction of the rec A and lex A genes, the umu D, umu C, din A, din B, din D, din F genes, amongst others, are expressed. While some of the genes involved in some of the SOS responses have been identified, for example umu D and umu C expression is necessary for bacteriophage and chromosomal mutagenesis, no function has been identified for other SOS-induced genes such as din A, din B and din D (Walker, 1984).

The SOS system is maintained in a repressed state by the action

of the lex A gene product which functions as a repressor of numerous genes associated with the SOS response, including the rec A, lex A, uvr A and umu C genes. These genes possess either one (rec A, uvr A, din A) or two (lex A, umu D, umu C) operator sequences to which the Lex A molecules are able to bind. Although repressed, a significant level of expression of the lex A and rec A genes occurs which produces sufficient Rec A protein for homologous recombination (Walker, 1984).

Induction of the SOS response occurs as a result of the presence of single-stranded regions in the DNA molecule. In addition to UV treatment, a variety of chemical agents have been shown to induce the SOS response, including methyl methanesulphonate, 4-nitroquinoline-1-oxide, N-methyl-N'-nitro-N-nitrosoguanidine and mitomycin C (Quillardet et al., 1982). These agents introduce premutagenic lesions in DNA which interfere with DNA replication, resulting in the production of single-stranded regions in the duplex molecule (Bagg et al., 1981; Walker, 1984). Cohen et al. (1983) suggested that a quaternary complex of single-stranded DNA, single-strand DNA binding protein, Rec A and adenosine triphosphate functions as the active machinery which induces the SOS response in vivo. The SOS system is presumably induced by the activation of Rec A as a consequence of the formation of this complex at single-stranded regions of DNA.

The reversible activation of Rec A allows the specific proteolytic cleavage of the Lex A protein. As the number of Lex A molecules cleaved by the protease activity of Rec A increases, the cellular pool of Lex A protein decreases, resulting in the expression of formerly Lex A repressed SOS genes. A number of factors determine whether specific SOS responses will be expressed: (i) the amount of inducing signal and its persistence, a function of the dose of inducing treatment, influences the amount of activated Rec A protein and the time it remains activated; (ii) the rate at which the repressor of a particular SOS gene is cleaved by the Rec A protease activity; (iii) the affinity of the Lex A repressor for the operator of a particular SOS gene; and (iv) the level of expression of each SOS gene required for its gene product to exhibit a physiological effect (Walker, 1984).

Thus, the SOS system may exist in a number of intermediate states of induction. Lex A binds less strongly to the lex A and uvr B operators than the rec A operator. Likewise, uvr A, din A and din B operators bind Lex A less strongly than the umu D and umu C genes which, in turn, bind Lex A less strongly than din D (Walker, 1984). A limited SOS-inducing signal would not derepress the rec A gene, thus preventing amplification of activated Rec A protein. Stronger SOS-inducing treatment would result in the expression of uvr⁺-dependent, error-free excision

repair systems. The derepression of the umu DC⁺-dependent, error-prone repair system would require a large, persistent SOS-inducing signal (Walker, 1984).

DNA mutagenesis, resulting from the induction of the SOS response by UV and chemical treatment, is due to the introduction of an incorrect base at the site of DNA damage. Three mechanisms have been postulated to explain SOS-induced mutagenesis: (i) the involvement of a new or modified DNA polymerase which has lost its 3'-5' proofreading function; (ii) a short oligonucleotide, homologous to the region containing the lesion, could be introduced via a recombinational event; and (iii) an insertase may incorporate an incorrect base (Walker, 1984). However, the most feasible explanation of the mechanism of SOS-related mutagenesis, is the involvement of a DNA polymerase. This hypothesis is consistent with the observation that the properties of a lesion influences the character of the base pair change and that SOS repair is able to perform on both single- and double-stranded DNA templates (Walker, 1984).

Once the damaged DNA is repaired, the inducing signal is removed and consequently, the protease activity of Rec A is reversed to its inactive state. This results in a build-up of uncleaved Lex A molecules which repress the SOS genes, beginning with those genes which have the highest affinity for the Lex A protein.

This allows the cell to rapidly return to the uninduced state as the inducing signal begins to decrease (Walker, 1984).

Bacteriophages have contributed considerably to the study of DNA repair mechanisms which exist in their hosts. Some bacteriophages, such as phage T4, possess their own DNA repair systems, while smaller phages, for example phage lambda, rely entirely on host-mediated DNA repair (Bernstein, 1981). Bacteriophages which rely on host-mediated DNA repair systems are extremely useful for the detection of specific repair processes which occur in the host cell; the alteration of the survival curve of UV-damaged phage, as a result of a genetic or physiological change in a host cell, indicates the existence of a repair mechanism in the wild type host (Devoret et al., 1975).

Three distinct types of DNA repair systems have been shown to restore damaged lambda DNA; namely host-cell reactivation, prophage induction and UV reactivation (Weigle reactivation) (Devoret et al., 1975). Host-cell reactivation of UV-irradiated lambda is performed by the excision repair system in E. coli. This system has been experimentally defined by the observation that UV-irradiated phage has a different plating efficiency depending on the genetic background of the host cell. This error-free repair system is able to restore 85% of damaged lambda DNA and is non-mutagenic with respect to the phage DNA (Devoret,

1975).

Prophage reactivation occurs when UV-irradiated lambda phage is introduced into a host carrying an homologous heteroimmune prophage. The increased survival of lambda, as a result of this system, is due to recombination between the homologous DNA of the UV-treated phage and that of the undamaged prophage (Devoret, 1975). Prophage reactivation repairs 50% of the lethal lesions, irrespective of host-cell reactivation. Although prophage reactivation depends on the expression of the host rec A gene, this repair system does not lead to mutagenesis of the rescued phage (Devoret, 1975; Bernstein, 1981).

Weigle (1953) discovered that the survival of UV-irradiated lambda can be enhanced by exposure of the host cell to a low dose of UV light prior to phage infection. This repair process is able to restore up to 50% of the lethal lesions. However, in a host cell capable of performing excision repair, Weigle reactivation accounts for the repair of only 8% of the total lesions since this repair system occurs subsequent to the excision of pyrimidine dimers (Devoret, 1975). Weigle reactivation is dependent on the expression of the host rec A and lex A genes (Devoret, 1975; Bernstein, 1981; Walker, 1984).

UV-irradiation of lambda DNA does not result in phage mutagenesis

unless it is accompanied by UV treatment of the host cell (Weigle, 1953). This phenomenon has been termed Weigle mutagenesis. The observation that both Weigle reactivation and Weigle mutagenesis occur in wild type and uvr⁻ cells, but are absent in cells lacking rec A or lex A gene functions, indicates that Weigle reactivation reflects the induction of an existing error-prone bacterial repair system (Defais et al., 1971).

Weigle reactivation and Weigle mutagenesis are considered to be one of the manifestations of the bacterial SOS response (Radman, 1975; Witkin, 1976; Walker, 1984). This conclusion is supported by the following observations: (i) the induction of Weigle reactivation and Weigle mutagenesis is inhibited by the presence of chloramphenicol after UV-irradiation of the host cell prior to infection with UV-damaged phage; and (ii) the mutant strain recA441 was able to perform Weigle reactivation and Weigle mutagenesis, in the absence of bacterial DNA damage, when grown at 42°C which allows the constitutive expression of SOS functions (Walker, 1984).

The use of inhibitors of DNA repair to study UV repair systems in E. coli is well established. Caffeine, a known inhibitor of excision repair in E. coli, is able to bind to single-stranded DNA and at locally denatured areas adjacent to pyrimidine dimers in double-stranded DNA (Rothman, 1980). Rothman postulated that

the binding of caffeine to DNA inhibited incision on the 5' side of the dimer by an endonuclease and the exonucleic excision of the dimer by DNA polymerase I. However, the difficulty of differentiating between the post-incision steps, due to the tightly co-ordinated nature of excision repair, led the author to suggest that caffeine inhibition of the incision step may induce compensating adjustments in the rates of other steps. Moreover, the observation that caffeine is able to exert growth-suppressing effects in unirradiated cells stresses the importance of taking the concentration of caffeine into account in order to prevent erroneous conclusions when measuring the effect of this inhibitor on DNA repair (Fong and Bockrath, 1979; Rothman, 1980).

Although the biochemical mechanism whereby sodium arsenite inhibits rec A-dependent SOS repair of UV-irradiated E. coli DNA has not been resolved, genetic studies support this hypothesis (Rothman et al., 1975). The authors noted that UV-treated wild type and excision repair deficient strains of E. coli exhibited a decreased survival in the presence of sodium arsenite. The magnitude of the sodium arsenite-induced increase in UV sensitivity is directly proportional to increasing UV dosage. Furthermore, sodium arsenite had no effect on the survival of UV-damaged rec A mutants. As in the case of caffeine, caution must be exercised in the elucidation of sodium arsenite inhibition of DNA repair as this chemical causes a loss of colony-forming

ability at high concentrations.

UV-sensitive (uvs) mutants of S. coelicolor A3(2), isolated by Harold and Hopwood (1970a; 1970b), provided the first evidence for the existence of DNA repair mechanisms in Streptomyces. The 23 uvs mutants led to the recognition of 6 loci, uvs A-F, in this Streptomyces species. Genetic analysis of the 6 loci revealed that uvs A, C, D are linked in a cluster on the S. coelicolor A3(2) chromosome, uvs B, E are located in a second cluster diametrically opposite the uvs A, C, D cluster, and uvs F occurred separately from the other loci (Harold and Hopwood, 1970b). Several of the authors' results indicated that uvs A, B, C and D may be involved in the same mechanism of DNA repair (Harold and Hopwood, 1970a). Mutations in these genes did not affect the recombination ability of S. coelicolor A3(2), suggesting that the uvs A, B, C and D loci in this micro-organism correspond to the uvr A, B, C and D genes of E. coli K12. However, the authors were unable to confirm this hypothesis as tests commonly employed to elucidate mechanisms of UV repair in E. coli were not available for similar use in S. coelicolor A3(2). Chemical inhibitors of excision repair in E. coli, such as caffeine, did not exhibit an effect on the UV survival of wild type S. coelicolor A3(2). Differentiation of the repair mechanisms operating on damaged DNA, by host-cell reactivation and Weigle reactivation of phage, was not possible due to the

absence of a suitable S. coelicolor A3(2) actinophage. Moreover, pyrimidine dimer excision by photoreactivation does not occur in S. coelicolor A3(2) (Harold and Hopwood, 1970a). The authors suggested that uvs E may be involved in a different repair function from the uvs A, B, C, D loci and uvs F may be required for the operation of a system capable of repairing a proportion of the DNA damage repairable by the uvs A, B, C, D system (Harold and Hopwood, 1970a).

S. cattleya NRRL 8057 produces the broad spectrum beta-lactamase resistant beta-lactam antibiotic thienamycin (Kahan et al., 1979). Aerial mycelium production is a characteristically unstable phenotype in S. cattleya. The high frequency loss of aerial mycelium production (Amy^+ to Amy^-) is associated with a simultaneous change from arginine prototrophy to arginine auxotrophy (Arg^-) as a consequence of a deficiency in the enzyme arginosuccinate synthetase (Kirby and Lewis, 1981). The genetic instability in S. cattleya is often reversible and is also associated with other phenotypic changes such as antibiotic production and growth rate.

As mentioned earlier, the fact that UV light is able to induce genetic instability in Streptomyces suggests that DNA repair systems may be involved in this phenomenon. Amy^- variants of S. cattleya occur both spontaneously and after UV treatment. Of the

spontaneously occurring Amy⁻ variants, 56% were Arg⁻, 11% were Arg⁻ Met⁻ and 5% were Met⁻. However, UV-irradiation of S. cattleya spores produced Amy⁻ isolates of which 91% were Arg⁻, 2% were Met⁻ and 1% were Arg⁻ Met⁻ (Coyne et al., 1984). Although the presence of a photoreactivation system was not observed in UV-irradiated S. cattleya spores, the presence of a shoulder in the UV survival curve and the reduction in spore survival in the presence of either caffeine or sodium arsenite, indicated that UV repair systems may occur in this Streptomyces strain (Coyne et al., 1984). However, a decrease in the viability of UV-irradiated spores in the presence of chemical inhibitors of DNA repair does not prove the presence of a repair system as an interaction between the inhibitors and UV-irradiation could cause a viability variation which would mimic the response. Therefore, to determine whether caffeine and sodium arsenite are acting at the level of DNA repair, it is necessary to employ a bacteriophage which may be subjected to UV-irradiation independently of the host organism and the chemicals.

3.2 METHODS

3.2.1 Bacteria and actinophage

S. cattleya NRRL 8057 was grown on malt-3 agar at 30°C. S.

cattleya spore suspensions were prepared in sterile distilled water as described in section 2.2.2. Actinophage VC11 was propagated on S. cattleya using the double-layer agar technique described in section 2.2.3.

3.2.2 Survival of UV-induced actinophage VC11

VC11 (500 ul) was suspended in 5 ml nutrient broth and placed in a 9 cm sterile glass Petri dish. The phage suspension was UV-irradiated, using an English Electric G8T5 tube which provided an irradiation level (254 nm) of 5 Jm^{-2} , for 10 sec intervals with agitation. The level of UV-irradiation was determined with a UV meter model 225 (Ultraviolet Products, San Gabriel, California). Phage samples (100 ul), beginning at 0 sec (no UV-irradiation), were removed every 10 sec and added to 900 ul soft nutrient agar at 42°C . Further 10-fold serial dilutions of phage were prepared by transferring 200 ul phage to 2 ml molten soft nutrient agar. Untreated S. cattleya spores (125 ul) were added to each phage dilution which was subsequently plated on nutrient agar supplemented with 0.5% (w/v) glucose and 8 mM $\text{Ca}(\text{NO}_3)_2$. The number of VC11 p.f.u. were scored after incubation at 30°C for 24 h.

Five ml of an S. cattleya spore suspension, contained in a 9 cm sterile glass Petri dish, was UV-irradiated for 10 sec with

agitation. UV-treated spores (125 μ l) were subsequently added to 10-fold dilutions of UV-irradiated VC11 phage in soft nutrient agar at 42°C and plated on nutrient agar containing 0.5% (w/v) glucose and 8 mM $\text{Ca}(\text{NO}_3)_2$. Following incubation at 30°C for 24 h, the number of VC11 p.f.u. was determined.

The percentage survival of UV-irradiated VC11 was calculated by expressing the number of p.f.u. of phage, following each 10 sec interval of UV-irradiation, as a fraction of the number of p.f.u. of untreated phage. UV survival curves of VC11 propagated on UV-treated and untreated S. cattleya spores were constructed by plotting the percentage survival of UV-irradiated phage as a function of increasing UV dosage.

3.2.3 Determination of the minimal inhibitory concentration of caffeine and sodium arsenite for S. cattleya

As high concentrations of caffeine and sodium arsenite are known to exert growth-suppressing effects on E. coli, the minimal inhibitory concentration (m.i.c.) of these chemicals for S. cattleya was established. Ten-fold serial dilutions of an S. cattleya spore suspension were plated on malt-3 agar containing caffeine at final concentrations of 1, 2, 3, 4, 5 and 6 mg/ml. Similarly, the effect of sodium arsenite on the viability of S.

cattleya spores was determined by propagating 10-fold serial dilutions of S. cattleya spores on malt-3 agar which included sodium arsenite at final concentrations of 150, 300, 450, 600, 750 and 900 ug/ml. The plates were scored for growth following incubation at 30°C for 24 h. The m.i.c. of each chemical for S. cattleya was regarded as the highest concentration at which S. cattleya was able to produce colonies.

3.2.4 Effect of caffeine and sodium arsenite on VC11

VC11 (500 ul) was suspended in 5 ml nutrient broth. Ten-fold serial dilutions of the phage suspension were subsequently prepared by transferring 200 ul phage to 2 ml soft nutrient agar at 42°C. S. cattleya spores (125 ul) were added to each phage dilution which was subsequently plated on nutrient agar supplemented with 0.5% (w/v) glucose, 8 mM $\text{Ca}(\text{NO}_3)_2$, and either 1 mg/ml caffeine or 150 ug/ml sodium arsenite. Similarly, 10-fold serial dilutions of VC11 were propagated on S. cattleya in the absence of caffeine and sodium arsenite. The plates were assayed for plaques following incubation at 30°C for 24 h. The phage e.o.p. in the presence of caffeine and sodium arsenite was determined by expressing the number of p.f.u. in the presence of either caffeine or sodium arsenite as a fraction of the number of VC11 p.f.u. in the absence of these chemicals.

3.2.5 Effect of chemical inhibitors of *E. coli* DNA repair on the survival of UV-irradiated actinophage VC11

UV-irradiation of VC11 and *S. cattleya* spores was accomplished as described in section 3.2.2. Survival of UV-irradiated phage, propagated on either untreated or UV-treated spores, was determined after growth on nutrient agar supplemented with 0.5% (w/v) glucose, 8 mM $\text{Ca}(\text{NO}_3)_2$, and either 1 mg/ml caffeine or 150 ug/ml sodium arsenite at 30°C for 24 h. The effect of caffeine and sodium arsenite on the survival of UV-irradiated phage was ascertained by comparison of the UV survival curves of the phage (constructed as in section 3.2.2) in the presence or absence of these chemical inhibitors of DNA repair systems in *E. coli*.

3.3 RESULTS

3.3.1 Survival of UV-irradiated VC11

Survival of UV-irradiated VC11 actinophage followed simple one-hit kinetics in the presence of untreated *S. cattleya* spores (Fig. 1). This result would be expected in the absence of a DNA repair system in the germinating *Streptomyces* spores. However, UV-irradiation of the host spores (50 Jm^{-2}) prior to phage infection increased the survival of UV-irradiated VC11 by 100% or

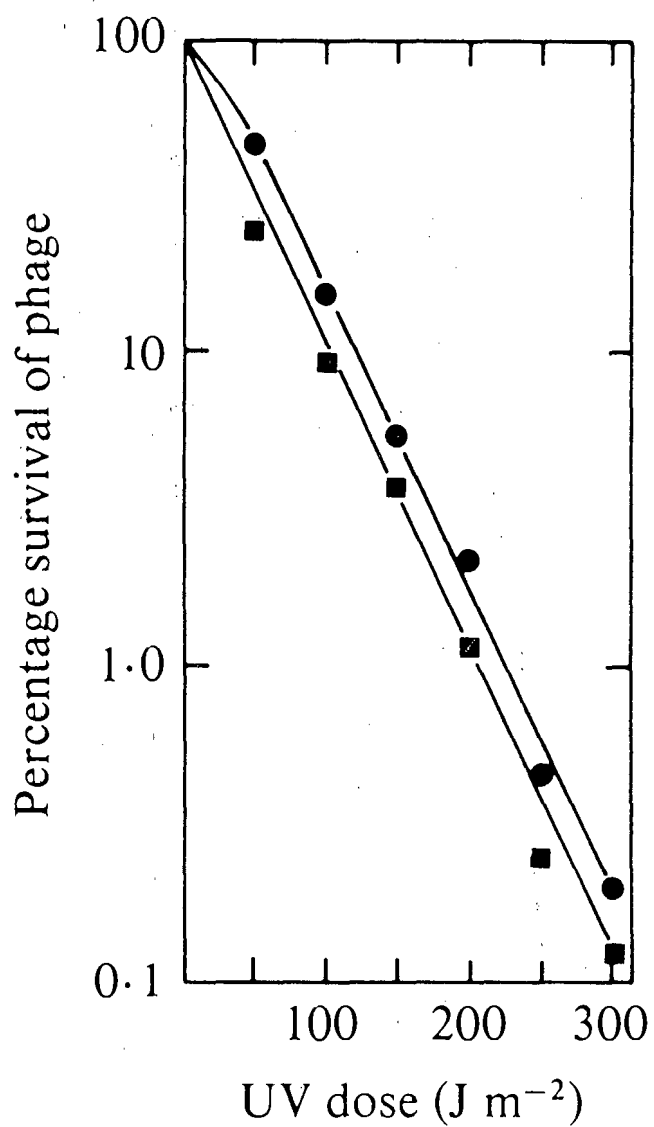


FIGURE 1. Survival of UV-irradiated VC11 phage. ■, No irradiation of *S. cattleya* spores; ●, phage plated on UV-irradiated *S. cattleya* spores (50 J/m²).

more and created a distinct shoulder to the survival curve (Fig. 1). This is characteristic of the induction of a repair mechanism in the UV-irradiated S. cattleya spores which is able to repair UV-damaged phage DNA.

3.3.2 Effect of caffeine and sodium arsenite on the viability of S. cattleya and VC11

It has been established that high concentrations of caffeine and sodium arsenite exert growth-suppressing effects in untreated E. coli (Rosman et al., 1975; Fong and Bockrath, 1979; Rothman, et al., 1980). The m.i.c. of caffeine and sodium arsenite for untreated S. cattleya was found to be 3 mg/ml and 450 ug/ml respectively. In order to prevent erroneous conclusions when measuring the effect of these chemicals on DNA repair systems in S. cattleya, experiments were performed with one-third of the m.i.c. of these substances for this Streptomyces species. At this concentration, caffeine and sodium arsenite reduced the viability of the S. cattleya spores by only 10%. The results of subsequent experiments were corrected for this inhibitory effect (determined from controls).

Moreover, as both caffeine and sodium arsenite are mutagenic and thus may impair vital cellular functions of S. cattleya, the e.o.p. of VC11 propagated on S. cattleya in the presence of

TABLE 1. EFFECT OF CAFFEINE AND SODIUM ARSENITE ON VC11
IN THE ABSENCE OF UV-IRRADIATION

Chemical	Phage titre ^a	Phage e.o.p. ^b
Absent	1.32×10^8	1.0
Sodium arsenite	1.95×10^8	1.4
Caffeine	8.20×10^7	0.6

a: The phage titre (p.f.u./ml) was determined from 5 separate experiments.

b: The phage e.o.p. was estimated by expressing the number of p.f.u./ml in the presence of either chemical as a fraction of the phage titre in the absence of the chemicals. The e.o.p. of VC11 propagated in the absence of the chemicals is equal to one.

caffeine and sodium arsenite at one-third of the m.i.c. for this streptomycete was determined in the absence of UV-irradiation of both the phage and host (Table 1). The presence of these chemicals was not found to exert a significant effect on the viability of the actinophage. Thus, an alteration in the survival of UV damaged VC11 in the presence of these substances would reflect an effect on DNA repair systems present in S. cattleya.

3.3.3 Effect of chemical inhibitors of DNA repair in E. coli on the survival of UV-irradiated actinophage VC11

The presence of caffeine resulted in a reduction in the survival of UV-irradiated VC11 propagated on untreated S. cattleya spores. No such effect was observed in the presence of sodium arsenite (Fig. 2).

Similarly, a reduction in the survival of UV-irradiated phage propagated on UV-damaged S. cattleya spores occurred in the presence of caffeine (Fig. 3). No change in the survival of UV-irradiated VC11 occurred in the presence of sodium arsenite.

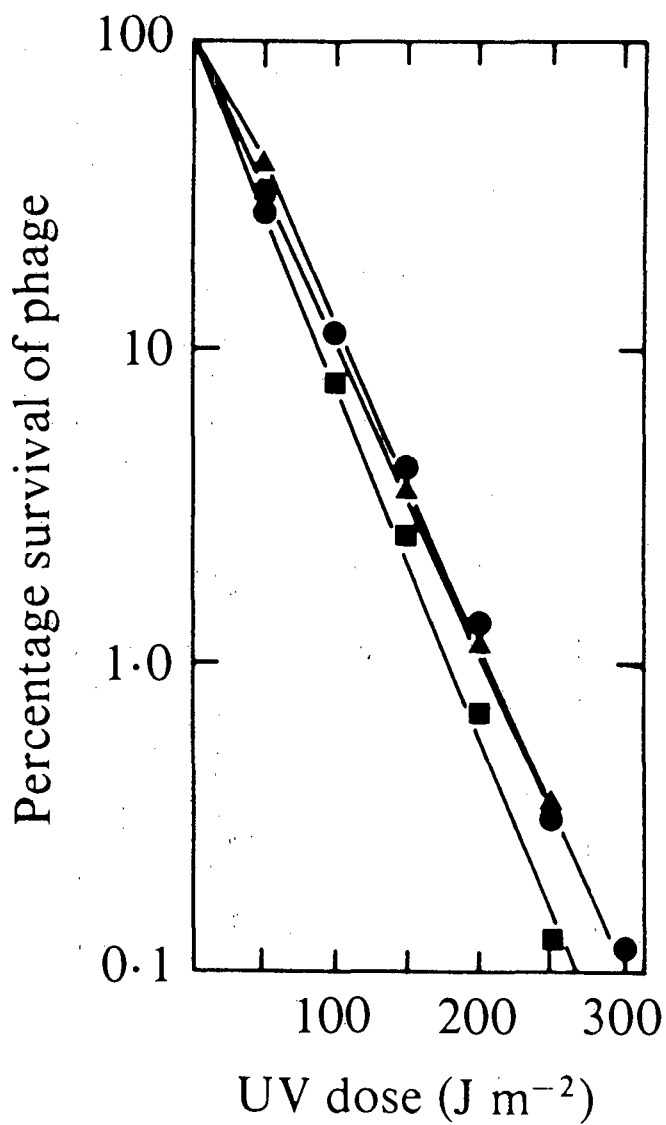


FIGURE 2. Effect of caffeine and sodium arsenite on the survival of UV-irradiated VCl1 phage. ●, No inhibitor; ▲, + sodium arsenite; ■, + caffeine.

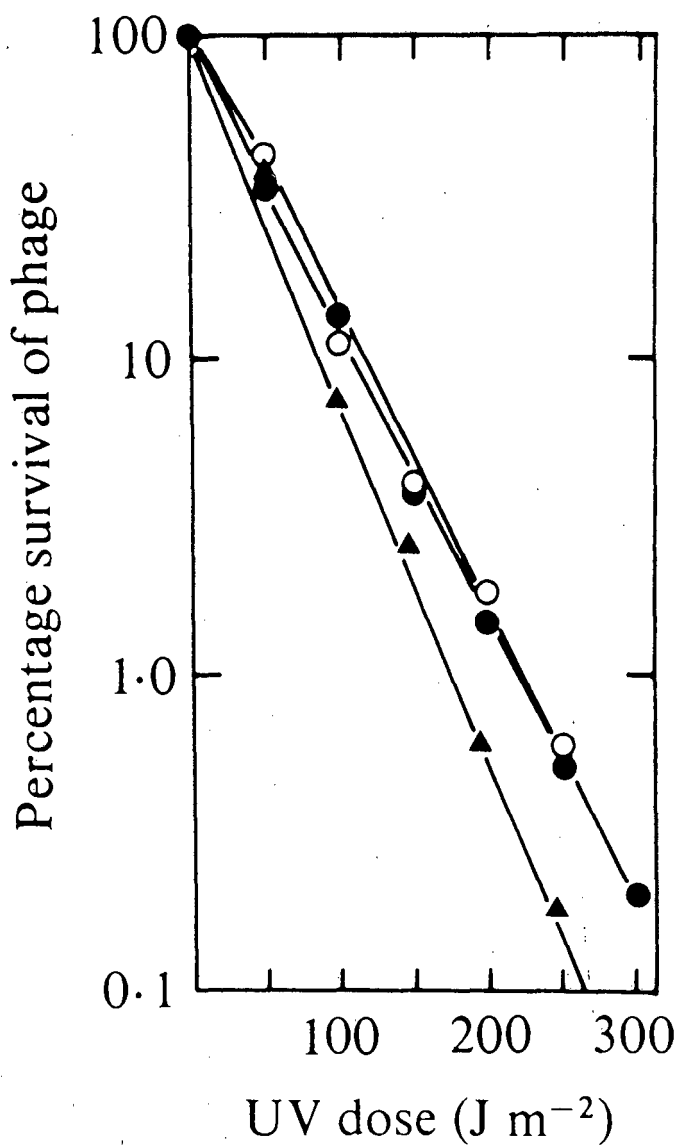


FIGURE 3. Effect of caffeine and sodium arsenite on the survival of UV-irradiated VC11 phage propagated on UV-treated (50 J/m²) *S. cattleya* spores. ●, No inhibitor; ▲, + caffeine; ○, + sodium arsenite.

3.4

DISCUSSION

The use of a bacteriophage allows the separation of DNA damage from host repair systems in order to determine whether these DNA repair mechanisms are constitutive or inducible. This is possible due to the dependence of the phage on host enzymes for DNA replication and repair.

The survival curves of UV-irradiated VC11, presented in Fig. 1, indicated that phage survival was enhanced by the action of a host repair system. In order to characterize the repair mechanism further, it was necessary to examine the effect of caffeine and sodium arsenite, which inhibit DNA repair systems in E. coli, on the survival of bacteriophage both in the presence and absence of UV induction of S. cattleya spores. Elucidation of the repair systems, namely host-cell reactivation and Weigle reactivation, using VC11 was not possible because UV-sensitive mutants of S. cattleya would be necessary for the identification of a host-cell reactivation system operating in this organism. However, a caffeine-induced decrease in the survival of UV-irradiated VC11 propagated on untreated spores would indicate the existence of host-cell reactivation in S. cattleya.

Although caffeine and sodium arsenite are known to inhibit DNA repair systems in E. coli (Rothman et al., 1975; Rothman, 1980),

it is not possible to state that these chemicals exert a similar effect in Streptomyces. Indeed, Harold and Hopwood (1970a) failed to detect an effect of these chemicals on the UV survival of wild type S. coelicolor A3(2). However, as the e.o.p. of VC11 is not significantly altered by the presence of caffeine and sodium arsenite at one-third of the m.i.c. of these chemicals for S. cattleya (Table 1), it is possible that a change in the survival of UV-damaged VC11 in the presence of these substances may indicate that S. cattleya DNA repair systems are influenced by caffeine and sodium arsenite.

The decreased survival of UV-damaged phage propagated on untreated S. cattleya spores in the presence of caffeine, reflected a constitutive, caffeine inhibitable, host-mediated UV repair system in this organism. Similarly, caffeine inhibited an inducible repair system in S. cattleya as shown by the decreased phage survival in the presence of UV-irradiated spores. These results indicate that both the constitutive and the inducible systems are inhibited by caffeine. It is not possible to state whether two separate repair systems exist in S. cattleya or whether they are constitutive and induced components of the same system.

Although the survival of UV-irradiated S. cattleya spores decreased in the presence of sodium arsenite (Coyne et al.,

1984), suggesting the presence of a sodium arsenite inhibitable DNA repair system in S. cattleya, this was not confirmed by bacteriophage studies. The survival of UV-treated VC11 remained constant when propagated on both UV-irradiated and untreated S. cattleya spores in the presence of sodium arsenite. Sodium arsenite has been implicated in the inhibition of the error-prone, SOS-induced DNA repair mechanism in E. coli. In E. coli, the SOS response persists for approximately two hours after induction (Radman, 1975; Witkin, 1976). Spore germination is required for actinophage attachment prior to infection of the host. The time interval between UV-irradiation of the S. cattleya spores and spore germination was between four and six hours. Thus it is likely that any 'SOS-like' response would have decayed before phage attachment. Although no rec A mutants have been isolated in Streptomyces, many species exhibit a high level of recombination which may indicate the presence of a similar system. Experiments involving UV-irradiation of germinating S. cattleya spores and sequential addition of UV-treated VC11 actinophage may detect such a system.

The occurrence of S. cattleya Amy^- Arg^- variants induced by UV-irradiation was almost completely eliminated by the presence of caffeine (Coyne et al., 1984). The effect of this chemical on the induction of genetic instability in S. cattleya indicates that a component of the caffeine inhibitable DNA repair system(s)

is responsible for the genetic instability in this organism. Moreover, the increase in genetic instability in the presence of sodium arsenite suggests that there may be a sodium arsenite-inhibited repair system, not detected using actinophage VC11, which is responsible for repairing the DNA damage which induces the genetic instability (Coyne *et al.*, 1984).

The interaction of UV-damage and DNA repair systems in genetic instability in *S. cattleya* provides the first insight into the physiological basis of this phenomenon in *Streptomyces*. There may be a complex interaction between replicative recombination, UV-damaged DNA and DNA repair systems in these organisms.

Variants having alterations in their secondary metabolism arise frequently in *Streptomyces* and are likely to differ from the wild type strains by characteristic changes (deletions, amplifications and rearrangements) within the chromosomal DNA (Schrenpf, 1982). Kanamycin-sensitive (Kan^S) variants occur in *S. rimosus* at a frequency of approximately 1% (Danilenko *et al.*, 1982). All the Kan^S variants were found to have become sensitive to neomycin (Neo^S) and paramycin (Par^S), while the initial level of resistance to streptomycin and gentamycin was retained. In addition, the $Kan^S Par^S Neo^S$ variants reverted to the initial antibiotic resistant phenotype at a frequency of 1×10^{-3} . The Kan^r determinant, located on the chromosome of *S. rimosus*, was

cloned into S. lividans 66. Transformed S. lividans exhibited unstable, cyclic transitions between the Kan^r and Kan^s phenotypes which were identical to the reversible, phenotypic changes displayed by the Kan^r determinant in S. rimosus (Danilenko and Potekhin, 1983). S. rimosus chromosomal DNA, isolated from variants which exhibited an increased level of Kan^r, displayed repeated tandem duplication of the 10 md fragment harbouring the Kan^r determinant (Danilenko et al., 1982).

Variants of Streptomyces reticuli and S. scabies, having lost the ability to produce melanin, had undergone a deletion of the structural gene for tyrosinase and contained varying amounts of amplified sequences within their chromosomal DNA (Schrempf, 1982). The amplified DNA sequences are found at low copy number in the wild type chromosome, possess the same G+C content as other chromosomal sequences, and have no known function. The fact that parts of the amplified sequence in S. reticuli share varying degrees of homology with several regions of the chromosomal DNA of almost 20 different species of Streptomyces, may indicate the presence of transposable elements in the genome of these Streptomyces strains (Schrempf, 1982).

On this basis, the possible role of recombinational events (transpositions) in cell differentiation and development is very real. It is possible that this type of regulation is widespread

among Streptomyces, a genus which undergoes several stages in development and forms a wide variety of secondary metabolites (Danilenko et al., 1981).

A molecular model proposed by Shapiro (1979), to explain the mechanism of transposition and replication of transposable elements, has a number of interesting features. Transposition of an element to a new site does not remove the element from its original site, resulting in cells accumulating multiple elements with time (sequence amplification) unless transposition is repressed. The initial interaction between the two DNA duplexes involves single-strand cleavage and ligation without base-pairing of complementary chains. This explains how replicative recombination can occur in the absence of detectable sequence homology and thus accounts for the wide versatility of DNA reorganizations mediated by insertion elements (Shapiro, 1982).

The generation of single-strand breaks, either directly (recombination and DNA repair) or indirectly (UV-irradiation), is a common feature. Indeed, incubation of S. cattleya spores in the presence of ^{32}P -labelled orthophosphate, which generates single-strand breaks in DNA molecules at -70°C , resulted in a 17-fold increase in the frequency of $\text{Amy}^{-}\text{Arg}^{-}$ variants (0.5% to 8.4%). As the effect of ^{32}P was not inhibited by caffeine, it is likely that single-strand breaks are the primary event causing

genetic instability and not an effect of a DNA repair system (Coyne et al., 1984).

A tentative explanation for the relationship between single-strand breaks and genetic instability may be that UV-irradiation induces a repair system which excises pyrimidine dimers from the DNA molecule and, in so doing, creates single-strand breaks which promote recombination (transposition) events which lead to genetic instability. In other words, the induction of a DNA repair system(s), while not a requirement for recombination, may result in an increase in the occurrence of genetic rearrangements (transposition events) by providing possible insertion sites. This explanation is supported by the fact that caffeine inhibits a DNA repair system(s) as well as the induction of Arg⁻ Amy⁻ variants of S. cattleya. Indeed, it has been shown that caffeine inhibits single-strand DNA break formation in E. coli by binding at locally denatured areas adjacent to pyrimidine dimers and thereby inhibiting enzymes responsible for both the endonucleolytic cleavage at the dimer and the exonucleolytic digestion of the dimer-containing strand (Rothman, 1980). Moreover, once single-strand breaks are formed, caffeine is no longer effective in the inhibition of E. coli DNA repair (Fong and Bockrath, 1979). This observation may explain the inability of caffeine to inhibit the occurrence of Amy⁻ Arg⁻ variants induced by ³²P. Finally, if one postulates the presence

of a 'SOS-like' repair system in S. cattleya, it may be possible to explain the increase of $\text{Amy}^- \text{Arg}^-$ variants induced by sodium arsenite inhibition of this system by the fact that the in vivo machinery for SOS induction in E. coli is a quaternary complex of single-stranded DNA, single-strand binding protein, Rec A and ATP (Cohen et al., 1983). Induction of this repair system by the presence of single-strand breaks in the DNA molecule would result in the repair of these lesions and, in so doing, decrease the probability of genetic instability via genomic rearrangement. Thus, the selection of specific mutations affecting UV repair may in turn affect genetic instability. This could create methods of reducing genetic instability in industrially-important Streptomyces.

However, the above argument is highly speculative. There is no biochemical data which indicate the mechanism whereby caffeine inhibits DNA repair in Streptomyces. As E. coli and Streptomyces are evolutionary distinct micro-organisms, no direct comparisons with respect to the nature of caffeine inhibition of DNA repair and the mechanisms of DNA repair are possible between these bacterial groups. Thus, in order to elucidate the modes of DNA repair in Streptomyces, it will be necessary to isolate DNA repair mutants which could be studied in conjunction with UV survival of phage and caffeine inhibition. These mutants would also allow the identification of specific genes and their

products which are involved in DNA repair in Streptomyces. Subsequently, groups of genes involved in Streptomyces DNA repair could be isolated and their genetic regulation studied using in vitro transcription-translation systems. In addition, a direct investigation as to the nature of DNA damage following UV-irradiation of Streptomyces should be performed. Moreover, it is necessary to directly measure the repair of damaged Streptomyces DNA (for example, by monitoring the loss of radioactivity from lesions or following the appearance or disappearance of strand breaks in DNA by sedimentation in sucrose gradients; Hanawalt et al., 1979) in order to elucidate the biochemical processes involved in the restoration of UV impaired Streptomyces DNA. Likewise, no 'SOS-like' repair system has been detected in Streptomyces, while Streptomyces rec⁻ mutants have not been reported. The latter observation implies that it is highly unlikely that a DNA repair system analogous to the E. coli SOS repair system exists in Streptomyces. Thus, a detailed investigation into the genetic and biochemical processes of recombination in Streptomyces would provide valuable data which may lead to the identification of additional DNA repair systems in Streptomyces and may contribute towards the understanding of the genetic instability phenomena in these micro-organisms.

CHAPTER 4

TRANSFECTION OF STREPTOMYCES CATTLEYA AND STREPTOMYCES
OLIVACEUS PROTOPLASTS WITH PHAGE VC11 DNA

SUMMARY

S. cattleya and S. olivaceus were sensitive to lysozyme hydrolysis at 37°C, yielding protoplasts after 90 min incubation. S. olivaceus produced viable colonies after incubation at 30°C for 48 h, while S. cattleya exhibited very small colonies following 6 days incubation. The regeneration frequency of S. olivaceus protoplasts was estimated to be 26.7%, while the S. cattleya protoplast regeneration frequency of 0.5% reflected the slow rate of colony-formation on R2 agar. Attempts to improve the regeneration frequency of S. cattleya protoplasts were unsuccessful. S. cattleya protoplasts were not transfected with VC11 DNA. However, 1 ug of VC11 DNA generated 5.3×10^2 S. olivaceus transfectants. Thus, VC11 DNA transfected S. olivaceus protoplasts at a frequency of 0.24×10^{-6} per phage DNA molecule and 1.3×10^{-5} per viable protoplast. Although transfection of S. cattleya protoplasts with VC11 DNA was unsuccessful, indirect 'transfection' of S. cattleya with phage DNA was accomplished by the regeneration of VC11 transfected S. olivaceus protoplasts in

the presence of S. cattleya spores to yield 55 'transfectants' per microgram of VC11 DNA.

4.1 INTRODUCTION

The advent of recombinant DNA technology has provided a powerful tool for the study of genetic control of antibiotic biosynthesis in Streptomyces, improvement of antibiotic yields and the development of novel, natural antibiotics. The ability to introduce genetic material into viable cells is a fundamental requirement for genetic analysis by recombination and complementation studies.

Genetic recombination via plasmid-mediated conjugation is known to occur in S. coelicolor A3(2), S. lividans 66 and S. rimosus (Hopwood, 1983). The autonomously replicating S. coelicolor A3(2) plasmids SCP1 and SCP2 are able to produce recombinants between two genetically marked strains at a frequency of 10^{-5} of the total progeny. In addition, integrated SCP1-mediated chromosomal transfer gives rise to recombinants at a frequency greater than 10^{-1} following crosses with SCP1⁻ strains (Hopwood, 1983). The plasmids SLP2 and SLP3 of S. lividans 66, and SRP1 and SRP2 of S. rimosus are able to promote genetic recombination via conjugation between strains of their respective Streptomyces hosts. Although

plasmid-mediated conjugation in Streptomyces requires contact between mycelium, the actual nature of the conjugation process is unknown. The observation that the plasmid SLPl.6 (9.4 kb) is able to promote conjugation between S. coelicolor A3(2) and S. lividans 66, while a 2 kb region of the plasmid pIJ101 is responsible for chromosomal mobilization and transfer, indicates that the conjugation process in Streptomyces is simpler than that in E. coli (Hopwood, 1983).

A second system of gene transfer, namely generalized transduction, is promoted by the packaging of random chromosomal DNA fragments in phage particles following lytic growth of temperate or virulent bacteriophage (Sherratt, 1981). Thus, a lysate produced by a generalized transducing phage contains two types of particles: a majority containing only phage DNA and a minority containing mainly host DNA.

Only two cases of generalized transduction have been reported in Streptomyces. The virulent phage ϕ SV1 was used to cotransduce various loci into auxotrophic mutants of S. venezuelae and thus restore prototrophy (Stuttard, 1983). Süss and Klaus (1981) demonstrated generalized transduction of several markers from S. lividans 66 into Streptomyces hygroscopicus by the temperate actinophage SH10. The authors noted that the efficiency of transduction, between 2.7×10^{-6} and 2.1×10^{-6} per p.f.u.,

depended on the amount of infecting phage, the age of the germinating spores and the selective marker. Attempts to demonstrate generalized transduction in S. coelicolor A3(2) by the extensively characterized phages ϕ C31, R4 and VP5 produced negative results (Lomovskaya et al., 1980). Thus, generalized transduction is of limited use as a tool for genetic analysis in Streptomyces.

Genetic transformation involves the uptake, and usually the expression and inheritance, of naked DNA (Sherratt, 1981). In general, bacteria cannot distinguish the source of DNA and consequently, DNA of any origin may be introduced into a host cell and, if coupled to a functional replicon, be stably maintained in the recipient. A second advantage of genetic transformation is that the transforming DNA can be subjected to mutagenesis or other in vitro manipulations prior to introduction into bacterial cells.

The limiting factor in the yield of transformants is the competence of the recipient cell population to take up transforming DNA (Stanier et al., 1976). Competence is a physiological state that fluctuates greatly during the cell cycle. The ability of E. coli to undergo transformation was found to depend on the presence of calcium ions which render the bacterium competent. The cells subsequently receive a heat shock

during which they take up exogenous DNA (Timmis, 1981).

Roelants et al. (1976) showed that radioactively-labelled linear DNA was taken up by Streptomyces virginiae mutant Nb.10 and Streptomyces kasugaensis. Genetic transformation of these Streptomyces strains seemed to be independent of the origin of the DNA and occurred between the late exponential growth phase and stationary phase. However, S. coelicolor A3(2), Streptomyces fradiae, Streptomyces kanamyceticus, S. rimosus, Streptomyces tenebrarius, S. griseus, S. virginiae ATCC 13161 and Micromonospora purpurea either degraded the exogenous DNA or exhibited a very poor transformation efficiency. Thus, no efficient system for the transformation of Streptomyces with exogenous DNA has been developed due to the lack of a well-defined competence stage in these micro-organisms.

The establishment of procedures for the formation and subsequent regeneration of Streptomyces protoplasts has enabled the study of Streptomyces molecular genetics by a variety of techniques which were previously unavailable to these bacteria. Sagara et al. (1971) reported the increased sensitivity of Streptomyces griseoflavus to lysozyme when grown in a medium containing high concentrations of glycine. Subsequently, Okanishi et al. (1974) noted that the optimal concentration of glycine was different for S. griseus (0.8%, w/v) and S. venezuelae (2%, w/v). Thus,

depending on the Streptomyces species, glycine was utilized at a concentration which inhibited growth but allowed a reasonable yield of mycelia (Hopwood et al., 1977). Following growth in the presence of glycine, the cell walls were removed by digestion with a combination of lysozyme and lytic enzyme no. 2 in a hypertonic solution (Okanishi et al., 1974). The hypertonic solution, containing Mg^{2+} and Ca^{2+} ions, stabilized the protoplasts once the cell walls had been removed. It was later found that lytic enzyme no. 2 was unnecessary as lysozyme treatment alone, although slower, was sufficient for the preparation of protoplasts (Baltz, 1978; Hopwood and Wright, 1979).

Okanishi et al. (1974) studied the reversion of S. griseus and S. venezuelae protoplasts to the normal filamentous state using various synthetic agar media. The authors showed that protoplast regeneration on agar was significantly affected by the concentration of $MgCl_2$, $CaCl_2$, phosphate and casamino acids, as well as by the concentration of sucrose, the nature of the nitrogen source and the type of buffer. The combination of appropriate concentrations of $MgCl_2$ and $CaCl_2$ contributed towards protoplast stability and reversion, while the presence of casamino acids enhanced the growth and regeneration rates. On the basis of their results, the authors developed two distinct hypertonic solid synthetic media for the regeneration of

protoplasts of the two Streptomyces species at frequencies of 50%. One of the two regeneration media developed by Okanishi et al. (1974), namely R2 agar, was found to generate higher protoplast reversion frequencies (Hopwood et al., 1977) and a modified R2 medium (R2YE) has subsequently been successfully utilized with a wide variety of strains of at least 10 Streptomyces species (Hopwood, 1981b).

A number of physical conditions have been found to be important for optimal regeneration of Streptomyces protoplasts. Baltz (1978) observed that asynchronous regeneration of S. fradiae protoplasts resulted in the first colonies exerting an inhibitory effect on the regeneration of nearby protoplasts. As this phenomenon was not due to antibiotic production by the mature colonies, the author postulated that the inhibitory effect was similar to that observed in B. subtilis in which the excretion of autolytic enzymes impedes cell wall synthesis during protoplast regeneration. Auto-inhibition seems to be species-specific as a similar phenomenon was observed in S. acrimycini, but not in S. coelicolor A3(2), S. parvulus, S. lividans and S. griseus (Hopwood et al., 1977). Subsequently, it was found that auto-inhibition in S. fradiae can be eliminated by dehydration of the regeneration agar by 15 to 20% prior to use and by propagation of the protoplasts in soft R2 agar overlays (Baltz and Matsushima, 1981). This technique resulted in more rapid,

synchronous regeneration of S. fradiae protoplasts and enhanced the regeneration frequency by a factor of 10. The dehydration of the regeneration agar may lead to rapid dehydration of the soft agar overlay thus preventing protoplast lysis due to excess liquid. In addition, dehydration may retard the diffusion of auto-inhibitory substances.

The temperatures at which the mycelia were propagated prior to protoplast formation, and at which the protoplasts were regenerated, were found to be critical for efficient protoplast regeneration (Baltz and Matsushima, 1981). The authors noted that optimum regeneration frequencies of S. fradiae protoplasts (100%) were obtained when the cells were grown at 29°C and protoplasts subsequently regenerated at the same temperature. S. fradiae propagated at higher temperatures yielded protoplasts which regenerated poorly at 29°C, while cells grown at 29°C produced protoplasts which regenerated less efficiently at higher temperatures, indicating that the negative effects of elevated temperatures are synergistic. In addition, although increased regeneration frequencies of protoplasts of S. albus G, S. ambofaciens and S. griseofuscus occurred at 29°C, each species exhibited a different optimum temperature for cell growth prior to protoplast formation (Baltz and Matsushima, 1981).

Finally, the physiological state of the mycelium prior to

protoplast formation is important for efficient regeneration (Hopwood, 1981b). Okanishi et al. (1974) reported efficient regeneration of S. griseus and S. venezuelae protoplasts prepared from mycelia in the mid-exponential growth phase. However, protoplasts of S. fradiae, S. albus G, S. ambofaciens and S. griseofuscus harvested between the exponential and stationary growth phases regenerated more efficiently than protoplasts prepared from mycelia in the mid- to late-exponential or late-stationary growth phases (Baltz, 1978; Baltz and Matsushima, 1981). These observations indicate that the optimum physiological state of mycelia required for efficient protoplast regeneration varies between Streptomyces species.

Polyethylene glycol (PEG) was initially used to induce plant protoplast fusion. PEG was found to be effective between molecular weights of 1,000 to 6,000, concentrated PEG solutions (50%) were more efficient than dilute solutions, solutions of PEG of different molecular weights were equally effective at the same concentration (w/v), and protoplast fusion occurred after a short period of treatment (Hopwood, 1981b).

Hopwood et al. (1977) reported a procedure for the fusion of Streptomyces protoplasts by treatment with PEG. The technique, based on the mammalian cell system, utilized 40% (w/v) PEG 1540 in the presence of 15% dimethyl sulphoxide (DMSO) which enhances

the efficiency of protoplast fusion at this PEG concentration. Subsequently, 50% PEG 1,000 was found to induce optimum protoplast fusion frequencies in the absence of DMSO (Hopwood and Wright, 1979; Baltz and Matsushima, 1981). As in the case of plant protoplasts and mammalian cells, a brief exposure (1 min) to PEG promoted protoplast fusion in Streptomyces (Hopwood et al., 1977).

High frequencies of intrastrain chromosomal recombination are attained by PEG-induced protoplast fusion in Streptomyces (Hopwood et al., 1977; Hopwood and Wright, 1978; Baltz and Matsushima, 1981). Hopwood et al. (1977) analyzed the segregation of non-selected markers amongst recombinants from six-factor crosses of S. coelicolor A3(2) derivatives. The authors found that multiple crossover classes represented approximately 2% of the total progeny following a mating, whereas up to 20% of the total progeny represented recombinant genotypes as a result of protoplast fusion. This recombination was independent of the sex factors SCP1 and SCP2 and thus, should be applicable to strains lacking sex factors. Examination of the spatial distribution of crossover events in double and quadruple crossover recombinants, indicated very little tendency for crossovers to occur preferentially in map intervals adjacent to one another rather than in distant map intervals, suggesting that genomes brought together by protoplast fusion are diploid

(Hopwood and Wright, 1978). This observation contrasts with conjugation which generates zygotes which are incomplete mero-diploids in that they contain a complete chromosome from the recipient parent and approximately one-fifth of the donor chromosome (Hopwood, 1981b). In addition, several rounds of recombination occur between protoplast fusion and regeneration to form haploid cells, and fusions between three and four protoplasts occur frequently allowing the inheritance of markers from three or four parent genomes (Hopwood and Wright, 1978).

Chromosomal recombination via protoplast fusion has useful applications for genetic analysis in Streptomyces. The technique may be extended to the improvement of industrial strains by virtue of the fact that high recombination frequencies are attained in the absence of sex factors and the relaxed linkage allows a nearly random assortment of genotypes (Hopwood et al., 1977). These features would allow the crossing of divergent lines obtained by successive mutant selection in order to generate an assortment of new genotypes, including those in which positive gene interactions gave rise to superior performance or in which groups of negatively interacting genes had been disrupted (Hopwood et al., 1977). The utilization of optimal fusion conditions to generate high proportions of recombinant progeny would make the introduction of counter-selectable markers into the parent strains unnecessary (Hopwood, 1981b).

Although conjugation and protoplast fusion allow chromosomal recombination, the genetic exchange mediated by these techniques is essentially limited to strains of the same species. Protoplast fusion between a single auxotroph (met) of S. fradiae and a double auxotroph (nic ade) of S. bikiniensis yielded nic⁺ ade and nic ade⁺ colonies at a low frequency between 10^{-5} and 10^{-6} (Hopwood, 1981b). Conjugation between these two strains resulted in the appearance of these phenotypes at a 100-fold lower frequency. In addition, protoplast fusions between S. coelicolor A3(2) and S. lividans 66 exhibited a very low and poorly reproducible level of recombination, while no recombinants were generated from protoplast fusions between S. coelicolor A3(2) and S. parvulus which is able to act as a host for the S. coelicolor A3(2) plasmids SCP1 and SCP2 (Hopwood, 1981b). This situation has precluded the establishment of interstrain gene pools which would increase the possibility of discovering new antibiotics by recombination between antibiotic structural and regulatory genes resulting in the detection of 'silent gene' products (Hopwood, 1981b).

The lack of naturally occurring competence in Streptomyces for the uptake of free DNA has been overcome by the development of a technique enabling protoplast transformation with foreign DNA which allows the cloning of DNA of any origin into Streptomyces (Bibb et al., 1978). The transformation of Streptomyces

protoplasts with plasmid DNA is mediated by the presence of PEG. The optimum concentration of PEG 1,000 was found to be 20% (w/v) in contrast to 50% PEG 1,000 necessary for maximum recombination via protoplast fusion, suggesting that PEG-promoted protoplast fusion and protoplast transformation occur via different mechanisms. The mechanism whereby PEG induces protoplast transformation is unknown. As in the case of protoplast fusion, plasmid transformation of Streptomyces protoplasts is induced by a brief exposure (1 min) to PEG. Subsequent regeneration of the transformed protoplasts results in 80% of the colonies possessing exogenous DNA (Hopwood, 1981b). The relationship between DNA concentration and the number of transformants is linear with a slope of one over many orders of magnitude, indicating that single plasmid molecules are able to transform (Hopwood, 1981b). Transformation of protoplasts with the unrestricted SCP2* and SLP1.2 plasmids yielded 10^7 transformants per microgram of DNA (Hopwood, 1981b), while 'shotgun' cloning with SLP1-derived vectors generated between 10^4 and 10^5 transformants per microgram DNA (Chater et al., 1982a).

PEG-induced transformation of protoplasts with chromosomal DNA does not generate recombinants at a detectable level due to the possible presence of extracellular DNase activity (Makins and Holt, 1981). When phospholipids are dispersed in an aqueous solution, vesicles known as liposomes form, encapsulating

discrete volumes of the aqueous phase inside a bilayered lipid membrane. The addition of a DNA solution to a film of dried phospholipid results in the formation of liposomes containing DNA which can be subsequently fused with Streptomyces protoplasts in the presence of PEG (Makins and Holt, 1981). Chromosomal DNA encapsulated in liposomes is protected from the action of DNases and is released into the cytoplasm of protoplasts following fusion. Subsequent protoplast regeneration results in the generation of genetically-characterized transformants. As the optimum PEG 1,000 concentration (50%, w/v) is identical to that required for protoplast fusion, the authors suggested that the liposome functions as an artificial protoplast and that the process of liposome-mediated transformation mimics protoplast fusion. Independent transformation of distantly-linked marker genes conferring antibiotic resistance or sensitivity, auxotrophy, or prototrophy occurred at high frequencies (2-10%) in both S. clavuligerus and S. coelicolor A3(2), suggesting that nearly the entire recipient population was transformed for at least one marker (Makins and Holt, 1981). Thus, liposome-mediated transformation of protoplasts with chromosomal DNA has provided another useful tool for the study of the molecular genetics of Streptomyces.

The use of bacteriophage DNA cloning vectors depends on the availability of a system whereby hybrid DNA molecules may be

introduced into the host cell. Two such systems, transfection of phage DNA and in vitro packaging of the hybrid molecule prior to phage infection, have been developed for lambda DNA cloning vectors in E. coli (Timmis, 1981). However, the genetic elucidation of actinophages has not progressed enough in order to establish an in vitro packaging system and consequently, a Streptomyces transfection system is necessary for the use of phage vectors in these microbes (Krügel et al., 1980).

The first report concerning transfection in Streptomyces involved the introduction of DNA of the actinophage PK-66 into protoplasts of S. kanamyceticus, S. violaceoniger and S. acidomyceticus (Okanishi et al., 1968). However, transfection was assessed by monitoring the phage titre in the medium and thus the system did not allow the recovery of the products of individual transfection events (Hopwood, 1981b). A Streptomyces transfection system, based on the protoplast transformation procedure, allowed the uptake of DNA of phage SH10 by S. lividans 66 protoplasts at an efficiency between 2×10^{-8} and 2×10^{-7} p.f.u. per phage DNA molecule (Krügel et al., 1980). Transfection of the S. lividans 66 protoplasts was mediated by the presence of 20% (w/v) PEG 6000. In addition, transfected protoplasts were regenerated in liquid R2 medium and transfection efficiencies were determined by propagating samples together with S. lividans spores on solid media. Subsequently, Suarez and Chater (1980a) developed a

procedure whereby protoplasts transfected in the presence of 20% PEG 1000 were regenerated by directly plating the protoplasts together with an excess of uninfected spores in soft agar overlays on solid R2 agar so that phage plaques were easily detected. The presence of 20% PEG 1000 facilitated maximum protoplast transfection after a short period (1 min) of treatment and plaque-formation on an indicator lawn allowed recognition of the phage particles released by individual transfectants (Suarez and Chater, 1980a). It is not known whether regeneration of the transfected protoplasts is necessary for the production of phage particles which are subsequently propagated on indicator bacteria (Hopwood, 1981b). The inclusion of yeast extract in the regeneration medium allowed earlier detection of phage plaques following PEG-mediated protoplast transfection (Suarez and Chater, 1981).

The linear, first-order relationship between phage DNA concentration and transfection indicates that a single DNA molecule is able to initiate a transfection event (Suarez and Chater, 1980a; Suarez and Chater, 1981). In addition, DNA uptake is not affected by DNA conformation as both linear and circular molecules transfect with equal efficiency (Suarez and Chater, 1980a). The observation that excess non-transfecting DNA reduces the frequency of transfection indicates that individual protoplasts are able to incorporate limited amounts of exogenous

DNA, while EDTA-inhibited transfection may be due to a divalent cation-dependent specific uptake system in the cell membrane (Suarez and Chater, 1980a). Although 5×10^5 transfectants per microgram of DNA was obtained, increased transfection frequencies in some phage-host systems were limited by protoplast numbers even though less than 1 protoplast in 10^5 had been transfected (Suarez and Chater, 1980a). Moreover, transfection of S. lividans 66 protoplasts with a DNA mixture containing equal amounts of ϕ C31 and R4 phage DNA resulted in mostly ϕ C31 transfectants. Since only 1 protoplast in 10^5 is normally transfected by either phage DNA, only this fraction of transfectants should have been mixed (Suarez and Chater, 1980a). On the basis of these observations, the authors suggested that only a minor sub-population of the protoplasts is competent for transfection in contrast to protoplast transformation whereby 20 to 30% of the protoplast population were transformed (Suarez and Chater 1980a).

The low transfection frequencies (10^5 transfectants as opposed to 10^7 transformants per microgram DNA), together with inhibition of transfection by non-specific competing DNA, would severely hinder 'shotgun' cloning experiments with phage vectors. Rodicio and Chater (1982) discovered that the presence of positively-charged DNA-free liposomes in association with 50% (w/v) PEG 1000, induced a 100-fold increase in the efficiency of protoplast

transfection. Although the mechanism whereby this phenomenon occurs is unknown, the authors speculated that PEG (at the same concentration necessary for efficient protoplast fusion) causes the small liposomes to fuse with the relatively large protoplasts, while the presence of a positive charge induces the DNA to bind to the liposomes thus concentrating the DNA in the areas of fusion between the liposomes and protoplasts (Rodicio and Chater, 1982). The frequency of 10^7 tranfectants per microgram phage DNA, obtainable with stimulation of protoplast transfection by small DNA-free liposomes, will allow the successful utilization of phage vectors in 'shotgun' cloning experiments. In addition, small DNA-free liposomes were found to affect plasmid transformation of protoplasts, generating reproducible frequencies of 10^7 transformants per microgram DNA.

The establishment of techniques allowing the introduction of plasmid and phage DNA into Streptomyces strains will facilitate the use of recombinant DNA technology for the elucidation of the molecular genetics of these micro-organisms. Antibiotic structural and regulatory genes may now be isolated and the genetic mechanisms of antibiotic biosynthesis studied. In addition, the introduction of antibiotic production genes from a specific Streptomyces strain into an unrelated strain may create new combinations of structural genes which would synthesize novel antibiotics.

The availability of the phage VC11, able to virulently infect S. cattleya, S. olivaceus and S. lividans TC10, has created the possibility of establishing a cloning system in these organisms. Construction of a VC11-derived cloning vector would allow the isolation of antibiotic production and regulation, as well as metabolic, genes from the commercially-important antibiotic producers S. cattleya and S. olivaceus and the introduction of these genes into the antibiotic non-producing S. lividans strain in which their functions may be studied. Transfection of S. cattleya and S. olivaceus protoplasts with VC11 DNA, and subsequent regeneration of the transfected protoplasts, was investigated as successful VC11 DNA transfection of these Streptomyces species is a fundamental requirement for the application of recombinant DNA technology to these commercially-important beta-lactam antibiotic-producing micro-organisms.

4.2. METHODS

4.2.1 Preparation of Streptomyces protoplasts.

Protoplasts of S. cattleya and S. olivaceus were prepared in the following manner. Twenty ml Schrempf medium (see Appendix) supplemented with 1% (w/v) glycine in a 250 ml conical flask was

inoculated with a loopful of Streptomyces spores and incubated on an orbital shaker for 48 h at 30°C. The mycelia were pelleted by centrifugation at 10,000 rpm for 10 min in a SS 34 rotor at 4°C and resuspended in 20 ml P medium (see Appendix) containing a final concentration of 1 mg/ml lysozyme. The suspension was incubated at 37°C with agitation and monitored for the appearance of protoplasts by phase-contrast microscopy after 1 h. Following the detection of protoplasts, the suspension was pelleted by centrifugation at 10,000 rpm for 10 min in a SS 34 rotor at 4°C and resuspended in 10 ml P medium. The mycelia were subsequently removed by centrifugation at 2,000 rpm for 5 min and the supernatant was pelleted by centrifugation at 10,000 rpm for 10 min. The protoplasts were resuspended in 2 ml P medium and stored at -20°C in 200 μ l aliquots.

4.2.2 Regeneration of Streptomyces protoplasts.

S. cattleya and S. olivaceus protoplasts were regenerated using the following protocol. Frozen protoplasts in P medium (200 μ l) were thawed at room temperature. Ten-fold serial dilutions of the protoplasts were prepared in either P medium or sterile distilled water. The protoplast dilutions were incubated at room temperature for 4 h. Subsequently, 100 μ l of each dilution was spread onto the surface of R2 agar (see Appendix) with a platinum loop and incubated at 30°C until the appearance of colonies.

The protoplast regeneration frequency for each Streptomyces strain was ascertained by estimating the number of colonies originating from non-protoplasted units as opposed to true protoplast regeneration, and subsequently expressing the number of viable protoplasts as a percentage of the total number of protoplasts. The number of non-protoplasted units was obtained by incubating a sample of the protoplast suspension in sterile distilled water prior to growth on regeneration agar. Due to the absence of a rigid cell wall, the protoplasts undergo osmotic lysis in the hypertonic environment while non-protoplasted units remain intact. The total number of protoplasts (viable and non-viable protoplasts) was estimated from haemocytometer counts. Thus, the regeneration frequency of S. cattleya and S. olivaceus protoplasts was determined by the formula: Protoplast regeneration frequency = $100 \times (b-c)/a$, where 'a' is the total number of protoplasts, 'b' is the number of c.f.u. generated after dilution in P medium and 'c' is the number of c.f.u. produced following dilution in water.

4.2.3 Transfection of Streptomyces protoplasts with phage VC11 DNA.

Protoplasts of S. cattleya and S. olivaceus were transfected with phage DNA by the same procedure. VC11 DNA (5 ug), prepared by the method described in section 2.2.10., suspended in 10 ul TE

buffer was added to 100 ul of thawed protoplasts in P medium. Within 30 sec, 500 ul transformation medium (see Appendix) containing a final concentration of 20% (w/v) PEG 1000 was added to the suspension which was subsequently mixed by pipetting 3 times in a 5 ml glass pipette. The suspension was incubated for 1 min at room temperature to allow DNA uptake. Aliquots (100 ul) of the transfection reaction mix were added to 3 ml soft R2 agar (see Appendix) at 42°C which contained 0.3 ml of a spore suspension of the corresponding Streptomyces species. The soft R2 agar was mixed on a whirlymix and overlayed onto the surface of R2 agar. Phage plaques were scored following incubation at 30°C for 1 to 2 days.

The transfection efficiency per DNA molecule was estimated by expressing the number of transfectants as a fraction of the number of VC11 DNA molecules where 5 ug VC11 DNA is equivalent to 1.1×10^{11} VC11 DNA molecules. The efficiency of transfection per protoplast was ascertained by expressing the number of transfectants as a fraction of the number of viable protoplasts. The number of viable protoplasts was determined by subtracting the number of c.f.u. in water from that in P medium ('b-c' of the formula used for calculating the frequency of protoplast regeneration; section 4.2.2).

To demonstrate the absence of intact phage particles, 10 ul (5

ug) VC11 DNA in TE buffer was added to 100 ul P medium and 500 ul transformation medium. The suspension was mixed by pipetting 3 times with a 5 ml glass pipette. One hundred ul of the suspension was added to 0.3 ml S. cattleya spores in 3 ml molten soft R2 agar and subsequently overlayed onto R2 agar. The plates were scored for the presence of plaques after incubation at 30°C for 24 h.

In order to ensure that spore germination was not inhibited by the presence of PEG, 100 ul P medium and 500 ul transformation medium were added to 3 ml soft R2 agar containing 0.3 ml Streptomyces spores at 42°C. The soft agar was mixed and subsequently overlayed on R2 agar. The plates were examined for confluent growth after incubation at 30°C for 1 to 2 days.

4.2.4 'Transfection' of S. cattleya spores with VC11 DNA

S. olivaceus protoplasts in P medium (100 ul) were thawed at room temperature. Subsequently, 5 ug VC11 DNA suspended in 10 ul TE buffer was added to the protoplast suspension, followed by 500 ul transformation medium containing a final PEG 1000 concentration of 20% (w/v). The suspension was mixed by pipetting 3 times in a 5 ml glass pipette and subsequently incubated for 1 min to allow uptake of phage DNA. Aliquots (100 ul) of VC11 transfected S. olivaceus protoplasts were added to 3 ml soft R2 agar at 42°C

which contained 0.3 ml of a S. cattleya spore suspension. The soft R2 agar was mixed on a whirlymix and overlayed onto the surface of solid R2 agar. Phage plaques were scored following incubation at 30°C for 1 to 2 days.

4.3 RESULTS

4.3.1 Preparation of protoplasts.

The presence of 1% (w/v) glycine prevented the formation of large mycelial masses during propagation of the Streptomyces strains prior to protoplast formation. Both S. cattleya and S. olivaceus were sensitive to lysozyme hydrolysis at 37°C. S. olivaceus and S. cattleya protoplasts were detected by phase-contrast microscopy following lysozyme treatment for 90 min at 37°C. However, the presence of mycelial fragments suggested that these Streptomyces species had not been completely protoplasted.

4.3.2 Protoplast regeneration

S. olivaceus colonies were detected after 48 h at 30°C, while S. cattleya exhibited very small colonies after 6 days incubation. The total number of regenerating colonies (diluted in P medium) and the number of colonies originating from non-protoplasted

mycelial fragments (diluted in water) are presented in Table 1.

The regeneration frequency of S. olivaceus protoplasts was estimated to be 26.7%. Attempts to improve the regeneration frequency of S. cattleya protoplasts (0.5%) by either propagating the mycelia and regenerating the protoplasts at 28°C or preparing S. cattleya protoplasts from young mycelia (18 h old) were unsuccessful (results not shown).

4.3.3 Transfection of Streptomyces protoplasts with VC11 DNA.

Transfection of S. cattleya protoplasts with VC11 DNA was unsuccessful. The failure of VC11 DNA to transfect this Streptomyces species cannot be attributed to PEG inhibition of mycelial growth as S. cattleya spores were viable on R2 agar in the presence of P medium and transformation medium.

However, VC11 DNA was able to transfect S. olivaceus protoplasts, generating 5.3×10^2 transfectants per microgram of VC11 DNA. The absence of phage plaques following incubation of VC11 DNA in the presence of S. cattleya spores, indicates that VC11 transfection of S. olivaceus protoplasts was not due to the

TABLE 1. PERCENTAGE REGENERATION OF STREPTOMYCES PROTOPLASTS

Strain	Total	<u>Number of c.f.u./ml</u>		Protoplast
	Protoplasts per ml.	P medium	Water	regeneration (%)
<u>S. cattleya</u>	5.0×10^8	2.5×10^6	8.0×10^4	0.5
<u>S. olivaceus</u>	8.6×10^8	1.9×10^9	1.7×10^9	26.7

presence of intact phage particles.

4.3.4 'Transfection' of *S. cattleya* spores with VC11 DNA.

The inability to transfect *S. cattleya* protoplasts with VC11 DNA was overcome by the regeneration of VC11 transfected *S. olivaceus* protoplasts in the presence of *S. cattleya* spores. Phage particles produced in the *S. olivaceus* protoplasts were subsequently able to infect the germinating *S. cattleya* spores, generating up to 55 'transfectants' per microgram of VC11 DNA.

4.4 DISCUSSION

Although protoplast regeneration frequencies of upto 50% or more have been attained with specific *Streptomyces* species (for example, 56% with *S. rimosus* R8; Pigac et al., 1982), regeneration of protoplasts of other *Streptomyces* strains, such as *S. coelicolor* A3(2), occurs at very low frequencies (Hopwood, 1981b). Thus, the regeneration frequency of *S. olivaceus* protoplasts is average for *Streptomyces* protoplasts, while the *S. cattleya* protoplast regeneration frequency is extremely low.

Baltz and Matsushima (1981) demonstrated that the temperature at

which mycelia were propagated prior to protoplasting, and at which protoplast regeneration was performed, affects the efficiency of protoplast regeneration. However, an attempt to increase the regeneration frequency of S. cattleya protoplasts by propagating the mycelia and regenerating the protoplasts of this streptomycete at 28°C failed to yield improved protoplast regeneration frequencies. Likewise, efficient regeneration of S. griseus and S. venezuelae protoplasts was obtained when protoplasts were prepared from mycelia in the mid-exponential growth phase, while S. fradiae and S. griseofuscus protoplasts regenerated efficiently when prepared from mycelia in the transition state between the exponential and stationary growth phases (Okanishi et al., 1974; Baltz, 1978). These observations indicate that the physiological state of the mycelia prior to protoplasting is important for efficient protoplast regeneration. However, an attempt to improve the efficiency of S. cattleya protoplast regeneration by preparing protoplasts from young mycelia was unsuccessful.

VC11 DNA transfected S. olivaceus protoplasts at a frequency of 0.24×10^{-6} per phage DNA molecule which is of the same order of magnitude as the transfection frequencies obtained with other phage-host systems (Suarez and Chater, 1980a). In some phage-host systems, increased concentrations of phage DNA saturate the viable protoplasts, resulting in a plateau in the

transfection frequency even though only a small proportion of the protoplasts were transfected; for example, R4 DNA transfected S. lividans 66 at a frequency of 1.15×10^{-5} per viable protoplast (DNA saturation), while ϕ C31 DNA yielded a transfection frequency of 41.6×10^{-5} per viable S. lividans 66 protoplast (no DNA saturation) (Suarez and Chater, 1980a; Lomovskaya *et al.*, 1980). The occurrence of a sub-population of competent protoplasts was confirmed by transfection of S. lividans 66 with an equal mixture of ϕ C31 and R4 DNA, each of which normally transfects 1 protoplast in 10^5 , which resulted in the majority of transfectants containing ϕ C31 (Suarez and Chater, 1980a). As only 1 in 10^5 transfectants should have been mixed, the authors concluded that only a sub-population of the viable protoplasts is competent for both R4 and ϕ C31 DNA. Although it is not known whether increased concentrations of VC11 DNA saturate viable S. olivaceus protoplasts, 1.1×10^{11} molecules of DNA yielded a transfection frequency of 1.3×10^{-5} per viable S. olivaceus protoplast. This value is similar to transfection frequencies reported for phage-host systems in which DNA saturation of viable protoplasts was reached.

Whereas protoplast regeneration is an essential prerequisite for the generation of transformant clones, it is not known whether regeneration of transfected protoplasts is necessary as these primary transfectants merely act as hosts for the production of

phage particles which are assayed on a lawn of indicator mycelia (Hopwood, 1981b). Thus, although the regeneration frequency of S. cattleya protoplasts was only 0.5%, VC11 DNA transfection of these protoplasts was attempted. However, no transfectants were obtained with 1.1×10^{11} VC11 DNA molecules and 2.4×10^6 viable S. cattleya protoplasts. The failure to transfect S. cattleya protoplasts with VC11 DNA may be attributed to either the phage DNA or the nature of the protoplasts. As VC11 DNA was able to transfect S. olivaceus protoplasts, while intact phage infection of S. cattleya is not affected by the presence of host-specified restriction systems (section 2.4), it is likely that the S. cattleya protoplasts were the limiting factor. If one assumes that only a fraction of the 2.4×10^6 viable S. cattleya protoplasts were competent, then it is possible that this fraction of protoplasts was too small. If the size of the sub-population of competent protoplasts is a function of the number of viable protoplasts, then efficient protoplast regeneration is an important prerequisite for generating increased transfection efficiencies. Alternately, the slow rate of S. cattleya protoplast regeneration, the small size of the resulting colonies and the low regeneration frequency indicate that S. cattleya is extremely sensitive to the protoplasting procedure which may damage vital cellular functions necessary for the production of phage particles. If the latter explanation is valid, then an improvement in the regeneration frequency of S.

cattleya protoplasts may not be possible.

The regeneration of VC11 DNA transfected S. olivaceus protoplasts in the presence of S. cattleya spores provided a means whereby phage DNA could be introduced into S. cattleya. Thus, it is possible to manipulate the VC11 DNA in vitro and subsequently introduce the phage DNA into S. cattleya via transfection of S. olivaceus. In addition, it may be possible to increase the low VC11 'transfection' efficiency of S. cattleya spores, which is a direct function of the transfection frequency of S. olivaceus protoplasts with phage DNA, by transfecting S. olivaceus protoplasts with VC11 DNA in the presence of small DNA-free liposomes and 50% PEG 1000 which has been shown to generate a 100-fold increase in the number of transfectants (Rodicio and Chater, 1982). This procedure should prove useful for the introduction of broad host range phage cloning vectors into recipient strains in which protoplast transfection systems have not been established.

CHAPTER 5

CLONING STRATEGIES FOR THE IDENTIFICATION OF THE ORIGIN OF
REPLICATION OF ACTINOPHAGE VC11SUMMARY

An attempt to identify the ori region of the VC11 genome by 'shotgun' cloning BglII restricted phage DNA into the BamHI site of the B. subtilis plasmid pHV60 was unsuccessful. Similarly, the phage ori region was not isolated following the destruction of the replicon of the Streptomyces plasmid pLJ702 and subsequent transformation of S. olivaceus protoplasts with BglII restricted VC11 DNA ligated to BclI digested plasmid DNA. However, transformation of S. olivaceus protoplasts with pLJ702 resulted in expression of the plasmid borne tyrosinase and thiostrepton-resistance genes in this organism indicating that pLJ702 may be utilized as a DNA cloning vector in S. olivaceus.

5.1 INTRODUCTION

Biochemical, biophysical and genetic studies of DNA sequences of

complex genomes are greatly facilitated by a variety of techniques, called molecular cloning of DNA, which permit propagation of single DNA segments of virtually any origin in bacterial cells (Vosberg, 1977). Recombinant DNA research has to a certain degree enabled in vitro genetic recombination to produce hybrid DNA molecules which do not exist in nature. In addition, DNA cloning techniques allow gene transfer between species which do not normally exchange genetic material.

DNA cloning is accomplished via several steps: foreign DNA is dissected into fragments which are then joined in vitro to the vector DNA molecules by means of well-characterized enzymes. The resulting recombinant molecules are introduced into the host cells by a transformation or transfection step. The clones harbouring the DNA fragment of interest are subsequently selected among the progeny of the transformed cells by a combination of genetic and physical methods based on the properties of the vector and the cloned foreign DNA (Vosberg, 1977). The ability of bacterial plasmids and bacteriophage to promote gene transfer and recombination in procaryotes establishes these vectors among the most powerful molecular tools in recombinant DNA research. Besides the introduction and autonomous replication of foreign DNA in the host cell, cloning vectors usually effect an increase in the number of copies of the DNA insert per cell.

Cloning of DNA fragments in bacterial hosts requires propagation of these fragments via replication. DNA replication is a complex chain of events involving a variety of different enzymes and proteins as well as specific signals on the DNA provided by the nucleotide sequence of the region where DNA replication begins (origin of replication). An isolated DNA fragment may be introduced into a bacterial cell, but it will normally not be propagated unless it is joined to a DNA molecule (the vector) that affords an origin of replication (ori region) (Vosberg, 1977). As mentioned earlier, two categories of vector molecules have been used almost exclusively in cloning experiments: bacterial plasmids and bacteriophages.

Although the majority of cloning experiments are performed in E. coli, elucidation of the physiological activities of micro-organisms that are unrelated to E. coli require the cloning to be accomplished in the bacterial species under investigation. Cloning vector systems are currently being developed for many classes of micro-organisms, including B. subtilis, Staphylococcus aureus, Streptomyces, yeast, fungi, plant and animal cell lines, and intact plants.

Enquist (1980) proposed three general strategies for the application of recombinant DNA technology to actinomycetes. The first approach involves cloning actinomycete DNA fragments in a

genetically well-characterized micro-organism such as E. coli or B. subtilis. This cloning strategy would lead to the purification of large quantities of DNA fragments and may prove an invaluable method for the elucidation of actinomycete gene structure. However, Streptomyces promoters do not function in E. coli and hence transcription of Streptomyces genes may only be accomplished by transcriptional read-through from E. coli promoters (Hopwood, 1982). Thus, this approach would not be practical when specific production of an actinomycete gene product is required. In addition, the industrial production of recombinant DNA gene products in E. coli presents a variety of problems (Enquist, 1980). Proper transcription and translation signals are required for efficient gene expression, post-translational modification including glycosylation or proteolytic cleavage are unlikely to occur correctly in an organism unrelated to actinomycetes, and the membrane constituents of a Gram-negative micro-organism such as E. coli includes endotoxin and unpalatable lipids which present problems for the production of medical and food products for human consumption. The second cloning strategy would overcome the problems of gene expression by cloning actinomycete genes in actinomycetes. This approach would require the utilization of actinomycete plasmids and actinophages as cloning vehicles. In addition, the third strategy involves the construction of shuttle vectors which would allow propagation of hybrid DNA molecules in

both E. coli (or B. subtilis) and an actinomycete. The shuttle vector could be a composite plasmid which would replicate as a plasmid in both hosts or a composite phage and plasmid able to multiply as a phage in one host and a plasmid in the other. Selectable markers should be present for the identification of clones harbouring the vector in either host. These bifunctional vectors would be useful in propagating and amplifying Streptomyces DNA in E. coli which could subsequently be retransformed into Streptomyces. In addition, these vectors would facilitate analysis of heterologous gene expression in both Streptomyces and E. coli.

Ideally, both a plasmid and a phage vector should be available for a given cloning system. Plasmids are much smaller than phage genomes and are continually replicated in the host (Enquist, 1980). In addition, plasmid vectors are especially useful for the cloning of genes whose expression and regulation is to be studied (Timmis, 1981). Alternatively, phage DNA replicates in a relatively short time and the newly synthesized DNA molecules are packaged into phage particles. Actinophage vectors are often better than plasmid vectors for cloning fragments that are unstable due to size or are incompatible with the host (over-expression of genes coding for ribosomal proteins and some membrane proteins is lethal for the host cell) (Enquist, 1980). Lysogenization of a host by a hybrid phage containing cloned

genes allows these genes to be stably introduced into a recipient host at low copy number which may be desirable for industrial or academic purposes (Chater et al., 1981a). Moreover, the copy number of the cloned DNA may be increased by induction of a heat-inducible mutant (Lomovskaya et al., 1980). Other advantages of phage vectors include their wide host range, the ease with which radioactively-labelled DNA probes may be utilized via in situ plaque-hybridization to detect phage containing a specific DNA sequence, and the use of efficient phage promoters to obtain high levels of transcription of cloned DNA (Lomovskaya et al., 1980; Chater et al., 1981a).

In general, the larger the inserted DNA fragment, the greater the possibility of deletions or rearrangements occurring as a result of selective pressure for better growth. Often the choice between plasmid or phage vectors depends on the ratio of the size of the foreign DNA fragment to the size of the vector. The best ratio is one or less for plasmids, while the ratio is dependent on the packaging capacity of the phage head (Enquist, 1980). The packaging constraints of phage vectors allows for the development of cloning systems selective for inserted DNA (Chater et al., 1981a). In addition to the commonly used selection markers such as antibiotic resistance and phage immunity (temperate phages), a variety of alternate markers may be employed in actinomycete cloning vectors including pigmentation, colony morphology and

lethal zygotis (Enquist, 1980).

As recent studies have indicated the widespread occurrence of plasmids in Streptomyces, it is almost certain that new potential plasmid vectors could be discovered by a survey of taxonomically related strains. Three plasmid groups have been utilized in the construction of cloning vectors thus far. These are SCP2 from S. coelicolor A3(2) (Bibb et al., 1977), the SLP1 plasmid series in S. lividans 66 (Bibb, 1981) and the S. lividans ISP5434 pLJ101 plasmid family (Kieser et al., 1982). However, as this study is concerned with the construction of a phage cloning vector for S. cattleya and S. olivaceus, the vast amount of literature which describes the development of plasmid cloning vectors and the successful utilization of these vectors for the isolation of antibiotic-biosynthetic genes will not be reviewed.

A number of virulent and temperate actinophages, infectious to a variety of Streptomyces species, have been isolated. The biological and genetic characteristics of these phages have been described in section 2.1. The broad host range temperate phages R4 and ϕ C31 have been extensively studied for possible use as Streptomyces cloning vectors. The isolation of deletion mutants, lacking DNA not essential for phage propagation, is a fundamental prerequisite for the construction of actinophage cloning vectors since the amount of foreign DNA which can be incorporated into

phage particles is usually limited. Moreover, the occurrence of unique restriction sites in a dispensable region of the phage genome would allow insertion of exogenous DNA without interfering with phage replication.

Packaging of DNA into phage particles occurs by one of two systems depending on the phage. The first system involves slicing DNA concatamers into segments which would fill a phage head. This system results in the generation of a short region of DNA which becomes redundant. Thus, deletion of a portion of the phage genome would not decrease the amount of DNA included in each particle, but would result in an increased amount of redundant DNA. Alternatively, DNA concatamers are cut during packaging by a phage-specific enzyme which recognizes a cohesive end site (cos) present once per genome length. Consequently, deletion of a section of the phage genome results in the packaging of smaller DNA segments (Lomovskaya et al., 1980).

Magnesium ions are necessary to maintain the structural integrity of the phage head. The presence of chelating agents such as EDTA or sodium pyrophosphate, which remove magnesium ions, results in the bursting of any lambda phage head with a DNA content in excess of 96% of the wild type (Arber et al., 1983). Consequently, phage deletion mutants may be selected on the basis of increased resistance to treatment with chelating agents

by virtue of the fact that deletion of a portion of the phage genome reduces the tightness of the packaged DNA thereby rendering the phage head more stable in the presence of these chemicals (Lomovskaya et al., 1980).

The temperate phage R4 possesses 3 unique restriction sites within a dispensable segment of the genome (Isogai et al., 1981). Although Streptomyces chromosomal DNA is not often cleaved by EcoRI, the unique PvuII restriction site could be utilized for insertion of various DNA fragments created with restriction enzymes which generate blunt-ends such as SmaI, AluI, HaeIII, BalI, HpaI and StuI. Moreover, the unique PstI site and EcoRI sites may be replaced by the insertion of target sites for other restriction enzymes at these positions.

Isogai et al. (1981) isolated 2 sodium pyrophosphate resistant deletion mutants of the phage R4. Both the R4 deletion mutants, designated R4 Δ 1 and R4 Δ 2, had lost the unique EcoRI restriction site. R4 Δ 1 had been deleted by 1.8 kb, while R4 Δ 2 had lost 2.7 kb of the wild type phage genome (Takahashi et al., 1982).

Two deletion mutants of R4 Δ 2 were subsequently isolated by Takahashi et al. (1982). Besides the original 2.7 kb deletion of R4 Δ 2, the mutants R4 Δ 21 and R4 Δ 22 were found to have deletions of 1.5 and 2.5 kb respectively. Moreover, R4 Δ 21 was non-lysogenic

in S. parvulus, while the wild type R4 phage and the other R4 deletion mutants remained lysogenic in this Streptomyces strain.

As mentioned earlier, the unique PvuII restriction site, located in a dispensable region of the genome of R4 deletion mutants, is amenable to the insertion of foreign DNA. This was demonstrated by the insertion of SmaI digested SPA38 phage DNA into the PvuII site of R4 Δ 2 and the subsequent cloning of the hybrid phage genome in S. lividans 66 and S. parvulus 2297 (Isogai et al., 1981; Takahashi et al., 1982). However, to simplify the recovery of cloned DNA fragments, a DNA decamer containing a BamHI restriction site was inserted into the PvuII site of the deletion mutants R4 Δ 2, R4 Δ 21 and R4 Δ 22 (Takahashi et al., 1982). The presence of a BamHI site is advantageous for gene cloning in Streptomyces since Streptomyces DNA contains a number of restriction sites recognized by the enzymes BamHI, SacI and SalI as a consequence of its high G+C content. In addition, the BamHI site can be utilized for cloning BglII, BclI and Sau3a generated DNA fragments.

As the genome size of wild type R4 phage is 53.7 kb, 12.5 kb larger than the genome of the phage ϕ C31, R4 phage is expected to be able to accommodate larger segments of foreign DNA (Takahashi et al., 1982). Moreover, if the deletions found to be clustered at the right hand side of the R4 genome comprise a continuous

segment of dispensable DNA, then this phage could be utilized as a replacement vector whereby at least 14 kb of foreign DNA may be accommodated.

The temperate actinophage ϕ C31 is genetically and physically the best characterized Streptomyces phage. The broad host range, availability of a restriction enzyme map and an efficient transfection system render this phage potentially useful as a DNA cloning vector.

A number of EDTA resistant derivatives of ϕ C31 Δ M, which contains a single spontaneous deletion of 1.63 kb following routine subculture, were isolated (Lomovskaya et al., 1980). These deletions were located in a 1.7 kb region near the centre of the phage genome. Analysis of the ϕ C31 Δ M derivatives indicated that the phages had lost part or all of the gene coding for a repressor protein involved in the maintenance of lysogeny (c mutants). Thus, the deletion mutants retained the ability to propagate via the lytic mode, while having lost the capacity to lysogenise Streptomyces host cells. In addition, a further group of ϕ C31 Δ M mutants possessing deletions in the right-end portion of the genome, retained the c^+ phenotype and had lost the att site which is required for integration into the host chromosome (Lomovskaya et al., 1980; Chater et al., 1981a).

ϕ C31cts Δ MA23, which contains a deletion of 2.98 kb (Chater et al., 1981a), was utilized in the construction of a chimaeric DNA molecule which can replicate as a phage in Streptomyces and as a multicopy plasmid in E. coli (Suarez and Chater, 1980b). The E. coli plasmid pBR322 is 4.362 kb in length and contains single EcoRI, BamHI and PstI restriction sites. ϕ C31cts Δ MA23 was treated with ligase to covalently close the cos site and subsequently, partially-digested with EcoRI to produce a single EcoRI cut per DNA molecule (ϕ C31 has no unique restriction sites, but contains 6 sites recognized by EcoRI). EcoRI digested pBR322 was ligated to partially-digested ϕ C31cts Δ MA23 and transfected into S. lividans. A plaque-hybridization test in which ³²P-labelled pBR322 was used as a probe, located a single hybrid phage containing pBR322 inserted into an EcoRI site 6.35 kb from the right-end of the genome of ϕ C31cts Δ MA23.

The pBR322- ϕ C31 hybrid phage has a number of features which make it suitable as a potential Streptomyces shuttle vector. The insertion of pBR322 into the ϕ C31cts Δ MA23 genome, while not interfering with phage replication, provides single BamHI and PstI restriction sites which may be utilized for insertion of foreign DNA. The hybrid molecule may be transformed into E. coli where it replicates as a plasmid and is able to express ampicillin and tetracycline resistance. Moreover, the chimaeric molecule, isolated from transformed E. coli cells, is able to

transfect S. lividans in which it is propagated as a phage (Suarez and Chater, 1980b).

In contrast to wild type ϕ C31 phage, the pBR322- ϕ C31cts Δ MA23 DNA was found to be restricted by S. albus P (Suarez and Chater, 1980b). S. albus P contains the SalPI restriction-modification system which is able to recognize the PstI site (an isoschizomer of SalPI) of pBR322. Moreover, the hybrid phage is unable to infect S. albus G mutants which are SalGI restriction deficient, modification proficient ($R^{-}M^{+}$) (Chater et al., 1981a). The increased sensitivity to certain host-specific restriction-modification systems provides an in vivo method for recognizing hybrid phage DNA molecules.

Deletion mutants of the ϕ C31cts Δ MA23:pBR322 hybrid DNA molecule were isolated via two procedures. The ϕ C31cts Δ MA23:pBR322 hybrid phage was hypersensitive to the presence of chelating agents due to the presence of 3% more DNA than in wild type ϕ C31 phage (Chater et al., 1981a). Secondly, restriction-modification resistant mutants, isolated following growth on S. albus G, were found to have lost a 3.5 kb portion of the pBR322 DNA sequence.

Although all the DNA deletions, obtained following treatment with chelating agents, were located at the right-end of the hybrid phage genome, two classes of deletion mutants were isolated

(Chater et al., 1981a). One group of mutants, found to have lost ϕ C31 DNA sequences to the right of pBR322, were unable to lysogenize Streptomyces but retained the ability to replicate in E. coli. The second class of deletion mutants, having lost a portion of DNA located on the right-end of the pBR322 genome, had retained the ability to lysogenize Streptomyces but had lost the capacity to replicate in E. coli.

Preliminary 'shotgun' cloning experiments, utilizing the first group of ϕ C31 Δ ts Δ MA23:pBR322 deletion mutants containing 5% less DNA than wild type ϕ C31, determined the cloning capacity of the hybrid phage (Chater et al., 1981a). The single BamHI and PstI restriction sites of pBR322 were able to accommodate up to 3.8 kb of foreign DNA without interfering with the ability of the vector to replicate in E. coli, while the replacement of the BamHI-PstI segment of pBR322 with exogenous DNA allowed cloning of larger DNA fragments (up to 7 kb). Although the removal of a large segment of the pBR322 genome prevented replication in E. coli, the restricted vector is unable to reconstitute itself in the absence of inserted DNA since the BamHI- and PstI-generated ends are not complimentary. Moreover, the relatively stringent packaging constraints of ϕ C31 (a minimum of 37.5 kb up to a maximum of 42.5 kb) may be circumvented by propagating the hybrid molecule as a plasmid in E. coli which would allow the deletion of dispensable DNA to create a molecule of approximately 33 kb.

Such a molecule would be unable to transfect Streptomyces unless additional DNA (4.5 to 9.5 kb) was inserted, providing a positive selection system for the insertion of exogenous DNA (Chater et al., 1981b).

ϕ C31 KC400 was constructed by subcloning a 1.9 kb BamHI fragment containing the viomycin-resistance (vph) gene from pLJ13 (Thompson et al., 1982) into the BamHI restriction site of ϕ C31c⁺ Δ MA23:pBR322 Δ W12, which contains a 4.7 kb deletion of non-essential DNA covering the right side of pBR322 and an adjacent portion of ϕ C31 DNA, followed by superinfection of a S. coelicolor J1501-derivative lysogenic for ϕ C31c⁺ Δ MA23:pBR322 Δ W12:vph with ϕ C31c⁺ Δ MA23:pBR322 Δ W17:tsr which resulted in the isolation of the phage-derivative ϕ C31c⁺ Δ MA23:pBR322 Δ W17:vph (Chater et al., 1982b). The vector may be propagated either as a phage in Streptomyces or a plasmid, designated pLJ505, in E. coli (Hopwood et al., 1983a). Although ϕ C31 KC400 contains the repressor gene required for maintenance of the lysogenic state, deletion of the attachment site renders this phage non-lysogenic (Chater et al., 1982b; Hopwood et al., 1983a). However, the att-defective phage vector is able to lysogenize Streptomyces when ϕ C31 lysogens are used as an indicator strain. The resident prophage provides a region of DNA sequence homology into which the superinfecting ϕ C31 KC400 vector can recombine (Chater et al., 1982b). Alternatively, ϕ C31 KC400,

containing foreign DNA may be reinfected into an indicator strain from which the cloned DNA was derived. Sequence homology between the DNA inserted in ϕ C31 KC400 and the original DNA sequence present in the indicator strain would allow the vector to integrate into the host genome (Harris et al., 1983; Hopwood et al., 1983a). DNA cloning in ϕ C31 KC400 is accomplished by inserting up to 6 kb of PstI-cleaved exogenous DNA into the PstI-PvuII restricted vector. Digestion of ϕ C31 KC400 with PstI restriction enzyme results in the removal of most of the pBR322 DNA sequence, while PvuII prevents its reinsertion into the vector (Hopwood et al., 1983a). Following ligation and transfection of S. lividans, the vector may be integrated into an indicator strain and, in so doing, would render the lysogenized host viomycin resistant, thus providing a directly selectable phenotype (Chater et al., 1982b; Hopwood et al., 1983a; Harris et al., 1983).

ϕ C31 KC400 is able to accommodate up to 6 kb of PstI-cleaved exogenous DNA. A number of ϕ C31 KC400 derivatives were constructed by Harris et al. (1983). ϕ C31 KC401 can incorporate up to 8 kb of foreign DNA due to a deletion of 1.79 kb in the c gene region. However, ϕ C31 KC401 requires the presence of a ϕ C31 c⁺ prophage in the indicator strain to become established and maintained as a prophage. The authors constructed a derivative of ϕ C31 KC401 by inserting a 6.03 kb PstI-generated fragment of

the plasmid SCP2 into the PstI site of the vector to produce ϕ C31 KC402. The integrated SCP2 DNA fragment provided 2 internal BamHI restriction sites capable of accommodating up to 9 kb of exogenous DNA. However, the insertion of foreign DNA into BamHI sites of ϕ C31 KC402 results in the loss of the viomycin-resistance marker. Finally, ϕ C31 KC404, a derivative of ϕ C31 KC400 lacking the 3.9 kb PstI fragment, can accommodate up to 6 kb of foreign DNA cleaved with PstI or BamHI restriction enzymes without the destruction of the viomycin-resistance gene.

Chater and Bruton (1983) utilized the cloning vector ϕ C31 KC400 to isolate the genes involved in the biosynthesis of methylenomycin A. The method employed, known as mutational cloning, allowed detection of the cloned antibiotic genes without the use of mutants. As the genes involved in the production of methylenomycin A are situated on the S. coelicolor A3(2) plasmid SCP1, the authors initially identified vectors containing fragments of this plasmid in order to minimise the initial amount of screening. This was accomplished by cloning PstI-cleaved total DNA from a Streptomyces parvulus derivative containing the SCP1 plasmid carrying the methylenomycin A genes from S. coelicolor A3(2) into the PstI site of ϕ C31 KC400 and subsequently transfecting the recombinant phage vector into S. lividans. Phage plaques containing SCP1 DNA were identified by their ability to transduce viomycin resistance into a SCP1⁺ strain of S. lividans or a NF strain of S. coelicolor (containing an integrated SCP1), but not into a SCP1⁻ strain of S. parvulus.

(Chater and Bruton, 1983). The presence of SCP1 DNA in some of these phages was confirmed by their ability to express methylenomycin A resistance (known to be linked to SCP1; Kirby *et al.*, 1975) in a SCP1⁻ *S. coelicolor* ϕ C31 lysogen. Secondly, the 2.44 kb insert isolated from these phages hybridized to DNA isolated from SCP1-containing *Streptomyces* strains, but not to SCP1⁻ *S. coelicolor* or SCP1⁻ *S. parvulus* DNA. Methylenomycin A production was investigated in *S. coelicolor* NF strains containing integrated ϕ C31 KC400 carrying SCP1 DNA on the basis that antibiotic production would be eliminated by the insertion of phage bearing homologous DNA internal to a transcriptional unit (lacking a promoter and the last transcriptional stop codon) (Chater and Bruton, 1983). Nine phage clones were subsequently found to contain DNA fragments internal to coding sequences for methylenomycin A production. These DNA fragments represent approximately 7 kb of DNA indicating that at least 7 to 10 average-sized polypeptides may be involved in methylenomycin A biosynthesis.

The system of mutational cloning, developed by Chater and Bruton (1983), may be employed for the isolation of DNA from large transcriptional units as the probability of obtaining internal fragments is greater. These DNA fragments may subsequently be utilized as radioactive probes for screening gene libraries or Southern blots. Furthermore, mutational cloning may be applied

to Streptomyces species for which genetics, mutants, or transformation/transfection systems are unavailable as cloned DNA may be introduced into these species via natural phage infection.

S. cattleya and S. olivaceus are producers of the commercially-important carbapenem antibiotics (Chapter 1). As no endogenous plasmids have been reported in these Streptomyces species, the isolation of the virulent actinophage VC11 has made it possible to study the genetics and mechanisms of antibiotic biosynthesis in these streptomycetes. However, the use of VC11 as a potential cloning vector for S. olivaceus and S. cattleya presents a number of disadvantages. VC11 DNA does not contain unique restriction sites for the insertion of foreign DNA; the 4 BglII sites being the least number of restriction sites recognized by a single endonuclease (section 2.3.5). In addition, infection of a host cell with the virulent phage ultimately results in the destruction of the organism via phage-mediated cell lysis.

The disadvantages of VC11 as a potential cloning vector may be overcome by construction of a DNA vector incorporating the ori region of the actinophage genome. Indeed, certain phages (for example, bacteriophage lambda) can generate mutants which are stably maintained as plasmids (Campbell, 1977). Plasmid formation involves controlled autonomous replication,

unaccompanied by expression of viral genes which are lethal to the host. Autonomous replication of lambda requires the expression of two phage-coded proteins, products of genes Q and P, which specifically promote replication initiated at the phage ori region. The lambda variants, designated lambda dv, resemble relaxed plasmids in that they are present at approximately 50 copies per chromosome (Campbell, 1977). In addition, lambda dv variants have lost 70-90% of the viral genome and include, besides genes Q, P and the phage ori region, the cro gene which also activates initiation of replication from the ori region and prevents excessive replication of lambda dv by reducing rightward transcription from the promoter P_R . Neither the N gene product which promotes longer transcripts from the promoters P_L and P_R by overcoming normal termination signals, nor the cI gene which is necessary for maintenance of the lysogenic state, are needed by lambda dv (Campbell, 1977).

Thus, construction of a cloning vector for S. cattleya and S. olivaceus would involve the portion of the VC11 genome containing the ori region together with an antibiotic selectable marker. The resulting plasmid would be introduced into host cells via protoplast transformation. The absence of the phage lytic genes would ensure the survival of the recipient strain, while the absence of the phage capsid would bypass the phage adsorption barrier, thus widening the host range of the vector. The phage

ori region would allow replication of the plasmid to yield a high copy number. The utilization of the thiostrepton-resistance gene from pLJ702, contained on a 1.05 kb BclI fragment, together with the VC11 ori region would produce a small plasmid capable of accommodating large DNA inserts. Finally, the presence of a small segment of phage DNA would reduce the number of restriction sites and in so doing, may provide unique sites for the incorporation of exogenous DNA.

Isolation of the VC11 ori region was attempted via two procedures. In the first approach, use was made of the B. subtilis plasmid pHV60 (Ehrlich et al., 1982a). This plasmid, identical to the plasmid pHV32, was isolated in the following manner. The S. aureus plasmid pC194 (2.8 kb), which specifies chloramphenicol resistance and is able to replicate in B. subtilis (Ehrlich et al., 1982b), was digested with the restriction enzyme HindIII and ligated to the similarly restricted E. coli plasmid pBR322 to produce plasmid pHV14 (7.2 kb) (Ehrlich, 1978). pHV14, which is able to replicate in both E. coli and B. subtilis, expresses chloramphenicol resistance in B. subtilis, and ampicillin and chloramphenicol resistance in E. coli. Insertion of pC194 into the pBR322 HindIII site had resulted in the inactivation of the pBR322 tetracycline-resistance gene (Ehrlich et al., 1982b). However, tetracycline resistant mutants of E. coli transformed with pHV14

were found to occur at a frequency of 10^{-7} (Primrose and Ehrlich, 1981). Three types of plasmid DNA were isolated from the tetracycline resistant E. coli transformants. One clone contained a plasmid which was smaller than the parental pHV14 and contained a single HindIII restriction site. This plasmid, designated pHV32 (5.8 kb), conferred ampicillin, tetracycline and chloramphenicol resistance to transformed E. coli cells (Primrose and Ehrlich, 1981). Analysis of pHV32 by restriction enzyme digestion indicated a large deletion within the pCl94 component of pHV14, extending somewhat beyond the HindIII junction into the pBR322 section of the hybrid molecule. Since pHV32 is able to encode chloramphenicol resistance, the authors suggested that the antibiotic-resistance gene of pCl94 is located proximal to the EcoRI site of pBR322 in pHV14 (Primrose and Ehrlich, 1981). Moreover, no chloramphenicol resistant B. subtilis cells transformed with pHV32 were isolated, indicating that the ori region of pCl94 had been deleted from pHV32 (Niaudet and Ehrlich, 1979).

pHV60 (pHV32; Fig. 1) is a convenient plasmid for the isolation of the ori region of a DNA molecule. The plasmid is able to replicate in E. coli, but not in B. subtilis. In addition, pHV60 expresses ampicillin, tetracycline and chloramphenicol resistance in E. coli, while the chloramphenicol-resistance gene is highly expressed in B. subtilis when associated with a viable

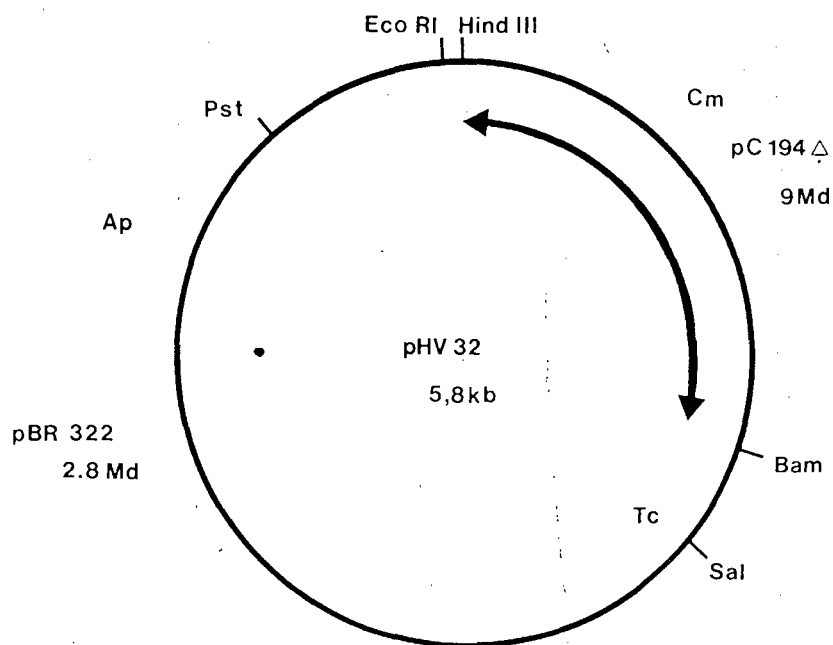


FIGURE 1. Restriction map of the plasmid pHV32 (pHV60). Ap and Tc denote the ampicillin and tetracycline resistant determinants of pBR322 respectively. Cm indicates the chloramphenicol resistance gene of pC194. pC194 Δ refers to deleted pC194. After Primrose and Ehrlich (1981).

replication region. Thus, 'shotgun' cloning of foreign DNA into pHV60 would allow direct selection of B. subtilis transformants harbouring an ori region via the expression of the chloramphenicol-resistant phenotype. Transformants containing pHV60 lacking an exogenous ori region would be non-viable in the presence of chloramphenicol. Additional advantages of pHV60 (pHV32) include (i) single EcoRI, SalI, BamHI, HindIII and PstI restriction sites, located outside the region containing the chloramphenicol-resistance gene, which are available for insertion of foreign DNA, (ii) the plasmid may be prepared in large amounts in E. coli, free of any contaminating replicon active in B. subtilis, using chloramphenicol amplification, and (iii) a high transforming efficiency of the chloramphenicol-resistance gene (over 10^6 transformants per microgram of DNA) when associated with an ori region facilitating the isolation of replication regions from complex genomes (Niaudet and Ehrlich, 1979).

The second strategy for the isolation of the VC11 ori region involved the Streptomyces plasmid pIJ702. The plasmid was derived in the following manner. Kieser et al. (1982) isolated four plasmids from S. lividans ISP5434. These plasmids, designated pIJ101 (8.9 kb), pIJ102 (4.0 kb), pIJ103 (3.9 kb) and pIJ104 (4.9 kb), were shown to be replicated at a high copy number. The copy number of these plasmids (40-300) appears to be

the highest reported for any bacterial plasmid in the absence of artificial amplification (chloramphenicol). Restriction enzyme analysis suggested that the smaller plasmids were related to the largest member (pIJ101) and recently originated from this plasmid following an in vivo deletion event (Kieser et al., 1982). The pIJ101 plasmid family is potentially useful as Streptomyces cloning vectors due to their high copy number and broad host range. Besides S. lividans 66 and S. coelicolor A3(2), the host range of these plasmids includes strains of S. acrimycini, S. albus, S. azureus, S. fradiae, Streptomyces glaucescens, S. griseus, S. parvulus, Streptomyces pristinaespiralis and S. rimosus (Kieser et al., 1982).

As further analysis of the biological functions of pIJ101 and its derivatives was hampered by the lack of selectable markers apart from pock formation, Kieser et al. (1982) constructed a number of in vitro insertion and deletion variants following insertion of antibiotic-resistance genes carried by the plasmids pIJ2 (neomycin-resistance), pIJ6 (thiostrepton-resistance) and pIJ13 (viomycin-resistance). The pIJ101 derivatives have a number of suitable restriction sites for the insertion of foreign DNA without interrupting plasmid replication and contain selectable markers specifying antibiotic resistance. These plasmids include pIJ360 and pIJ361 (thiostrepton and viomycin resistance), and pIJ350 (thiostrepton resistance).

A 1.55 kb BclI fragment, harbouring the tyrosinase gene cloned from Streptomyces antibioticus chromosomal DNA, was subcloned from the low copy number plasmid pLJ700 into the high copy number plasmid pLJ350 (Katz et al., 1983). pLJ350 (4.1 kb) contains 3 BclI sites, codes for thiostrepton resistance and has a broad host range. The various recombinant plasmids were 5.65 kb in length and contained the 1.55 kb tyrosinase fragment integrated in both orientations in the 2 available BclI cloning sites of pLJ350 (the third BclI site is located in a region that is essential for plasmid replication).

Restriction endonuclease analysis of one of the recombinant plasmids bearing the tyrosinase genes, designated pLJ702 (Fig. 2), indicated that single restriction sites for the enzymes SphI, BglII and SstI are present within the tyrosinase gene and thus allow clone selection on the basis of insertional inactivation of this gene (Katz et al., 1983). Transformants harbouring pLJ702 form black colonies on tyrosine-supplemented media due to melanin production from tyrosine, whereas recombinant plasmids containing DNA inserts within the tyrosinase gene remain white. The high copy number plasmid pLJ702 is non-conjugative, codes for thiostrepton resistance and is able to transform a variety of Streptomyces strains including S. coelicolor A3(2), S. griseus CUB94, S. parvulus, S. albus G, S. vinaceus, S. acrimycini, and S. clavuligerus. However, the tyrosinase gene was not strongly expressed in S. clavuligerus (Katz et al., 1983).

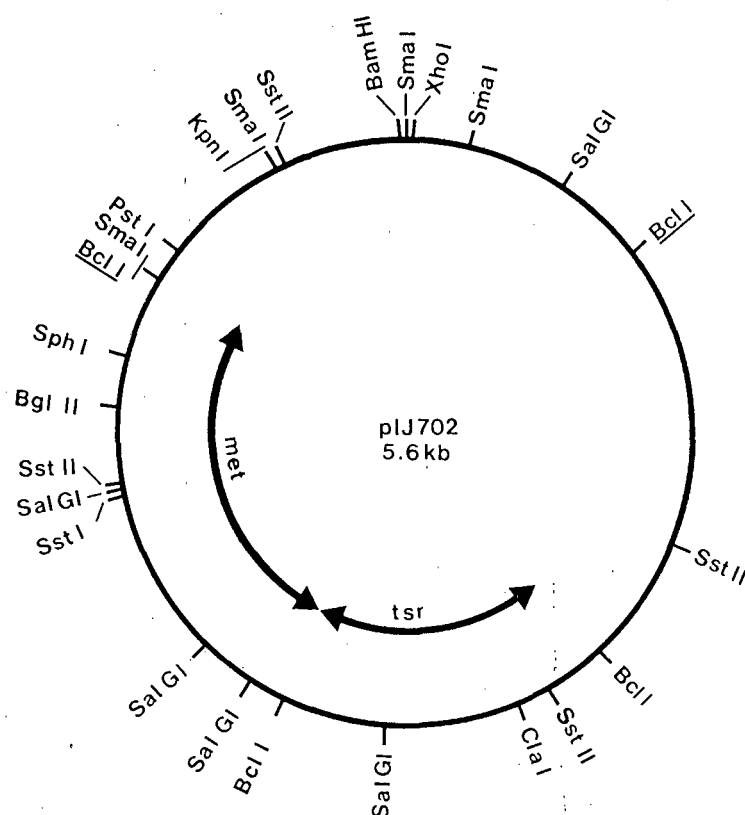


FIGURE 2. Restriction map of the plasmid pIJ702. tsr refers to the thiostrepton-resistant determinant contained on a 1.05 kb BclI fragment; met indicates the tyrosinase gene borne on a 1.55 kb BclI segment. The plasmid ori region is included within the 1.71 kb BclI fragment extending between the two underlined BclI sites. After Katz *et al.* (1983).

The major part of the replication region of pLJ702 is a 1.71 kb DNA fragment containing sites recognized by the restriction enzymes BamHI, SmaI, XhoI, SalI and BclI (Fig. 2) (Kieser et al., 1982; Katz et al., 1983). Destruction of the replication region of pLJ702 by restriction enzyme digestion would render the vector non-viable. Subsequent ligation of digested pLJ702 in the presence of BglII restricted VC11 DNA would produce a variety of hybrid molecules. However, the possibility of reconstituting a functional pLJ702 ori region is minimal due to the number of DNA fragments generated following digestion of this region with a multitude of restriction enzymes. Thus, direct selection of thiostrepton resistant Streptomyces transformants would enable the isolation of the VC11 ori region since hybrid molecules lacking a functional replicon would be non-viable in the presence of thiostrepton.

5.2 METHODS

5.2.1 Isolation of the VC11 ori using the plasmid pHV60

5.2.1.1 Bacterial strains

The plasmids pHV60 and pHV33 were contained in the E. coli K-12

derivative HVC45, thiA¹, leu⁶, thi¹, lac Y¹, ton A²¹, sup E⁴⁴, str^R, hsd R (Niaudet and Ehrlich, 1979). Cloning experiments were performed in the B. subtilis strain BD224/LA46, trp 32, thr 5, recE⁻ originally isolated by Dr. S. Reid of the Department of Microbiology, Witwatersrand University, South Africa.

5.2.1.2 Preparation of plasmid DNA

The plasmids pHV60 and pHV33 were isolated from E. coli HVC45 by the procedure developed by Ish-Horowicz and Burke (1981). Luria broth (200 ml) was inoculated with E. coli HVC45 containing either pHV60 or pHV33 and incubated overnight at 37°C with agitation. The cells were harvested from the saturated culture by centrifugation at 5,000 rpm for 10 min in a GSA rotor at 4°C. The pelleted cells were resuspended in 3 ml of 50 mM glucose, 25 mM Tris, pH 8.0, 10 mM EDTA and incubated for 5 min at room temperature. Six ml of 0.2 N NaOH, 1% (w/v) SDS was gently mixed with the suspension which was subsequently incubated at 0°C for 5 min. Precooled 5 M KOAc pH 4.8 (4.5 ml) was added to the suspension. Following gentle mixing, the suspension was incubated at 0°C for 10 min. The precipitated protein, SDS and chromosomal DNA were subsequently removed by centrifugation at 12,000 rpm for 15 min at 4°C in a SS 35 rotor. Residual protein was extracted from the supernatant by treatment with an equal

volume of phenol saturated with TE buffer. Following gentle mixing to form an emulsion, the aqueous phase was separated from the organic phase by centrifugation at 10,000 rpm for 5 min at 4°C. An equal volume of ether was subsequently added to the aqueous phase to remove residual phenol. The ether and DNA suspension were mixed to form an emulsion and the aqueous phase was removed following centrifugation at 10,000 rpm for 1 min at 4°C. After the addition of 2 volumes of cold 96% ethanol, the DNA suspension was incubated at room temperature for 15 min. Subsequently, the plasmid DNA was precipitated by centrifugation at 12,000 rpm for 15 min at 4°C and resuspended in 4.2 ml TE buffer. CsCl (4.5 g) and 0.2 ml of 10 mg/ml ethidium bromide were added to the DNA suspension and the refractive index of the solution was adjusted to 1.394 with the use of a hand-held 0-50% sugar refractometer. The DNA was purified by isopycnic centrifugation at 55,000 rpm for 12 h at 15°C in a Beckman VTi 65.2 rotor. The plasmid DNA band, visualized with UV light at 270 nm, was removed from the CsCl gradient using a wide bore disposable syringe. The ethidium bromide was extracted from the DNA suspension by 3 cycles of NaCl-saturated isopropanol treatment. Finally, the CsCl was removed by overnight dialysis against TE buffer at 4°C. The procedure yielded a plasmid DNA concentration of 79.7 ug/ml.

5.2.1.3 Restriction of pHV60 and VC11 DNA

VC11 DNA (16 ug), prepared by the method described in section 2.2.10, was digested in a final volume of 20 ul low salt digestion buffer (see Appendix) containing 5 mM spermidine with 16 u BglII restriction enzyme for 18 h at 37°C. pHV60 DNA (8 ug) was digested for 18 h at 37°C with 16 u BamHI restriction enzyme in a final volume of 20 ul medium salt digestion buffer (see Appendix). The restriction enzymes were supplied by Boehringer-Mannheim.

5.2.1.4 Electrophoresis of restricted pHV60 and VC11 DNA

Digested VC11 DNA (8 ug) and 4 ug digested pHV60 DNA were analyzed by comparison with undigested phage and plasmid DNA respectively. Gel loading buffer (2.5 ul) (see Appendix) was added to each DNA sample (10 ul). Electrophoresis was performed in a vertical direction at 100 V (constant voltage) for 3 h through 0.8% (w/v) Tris-Borate-EDTA-buffered agarose gel containing 0.5 ug/ml ethidium bromide. The DNA was subsequently visualized with a 254 nm UV lamp and photographed with a Polaroid Land camera.

5.2.1.5 Ligation of restricted pHV60 and VC11 DNA

The pHV60 and VC11 DNA restriction enzyme digestion reactions

were halted by removal of the restriction endonucleases with 3 cycles of treatment with TE buffer-saturated phenol in the following manner. An equal volume of TE buffer-saturated phenol was added to the DNA suspension, mixed on a whirlymix to form an emulsion and centrifuged at 12,000 rpm for 4 sec in a microfuge in order to separate the two phases. After removal of the aqueous phase, following the final round of phenol extraction, residual phenol was removed by 2 cycles of ether extraction. Two volumes of ether were added to the DNA suspension, whirlymixed to form an emulsion and centrifuged at 12,000 rpm for 4 sec in a microfuge. Residual ether was subsequently extracted by evaporation in a Savant speed vac concentrator for 5 min.

Sodium acetate pH 8.0 was added to each DNA suspension to a final concentration of 0.3 M. Three volumes of cold 96% ethanol were added and the DNA suspensions were incubated at -70°C for 30 min. The DNA was precipitated by centrifugation at 12,000 rpm in a microfuge for 5 min at 4°C . The supernatant was discarded and a second aliquot of cold 96% ethanol was added without disturbing the pelleted DNA. Following centrifugation at 12,000 rpm in a microfuge for 2 sec at 4°C , the ethanol was discarded and the DNA pellet dried in a Savant speed vac concentrator for 15 min. Subsequently, the DNA pellets were each resuspended in 7 μl TE buffer.

VC11 DNA (6.9 μg) and 5 μg pHV60 DNA (3:1 molar ratio) were

ligated in 15 μ l ligation buffer (see Appendix) with 10 μ l ligase (Boehringer-Mannheim). These relatively high DNA concentrations were used in order to select for concatamers which are necessary for transformation of B. subtilis. BglII restricted VC11 DNA (1 μ g) was religated in 5 μ l ligation buffer with 2.5 μ l ligase. Similarly, 1 μ g BamHI digested pHV60 DNA was religated with 2.5 μ l ligase. The ligation reactions were performed at 12°C for 18 h.

5.2.1.6 Preparation of competent B. subtilis BD224/LA46 cells

Competent B. subtilis cells were prepared by the method developed by Anagnostopoulos and Spizizen (1961). A tryptose blood agar base slant supplemented with tryptophan and threonine was inoculated with an overnight B. subtilis culture grown at 37°C on Luria agar containing the auxotrophic requirements of the B. subtilis strain. Following incubation at 37°C for 12 h, 5 ml growth medium 1 (GM1) (see Appendix) was added to the slant and whirlymixed to resuspend the bacterial cells. GM1 (15 ml) was inoculated with the resuspended cells to an absorbance (A_{650}) of 0.3. The suspension was incubated for 4.5 h with shaking at 37°C. The culture was subsequently added to 150 ml GM2 (see Appendix) and incubated for a further 90 min at 37°C with agitation. The cells were sedimented by centrifugation in a GSA rotor at 10,000 rpm for 10 min at 4°C and resuspended in 15 ml supernatant containing a final concentration of 8% (v/v) glycerol. Aliquots (1 ml) were placed in sterile microfuge

tubes, frozen rapidly in liquid nitrogen and stored at -70°C .

5.2.1.7 Transformation of competent *B. subtilis* BD224/LA46

Competent *B. subtilis* cells were transformed by the method of Anagnostopoulos and Spizizen (1961). Frozen competent *B. subtilis* cells (1 ml) were thawed at 37°C . Nine ml *B. subtilis* transformation medium (see Appendix) was added to the cell suspension. Competent cells suspended in transformation medium (0.1 ml) were added to 4 microfuge tubes, each of which contained 0.5 μl (0.4 μg), 1.0 μl (0.8 μg), 2.5 μl (2.0 μg) and 5 μl (4.0 μg) of pHV60 - VC11 ligated DNA. The mixtures were allowed to express for 30 min at 37°C and subsequently spread on Luria agar containing 5 $\mu\text{g}/\text{ml}$ chloramphenicol. The plates were scored for growth after incubation at 37°C for 15 h.

Similarly, *B. subtilis* was transformed with 0.5 μg pHV33 and 0.5 μg pHV60 DNA. Finally, 0.1 ml untransformed competent cells were scored for growth following incubation at 37°C for 15 h.

5.2.2 Isolation of the VC11 ori using the plasmid pLJ702

5.2.2.1 Extraction of pLJ702 DNA from *S. lividans* 3131

Malt-3 broth (200 ml), inoculated with *S. lividans* 3131, was

incubated at 30°C for 48 h with agitation. The cells were pelleted by centrifugation at 7,000 rpm for 10 min at 4°C in a GSA rotor and resuspended in 5 ml precooled 25% (w/v) sucrose, 50 mM Tris, pH 8.0. Following the addition of 100 mg lysozyme, the suspension was incubated on an orbital shaker at 37°C for 1 h. One ml 0.25 M EDTA was added to the suspension which was subsequently incubated at 0°C for 5 min. Five ml 50 mM Tris, pH 7.5, 2% (w/v) SDS, 10 mM EDTA was added to the suspension. Following incubation at 0°C for 10 min, 4 ml 5 M NaCl was mixed gently with the lysed cell suspension which was subsequently incubated at 0°C for 1 h. The precipitated protein, SDS and chromosomal DNA were removed by centrifugation at 15,000 rpm in a SS 35 rotor for 30 min at 4°C. CsCl was added to the supernatant to a final concentration of 1 mg/ml and subsequently, 0.8 ml of 10 mg/ml ethidium bromide was introduced to the suspension. Residual debris was extracted by centrifugation at 10,000 rpm for 5 min. The refractive index of the solution was adjusted to 1.394 with a hand-held 0-50% sugar refractometer. Plasmid DNA was further purified and concentrated by isopycnic centrifugation at 55,000 rpm through a CsCl gradient for 18 h in a Beckman VTi 65.2 rotor at 15°C. The plasmid DNA band, visualized by UV light at 270 nm, was removed from the CsCl gradient with a wide bore disposable syringe. The ethidium bromide was extracted by 2 cycles of treatment with NaCl-saturated isopropanol. The DNA suspension was diluted 3-fold with TE buffer. Three volumes of

cold 96% ethanol were added and the DNA suspension was incubated at -70°C for 30 min. The precipitated plasmid DNA was pelleted by centrifugation at 18,000 rpm in a SS 35 rotor for 15 min at 4°C and resuspended in 500 μl TE buffer to yield a pLJ702 DNA concentration of 676 $\mu\text{g}/\text{ml}$.

5.2.2.2 Restriction of pLJ702 and VC11 DNA

pLJ702 DNA (5.5 μg) was digested at 37°C for 4 h in 10 μl low salt digestion buffer with 32 u of the restriction enzyme KpnI. Two hundred mM KCl (1.22 μl) (final concentration 20 mM) and 40 u SmaI restriction enzyme were added to the reaction mix which was incubated for a further 18 h at 37°C . Sodium acetate was added to a final concentration of 0.3 M, followed by 3 volumes of cold 96% ethanol. The solution was incubated at -70°C for 30 min and the DNA precipitated by centrifugation at 12,000 rpm in a microfuge for 5 min at 4°C . The supernatant was discarded, a second aliquot of cold 96% ethanol was gently added so as not to resuspend the DNA pellet and recentrifuged at 12,000 rpm for 1 min at 4°C . The ethanol was removed and the DNA pellet was subsequently dried in a Savant speed vac concentrator for 15 min. The pLJ702 DNA resuspended in 3 μl TE buffer, was digested in 10 μl medium salt digestion buffer with BclI (45 u), BamHI (36 u) and PstI (36 u) restriction enzymes for 5 h at 37°C . The digested pLJ702 DNA was subsequently ethanol precipitated as

above and resuspended in 10 ul TE buffer.

VC11 DNA (4.5 ug), prepared by the protocol described in section 2.2.10, was digested with 40 u BglII restriction enzyme in 10 ul low salt digestion buffer containing 5 mM spermidine for 5 h at 37°C. Subsequently, the phage DNA was ethanol precipitated and resuspended in 10 ul TE buffer.

Restricted pLJ702 (1 ug) and VC11 DNA was analyzed on a 1.2% (w/v) Tris-Borate-EDTA-buffered agarose gel containing 0.5 ug/ml ethidium bromide by comparison with unrestricted plasmid and phage DNA, and sized according to a HindIII-digested lambda DNA standard. Electrophoresis was performed in a horizontal direction at 38 V (constant voltage) for 18 h. The DNA was visualized using a 254 nm UV light and photographed with a Polaroid Land camera.

5.2.2.3 Ligation of restricted pLJ702 and VC11 DNA

Three separate VC11-pLJ702 DNA ligation reactions were performed at various phage : plasmid DNA ratios: 2 ug VC11 and 0.5 ug pLJ702 DNA (4:1), 1 ug VC11 and 1 ug pLJ702 DNA (1:1), and 0.5 ug VC11 and 2 ug pLJ702 DNA (1:4). The ligation reactions were executed in a final volume of 10 ul ligation buffer with 5 u ligase for 48 h at 12°C. Restricted pLJ702 DNA (1 ug) was

religated by the same procedure.

5.2.2.4 Determination of the minimum inhibitory concentration of thiostrepton for *S. olivaceus*

One hundred μl of 10^{-3} and 10^{-4} dilutions of a spore suspension of *S. olivaceus* was spread on malt-3 agar supplemented with the antibiotic thiostrepton at the following concentrations ($\mu\text{g/ml}$): 1, 5, 10, 15, 20, 35 and 50. In addition, dilutions of *S. olivaceus* spores were propagated on malt-3 agar lacking antibiotic. The plates were incubated for 48 h at 30°C . Following the appearance of colonies in the absence of antibiotic, the number of cfu at each thiostrepton concentration was determined from duplicate experiments. The minimum inhibitory concentration (m.i.c.) of thiostrepton for *S. olivaceus* was taken as the highest thiostrepton concentration at which *S. olivaceus* was able to produce colonies.

5.2.2.5 Transformation of *S. olivaceus* protoplasts

S. olivaceus protoplasts, prepared by the method described in section 4.2.1 and stored at -20°C , were thawed at room temperature. Aliquots (100 μl) of *S. olivaceus* protoplasts were added to each of the 3 VCII-pLJ702 DNA ligations. Within 30 sec, 500 μl of *Streptomyces* transformation medium (see Appendix) was

introduced. The DNA-protoplast suspension was mixed by pipetting 3 times in a 5 ml glass pipette and subsequently incubated for 1 min at room temperature to allow DNA uptake. Aliquots (100 μ l) of 10^0 , 10^{-1} and 10^{-2} dilutions, performed in P medium were spread with a platinum loop onto R2 agar and incubated at 30°C for 24 h to allow expression of the transformed DNA. Subsequently, 3 ml soft R2 agar supplemented with thiostrepton was overlaid on the R2 agar to produce a final antibiotic concentration of 5 μ g/ml. The plates were incubated for a further 5 days at 30°C.

In addition, S. olivaceus protoplasts were transformed with 1 μ g undigested pIJ702 DNA by the same procedure described above. Untransformed S. olivaceus protoplasts (100 μ l) were added to 500 μ l Streptomyces transformation medium and 100 μ l of this suspension was subsequently propagated on R2 agar. Following incubation at 30°C for 24 h, 3 ml soft R2 agar containing thiostrepton was added as previously described.

5.2.2.6 Analysis of thiostrepton resistant S. olivaceus transformants

5.2.2.6.1 Expression of thiostrepton resistance by S. olivaceus transformants

Ten-fold serial dilutions of spores of S. olivaceus transformed

with either pIJ702 or hybrid DNA, suspended in 400 μ l sterile 20% (v/v) glycerol, were prepared in sterile distilled water. One hundred μ l of each dilution was subsequently propagated on malt-3 agar supplemented with thiostrepton at a final concentration of 5 μ g/ml. The plates were incubated at 30°C until the colonies had sporulated. The spores were harvested and the procedure repeated. Thus, the ability of the S. olivaceus transformants to grow in the presence of thiostrepton was determined by successive passage on malt-3 agar containing thiostrepton.

5.2.2.6.2 Extraction of transformed S. olivaceus DNA

Three thiostrepton resistant S. olivaceus transformants, designated 2RA, 2RB and OL7 for convenience, were each inoculated into 200 ml malt-3 broth supplemented with 2.5 μ g/ml (final concentration) thiostrepton. The cultures were incubated at 30°C for 4 days with agitation. The mycelia were harvested by centrifugation at 7,000 rpm in a GSA rotor for 10 min at 4°C and the DNA was subsequently extracted by the method described in section 5.2.2.1. However, following isopycnic centrifugation, no plasmid DNA bands were visible by illumination of the CsCl gradients with UV light (270 nm). Thus, 1 ml of each of the CsCl gradients, below the visible chromosomal DNA band, was removed with a disposable wide bore syringe. Similarly, the chromosomal

DNA bands of 2RA and 2RB were removed and pooled. The ethidium bromide was extracted with NaCl-saturated isopropanol and, following ethanol precipitation, each sample was resuspended in 200 ul TE buffer.

5.2.2.6.3 Hybridization of ^{32} P-labelled VC11 DNA to transformed *S. olivaceus* DNA

One hundred ul of 2RA, 2RB and OL7 was each digested with 20 u ClaI restriction enzyme in 120 ul low salt digestion buffer. Pooled transformed *S. olivaceus* chromosomal DNA (10 ug) was restricted with 50 u SalI endonuclease in 120 ul high salt digestion buffer. Similarly, 10 ug untransformed *S. olivaceus* chromosomal DNA was digested with 50 u SalI restriction enzyme. VC11 DNA (1.6 ug) was digested with 12 u BglII restriction enzyme in 120 ul low salt digestion buffer containing 5 mM spermidine. All reactions were performed at 37°C for 5 h.

The restriction enzyme reactions were terminated by the addition of 30 ul gel loading buffer to each sample. Electrophoresis of the samples was performed in a horizontal direction through 1.2% (w/v) Tris-acetate-buffered agarose gel containing 0.5 ug/ml ethidium bromide at 40 V (constant voltage) for 18 h with recirculation of the buffer. The DNA was subsequently visualized by UV light at 254 nm and photographed with a Polaroid Land

camera.

Transfer of the DNA from the agarose gel to gene screen (New England Nuclear) was accomplished by the technique developed by Smith and Summers (1980). The agarose gel was washed twice in 2 volumes of 0.25 M HCl for 15 min with agitation. The gel was rinsed with distilled water and subsequently washed twice with 2 volumes of 0.5 M NaOH, 1.5 M NaCl with agitation for 15 min. The gel was neutralized by 2 washes in 2 volumes of 1 M NH_4OAc , 0.02 M NaOH for 30 min with agitation. The gel was subsequently inverted and placed on a dry flat surface. Gene screen, previously soaked in neutralizing buffer for 2 min, was placed on top of the agarose gel, followed by 3 sheets of Whatman 3 MM filter paper saturated in the same buffer. A 2 inch stack of dry paper towels, a glass plate and finally a 1 kg weight were subsequently placed on top of the filter paper. Transfer of the DNA fragments to the gene screen was complete after 2 h. The gene screen was rapidly rinsed in 2 x SSC and allowed to air dry on a dry piece of filter paper. Subsequently, the gene screen was placed between 2 sheets of filter paper and baked under vacuum for 2 h at 80°C.

Nick-translation of VC11 DNA was performed according to the protocol and kit supplied by Amersham. VC11 DNA (1.6 ug) was labelled with ^{32}P in a 100 ul reaction mix containing 20 ul

solution 1 (100 μ M dATP, 100 μ M dGTP, 100 μ M dTTP in a concentrated nick-translation buffer containing Tris, pH 7.8, MgCl_2 and 2-mercaptoethanol), 5 μ l [α - ^{32}P] dCTP (10 mCi/ml) and 10 μ l Solution 2 (5 u DNA polymerase I and 100 pg DNase I in a buffer solution containing Tris, pH 7.5, MgCl_2 , glycerol and bovine serum albumin). The nick-translation reaction was performed for 2 h at 12°C.

The nick-translated VC11 DNA was separated from unincorporated nucleotides by the spun column procedure described in Maniatis (1982a). A small amount of sterile glass wool was used to plug the bottom of a sterile 1 ml disposable syringe. A column (0.9 ml bed volume) of Sephadex G-50 equilibrated in TE buffer containing 0.1 M NaCl was prepared in the 1 ml syringe in the following manner. Sephadex G-50 was added to the syringe which was subsequently inserted into a 17 x 100 mm Falcon disposable test tube and centrifuged for 4 min at 1,600 g in a Hettich benchtop centrifuge. Sephadex G-50 was added until the packed column volume reached the 0.9 ml mark on the syringe. The Sephadex G-50 column was washed twice with 100 μ l TE buffer containing 0.1 M NaCl. The 100 μ l nick-translation reaction mix was subsequently added to the column and collected in a decapped sterile Eppendorf tube at the bottom of the 17 x 100 mm Falcon test tube following centrifugation at 1,600 g for 4 min.

The number of counts per minute (cpm) of the nick-translated probe was determined by placing 1 μ l of the ^{32}P -labelled VC11 DNA in 5 ml scintillation fluid and counting in a Packard Tri-Carb 460 scintillation counter for 2 min. The total cpm of the probe DNA was subsequently calculated by multiplying the cpm/ μ l by the total volume of nick-translated DNA.

DNA hybridization was performed by the method described in Maniatis (1982b). The baked gene screen was saturated with 6 x SSC for 2 min and placed in a plastic bag to which 60 ml prehybridization solution (6 x SSC, 0.5% SDS, 5 x Denhardt's solution and 100 μ g/ml denatured salmon sperm DNA) was added. The salmon sperm DNA had been denatured by incubation in a boiling water bath for 10 min followed by rapid cooling at 0°C . The sealed plastic bag was incubated at 68°C for 4 h with agitation. Subsequently, the prehybridization fluid was discarded and replaced with 15 ml hybridization solution which contained 6 x SSC, 10 mM EDTA, 5 x Denhardt's solution, 0.5% SDS and 100 μ g/ml denatured salmon sperm DNA. 100 μ l ^{32}P -labelled VC11 DNA (1.2×10^7 cpm), heat denatured by the same protocol used for salmon sperm DNA, was added to the plastic bag. The sealed plastic bag was subsequently incubated at 60°C for 18 h with agitation. The hybridization reaction was performed at $T_m = -42^{\circ}\text{C}$.

In order to remove non-specifically bound radioactivity, the gene

screen was washed in 2 x SSC, 0.1 % SDS at 60°C for 15 min with agitation and subsequently, in 0.2 x SSC, 0.1% SDS at 60°C for 1 h with agitation. Thus, the gene screen was washed at $T_m = -17^{\circ}\text{C}$. The gene screen was placed under Kodak XAR X-ray film between intensifying screens for 2 weeks at -70°C .

5.2.2.6.4 Hybridization of ^{32}P -labelled DNA of the *S. olivaceus* transformant 2RB to pLJ702 and VC11 DNA

Single BglI, BglII and BclI digests of 5 ug VC11 DNA were performed with 10 u of each restriction enzyme in 20 ul of either low salt (BglII) or medium salt digestion buffer (BclI and BglI) in the presence of 5 mM spermidine. Similarly, 5 ug pLJ702 DNA was restricted with 10 u BclI and 2 ug lambda DNA was digested with 10 u HindIII. The DNA samples were restricted at 37°C for 5 h. Double pLJ702 DNA restriction digests were performed in the following manner. pLJ702 DNA (5 ug) was digested with 10 u of either ClaI or BglII restriction enzyme at 37°C for 12 h. Subsequently, 500 mM NaCl was added to each 10 ul reaction mix to produce a final salt concentration of 50 mM (medium salt digestion buffer) and the DNA was then digested with 10 u BclI restriction enzyme at 37°C for 5 h.

Restricted pLJ702, lambda and VC11 DNA was analyzed on 1.2% (w/v) Tris-acetate-buffered agarose gel containing 0.5 ug/ml ethidium bromide. Electrophoresis was performed in a horizontal direction

at 38V (constant voltage) for 15 h with recirculation of the buffer. Subsequently, the DNA was visualized with UV light at 254 nm and photographed with a Polaroid Land camera.

The DNA was transferred to gene screen by the method described in section 5.2.2.6.3. 2RB DNA (50 ul) and 10 ul lambda DNA were nick-translated with ^{32}P (1.6×10^8 cpm and 5.5×10^5 cpm, respectively) and hybridized at $T_m = -51^\circ\text{C}$ to pLJ702, lambda and VC11 DNA by the procedure described in section 5.2.2.6.3. Non-specifically bound radioactivity was removed at $T_m = -22^\circ\text{C}$ by three 15 min washes with 2 x SSC, 0.1% SDS at 65°C , followed by 0.2 x SSC, 0.1% SDS at 65°C for 1 h with agitation. The gene screen was placed under Kodak XAR X-ray film between intensifying screens for 2 weeks at -70°C .

5.2.2.6.5 Hybridization of ^{32}P -labelled DNA of the *S. olivaceus* transformant OL7 to pLJ702 DNA

pLJ702 DNA (2 ug) was digested with 10 u BclI restriction endonuclease in 90 ul medium salt digestion buffer. Double digests of 2 ug pLJ702 DNA with either BamHI and BclI or ClaI and BglII restriction enzymes were performed using 10 u of each endonuclease in 90 ul of either medium salt or low salt digestion buffer, respectively. Similarly, a triple digest of 2 ug pLJ702 DNA was executed with 10 u of the restriction enzymes ClaI, BglII and KpnI in 90 ul low salt digestion buffer. The digestion

reactions were performed at 37°C for 5 h. The restricted DNA was analyzed on a 1.2% (w/v) Tris-acetate-buffered agarose gel containing 0.5 ug/ml ethidium bromide. Electrophoresis was executed in a horizontal direction at 38 V (constant voltage) for 15 h with recirculation of the buffer. The DNA was visualized with UV light at 254 nm and photographed with a Polaroid Land camera.

The DNA was transferred to gene screen by the procedure described in section 5.2.2.6.3. Contaminating *S. olivaceus* chromosomal DNA was removed from OL7 by centrifugation through a second CsCl gradient.

OL7 DNA (15 ul) was labelled with [α - ^{32} P] dCTP by nick-translation (section 5.2.2.6.3). Radioactively-labelled OL7 DNA (8.1×10^7 cpm) was hybridized at $T_m = -50^\circ\text{C}$ to restricted pLJ702 DNA using the protocol described in section 5.2.2.6.3. Non-specifically bound radioactivity was removed at $T_m = -16^\circ\text{C}$ by washing the gene screen in 2 x SSC, 0.1% SDS at 65°C for 1 h, followed by 0.1 x SSC, 0.1% SDS at 65°C for 90 min with agitation. The gene screen was placed under Kodak XAR X-ray film between intensifying screens for 24 h at -70°C.

5.3 RESULTS AND DISCUSSION

5.3.1 Isolation of the VC11 ori using the plasmid pHV60

pHV60 DNA possesses a unique BamHI restriction site which is available for insertion of foreign DNA. Although the vector is unable to replicate autonomously in B. subtilis, it is able to express chloramphenicol resistance when associated with a replicon active in this micro-organism (Niaudet and Ehrlich, 1979).

VC11 DNA was restricted with BglII, while the restriction enzyme BamHI was used to digest the plasmid pHV60 (Fig. 3). BglII digestion of VC11 DNA yielded 6 fragments of 23.2; 12; 11.1; 7.6; 2.6; 0.65 kb in length (the smallest DNA fragment is not visible). Digestion of the single BamHI restriction site of pHV60 produced a linear DNA molecule of approximately 5.1 kb. Both restriction enzymes yield DNA fragments with identical protruding 5' cohesive termini which may be ligated to form a hybrid site that is not recognized by either endonuclease.

Untransformed competent B. subtilis cells were found to produce tiny colonies in the presence of chloramphenicol. Similarly, B. subtilis transformed with either pHV60, VC11 or ligated pHV60-VC11 DNA exhibited rudimentary colony formation. However,



FIGURE 3. Restriction enzyme digestion of pHV60 and VC11 DNA. Lanes: A, pHV60 undigested; B, pHV60 digested with BamHI; C, lambda DNA digested with HindIII; D, VC11 DNA digested with BglII; E, undigested VC11 DNA.

transformation of B. subtilis with pHV33, which possesses a replication region and consequently can replicate and express chloramphenicol resistance in B. subtilis (Primrose and Ehrlich, 1981), resulted in a large number of normal-sized colonies when grown for 15 h in the presence of chloramphenicol.

Although BglII digested VC11 DNA was 'shotgun' cloned into the BamHI restriction site of pHV60, the introduction of a single hybrid DNA molecule containing the phage ori region into a B. subtilis cell would have allowed normal cell growth in the presence of chloramphenicol. In other words, it should have been possible to isolate the VC11 ori region from a single transformation event. Thus, the results imply that the ori region of VC11 is non-functional in B. subtilis. The inability of the phage replicon to function normally in Bacillus, which has been proposed as an alternate Gram-positive host for cloning Streptomyces genes (Enquist, 1980), reflects the highly specific nature of the regulatory sequences of Streptomyces.

5.3.2 Isolation of the VC11 ori using the plasmid pLJ702

Isolation of the VC11 ori region was attempted in an organism which is susceptible to infection by VC11 phage (section 2.4). Thus, DNA cloning was performed in S. olivaceus, previously shown to be transfectable with VC11 DNA (section 4.4).

A cloning strategy based on the pHV60 system in *B. subtilis* was utilized for the isolation of the VC11 replicon. Although replication of the *Streptomyces* plasmid pLJ702 has not been reported in *S. olivaceus*, the plasmid has a high copy number, a broad host range and the region of the genome responsible for replication of the plasmid has been mapped to a 1.71 kb DNA fragment (Kieser *et al.*, 1982; Katz *et al.*, 1983).

Digestion of pLJ702 DNA with the restriction enzymes *Kpn*I, *Sma*I, *Bcl*I, *Bam*HI and *Pst*I resulted in the destruction of the replication region of the plasmid genome. Analysis of restricted pLJ702 DNA by electrophoresis revealed the presence of 3 *Bcl*I restriction fragments: 1.48; 1.25 and 1.12 kb in length (Fig. 4). The largest *Bcl*I fragment (1.71 kb; Fig. 2) containing the plasmid replicon was digested into 6 segments of which 4 (0.62; 0.40; 0.38 and 0.20 kb) were visible. VC11 DNA was digested with *Bgl*II restriction enzyme (Fig. 4), yielding 4 DNA fragments which have protruding 5' cohesive termini compatible with those generated by *Bcl*I digestion. As the thiostrepton-resistance gene of pLJ702 is contained in the 1.12 kb *Bcl*I DNA fragment, 'shotgun' cloning of *Bgl*II restricted phage DNA into the *Bcl*I restriction sites of pLJ702 may lead to the construction of a hybrid molecule containing a VC11 DNA fragment bearing the phage *ori* region which would allow replication of the plasmid and expression of the thiostrepton-resistance gene in *S. olivaceus*.



FIGURE 4. Restriction enzyme digestion of pIJ702 and VC11 DNA. Lanes: A, undigested VC11 DNA; B, BglII digested VC11 DNA; C, lambda DNA digested with HindIII; D, pIJ702 digested with BamHI, BclI, KpnI, PstI and SmaI; E, pIJ702 undigested.

Antibiotic-resistant S. olivaceus transformants were selected in the presence of 5 ug/ml thiostrepton which prevents growth of wild type S. olivaceus. pLJ702 was found to transform S. olivaceus protoplasts with an efficiency of 2×10^3 cfu/ug of DNA. Five thiostrepton-resistant transformants were obtained following transformation of S. olivaceus with ligated VC11-pLJ702 DNA, while religated pLJ702 did not produce antibiotic-resistant colonies, indicating that the plasmid replicon was non-functional due to restriction enzyme digestion.

Expression of the thiostrepton-resistance phenotype by the 5 S. olivaceus transformants harbouring hybrid VC11-pLJ702 DNA molecules was found to be unstable following successive passage on antibiotic-containing medium. Three of the transformants failed to produce more than 2 - 3 colonies following each passage, indicating a low level of expression of the thiostrepton-resistance phenotype. The remaining 2 transformants (designated 2RA and 2RB for convenience) were more stable in the presence of the antibiotic, producing between 7 and 10 thiostrepton-resistant colonies per propagation cycle.

Electrophoretic analysis of 2RA and 2RB DNA (fractionated from the section of the CsCl gradient below the chromosomal DNA band) revealed the presence of chromosomal DNA and the absence of a ClaI restriction fragment (pLJ702 possesses a single ClaI site, while VC11 DNA contains no ClaI recognition sequences)

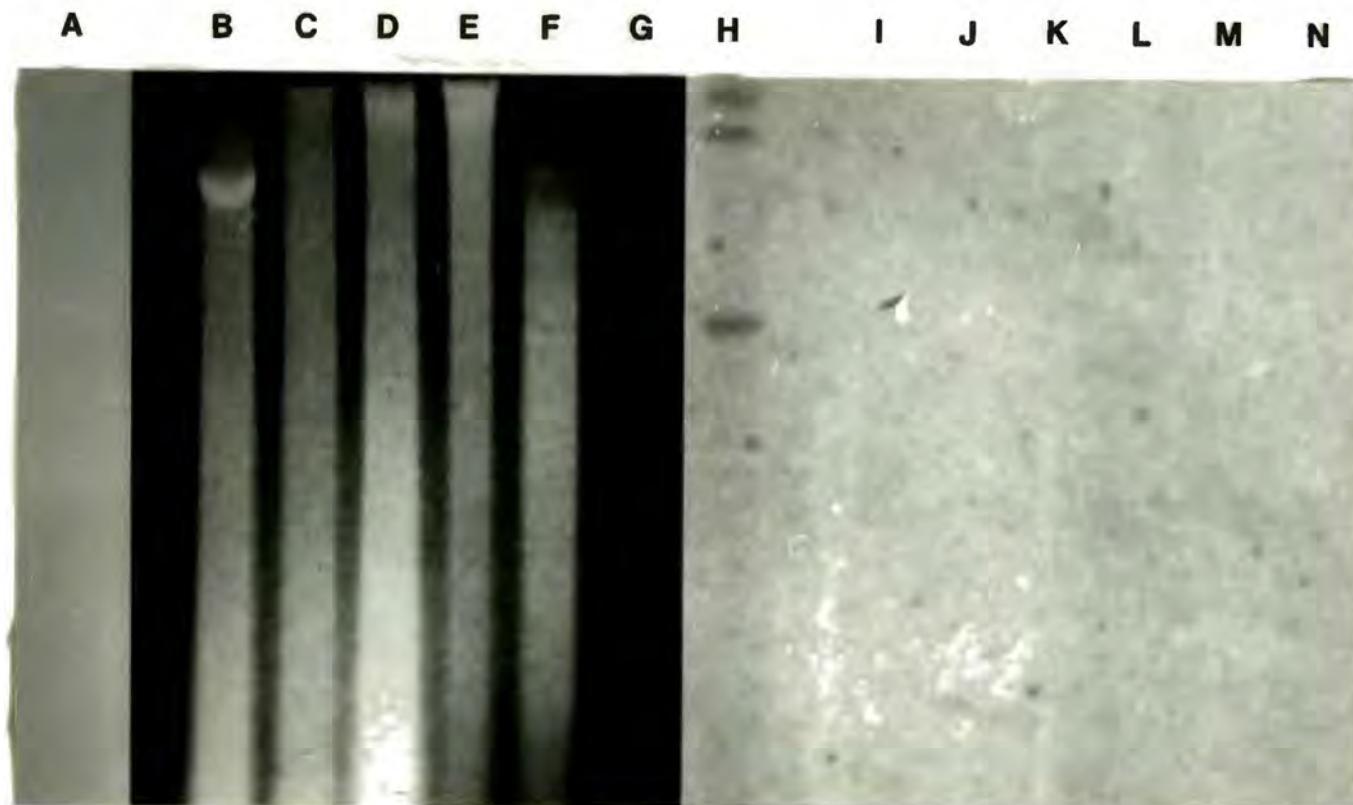


FIGURE 5. Restriction enzyme digestion of 0L7, 2RA, 2RB, 2RA+2RB chromosomal DNA, *S. olivaceus* chromosomal DNA, VC11 DNA and hybridization to ^{32}P -labelled VC11 DNA. Lanes: A+H, *Bgl*II digested VC11 DNA; B+I, 0L7 digested with *Cla*I; C+J, 2RA digested with *Cla*I; D+K, 2RB digested with *Cla*I; E+L, undigested 2RA and 2RB chromosomal DNA, F+M, *Sal*I digested 2RA

corresponding to plasmid DNA (Fig. 5). This observation can be explained by a low copy number of the hybrid plasmid due to inefficient replication by the phage replicon.

The S. olivaceus transformants 2RA and 2RB were analyzed by DNA hybridization to ascertain whether phage DNA sequences are present. However, ^{32}P -labelled VC11 DNA failed to hybridize to DNA from 2RA and 2RB, indicating the absence of phage sequences in these clones (Fig. 5). In addition, the transformant 2RB was radioactively-labelled and utilized to probe restricted pLJ702 and VC11 DNA by DNA hybridization (Fig. 6). Once again, no homology between 2RB and VC11 DNA was detected. However, extensive homology between 2RB and pLJ702 DNA was observed. While 2RB contained DNA sequences homologous to the 3 largest BclI restriction fragments of pLJ702 DNA, very little hybridization to the smallest BclI fragment bearing the thiostrepton-resistance gene occurred.

The presence of a cos site in the VC11 genome (section 2.3.5) suggests that the mechanism of VC11 replication is similar to the lytic cycle of phage lambda (Lomovskaya et al., 1980). Although the genes involved in replication of the lambda genome are clustered on the phage DNA (Campbell, 1977), BglII restriction of VC11 DNA may have resulted in the separation of gene functions vital to replication of the phage genome. Likewise, the presence

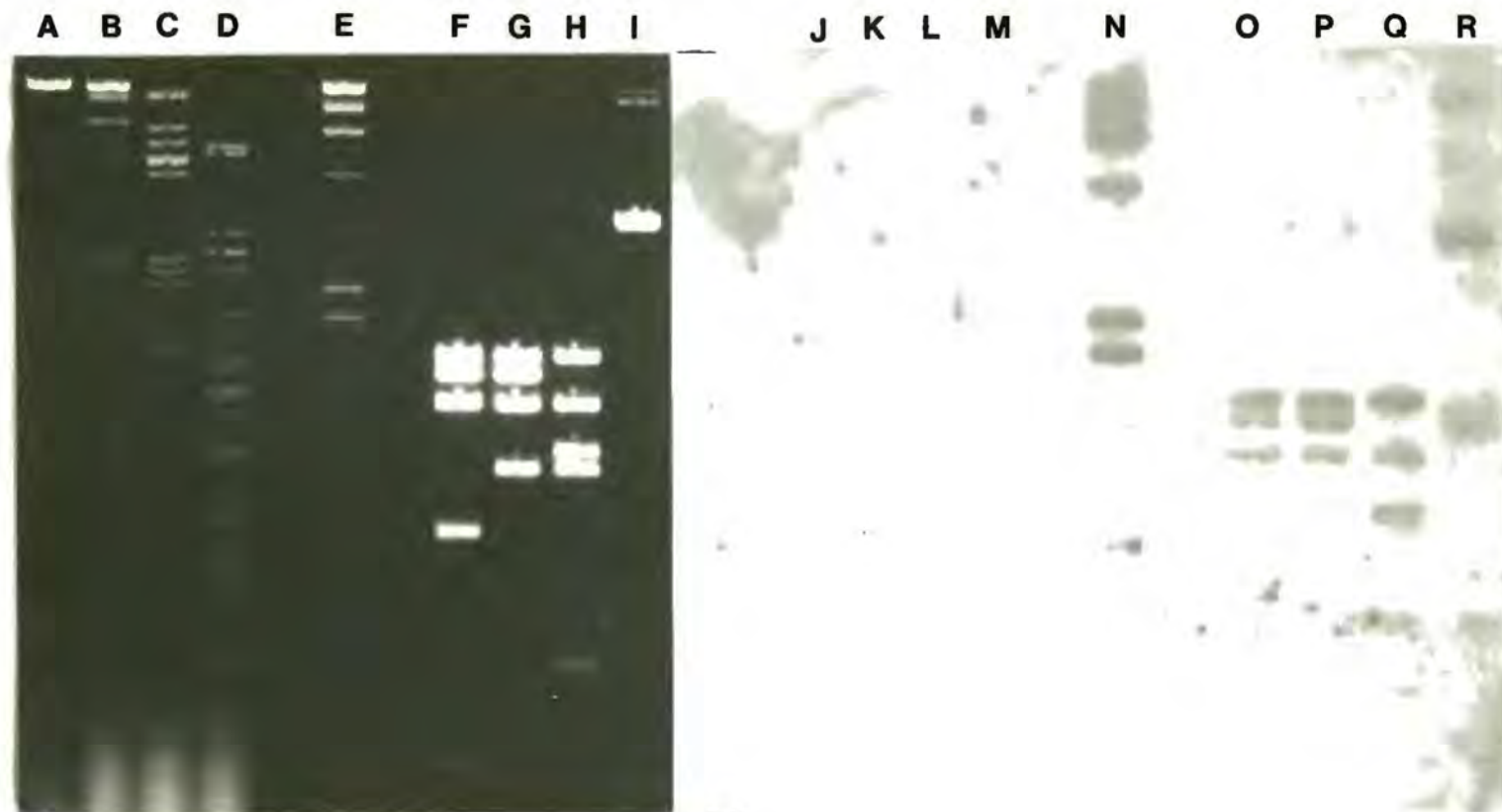


FIGURE 6. Hybridization of ^{32}P -labelled 2RB DNA to restricted pLJ702 and VC11 DNA. Lanes: A+J, undigested VC11 DNA; B+K, BglII digested VC11 DNA; C+L, BclI digested VC11 DNA; D+M, BglI digested VC11 DNA; E+N, HindIII digested lambda DNA; F+O, pLJ702 digested with BclI and ClaI; G+P, pLJ702 digested with BclI; H+Q, pLJ702 digested with BclI and BglII; I+R,

of a BglII restriction site within the ori region of VC11 would have prevented the isolation of the phage replicon using pHV60. The presence of thiostrepton may have exerted a selective pressure on the S. olivaceus transformants for the preservation of the plasmid pLJ702 (especially the thiostrepton-resistance gene). The inability of the VC11 ori region to replicate and thus maintain the plasmid in the host cell, may have resulted in the rearrangement and integration of pLJ702 DNA sequences into the chromosome of S. olivaceus. Rearrangement of the thiostrepton-resistance gene may account for its low expression and stability in the presence of the antibiotic. However, this interpretation does not explain the low degree of hybridization between the BclI restriction fragment containing the thiostrepton-resistance gene and 2RB DNA. It is extremely likely that the low stringency of hybridization would have allowed irreversible non-specific hybridization between the G+C rich 2RB DNA and similar portions of pLJ702. The high stringency of the washes would explain the failure to detect homology between 2RB and VC11 DNA, or the thiostrepton-resistance gene of pLJ702, as both are relatively A+T rich and consequently possess low melting temperatures.

S. olivaceus transformed with intact pLJ702 exhibited a confluent lawn of thiostrepton-resistant colonies following serial passage on thiostrepton-containing medium, indicating strong expression

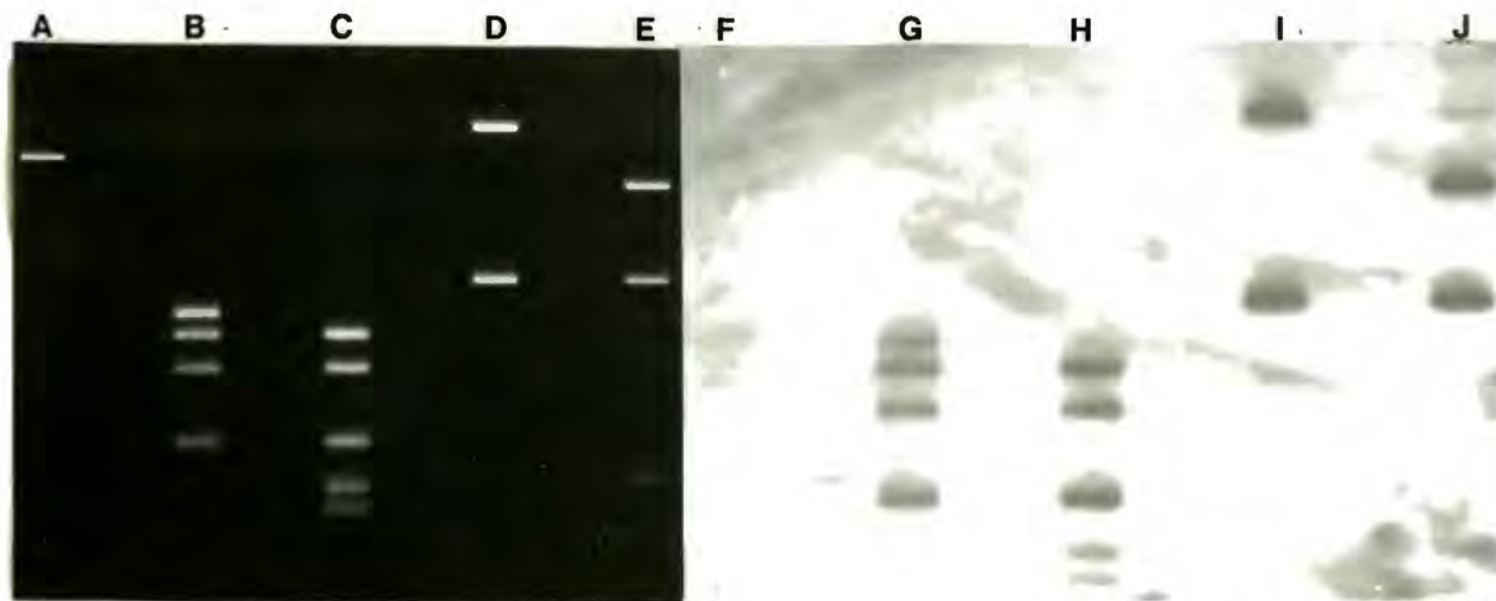


FIGURE 7. Hybridization of ^{32}P -labelled OL7 DNA to restricted pIJ702. Lanes: A+F, undigested pIJ702; B+G, BclI digested pIJ702; C+H, pIJ702 digested with BamHI and BclI; D+I, pIJ702 digested with BglII and ClaI; E+J, pIJ702 digested with BglII, ClaI and KpnI.

of the thiostrepton-resistance gene and stable maintenance of the plasmid. In addition, the S. olivaceus transformants (designated OL7) were very dark in colour due to melanin production by the tyrosinase gene of pLJ702. Analysis of DNA extracted from the S. olivaceus transformant OL7 by agarose gel electrophoresis showed the presence of a plasmid DNA band (Fig. 5). In order to determine the nature of the plasmid present in this transformant, ³²P-labelled OL7 DNA was analyzed by DNA hybridization to restricted pLJ702 (Fig. 7). Hybridization of the radioactive transformant DNA to all the restriction fragments of pLJ702 indicates that the plasmid present in the S. olivaceus transformant OL7 is indeed pLJ702. The ability of pLJ702 to transform S. olivaceus and express thiostrepton resistance and melanin production in this Streptomyces species, will allow the elucidation of the genetics and biochemistry involved in the synthesis of the commercially-important olivanic acids by recombinant DNA technology.

CHAPTER 6

DETECTION OF THE TUF GENE IN STREPTOMYCES CATTLEYA AND STREPTOMYCES OLIVACEUS

SUMMARY

A single copy of the tuf gene which encodes the polypeptide chain elongation factor Tu was identified in S. cattleya and S. olivaceus by DNA hybridization. Although restriction enzyme digests indicate differences in the nucleotide sequence of the tuf gene region in these Streptomyces species, the Streptomyces tuf gene bears strong sequence homology to the carboxyl-terminal half of the E. coli tufA gene. The detection of the tuf gene of S. cattleya and S. olivaceus has several potential applications for the study of the molecular genetics of these important beta-lactam antibiotic-producing Streptomyces species.

6.1 INTRODUCTION

Translation is the process whereby various amino acids are brought by their cognate transfer RNA (tRNA) molecules to the ribosome where they are polymerized into protein in the sequence

encoded by the messenger RNA (mRNA). The process of translation has been extensively reviewed by Lucas-Lenard and Lipman (1971), Haselkorn and Rothman-Denes (1973) and Lengyel (1974).

Protein synthesis is performed on 70S ribosomes in procaryote cells. They are composed of two subunits: 50S and 30S; the former containing 34 ribosomal proteins (r proteins) (designated L1-L34) and 2 species of ribonucleic acids (rRNA) (23S and 5S rRNA), while the latter subunit consists of 21 r proteins (S1-S21) and 16S rRNA (Kaziro, 1978). Ribosomes have 2 tRNA binding sites adjacent to each other. The peptidyl (P) site is located toward the 5' end of mRNA and the other, designated the aminoacyl (A) site, towards the 3' end.

tRNA is a polynucleotide of 30 kD and is composed of 73 to 93 nucleotides (Kaziro, 1978). Procaryotic cells contain approximately 60 different species of tRNAs, indicating that more than one tRNA species exists for each amino acid. The nucleotide sequences of all the tRNAs that have been currently investigated can be arranged in a cloverleaf structure, consisting of 4 double-stranded helical regions and 4 single-stranded loops. The translation of nucleotide sequences of mRNA into amino acid sequences is achieved through interactions of mRNA codons (3 adjacent nucleotides) with anticodons (nucleotides complimentary to codons) in one of the loops of tRNA, designated the anticodon

loop. Amino acids are bound to their cognate tRNA molecules by a process known as aminoacylation of tRNA. The reaction occurs in two steps and is catalyzed by an aminoacyl-tRNA synthetase. There is one specific aminoacyl-tRNA synthetase for each of the 20 amino acids in procaryote cells. While the mode of recognition between each specific aminoacyl-tRNA synthetase and tRNA molecule is unknown, it is thought to involve the anticodon (Lengyel, 1974).

The process of translation may be divided into 3 consecutive phases: peptide chain initiation, peptide chain elongation and peptide chain termination. Peptide chain initiation and termination are single events in the production of specific proteins in contrast to the repeating process of amino acid addition in peptide chain elongation.

The peptide chain initiation tRNA in procaryotes, an N-formylated derivative of a methionyl-tRNA (fMet-tRNA_f), does not participate in peptide chain elongation. Initiation factor IF3 binds the 30S ribosomal subunit to the initiation codon (AUG or GUG) of the mRNA. Subsequently, IF2 and IF1 associate fMet-tRNA_f and guanosine 5'-triphosphate (GTP) with the 30S ribosome initiation complex. Finally, the 50S ribosomal subunit binds to the 30S subunit to reconstitute the 70S ribosome, resulting in the release of the 3 initiation factors. The release of IF2 depends

on the hydrolysis of GTP and the presence of IF1 (Lengyel, 1974). Thus, the end-product of the initiation phase of translation is a 70S ribosome containing fMet-tRNA_f in the P site.

During peptide chain elongation, the polypeptide chain grows as the ribosome moves along the mRNA molecule towards the 3'-terminal nucleotide in steps of three nucleotides. One aminoacyl residue is attached to the protein chain during each step, leading to the synthesis of a polypeptide from the amino-terminal to the carboxy-terminal end.

Elongation factor Tu (EF-Tu) complexed with GTP interacts with an aminoacyl-tRNA, specified by the codon adjacent to the 3' side of the initiation codon, to form a ternary complex. Aminoacyl-tRNA·EF-Tu·GTP is subsequently transferred to the A site of the 70 S ribosome, GTP is hydrolyzed and EF-Tu·GDP is released. The fMet residue of the fMet-tRNA_f in the P site is released from its linkage to tRNA, forming a peptide bond with the alpha-amino group of the aminoacyl-tRNA in the A site. The enzyme peptidyl transferase, specified by the 50 S ribosomal subunit, catalyses the formation of the peptide bond. Elongation factor G (EF-G) and GTP are bound to the ribosome to facilitate translocation of the translation machinery. The discharged tRNA is released from the P site, the dipeptidyl-tRNA is moved from the A site to the P site and the ribosome is shifted 3

nucleotides further along the mRNA in the direction of the 3'-terminal end. As a result, GTP is cleaved to GDP + Pi which, together with EF-G, is released from the ribosome. Elongation factor Ts (EF-Ts) catalyses the release of GDP from EF-Tu·GDP, resulting in the formation of EF-Tu·EF-Ts which in turn interacts with GTP to generate EF-Tu·GTP and free EF-Ts. Finally, the ternary complex aminoacyl-tRNA·EF-Tu·GTP is formed and the third aminoacyl-tRNA is attached by repeating the cycle.

Peptide chain termination is triggered when, in the course of the movement of the ribosome along the mRNA, a chain terminator codon (UAA, UAG or UGA) is reached at the ribosomal A site. Thereupon, the release factor 1 (RF1) in the presence of UAA or UAG, or RF2 in the presence of UAA or UGA, together with RF3 and GTP result in the disassociation of the polypeptide chain from the tRNA in the P site and subsequently the mRNA·ribosome·tRNA complex falls apart.

EF-Tu is able to form a ternary complex, in a 1:1:1 molar ratio, with any aminoacyl-tRNAs except the initiator fMet-tRNA. The substrate specificity of EF-Tu assures that, as long as available, only charged tRNAs specified by the appropriate codons are bound to the ribosome. Only EF-Tu·GTP and not EF-Tu·GDP can interact with aminoacyl-tRNA to form a ternary complex. In addition, only EF-Tu·GTP and not EF-Tu·GDP is able to associate

with ribosomes. Thus, the hydrolysis of GTP to generate EF-Tu*GDP is required to release this complex from ribosomes. From these observations it appears that the reactivity of EF-Tu is modulated through sequential transitions of its conformation as a result of the presence of GTP or GDP (Kaziro, 1978).

Chemical studies of the structure of EF-Tu have revealed that the protein contains 2 reactive and 1 unreactive sulphydryl groups. One reactive sulphydryl (SH_1) is essential for the binding of GDP or GTP and is protected by the guanine nucleotides in the presence of Mg^{2+} , while the other reactive sulphydryl group (SH_2) is necessary for the binding of aminoacyl-tRNA which protects the site only when EF-Tu is complexed with GTP. SH_2 is also essential for interaction with ribosomes in the presence of GTP. The third sulphydryl group is buried in the interior of the molecule and is thus non-reactive (Kaziro, 1978).

From studies on the conformation of EF-Tu, it was concluded that a reversible local conformational change is induced in the proximity of the SH_2 group by the ligand conversion from GDP to GTP at the site close to SH_1 (Kaziro, 1978). Thus, EF-Tu may be regarded as a type of allosteric protein in that its ability to interact with aminoacyl-tRNA is turned on or off by a ligand change at a separate site. In contrast to other allosteric proteins which are composed of several identical or non-identical

subunits, EF-Tu is a monomeric protein consisting of a single polypeptide chain with a molecular weight of approximately 47,000 (Kaziro, 1978).

The role of GTP in EF-Tu-promoted binding of aminoacyl-tRNA can be summarized as follows. A unique conformation of EF-Tu induced by GTP can select aminoacylated tRNAs in preference to uncharged tRNA. The ternary complex aminoacyl-tRNA·EF-Tu·GTP is subsequently transferred to a precise location on the 50 S ribosomal subunit through the conformation of EF-Tu·GTP which favours interaction between EF-Tu and ribosomes. Hydrolysis of GTP promotes a conformational change of EF-Tu which discourages the association between EF-Tu and ribosomes, and in addition, results in an irreversible shift of the equilibrium toward the binding of aminoacyl-tRNA to ribosomes (Kaziro, 1978).

The E. coli phage Q β induces an enzyme which is responsible for specifically replicating the RNA genome of the phage by copying both the 'plus' and 'minus' RNA strands (Blumenthal et al., 1972). The phage-specific Q β replicase has been shown to consist of 4 subunits: subunit II has a molecular weight (mw) of 65,000 and is coded for by the phage, while the remaining three subunits are present in uninfected E. coli cells. Subunit I (mw 70,000) was found to be identical to the E. coli 30 S ribosomal protein S1 (Wahba et al., 1974), while subunit IV (mw 35,000) was

demonstrated to be identical to EF-Ts (Blumenthal et al., 1972). In addition, the latter group reported that subunit III (mw 45,000) of the Q β replicase is identical to EF-Tu as both polypeptides co-electrophoresed on sodium dodecyl sulphate gels, EF-Tu was precipitated by anti-Q β replicase serum, and subunit III was able to bind guanine nucleotides and phenylalanyl-tRNA. In addition, Q β replicase activity could be reconstituted from subunits I and II with EF-Tu and EF-Ts. The authors postulated that the two elongation factors perform similar functions to those during protein synthesis as both phage RNAs and tRNAs possess the sequence CCA at the 3' end which can be presented in a tRNA-like structure. Thus, the elongation factors in Q β replicase may initiate replication by binding the enzyme to the 3' end of the phage RNA, or alternatively, may participate in the RNA elongation reaction (Blumenthal et al., 1972).

EF-Tu has been found to participate in the regulation of rRNA synthesis (Travers et al., 1970; Travers, 1973). Although rRNA genes constitute only 0.2 to 0.4% of the total genome of E. coli, rRNA synthesis in rapidly growing cells accounts for up to 40% of the instantaneous rate of total RNA synthesis (Travers et al., 1970). The elongation factors EF-Tu and EF-Ts were shown to be responsible for the preferential stimulation of rRNA synthesis by RNA polymerase in the presence of competing mRNA in vitro. In addition, subunits III and IV of the Q β replicase of the E. coli

RNA phage Q β , previously identified as EF-Tu and EF-Ts respectively, are able to induce a similar effect. Travers (1973) identified an open and closed form of the rRNA promoter in vitro. The author reported that EF-Tu and EF-Ts generated a 5-fold increase in the in vitro synthesis of rRNA from the closed promoter, while the elongation factors did not exhibit a similar effect on the open form of the rRNA promoter. In addition, EF-Tu was found to control the specificity of transcription of the closed form of the rRNA promoter in the absence of EF-Ts.

In order to explain the direct effect of EF-Tu and EF-Ts on RNA polymerase, two conformational states of RNA polymerase, designated $(E\sigma)_m$ and $(E\sigma)_s$, were postulated (Travers, 1973). $(E\sigma)_m$ is able to initiate RNA synthesis at mRNA promoters and at open rRNA promoters, while $(E\sigma)_s$ initiates efficient RNA synthesis at closed rRNA and tRNA promoters. Although the two forms are assumed to be in equilibrium, $(E\sigma)_m$ is favoured at high KCl concentrations while neither form is favoured at low KCl concentrations. As EF-Tu and EF-Ts stabilise the $(E\sigma)_s$ form, this form predominates over the $(E\sigma)_m$ form at low KCl concentrations. Thus, in extreme situations when one form of the RNA polymerase is limited, synthesis of the corresponding RNA is significantly restricted.

Travers (1973) noted that the synthesis of rRNA was

preferentially inhibited relative to mRNA when wild type E. coli was starved for an amino acid or the cell growth rate was limited. Inhibition of rRNA synthesis was due to the production of guanosine tetraphosphate (ppGpp), specified by the rel gene, which acts on both EF-Tu and RNA polymerase in the presence of the closed form of the rRNA promoter. On the basis of several observations, the author concluded that the rRNA promoter is normally closed in vivo and that rRNA synthesis can be regulated by controlling the activity of $(E\sigma)_s$ through the action of EF-Tu and ppGpp. This control of rRNA synthesis by ppGpp and the elongation factors Tu and Ts emphasizes the coupling between protein synthesis and the transcription of the RNA destined to become a component of the protein synthesizing machinery of the cell (Travers, 1973).

Several specialized lambda transducing phages, including lambda fus2 and lambda fus3, have been isolated which carry bacterial DNA from the str-spc region at 72 minutes on the E. coli chromosome (Jaskunas et al., 1975a; Nomura, 1976; Jaskunas et al., 1977). UV-irradiated E. coli cells were infected with these phages. UV treatment damages bacterial DNA and decreases RNA and protein synthesis to a negligible level without seriously damaging the RNA and protein synthesizing machinery. In addition, the presence of a lambda lysogen prevented expression of the genes of the transducing phage. Thus, synthesis of any

protein after infection of intact transducing phage is a strong indication of the presence of an E. coli structural gene for that protein on the transducing phage DNA. Proteins synthesized after phage infection were labelled with ^{35}S -methionine and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis followed by autoradiography (Nomura, 1976). Likewise, E. coli chromosomal genes carried by these phages were identified by an in vitro approach which entailed the use of the phage DNA as template for the synthesis of RNA and protein. Demonstration of the synthesis of a specific protein or RNA is proof for the presence of the corresponding gene on the transducing phage genome (Nomura, 1976).

Using the above techniques, the genes for 27 r proteins were identified on the genome of lambda fus2 (Jaskunas et al., 1977). In addition, the genes coding for the alpha subunit of RNA polymerase, EF-G (fus) and EF-Tu (tufA) were found to be included in the genome of this specialized lambda transducing phage (Jaskunas et al., 1975b; Nomura, 1976). By analyzing insertion or deletion mutants of lambda fus3, and restriction enzyme fragments of the phage genome, the 27 r protein genes, the gene encoding the alpha subunit of RNA polymerase, the fus gene and the tufA gene were mapped (Jaskunas et al., 1977; Lindahl et al., 1977). The gene cluster was found to be organized into 4 transcription units: p_{str} (S12, S7), EF-G, EF-Tu; p_{S10} S10, L3,

(L4, L2, L23), (L22, S19), S3, (S17, L16, L29); p_{spc} L14, L24, L5, S14, S8, L6, L18, S5, (L30, L15); p_{α} S13, S11, S4, alpha, L17; where groups of genes in parenthesis indicate that their order has not been determined (Lindahl *et al.*, 1977).

Similarly, the specialized lambda transducing phage lambda rif^d18, originally isolated by Kirschbaum and Konrad (1973), was shown to contain E. coli chromosomal DNA sequences from the rif region at 88 minutes on the E. coli chromosomal map (Nomura, 1976). *In vivo* and *in vitro* analysis of the DNA of lambda rif^d18 indicated that the phage included a cluster of 4 r protein genes together with the genes for the β and β' subunits of RNA polymerase (Kirschbaum and Konrad, 1973; Nomura, 1976). In addition, Jaskunas *et al.* (1975b) discovered that *in vitro* protein synthesis using lambda rif^d18 DNA as a template generated an identical protein to the reference EF-Tu on sodium dodecyl sulphate-polyacrylamide gels. Moreover, the eluted protein produced by lambda rif^d18 was found to react with antisera specific against EF-Tu. From these results, the authors concluded that a second EF-Tu structural gene, designated tufB, is present at 88 minutes on the E. coli chromosome.

Furano (1978) utilized radioactive tuf mRNA to detect the tuf gene in E. coli chromosomal DNA fragments digested with various endonucleases. By this approach, the author was able to directly

detect two copies of the tuf gene on the chromosome of both E. coli K-12 and E. coli B, thus confirming duplication of the gene encoding EF-Tu in E. coli.

Two-dimensional chromatographic analysis of tryptic digests of the tufB gene product revealed 50 radioactive spots, while an additional spot was observed following tryptic digestion of the tufA gene product (Furano, 1977). Analysis of the amino acid sequence of EF-Tu has shown that except for a single difference in the carboxyl-terminal peptide (glycine or serine occurred at a ratio of 0.7 to 3), the tufA and tufB gene products are essentially identical (Arai et al., 1980).

No significant difference in the activities of the tufA and tufB gene products during peptide chain elongation was observed (Miller et al., 1978). Both elongation factors were able to bind Ef-Ts, GDP and phe-tRNA with similar affinities. Although the tufA gene product was able to bind ribosomes more tightly, both of the elongation factors promoted the binding of phe-tRNA to ribosomes at equal rates.

The nucleotide sequence of the tufA gene (Yokota et al., 1980) and the tufB gene (An and Friesen, 1980) was determined in order to elucidate the significance of two genes coding for EF-Tu in E. coli. The nucleotide sequence of both genes agreed with the

amino acid sequence of EF-Tu obtained by Arai *et al.* (1980). The sole ambiguity was found to occur at the carboxyl-terminus of the EF-Tu structural genes. Yokota *et al.* (1980) reported a glycine codon at this position in tufA, while a serine codon was found to occur in tufB (An and Friesen, 1980). As Arai *et al.* (1980) reported the predominance of glycine over serine at the carboxyl-terminus of EF-Tu isolated from E. coli, the results of the nucleotide sequence studies indicate that the tufA gene product predominates over EF-Tu produced by the tufB gene.

Although EF-Tu produced by the tufA and tufB genes is essentially identical both structurally and functionally, 9 nucleotide differences in the first 300 base pairs, 3 differences in the last 16 base pairs and 1 difference in the 850 base pairs of the central portion of the structural genes were identified (Yokata *et al.*, 1980; An and Friesen, 1980). Except for the initiation codon (GTG for tufA and ATG for tufB) and the last codon which had nucleotide differences at the first portion of the codon, all the other nucleotide differences occurred in silent third codon positions. The nucleotide sequences on the 5' side of the initiation codon of the two genes were found to differ completely, while the nucleotide sequences on the 3' side of the EF-Tu genes, although similar, differ by small deletions and insertions.

The fact that the gene products of tufA and tufB are essentially identical both in structure and function suggests that there is strong selective pressure to maintain the structure of EF-Tu within narrowly-defined limits due to the complex nature of EF-Tu functions during polypeptide chain elongation (An and Friesen, 1980). The distribution of nucleotide differences between tufA and tufB indicates that recombination may have occurred between these two genes. If so, then it is possible that only the amino- and carboxy-terminal regions of EF-Tu must be preserved (An and Friesen, 1980).

Nucleotide sequences of r protein genes carried by lambda rif^{d18} (Post et al., 1979) and those included in the genome of lambda fus3 (Post and Nomura, 1980) have indicated a highly non-random use of codons for several amino acids. The codons preferentially utilized are those recognized by the most abundant of the isoaccepting species of tRNA (Post and Nomura, 1980). Efficient synthesis of r proteins is essential in rapidly growing E. coli which has been shown to contain over 1.5×10^5 ribosomes per cell at a cell doubling time of 22 minutes. Since the number of ribosomes also doubles every 22 minutes, the concentration of usable, charged tRNA could limit the rate of protein synthesis. Thus, use of abundant tRNA species could make translation of r proteins more efficient by increasing the usable charged tRNA concentration and minimizing one possible rate-limiting factor

for polypeptide chain elongation (Post and Nomura, 1980). In addition, use of abundant tRNA species could minimize translational errors which occur as a result of more effective competition by incorrect aminoacyl-tRNAs due to limiting concentrations of charged tRNA (Post et al., 1979; Post and Nomura, 1980).

Since the nucleotide sequence of both tufA and tufB indicated a non-random usage of codons which is similar to that in r protein genes, the above argument may apply to the two EF-Tu genes (Yokota et al., 1980; An and Friesen, 1980). EF-Tu is not only related to the r proteins in its biological function, but also in its genetic location and expression. EF-Tu, synthesized at 4 to 5 copies per ribosome, represents approximately 10% of the total cellular protein in rapidly growing cells (Yokota et al., 1980; van der Meide et al., 1983). Whereas EF-Ts, EF-G and the ribosomes are maintained at a constant 1:1 molar ratio during steady-state growth of E. coli (Gordon, 1970; Gordon and Weissbach, 1970), the molar ratio of EF-Tu to ribosomes varies from 8:1 at 2 cell doublings per hour to 14:1 at 0.2 to 0.5 cell doublings per hour (Furano, 1975). Indeed, the observed levels of cellular EF-Tu were found to parallel those of tRNA which is consistent with the role of EF-Tu in protein synthesis as efficient translation would require that all the aminoacyl-tRNA in cells be bound to EF-Tu (Furano, 1975). Thus, it may be

envisaged that two copies of the gene coding for Ef-Tu is necessary to accommodate such high levels of this protein during rapid cell growth (An and Friesen, 1980).

Pedersen *et al.* (1976) reported that although the fus and tufA genes are under the control of a common promoter (Lindahl *et al.*, 1977), 3 to 4 copies of EF-Tu are produced per EF-G molecule. Sequence studies of the intercistronic region between the fus and tufA genes indicated a large region of two-fold symmetry followed by a Shine-Dalgarno sequence 9 to 12 bases upstream from the tufA initiation codon (Yokota *et al.*, 1980). The authors suggested that the hairpin structure of the mRNA preceeding the Shine-Dalgarno sequence may be responsible for the increased translation efficiency of tufA. In addition, the presence of a possible binding site for RNA polymerase within the coding region of the fus gene, together with the ability of the cloned tufA gene to be weakly expressed both *in vivo* and *in vitro* (Shibuya *et al.*, 1979), suggest that the tufA gene may possess a secondary promoter (Yokota *et al.*, 1980).

The lack of sequence homology between the regions on the 5' side of the tufA and tufB genes suggests that there are no common regulatory sequences between these two EF-Tu genes (An and Friesen, 1980). Pedersen *et al.* (1976) found that the tufA gene is three times more active than the tufB gene. ppGpp,

synthesized by the *E. coli* relA gene during amino acid or charged tRNA deprivation, was shown to control the biosynthetic rate of rRNA and r protein (Dennis and Nomura, 1974; Dennis and Nomura, 1975), EF-G and EF-Tu (Furano and Wittel, 1976), tRNA (Blumenthal et al., 1976), and EF-Ts (Reeh et al., 1976). Moreover, Reeh et al. (1976) demonstrated that the tufA and tufB genes differ in their response to the stringent regulatory mechanism. The above observations imply that there is regulatory communication between the two EF-Tu genes. Indeed, van der Meide et al. (1983) reported that elevation of the intracellular concentration of EF-Tu suppresses the expression of the tufB gene, but not expression of the tufA gene. As the EF-Tu population is maintained in ternary complexes with aminoacyl-tRNA and GTP under normal conditions, the authors suggested that free EF-Tu (generated by an excess of EF-Tu) would exert this regulatory action on tufB in order to restore the 1:1 molar ratio between EF-Tu and tRNA. Thus, it seems that there is regulatory communication between the tufA and tufB genes via EF-Tu which allows the balance between the levels of EF-Tu and tRNA to be maintained.

As naturally occurring, genetically stable duplications of protein-encoding genes are rare in procaryotes, Filer and Furano (1981) examined various species of both Gram-negative and Gram-positive bacteria in order to assess whether duplicate tuf

genes are present in other procaryotes. The nucleotide sequence of the tufA and tufB genes in E. coli, as well as the structure and function of the EF-Tu protein produced by these genes, have indicated that the E. coli tuf genes have undergone negligible evolutionary divergence. Furthermore, Furano (1978) found that the duplicate tuf genes in Salmonella typhimurium are almost identical to the E. coli EF-Tu genes in contrast to the more than 50% sequence divergence observed between the total genomes of these bacteria (Filer and Furano, 1980). Radioactively-labelled DNA fragments bearing either the cloned amino- or carboxyl-terminus of the E. coli tuf gene were utilized in DNA hybridization studies to facilitate the identification of the tuf genes of other micro-organisms. From their results, the authors reported that duplication of the tuf gene is widespread amongst Gram-negative bacteria, while the two Gram-positive genera B. subtilis and Micrococcus luteus possessed only one copy of the tuf gene.

The detection of a single tuf gene in B. subtilis by DNA hybridization (Filer and Furano, 1981) confirmed previous mutational studies in this Gram-positive micro-organism. Dubnau et al. (1976) isolated four temperature-sensitive mutants of B. subtilis by localized mutagenesis in the major ribosomal gene cluster. Determination of the positions of the mutations indicated that the tuf gene was closely associated with the strA

locus and the EF-G gene in *B. subtilis*. A temperature-sensitive mutation (ts-5) affecting the activity of Ef-Tu was found to result in more than 90% loss in EF-Tu activity after heating. If more than one tuf gene is present in *B. subtilis*, only 50% loss of EF-Tu activity should have occurred. The antibiotic kirromycin, which prevents the conformational transitions of EF-Tu necessary for recycling on the ribosomes, was utilized for the isolation of spontaneous kirromycin-resistant mutations in *B. subtilis* which were subsequently mapped very close to ts-5 between the strA and spcA loci on the chromosome (Smith and Paress, 1978). All the EF-Tu from kirromycin-resistant strains exhibited altered migration in isoelectric-focussing gels. indicating that only one tuf gene is expressed in vivo.

Thus, the single tuf gene in *B. subtilis* is genetically linked to the strA and rif loci (Henner and Hoch, 1980). As already mentioned, the tufA gene is linked to the strA locus, while the tufB gene and the rif locus are linked in *E. coli*. However, the strA and rif loci, located at 72 and 88 minutes on the chromosome respectively, are genetically unlinked in this micro-organism (Bachmann and Low, 1980). *Pseudomonas putida* was found to contain duplicate tuf genes which are genetically linked (Filer and Furano, 1981). Moreover, the *Pseudomonas* str and rif loci are also linked on the chromosome (Holloway et al., 1979). The nucleotide alignments of the two types of 5 S RNA secondary

structure which occur in procaryotes have allowed the construction of a phylogenetic tree of the 5 S RNA species (Hori and Osawa, 1979). From these studies the authors found that Pseudomonas species represent an intermediate state between the evolutionary events that separate the Gram-positive genera from the Enterobacteriaceae. Thus, a single translocation event may have caused the transposition of one of the tuf genes and a contiguous section of the chromosome leading to duplication of the tuf gene and separation of the ribosomal gene cluster in Gram-negative bacteria (Filer and Furano, 1981).

In addition, Filer and Furano (1980; 1981) demonstrated that portions of the E. coli tuf gene are highly conserved amongst various taxonomically unrelated procaryotes which are thought to have been phylogenetically separated 1 to 2 billion years ago. The authors found that close to 50% or more of the conserved nucleotide sequences, in the majority of the micro-organisms tested, are homologous to the fragment encoding the carboxyl-terminal half of EF-Tu, while 60 to 70% of the conserved nucleotide sequences of Aerobacter, Pseudomonas, and Caulobacter are homologous to this portion of the E. coli tuf gene (Filer and Furano, 1980). The anaerobic purple sulphur photosynthetic bacterium Chromatium vinosum, contained only sequences homologous to the carboxyl-terminal half of the E. coli tuf gene.

As the probability of a 20 nucleotide homologous sequence arising by chance between two independent genes is 10^{-12} , the possibility of detecting such a sequence in a typical procaryotic genome would be $(10^{-6})^n$ in n different independent procaryotes (Filer et al., 1981). Thus, if two nucleic acid sequences have a degree of similarity greater than that which can occur by chance, then the genes encoding these sequences are homologous.

It is conceivable that different ancestral DNA sequences could converge in time due to selection for a particular structure necessary for performing a specific function. However, extensive taxonomic studies on structural RNAs (mRNA and 5 S RNA) in procaryotes have failed to provide any evidence for convergent evolution affecting these molecules. Moreover, because the genetic code is degenerate, it is most unlikely that convergence based on selection of a given amino acid sequence could generate identical protein-encoding sequences (Filer et al., 1981).

On the basis of the degenerate genetic code, which ensures that approximately 30 to 40% of potential base replacements would not be reflected in the encoded protein, the DNA sequence of a given gene changes faster during evolution than the amino acid sequence of the protein encoded by that gene (Filer et al., 1981). Robertson and Jeppesen (1972) studied the nucleotide sequences of three closely related RNA bacteriophages of E. coli.

Bacteriophages MS2 and R17 have identical coat protein sequences, while the coat protein sequence of f2 differs by one amino acid. However, the authors found that the nucleotide sequences of the gene encoding the protein coat of these phages differed by 4%. The nucleotide sequences of the mRNAs of the sea urchin Strongylocentrotus purpuratus and Lytechinus pictus, which code for the highly conserved histone H4, have been shown to have undergone considerable divergence (Grunstein et al., 1976). The authors reported that 11.5% of the bases had undergone substitution at a rate calculated to be 3×10^{-9} base changes per codon per year.

In addition to accumulating silent nucleotide substitutions, genes also accumulate nucleotide substitutions that lead to amino acid changes. As many of these changes are neutral in terms of evolutionary selection, the homologous genes of even closely related organisms exhibit nucleotide divergence. Thus, although E. coli and S. typhimurium diverged from a common ancestor relatively recently in evolutionary terms, 5 amino acid differences between the trpE polypeptides were identified in the first 33 residues (Lee et al., 1978). Each of these amino acid differences was due to a single base-pair change in either the second or third position of the respective codon with the exception of residue 28 which has a base substitution in the first and third codon positions. In addition, the authors

reported 13 base substitutions which do not result in amino acid differences.

On the basis of the above observations, Filer *et al.* (1981) proposed two hypotheses regarding the widespread occurrence of nucleotide sequences homologous to the *E. coli tuf* gene in taxonomically unrelated micro-organisms. The authors suggested that the *tuf* gene sequence homology occurred as a result of chromosomal transfer between procaryotes after they diverged from a common ancestor. The widespread occurrence of plasmids in procaryotes, some of which are able to mobilize the chromosome of their hosts as well as replicate in taxonomically unrelated genera, and the occurrence of illegitimate recombinational events that allow chromosomal exchange between non-homologous DNA sequences, could promote the spread of chromosomal DNA among various unrelated micro-organisms. Alternatively, portions of the *tuf* gene are highly conserved amongst evolutionary distinct genera due to very strong selective pressure for the conservation of the amino acid sequence of EF-Tu which has a highly specialized role in protein chain elongation (Filer *et al.*, 1981).

In order to evaluate the possibility of intergeneric chromosomal DNA transfer, Filer *et al.* (1981) determined whether sequences homologous to the *E. coli* DNA flanking the *tufA* gene were also

present in unrelated genera. Conservation of the DNA sequences flanking the tufB gene were not investigated as the single tuf gene in B. subtilis is genetically linked to genes which correspond to those linking the tufA gene in E. coli. Thus, if chromosomal transfer involving the tuf gene occurred, it would have involved the portion of the chromosome which includes the tufA gene. In addition, the authors investigated whether the tuf-homologous sequences in one genus were identical to those in an unrelated genus. Three plasmids were radioactively-labelled and utilized in DNA hybridization studies (Filer et al., 1981). pTI contained 600 nucleotides encoding the carboxyl-terminal half of EF-Tu as well as 500 nucleotides of flanking downstream DNA, pGI included 600 base pairs of the fus gene which encode 200 amino acids near the carboxyl-terminus of EF-G, and pT/GI possessed sufficient DNA to encode the first 100 amino acids of the amino-terminus of EF-Tu and approximately 8 amino acids from the carboxyl-end of EF-G. The authors found that the degree of sequence homology to the DNA adjacent to the E. coli tufA gene is either non-existent or much less than in the case of the tuf gene sequence. In addition, the various procaryotes were found to share a common subset of nucleotide sequences homologous to the E. coli tufA gene. These results suggest that strong selective pressure and not recent intergeneric chromosomal transfer is responsible for the presence of tuf-homologous nucleotide sequences. As the genetic code is degenerate, the conservation

of these nucleotide sequences implies that in some instances codon-sequence is selected for (Filer et al., 1981).

Stringfellow et al. (1980) compared the E. coli EF-Tu with that of the dimorphic Gram-negative bacterium Caulobacter crescentus. Both polypeptides had identical molecular weights, were cleaved by trypsin to yield a 39,000 dalton fragment, and had a greater affinity for GDP than GTP. However, only C. crescentus EF-Ts was able to catalyze GDP exchange with heterologous EF-Tu. In addition, EF-Tu and EF-Ts of C. crescentus could substitute for the E. coli elongation factors to generate functional QB replicase. Mikulik et al. (1982) studied the molecular properties of EF-Tu·GDP from the kirromycin-producing strain of Streptomyces collinus. EF-Tu produced by S. aureofaciens, E. coli and S. collinus were found to share common antigenic determinants. In addition, the authors demonstrated that in spite of differences in the molecular weights between EF-Tu·GDP of E. coli (46,000) and S. collinus (52,000), both elongation factors are functionally interchangeable in poly(U) translation on ribosomes of E. coli and S. collinus, respectively. It is interesting to note that EF-Tu of S. collinus was found to be sensitive to the EF-Tu-specific antibiotic kirromycin and is not involved in the mechanism of self-resistance to the antibiotic (Mikulik et al., 1982).

Morikawa et al. (1978) analyzed the E. coli EF-Tu·GDP complex by high resolution X-ray crystallography in order to elucidate the three-dimensional structure of the molecule. The authors reported that the E. coli EF-Tu·GDP complex has a domain structure with weak connections between domains, suggesting flexibility of the molecule. This result is in agreement with the physical chemical evidence which indicates that EF-Tu undergoes conformational changes induced by GTP or GDP (Kaziro, 1978). In addition, the binding site for GDP and GTP is located approximately 20 residues from the carboxy-terminus, while the tRNA binding site is situated at the amino-terminus of EF-Tu (Morikawa et al., 1978). As GTP alters the conformation and reactivity of EF-Tu to allow the binding of aminoacyl-tRNA and ribosomes, the ability of EF-Tu to bind GTP is a fundamental requirement for polypeptide chain elongation and thus may explain the sequence homology between the carboxyl-portion of the tuf genes of diverse procaryotes.

At present, no genetic information concerning the tuf gene of Streptomyces is available. As the carboxyl-terminal end of the E. coli tuf gene is highly conserved among diverse procaryotes, this portion of the tuf gene was utilized to ascertain whether homologous DNA sequences exist in S. cattleya and S. olivaceus.

6.2 METHODS

6.2.1 Isolation of chromosomal DNA

S. cattleya and S. olivaceus chromosomal DNA was prepared by the same protocol. A 1 l flask containing 200 ml of malt-3 broth was inoculated with a loopful of mycelia and incubated at 30°C for 2 days with agitation. The bacterial culture was harvested by centrifugation at 10,000 rpm for 10 min in a GSA rotor at 4°C. The mycelia were resuspended in 5 ml 25% (w/v) sucrose, 10 mM Tris, pH 8.0. Lysozyme (1 mg/ml final concentration) was added to the suspension which was subsequently incubated at 37°C with agitation for 1 h. The mycelial suspension was placed on ice and the following solutions were added: 2.5 ml 0.2 M EDTA; 5 ml 10 mM Tris, pH 8.0; 0.2 M EDTA, 2% (w/v) SDS. After 5 min incubation at 0°C, the denatured protein was removed by 3 cycles of treatment with TE buffer-saturated phenol. The phenol was removed by 2 cycles of ether treatment. Sodium acetate was added to a final concentration of 0.3 M, followed by 3 volumes of cold 96% ethanol. The solution was incubated at -70°C for 30 min and the DNA precipitated by centrifugation at 12,000 rpm in a microfuge for 5 min at 4°C. The supernatant was discarded a second aliquot of cold 96% ethanol was gently added so as not to resuspend the DNA pellet and recentrifuged at 12,000 rpm for 1 min at 4°C. The ethanol was discarded and the DNA pellet was

subsequently dried in a Savant speed vac concentrator for 15 min. Finally, each chromosomal DNA pellet was resuspended in 50 μ l TE buffer to yield a final concentration of 4.7 mg S. olivaceus DNA and 11.7 mg S. cattleya DNA per ml.

Chromosomal DNA of B. subtilis SB19WT (obtained from the U.C.T. Microbiology Department Stock Collection) was isolated in the following manner. Luria broth (100 ml) was inoculated with B. subtilis cells and incubated on an orbital shaker at 37°C for 12 h. The bacteria were harvested by centrifugation at 10,000 rpm for 10 min at 4°C in a GSA rotor. The cells were resuspended in 5 ml 25% (w/v) sucrose, 10 mM Tris, pH 8.0. Lysozyme (40 mg) was added to the suspension which was subsequently incubated at 37°C with agitation for 15 min. Proteinase K (40 mg) was added, followed by 5 ml 2% (w/v) SDS, 0.1 M EDTA and the suspension was reincubated at 37°C for 1 h. The suspension was subsequently treated with TE buffer-saturated phenol, ether and ethanol as for Streptomyces chromosomal DNA. Each DNA pellet was resuspended in 20 μ l TE buffer to yield a final concentration of 6.7 mg/ml.

6.2.2 Restriction endonuclease digestion

Streptomyces chromosomal DNA samples (10 μ g) were digested separately with 50 u of BamHI, SalI and HindIII restriction enzymes at 37°C for 6 h. Similarly, 10 μ g B. subtilis

chromosomal DNA was digested with 50 u EcoRI and lambda DNA (1 ug) was restricted with 10 u HindIII restriction endonuclease.

6.2.3. Electrophoresis

DNA restriction fragments were separated in a 1.2% (w/v) agarose gel prepared in Tris-acetate buffer containing 0.5 ug/ml ethidium bromide. Electrophoresis was performed in a horizontal direction at 35 V (constant voltage) for 14 h with recirculation of the buffer. The DNA was visualized with UV light at 254 nm and photographed with a Polaroid Land camera.

6.2.4 Nick-translation

The 5'- and 3'-termini of the E. coli tufA gene, which code for the amino- and carboxy-termini of EF-Tu respectively, were supplied by Dr. D. Filer. The portions of the E. coli tufA gene had been isolated from the transducing phage lambda fus3 and cloned into either pMB9 or pBR322 to generate the recombinant plasmids pTI and pT/GI respectively (Filer et al., 1981). pTI consists of a SmaI DNA fragment containing 600 nucleotides of the 3'-terminus of the tufA gene, while pT/GI contains a SmaI-EcoRI DNA fragment bearing 300 nucleotides of the 5'-end of the E. coli tufA gene. pTI and pT/GI were labelled with [α -³²P] dCTP by nick-translation using a kit obtained from Amersham. One ug (10

ul) of DNA and 5 ul of [α - ^{32} P] dCTP (10 mCi/ml) were used in the nick-translation reaction which was performed according to the suppliers' instructions (section 5.2.2.6.3). The ^{32} P-labelled DNA was subsequently separated from unincorporated nucleotides by the spun column technique described in Maniatis (1982a) (section 5.2.2.6.3).

6.2.5 DNA hybridization

The technique developed by Smith and Summers (1980) was utilized for the transfer of the chromosomal DNA from agarose gel to gene screen (New England Nuclear) (section 5.2.2.6.3). DNA hybridization was performed according to the method described in Maniatis (1982b) (section 5.2.2.6.3). The hybridization reaction, which included either the ^{32} P-labelled, denatured carboxyl- or amino-terminus of the E. coli tufA gene (approximately 7×10^7 cpm) and lambda DNA (1×10^5 cpm), was allowed to proceed at $T_m = -43^\circ\text{C}$ for 18 h at 60°C with agitation. The gene screen was subsequently subjected to three 30 min washes in 2 x SSC, 0.1% SDS at 60°C ($T_m = -35^\circ\text{C}$) with agitation in order to remove non-specifically bound radioactivity and subsequently placed under Kodak XAR X-ray film between intensifying screens for 3 days at -70°C . The size of each DNA restriction fragment which hybridized to the radioactively-labelled E. coli tufA gene was extrapolated from a lambda DNA-HindIII standard.

6.3 RESULTS AND DISCUSSION

Streptomyces DNA sequences homologous to the 3'-terminus of the E. coli tufA gene were identified. S. cattleya DNA digested with HindIII and S. olivaceus DNA digested with BamHI and HindIII endonucleases exhibited single hybridization bands which may indicate the presence of a single copy of a tuf gene in these streptomycetes (Figure 1). Although these DNA restriction fragments are able to accommodate several tuf genes (the E. coli tufA gene is 1.2 kb in length), this suggestion is supported by the occurrence of a 527 base-pair fragment generated by SalI digestion of S. olivaceus chromosomal DNA which hybridizes to the 3'-end of the E. coli tufA gene. The two additional SalI digestion fragments of S. olivaceus DNA (3.9 and 3.3 kb) exhibited a low hybridization intensity indicating that they harbour the small remaining portions of the Streptomyces tuf gene. If two unlinked copies of the tuf gene occur in Streptomyces, then one would expect 2 or more DNA fragments exhibiting a high degree of hybridization, while two linked genes encoding EF-Tu in Streptomyces should have generated at least 3 or more hybridizing DNA fragments. The presence of a single tuf gene in Streptomyces is in agreement with Filer and Furano (1981) who noted that duplication of the tuf gene is widespread amongst Gram-negative bacteria, while two Gram-positive organisms examined possess only one copy of the gene. Although sequence

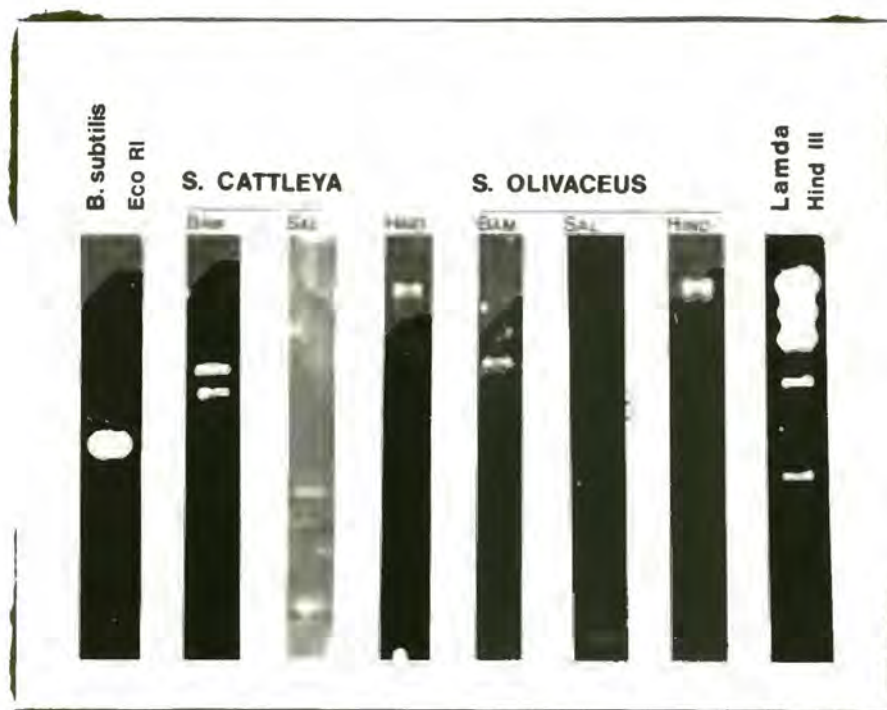


FIGURE 1. Hybridization of ^{32}P -labelled pTI to restricted *B. subtilis*, *S. cattleya* and *S. olivaceus* chromosomal DNA. The two arrows indicate the position of two faint hybridization bands when *S. olivaceus* DNA is digested with *Sal*I restriction enzyme.

homology between the 5'-terminus of the E. coli tufA gene and B. subtilis DNA has been observed (Filer and Furano, 1980), hybridization between the portion of the gene encoding the amino-terminus of E. coli EF-Tu and Streptomyces chromosomal DNA was not evident using identical hybridization conditions (results not shown).

Thus, the S. cattleya tuf gene includes a single BamHI and SalI restriction site, while the S. olivaceus tuf gene possesses two SalI sites (Table 1). Although the restriction sites of the tuf gene region in S. cattleya and S. olivaceus exhibit differences which imply a variation in DNA sequence, the tuf gene of these Streptomyces species bears strong sequence homology to the carboxyl-terminal half of the E. coli tufA gene. Filer and Furano (1980; 1981) have demonstrated that the tuf genes of a number of diverse genera of procaryotes possess homologous sequences to the E. coli tuf gene, especially with respect to the carboxyl-terminal portion of the gene. The capacity of S. collinus EF-Tu to mediate translation of poly(U) on E. coli ribosomes (Mikulik et al., 1982), as well as the observation that the EF-Tu:EF-Ts complex from B. subtilis and C. crescentus can substitute for the E. coli EF-Tu:EF-Ts complex to form an active replicase for the phage Q β (Filer and Furano, 1981; Stringfellow et al., 1980), demonstrates that the EF-Tu produced by the tuf genes of taxonomically unrelated micro-organisms perform

TABLE 1. RESTRICTION ENZYME RECOGNITION SEQUENCES WITHIN THE STREPTOMYCES TUF GENE

Restriction endonuclease	Recognition sequence	<u>Number of restriction sites</u>		
		<i>S. cattleya</i>	<i>S. olivaceus</i>	<i>E. coli</i> ^a
Bam HI	G GATCC	1	0	0
Sal I	A AGCTT	1	2	0
Hind III	G TCGAC	0	0	0

a: Restriction sites of the *E. coli* *tuf A* gene was obtained from the nucleotide sequence determined by Yokota *et al.* (1980).

identical functions to those of the E. coli EF-Tu. The absence of homology between the DNA sequences flanking the tufA gene of E. coli and other micro-organisms indicates a strong selective pressure for the conservation of part of the EF-Tu molecule produced by a variety of procaryotes (Filer et al., 1981). This is supported by the X-ray crystallographic studies of Morikawa et al. (1978) who demonstrated that the carboxyl-terminal end of EF-Tu binds GTP and GDP. As GTP alters the conformation of the EF-Tu molecule to allow the binding of aminoacyl-tRNA and ribosomes during peptide chain elongation (Kaziro, 1978), conservation of this portion of the elongation factor is essential.

The detection of the tuf gene in S. cattleya and S. olivaceus has several potential applications for the study of the molecular genetics of these important antibiotic-producing Streptomyces species. The tuf gene of B. subtilis, like that of Pseudomonas and E. coli, is closely associated with the ribosomal protein gene cluster (Smith and Paress, 1978; Filer and Furano, 1980). As the rif-str-spc loci are linked on the chromosomal map of S. coelicolor A3(2) (Hopwood et al., 1973), it seems that the Streptomyces ribosomal protein genes are located in a single cluster similar to that in B. subtilis (Dubnau et al., 1976; Smith and Paress, 1978). Thus, it is highly likely that the isolation of the tuf gene region of Streptomyces chromosomal DNA

would facilitate the isolation of the ribosomal protein genes which could then be studied by similar techniques to those utilized for the analysis of these important genes in E. coli. The isolation of the Streptomyces tuf gene would allow the study of the first promoter of a non-antibiotic structural gene of Streptomyces. Moreover, as the E. coli tuf genes are highly expressed (EF-Tu comprises between 5 and 10% of the total cellular protein content in rapidly growing cells; Yokota et al., 1980; van der Meide et al., 1983), utilization of the promoter of the Streptomyces tuf gene in an expression vector would be advantageous for the elucidation of gene function in these highly complex procaryotes.

CHAPTER 7

DISCUSSION

The aim of this study was to establish a base for the application of recombinant DNA technology to S. cattleya and S. olivaceus which would ultimately lead to an understanding of the genetic and biochemical mechanisms of antibiotic production in these beta-lactam antibiotic-producing streptomycetes. Thus, four areas of investigation were approached.

The first involved the isolation of an actinophage which was infectious to both S. cattleya and S. olivaceus. The availability of such a phage, besides its potential use as a DNA cloning vector for these Streptomyces species, would allow the study of UV repair systems, the identification of nonsense suppressors and the investigation of the genetics and biochemistry of differentiation (Dowding, 1973).

Following the screening of soil samples for an actinophage which exhibited a high e.o.p. on S. cattleya and S. olivaceus, the virulent phage VC11 was isolated. VC11 fits the B1 group of Bradley's morphological phage classification system, consisting of an icosahedral head (47 x 47 nm) and a long non-contractile

tail (196 x 7.8 nm). The phage infects a taxonomically-broad range of Streptomyces strains. In addition, although Flett et al. (1979) identified a restriction-modification system in both S. cattleya and S. olivaceus, these systems do not affect VC11 infection of these hosts.

As is the case with other actinophages, the genome of VC11 consists of a double-stranded DNA molecule which is 40.1 kb in length, while the 50.4% G+C content of the VC11 genome is at the lower limit for G+C contents of Streptomyces chromosomes, plasmids and actinophage DNAs. The presence of a cos site indicates that the genome of VC11 is replicated by the rolling circle model (Lomovskaya et al., 1980). Restriction enzyme analysis of the phage genome revealed the presence of numerous recognition sequences for 16 endonucleases, while restriction sites for a further 21 restriction enzymes were absent. Moreover, VC11 DNA includes 5 BglII restriction fragments which are 11.1, 7.6, 2.6, 23.2 and 0.65 kb in length.

The availability of a transformation/transfection system for S. cattleya and S. olivaceus is a fundamental requirement for the application of recombinant DNA technology to these streptomycetes. The development of a technique which allows the introduction of DNA into Streptomyces protoplasts has overcome the lack of naturally occurring competence in these

micro-organisms. As successful transformation/transfection of Streptomyces protoplasts depends on efficient protoplast regeneration to form viable colonies, the formation and subsequent regeneration of S. cattleya and S. olivaceus protoplasts was investigated. Both S. cattleya and S. olivaceus were sensitive to lysozyme hydrolysis yielding protoplasts after 90 min. The regeneration frequency of S. olivaceus protoplasts was 26.7%. However, the S. cattleya protoplast regeneration frequency of 0.5% is extremely low and reflects the slow rate of colony-formation on R2 agar (6 days). Although it has been demonstrated that the efficiency of protoplast regeneration is affected by the temperature at which mycelia were propagated prior to protoplasting and at which protoplast regeneration was performed (Baltz and Matsushima, 1981), and the physiological state of the mycelia prior to protoplast formation (Okanishi et al., 1974; Baltz, 1978), attempts to increase the regeneration frequency of S. cattleya protoplasts by either propagating the mycelia and regenerating the protoplasts at 28°C or preparing S. cattleya protoplasts from young mycelia (18 h old) were unsuccessful.

As the actinophage VC11 was to be utilized as a cloning vector for S. cattleya and S. olivaceus, VC11 DNA transfection of protoplasts of these streptomycetes was investigated. VC11 DNA transfected S. olivaceus protoplasts at a frequency of 0.24 x

10^{-6} per phage DNA molecule and 1.3×10^{-5} per viable protoplast. Although these values are similar to the transfection frequencies obtained with other phage-host systems in which only a sub-population of the viable protoplasts is competent, it is not known whether increased concentrations of VC11 DNA saturate viable S. olivaceus protoplasts. Thus, investigation of the effect of increased VC11 DNA concentrations on the transfection frequency of S. olivaceus protoplasts would indicate whether a similar phenomenon occurs in S. olivaceus. An attempt to transfect 2.4×10^6 viable S. cattleya protoplasts with 1.1×10^{11} VC11 DNA molecules was unsuccessful. As VC11 DNA was successfully introduced into S. olivaceus protoplasts, the failure to transfect S. cattleya protoplasts may possibly be explained if one assumes that only a small sub-population of the low number of viable S. cattleya protoplasts are competent. However, it is more likely that protoplasting has a detrimental affect on the viability of S. cattleya as indicated by the slow rate of protoplast regeneration, the small colony size and the low regeneration frequency. The inability to transfect S. cattleya protoplasts with VC11 DNA was overcome by the regeneration of S. olivaceus transfectants in the presence of S. cattleya spores. This procedure, which yielded 55 'transfectants' per microgram of VC11 DNA, will allow in vitro manipulation of the phage DNA prior to its introduction into S. cattleya following transfection of S. olivaceus. In addition,

this method should prove useful for the introduction of broad host range phage cloning vectors into recipient strains in which protoplast transfection systems have not been established.

The third area of investigation involved the development of a phage vector for S. cattleya and S. olivaceus. However, selection of sodium pyrophosphate resistant deletion mutants of VC11 for possible use as DNA cloning vectors is not feasible due to the virulent nature of this actinophage. Moreover, the distribution of restriction sites in the VC11 genome is not conducive to the insertion of exogenous DNA. Thus, an attempt was made to isolate the replicon of the VC11 genome which could be utilized, in conjunction with an antibiotic-resistance marker, as a DNA cloning vector. As the location of the VC11 ori region on the phage genome is unknown, two cloning strategies were employed which would theoretically allow direct selection of the VC11 replicon. However, both attempts were unsuccessful. The first strategem involved the B. subtilis plasmid pHV60 which relies on the insertion of a functional replicon in order to express chloramphenicol resistance in this micro-organism. The failure to detect chloramphenicol-resistant B. subtilis transformants may be due to the inability of the phage replicon to function in this bacterial group. Although infection of B. subtilis with intact phage was not attempted, it is unlikely that B. subtilis possesses surface receptors recognized by phage VC11.

Thus, the Streptomyces plasmid pLJ702, lacking a functional replicon, was utilized for the isolation of the VC11 ori region by direct selection of thiostrepton-resistant S. olivaceus transformants. As this approach was also unsuccessful, it is likely that either the VC11 ori region is cleaved by digestion with the restriction endonuclease BglII or additional genes involved in the regulation of VC11 DNA replication are not borne on the same BglII DNA fragment as the phage replicon. Elucidation of the organization of the VC11 ori region may be possible by the removal of portions of the phage DNA using partial restriction enzyme digestion and subsequently assaying the deleted genome for its ability to replicate following transfection of S. olivaceus protoplasts. However, this approach still requires some form of phenotypic expression, such as plaque-formation or antibiotic-resistance, in order to detect DNA replication. Moreover, the distribution of restriction sites in the VC11 genome would introduce additional complications. Thus, due to the complex nature of such an investigation, no further attempts at isolating the VC11 replicon for use as a DNA cloning vector were made. In addition, the application of recombinant DNA technology to S. olivaceus is now possible due to the ability of the high copy number plasmid pLJ702 to transform and express thiostrepton-resistance and tyrosinase production in this streptomycete.

As UV light induces genetic instability in S. cattleya with respect to antibiotic production, aerial mycelium formation, arginine biosynthesis and growth rate, the actinophage VC11 was utilized in the investigation of DNA repair systems in this streptomycete. The caffeine-mediated decrease in survival of UV-damaged VC11 propagated on untreated and UV-irradiated S. cattleya spores indicated the presence of a constitutive and an inducible repair system(s). As mentioned in section 3.4, a more detailed investigation into the nature of the damage caused by UV light, the genetic and biochemical mechanisms of S. cattleya DNA repair and the mode of caffeine inhibition of DNA repair is necessary in order to make an accurate assessment as to the relationship between DNA repair and genetic instability in Streptomyces. The elucidation of such a relationship would provide valuable information which would allow a better understanding of the mechanisms of secondary metabolite regulation. Already, it should be possible to utilize the actinophage VC11 for the identification of mutants which possess altered DNA repair systems and consequently, increased genetic stability. The availability of such mutants would be invaluable for the production of industrially-important antibiotics.

To elucidate the transcriptional control of Streptomyces genes, it is necessary to investigate the nature of Streptomyces promoters, regulatory sequences, RNA polymerases and termination

signals. Similarly, the study of ribosomal protein gene expression and regulation, and the nature of the mRNA nucleotide sequences involved in ribosome binding, would allow the mechanisms of translation in Streptomyces to be understood. The identification of the S. cattleya and S. olivaceus tuf genes has provided the opportunity for such investigations. As the product of the Streptomyces and E. coli tuf genes are functionally identical (Mikulik et al., 1982), a comparison of the nucleotide sequences of the E. coli tuf genes to that of the Streptomyces tuf gene may provide additional information with respect to the organizational and regulatory features of Streptomyces genes. Likewise, isolation of the Streptomyces ribosomal protein genes is now possible as the tuf genes of E. coli, Pseudomonas and B. subtilis have been shown to be closely associated with the ribosomal protein gene clusters in these organisms (Smith and Paress, 1978; Filer and Furano; 1980). By comparing the Streptomyces transcription-translation systems to those in E. coli and B. subtilis, it may be possible to overcome the barriers which prevent expression of Streptomyces genes in E. coli and so allow the study of Streptomyces gene function in this genetically well-characterized micro-organism.

APPENDIX

General media and buffers

Denhardt's solution (50x)

Ficoll	5 g
polyvinylpyrrolidone	5 g
distilled water	500 ml

Filter through 0.22 μ m millipore filter

Store at -20°C

Gel loading buffer

bromophenol blue	0.25% (w/v)
sucrose	40% (w/v)
distilled water	100 ml

Growth medium 1 (GM1)

Spizizen salts	20 ml
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Autoclave

Add sterile solutions:

20% (w/v) glucose	2.5 ml
1% (w/v) hydrolysed casein	5 ml
1% (w/v) yeast extract	6 ml
0.1% (w/v) L-tryptophan	5 ml
0.1% (w/v) L-threonine	5 ml
100 mM MgSO_4	1.5 ml
sterile distilled water to	100 ml

Growth medium 2 (GM2)

Spizizen salts	100 ml
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Autoclave

Add sterile solutions:

20% (w/v) glucose	12.5 ml
1% (w/v) hydrolysed casein	5 ml
1% (w/v) yeast extract	12.5 ml
100 mM MgSO_4	25 ml
1 mM $\text{Ca}(\text{NO}_3)_2$	1.25 ml
thymine	6.25 mg
sterile distilled water to	500 ml

Hybridization solution

20x SSC	4.5 ml
0.1 M EDTA	1.5 ml
50x Denhardt's solution	1.5 ml
5% SDS	1.5 ml
1 mg/ml denatured Salmon sperm DNA	1.5 ml

distilled water 4.3 ml

Ligation buffer (10x stock)

	(g/l)
Tris (0.5 M)	60.6
MgCl ₂ .6H ₂ O (0.1 M)	20.3
dithiothreitol (0.1 M)	15.4
spermidine (10 mM)	2.5
ATP (10 mM)	5.5

Luria agar

Luria broth containing 15 g Bacto nutrient agar

Luria broth

Bacto tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g
distilled water to	1 l

Malt-3 agar

Malt-3 broth containing 15 g Bacto nutrient agar

Malt-3 broth

Difco malt extract	20 g
Bacto yeast extract	5 g
distilled water to	1 l

Nutrient agar

Nutrient broth containing 15 g Bacto nutrient agar

Nutrient agar (soft)

Nutrient broth containing 5 g Bacto nutrient agar

Nutrient broth

Bacto nutrient broth	8 g
distilled water	1 l

P medium

sucrose	103 g
MgCl ₂ .6H ₂ O	2 g
K ₂ SO ₄	0.25 g
distilled water	790 ml

Autoclave

Add sterile solutions:

3.68% (w/v) CaCl ₂ .2H ₂ O	100 ml
0.25 M TES buffer (pH 7.2)	100 ml

0.5% (w/v) KH_2PO_4	10 ml
Trace elements solution	2 ml

Prehybridization solution

20x SSC	18 ml
5% (w/v) SDS	6 ml
50% (w/v) Denhardt's	6 ml
1 mg/ml denatured Salmon sperm DNA	6 ml
distilled water	24 ml

R2 agar

sucrose	103 g
glucose	10 g
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	10 .1 g
K_2SO_4	0.25 g
Bacto yeast extract	5 g
Casamino acids	0.1 g
NaNO_3	0.3 g
sodium glutamate	3 g
Adjust pH with KOH	7.6
distilled water	803 ml
Bacto nutrient agar *	20 g

Autoclave

* Soft R2 agar 6.5 g

Add sterile solutions:

0.25 M TES buffer (pH 7.2)	100 ml
3.68% (w/v) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	80.2 ml
0.5% (w/v) KH_2PO_4	10 ml
1 N NaOH	5 ml
Trace elements solution	2 ml

Restriction endonuclease digestion buffers

Low salt buffer* (10x)

	(g/l)
Tris (100 mM)	12.1
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (100 mM)	20.3
dithiothreitol (10 mM)	1.5
Adjust pH with HCl to	7.5

Medium salt buffer* (10x)

	(g/l)
Tris (100 mM)	12.1
NaCl (500 mM)	29.2
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (100mM)	20.3
dithiothreitol (10 mM)	1.5
Adjust pH with HCl to	7.5

High salt buffer* (10x)	
	(g/l)
Tris (100 mM)	12.1
NaCl (1000 mM)	58.4
MgCl ₂ .6H ₂ O (100 mM)	20.3
dithiothreitol (10 mM)	1.5
Adjust pH with HCl to	7.5

* Restriction endonuclease buffers were supplemented with 50 mM spermidine (12.5 g/l) when restriction digests of actinophage VC11 DNA were performed

Schrempf medium

sucrose	103 g
Casamino acids	24 g
MgCl ₂ .6H ₂ O	0.4 g
glucose	5 g
glycine	10 g
distilled water	800 ml
Adjust pH with 2 N NaOH to	7.4
Adjust volume to	1 l

SM buffer

	(g/l)
Tris (20 mM)	2.4
MgSO ₄ .7H ₂ O (1 mM)	0.25
NaCl (100 mM)	5.8
gelatin (0.1%, w/v)	1
Adjust pH with HCl to	7.5

Spizizens minimal salts (SMS)

(NH ₄) ₂ SO ₄	10 g
K ₂ HPO ₄	70 g
KH ₂ PO ₄	30 g
tri-sodium citrate	5 g
MgSO ₄ .7H ₂ O	1 g
distilled water to	1 l

Dissolve salts in order.

Dissolve MgSO₄.7H₂O seperately and

subsequently add to salts solution whilst stirring.

Store over chloroform.

SSC (20x)

NaCl	175.3 g
tri-sodium citrate	88.2 g
distilled water	800 ml
Adjust pH with 10 N NaOH to	7.4
Adjust volume to	1 l

Trace element solution

ZnCl ₂	40 mg
FeCl ₃ ·6H ₂ O	200 mg
CuCl ₂ ·2H ₂ O	10 mg
MnCl ₂ ·4H ₂ O	10 mg
NaB ₄ O ₇ ·10H ₂ O	10 mg
(NH ₄) ₆ MO ₇ O ₂₄ ·4H ₂ O	10 mg
distilled water	1 l

Transformation medium (B. subtilis)

Spizizen salts	20 ml
10 ⁻⁴ M MnSO ₄	0.1 ml
distilled water	68.9 ml

Autoclave

Add sterile solutions:

0.5% (w/v) glucose	2.5 ml
20 mM MgCl ₂ ·6H ₂ O	2 ml
1 mM EGTA	1 ml
2% (w/v) casein hydrolysate	1 ml
5 mg/ml L-threonine	1 ml
5 mg/ml L-tryptophan	2.5 ml

Transformation medium (Streptomyces)

sucrose	2.5 g
CaCl ₂ ·2H ₂ O	1.47 g
K ₂ SO ₄	0.044 g
Tris-maleic acid (0.05 M; pH 8.0)	*
Trace element solution	0.2 ml
distilled water	80 ml
PEG 1000 (25%, w/v)	+
Adjust volume to	100 ml

* Tris-maleic acid (0.05 M; pH 8.0)

0.2 M Tris-maleate acid (solution A)

Tris	24.2 g
maleic acid	23.2 g
distilled water to	1 l

1.0 N NaOH (solution B)

Add to transformation medium:

solution A	25 ml
solution B	6.9 ml

+ PEG 1000 (25%, w/v)

Heat 25 g PEG 1000 until molten

Add to transformation medium

Tris-acetate buffer (50x)

Tris (40 mM)	242 g
glacial acetic acid (20 mM)	57.1 ml
EDTA (2 mM)	37.2 g
distilled water to	1 l

Tris-borate buffer (10x)

Tris (89 mM)	108 g
boric acid (89 mM)	55 g
EDTA (2.5 mM)	8.4 g
distilled water to	1 l

Tris-EDTA buffer

Tris (10 mM)	1.21 g
EDTA (1 mM)	0.34 g
distilled water to	1 l
Adjust pH with concentrated HCl to	8.0

Tryptose blood agar base

Bacto tryptose blood agar base	33 g
distilled water to	1 l

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