GENE CLONING STUDIES IN TWO NOCARDIOFORM BACTERIA

by

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CERTIFICATION OF SUPERVISOR

In terms of paragraph 9 of "General Regulations for the Degree of PhD" I, as supervisor of the candidate R T Hill, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.

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ABBREVIATIONS

A	adenine
Ap	ampicillin
ATCC	American Type Culture Collection
bp	base pair(s)
C	cytosine
cfu	colony forming unit(s)
cpm	counts per minute
CsCl	caesium chloride
D	Dalton(s)
d	day(s)
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
EtdBr	ethidium bromide
g	standard gravitational acceleration
G	guanine
GPC	gel permeation chromatography
h	hour(s)
ISP	International *Streptomyces* Project
kb	kilobase(s)
kD	kilodalton(s)
Km	kanamycin
LB	Luria-Bernardi
min	minute(s)
$\underline{M}_r$	relative molecular mass
nmr	nuclear magnetic resonance
ori	origin of replication
p	plasmid
P_r	λ rightward promoter
PAGE	polyacrylamide gel electrophoresis
r	(superscript) resistance
RNA	ribonucleic acid
s	second(s)
s	(superscript) sensitivity
SDS	sodium dodecyl sulphate
SEP	*Streptomyces-E. coli*-like promoter(s)
sp	species
st	streptomycin
T	thymine
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
Ts	thiostrepton

u	units of enzyme activity
v/v	volume/volume
v/w	volume/weight
α	alpha
β	beta
δ	chemical shift (nmr)
γ	gamma
Δ	delta
μ	micro

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ABSTRACT

Nocardioforms are Gram-positive, aerobic actinomycetes and are a metabolically diverse group which produce antibiotics, useful enzymes, are important in the biotransformation of organic compounds and the decomposition of organic wastes and are important medically.

A gene cloning vector designated pLR591 was constructed from the broad host range, multicopy *Streptomyces* plasmid pIJ702 and the positive selection *Escherichia coli* plasmid pEcoR251. This plasmid has useful features for the construction of actinomycete genomic libraries. Cloning of DNA into the unique *Bgl*II endonuclease site of pLR591 inactivated the lethal *Eco*RI gene derived from pEcoR251, thereby selecting for recombinant plasmids containing inserted DNA. The thiostrepton resistance gene derived from pIJ702 was shown to be functional in *Streptomyces lividans* enabling selection of recombinant pLR591 plasmids containing foreign DNA in *S. lividans*. The vector pLR591 therefore functions as a positive selection *Streptomyces*-*E. coli* shuttle vector facilitating construction of actinomycete genomic libraries in *E. coli* and subsequent transfer of recombinant plasmids into *S. lividans*.

The plasmid pLR591 was used to construct genomic libraries of the nocardioforms *Nocardia corallina* JL10 and *Nocardia* sp ATCC 21145. The library of *N. corallina* JL10 contained approximately 9,000 clones and that of *Nocardia* sp ATCC 21145 approximately 25,000 clones and the probability

of any DNA sequence being represented in the libraries was approximately 99.9% in both cases. Both genomic libraries were transformed into *S. lividans* TK24. Construction of these libraries confirmed the value of pLR591 as a useful positive selection *Streptomyces*-*E. coli* shuttle vector.

The plasmids pNIL100, pNIL200 and pNIL400 which coded for the production of pigment in *E. coli* transformants were isolated; pNIL100 was isolated from the genomic library of *N. corallina* JL10 and pNIL200 and pNIL400 were isolated from the genomic library of *Nocardia* sp ATCC 21145. The plasmids pNIL100, pNIL200 and pNIL400 were mapped by restriction endonucleases and the DNA inserts in these plasmids were shown to originate from the appropriate *Nocardia* species by Southern hybridisation studies. The plasmid pNIL200 was shortened to produce a series of derivative plasmids also coding for pigment production in *E. coli*. These pigment-producing clones have great potential for the development of a chromogenic cloning vector analagous to the *lac* system of the pUC and M13 vector series. A DNA insert from pNIL200 was subcloned into pUC18 and pUC19 (in both orientations) and was found to produce greater amounts of pigment in one orientation than the other, indicating that the *Nocardia* DNA insert present in pNIL200 contained a promoter region involved in control of expression of the pigment gene/s which was expressed in *E. coli*. No pigment was detected in *Streptomyces griseus* transformants containing pNIL100, pNIL200 and pNIL400. The pigment produced by *E. coli* transformants containing pNIL200 was extracted, purified by preparative column chromatography and characterised by

visible- and UV-light spectroscopy and by infra-red and nuclear magnetic resonance spectroscopy and appeared to be carotenoid in nature.

The utilisation of the acyclic triterpenoid compound squalene by *Nocardia* sp was studied as nocardioforms are likely candidates for the biotransformation of squalene to produce useful chemical precursors. Three of the ten *Nocardia* sp tested were able to utilise squalene and the degradation products produced by these three strains were investigated by thin layer chromatography. A genomic library of one of these strains, *Nocardia* sp ATCC 21145 was screened in *E. coli* and *S. lividans* for squalene-utilising clones, but none were detected. Induction of proteins by squalene was investigated in *Nocardia* sp ATCC 21145 but no new or increased amounts of cytoplasmic or extracellular proteins were detected.

This work has resulted in the development of a useful positive selection *Streptomyces*-*E. coli* shuttle vector with wide application in actinomycete genomic library construction and which proved valuable in the construction of two nocardioform genomic libraries. These libraries yielded clones which coded for pigment production in *E. coli* and which were the first genes cloned from *Nocardia* sp. In addition, the potential of nocardioforms in biotransformation of squalene was investigated.

Also included in this study was an assessment of a number of bacterial species as potential rubber-degrading

microorganisms. *Nocardia* sp and *Streptomyces* sp, in particular, were considered as these microorganisms have previously been implicated in the biodeterioration of rubber.

CHAPTER ONE

GENERAL INTRODUCTION

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1.1 Classification of the nocardioforms *Nocardia* and *Rhodococcus*

Nocardioforms are a group of Gram-positive, aerobic, non-spore forming actinomycetes that produce elongated or filamentous structures which usually fragment into bacillary and coccoid elements. In addition to the genera *Nocardia* and *Rhodococcus*, the nocardioform actinomycete aggregate taxon includes organisms classified in the genera *Caseobacter*, *Corynebacterium*, *Mycobacterium* and the '*aurantiaca*' taxon (Goodfellow and Cross, 1984).

Nocardiae and rhodococci have had a long and confused taxonomic history. The history of the taxon *Nocardia* has been reviewed by Lechevalier (1976) and that of rhodococci by Bousfield and Goodfellow (1976). The application of modern taxonomic methods and an emphasis on chemical properties rather than morphological ones has recently resulted in both genera becoming more clearly circumscribed. Taxonomic studies on nocardiae and rhodococci that have contributed to this clarity include those of Goodfellow and Alderson (1976), Mordarski *et al.* (1977; 1980) and Goodfellow *et al.* (1982a; 1982b).

The genus *Nocardia* comprises aerobic non-motile Gram-positive, acid-fast to partially acid-fast actinomycetes that produce a primary mycelium which fragments into bacillary and coccoid elements, have cell walls containing *meso*-diaminopimelic acid, arabinose and galactose (wall chemotype IV *sensu* Lechevalier and Lechevalier, 1970) and contain mycolic acids (Goodfellow and Minnikin, 1981). Nocardiae usually produce an aerial mycelium that may be differentiated into arthrospores although this is dependent on the composition of the growth medium (Williams *et al.*, 1976). The guanosine plus cytosine (G + C) content of the DNA ranges from 64 to 72 mol % and the type species is *Nocardia asteroides* (Goodfellow and Cross, 1984).

The genus *Rhodococcus* was resurrected by Goodfellow and Alderson (1977) and accommodates nocardioform actinomycetes previously classified in the '*rhodochrous*' complex or as '*Mycobacterium*' *rhodochrous*, '*Gordona*', '*Jensenia*' or *Corynebacterium*. The genus *Rhodococcus* comprises aerobic, non-motile, Gram-positive actinomycetes that show considerable morphological diversity and can be acid-fast. Cocci or short rods, the first stage of the morphogenetic cycle, germinate into rods, form filaments with side projections, show elementary branching or may produce extensively branched hyphae, and some strains form feeble aerial hyphae. Fragmentation of any of these elements produces cocci or short rods which form the next generation. Colonies are usually pigmented buff, orange or red, although colourless strains do occur. The wall structure is chemotype IV, the G + C content of the DNA ranges from 63 to 72 mol % and the type species is *Rhodococcus rhodochrous* (Goodfellow and Cross, 1984).

1.2 Important aspects of the genera *Nocardia* and *Rhodococcus*

Nocardiae are important medically as agents of nocardiosis, primarily a pulmonary infection which may become systemic, and of actinomycetoma, a chronic granulomatous infection. Nocardiosis is usually caused by *N. asteroides*, although *Nocardia brasiliensis* and *Nocardia otitidis-caviarum* have also been implicated in this disease, whereas nocardial mycetoma is generally caused by *N. brasiliensis* (Beaman, 1976). Nocardiosis is generally an opportunistic infection in patients who are immunocompromised or who have some underlying disease, although Beaman *et al.* (1976) found that *Nocardia* was the primary pathogen in 15 to 40% of cases studied. *Rhodococcus* sp are rarely encountered as primary pathogens but there are several documented cases of serious rhodococcal infections in immunocompromised patients and those with chronic lung disease (Schaal and Beaman, 1984). Medical aspects of the genera *Nocardia* and *Rhodococcus* are discussed in reviews by Beaman (1976) and Schaal and Beaman (1984). Nocardiae and rhodococci have also been implicated in various plant and animal diseases, and Goodfellow and Williams (1983) list examples of these.

Nocardia and *Rhodococcus* sp have, in addition to their medical importance, numerous metabolic features of potential industrial importance. Various *Nocardia* sp have been reported to produce substances which inhibit the growth of microorganisms and may be of value as antibiotics.

Nocardorubin, a compound with a chinoid structure, was isolated from a *Nocardia* sp and showed inhibitory activity against a range of Gram-positive microorganisms (Aiso *et al.*, 1954). *Nocardia interforma* produces a group of antibiotics known as formycins (Hori *et al.*, 1964; Koyama and Umezawa, 1965). A range of nocardicins, some of which are active against Gram-negative bacteria, has been isolated from *Nocardia uniformis* subsp *tsuyamanensis* (Aoki *et al.*, 1976; Hosoda *et al.*, 1977). *Rhodococcus* sp synthesize the antibiotic hygromycin (Wakisuki *et al.*, 1980). '*Nocardia*' *orientalis* ATCC 19795, recently reclassified as *Amycolatopsis orientalis* (Lechevalier *et al.*, 1986) produces the therapeutically important antibiotic vancomycin (Barna and Williams, 1984). '*Nocardia*' *mediterranea* ATCC 13685, the producer of the antibiotic rifamycin, is no longer regarded as a member of the *Nocardia* genus (Goodfellow and Cross, 1984).

In addition to antibiotic production, certain nocardiae and rhodococci are of interest in the production of enzymes, the biotransformation of organic compounds to produce new derivatives, and the metabolism of recalcitrant compounds.

Production of the enzyme L-phenylalanine dehydrogenase by *Rhodococcus* sp M4 and its application in the production of L-phenylalanine from phenylpyruvate has been studied by Hummel *et al.* (1987). Campagna and Buckmann (1987) found that *Rhodococcus* sp M4 was superior to the supposedly high producing *Sporosarcina ureae* in the production of

L-phenylalanine in 50 l fermenters, and this process may be suitable for biotechnological application.

Biotransformation is the preparation of new derivatives from known substrates by the metabolic action of microorganisms. Members of the genera *Nocardia* and *Rhodococcus* are of particular interest in this regard because of their ability to perform highly selective chemical modifications of complex compounds, sometimes resulting in products which are not degraded further and therefore accumulate and can be relatively easily purified. Numerous examples of biotransformations by nocardiae and rhodococci are given in reviews by Raymond and Jamison (1971), Tàrnok (1976) and Peczyńska-Czoch and Mordarski (1984), and there are several recent reports of biotransformations by these organisms. The conversion of cholesterol to substances which are precursors of steroid hormones or oral contraceptives by *Rhodococcus* sp has been described (Ferreira *et al.*, 1984). Setchell *et al.* (1985) isolated a *Rhodococcus* sp which was able to use squalene as a sole carbon source and a single major transformation product was isolated and identified as a novel unsaturated ketone. Production of 1,2-epoxytetradecane from 1-tetradecane by *Nocardia corallina* B-276, previously reported by Furuhashi *et al.* (1981), was optimised by Furuhashi and Takagi (1984) to improve yields of 1,2-epoxytetradecane from 4 g/l to 80 g/l.

The metabolic diversity of nocardiae and rhodococci is also useful in the decomposition of organic wastes of industrial origin, and it is likely that these organisms are important in the degradation of pesticide residues in the natural environment. Pollutants such as oil spills, biocides and carcinogenic hydrocarbons may be subjected to microbial attack by a range of microorganisms including nocardiae and rhodococci, and be converted into less toxic products as a result of biotransformation and biodegradation.

Peczyńska-Czoch and Mordarski (1984) review some examples of the biotransformation by *Nocardia* strains of aliphatic and aromatic hydrocarbons and 'cooxidation' (oxidation of a 'non-growth' substrate in the presence of another substrate which is utilised as a carbon and nitrogen source) of some recalcitrant substrates. More recent studies include those done on the degradation of polychlorinated phenols by *Rhodococcus chlorophenolicus*. Polychlorinated phenols are commercially produced chemicals used by the wood-preserving industry, are extremely toxic, and are frequently found in high concentrations in soil surrounding wood-preserving facilities. Degradation of polychlorinated phenols by *R. chlorophenolicus* has been demonstrated, and shown to occur by mechanisms of dechlorination and *para*-hydroxylation. Eleven chlorophenols were degraded by this organism (Apajalahti and Salkinoja-Salonen, 1986; 1987). *R. chlorophenolicus* was also found to metabolise five different chlorinated guaiacols, which are pollutants produced during the chlorine bleaching of pulp (Häggblom *et al.*, 1986). Another class of industrial pollutants, phthalate esters, which are used as plasticisers, are degraded by *Rhodococcus erythropolis* (Kurane *et al.*, 1979; 1980) and the phthalate ester hydrolysing enzyme which catalyses this degradation has been purified and partially characterised (Kurane *et al.*, 1984).

Reports on the degradation of pesticides by nocardiae and rhodococci include the degradation of dalapon (2,2-dichloropropionic acid) by selected *Nocardia* strains (Hirsch and Alexander, 1960) and the conversion of DDT (1,1,1-trichloro-2,2-di-4-chlorophenylethane) to DDD (1,1-dichloro-2,2-di-4-chlorophenylethane) by *R. erythropolis* (Chacko *et al.*, 1966). More recently, Cook and Hütter (1986) described the dechlorination of deethylsimazine, a product of the aerobic degradation of the s-triazine herbicides simazine and atrazine, by cell-free extracts of *Rhodococcus corallinus*.

Nocardia and *Rhodococcus* strains have been implicated in the degradation of some complex, recalcitrant polymers, and are among the relatively few prokaryotes which degrade lignin and lignin-related compounds (Trojanowski *et al.*, 1977; Rast *et al.*, 1980; Crawford *et al.*, 1981). *Nocardia* sp have been found growing on rubber joints in water and sewage pipes and have been implicated in the deterioration of the rubber polymer (Leeflang, 1963; Hutchinson *et al.*, 1975). Tsuchii *et al.* (1985) isolated a *Nocardia* sp which grew well on unvulcanised natural rubber and synthetic isoprene rubber, and could also utilise various vulcanised natural rubber products as sole carbon sources, and degrade the polymers, resulting in oligomers of lower molecular mass ($\underline{M}r$).

The metabolic versatility of *Nocardia* and *Rhodococcus* sp is likely to result in these genera becoming important in biotechnology. This factor, the medical importance of nocardiae and rhodococci, and the recent clarity in the taxonomy of *Nocardia* and *Rhodococcus* are likely to stimulate further research on these interesting and important microorganisms.

1.3 Genetics of *Nocardia* and *Rhodococcus*

Knowledge of the fundamental genetics of nocardioforms, including nocardiae and rhodococci, lags behind that of the genetically well characterised Eubacteriales as nocardioforms are more difficult to study because of their slow growth, tendency to clump and ability to form coenocytic structures (Brownell and Denniston, 1984). The confused state, until recently, of the taxonomy of *Nocardia* and *Rhodococcus* has possibly also inhibited genetic studies on these genera, although there has been an attempt to elucidate the taxonomy of these genera by studies on *R. erythropolis* at the genetic level (Adams and Brownell, 1976).

Studies on the genetics of *N. asteroides* using a variety of auxotrophic mutants revealed genetic recombination between strains of *N. asteroides* and enabled the construction of a preliminary linkage map for a group of *N. asteroides* strains (Kasweck *et al.*, 1981; Kasweck and Little, 1982). Genetic recombination has also been demonstrated in

'N.' mediterranea and *'Nocardia' restricta* and is reviewed by Brownell and Denniston (1984).

The genetics of the transfer of the characteristic of hydrogen autotrophy (*aut*$^+$ marker) from strains of *Nocardia opaca* able to grow as hydrogen-oxidising bacteria (Aut$^+$ phenotype) to Aut$^-$ strains of *N. opaca* and *R. erythropolis* has been studied by Schlegel and co-workers. Initial indications were that the *aut*$^+$ marker was probably located on a plasmid (Reh and Schlegel, 1981). However, further study showed that plasmid-free Aut$^+$ strains could function as donors of the Aut character, which apparently resides on the chromosome and functions as an independent self-transmissible genetic element (Sensfuss *et al.*, 1986).

The nocardioform *R. erythropolis* has been well characterised genetically. Recombination between *R. erythropolis* strains was first investigated by Adams and Bradley (1963), and approximately 65 genetic traits have been used in the development of a linkage map of *R. erythropolis* (Brownell and Denniston, 1984). Two gene products, coded by the genes *mat*-E and *mat*-C, have been shown to be required for mating and transformation between compatible *Rhodococcus* strains, but the mechanism of recombination and the role of the mating factors are still unknown (Brownell and Denniston, 1984).

Further genetic analysis of *R. erythropolis* is likely to be facilitated by the discovery of a generalised transducing bacteriophage, named Q4, for *R. erythropolis* (Dabbs, 1987).

This bacteriophage mediated the transduction of a number of unlinked markers in *R. erythropolis* and transduction to prototrophy of auxotrophic markers was over 50 times the spontaneous reversion rate. The phage Q4 was also used to order loci in the three-factor cross.

Gene cloning techniques have not yet been applied to the genera *Nocardia* and *Rhodococcus*. Future application of these techniques should be facilitated by work towards the development of a *Rhodococcus* actinophage gene cloning system, by identification of various plasmids coding for resistance to heavy metals and an antibiotic in *Rhodococcus* and *Nocardia* sp, and by procedures to produce protoplasts in these species.

The temperate phage ΦEC was isolated from soil enriched with *R. erythropolis* and was also shown to grow on four other strains of the genus *Rhodococcus*: *R. canicruria*, *R. calcaria*, *R. globerula* and *R. rhodochrous* (Brownell *et al.*, 1967). The genome of this linear, double-stranded DNA phage of approximately 43 kb has been characterised by physical and enzymatic methods (Brownell *et al.*, 1980). The phage ΦEC has been extensively studied with a view to its use as a cloning vector with possible application for gene cloning of *Nocardia*, *Mycobacterium* and *Rhodococcus*, employing *R. erythropolis* as the host. Three potential cloning sites within the ΦEC genome were revealed and deletion mutants were isolated which reduced the ΦEC chromosome from 47 kb to 37 kb (Brownell *et al.*, 1982).

Plasmids which may be useful in the development of cloning vectors for *Nocardia* and *Rhodococcus* species include the set of related plasmids bearing genes conferring increased resistance to sodium arsenate and arsenite, cadmium chloride and chloramphenicol obtained from *R. erythropolis*. These large plasmids were made up of resistance determinants from the host organism together with part of the genome of nocardiophage Q4 (Dabbs and Sole, 1988). Other resistance plasmids are pHG31 and pHG33 of *N. opaca*, which carry the genetic information for thallium resistance (Sensfuss *et al.*, 1986). Plasmids were also detected in three of seven mating strains of *N. asteroides*. These plasmids could not be correlated with antibiotic susceptibility, carbon utilisation, nitrogen source, bacteriocin production or mating type and remained cryptic (Kasweck *et al.*, 1982). A cryptic plasmid was also detected in a strain of *N. corallina*. This 2.7 kb high copy number plasmid, designated pKU100, is the smallest plasmid yet isolated from an actinomycete and contained a number of unique restriction sites, making it suitable for development as a cloning vector (Kirby and Usdin, 1985).

Gene cloning procedures involving transformation and transfection of cells frequently require the production of protoplasts. Several *Rhodococcus* sp have been shown to produce transfectable protoplasts (Brownell, 1981). An efficient procedure for protoplasting of *R. erythropolis* by pretreatment with glycine has been described (Brownell *et al.*, 1982). Protoplasts have also been prepared in

'N'. mediterranea (Schupp and Divers, 1986) and *'N'. orientalis* (Matsushima *et al.*, 1987).

The development of gene cloning systems in the genera *Nocardia* and *Rhodococcus* would be a logical progression in the understanding of the genetics of these nocardioforms and could be valuable in investigations of medically important nocardiae and in the exploitation of the numerous properties of biotechnological potential of *Nocardia* and *Rhodococcus* sp. Such systems could also conceivably be important in the genetic investigation of the closely related nocardioform genus *Mycobacterium*, which contains the important human pathogens *Mycobacterium tuberculosis* and *Mycobacterium leprae*.

1.4 *N. corallina* JL10, *Nocardia* sp ATCC 21145 and the scope of this study

The two nocardioforms from which genes were cloned in this investigation were *N. corallina* JL10 and *Nocardia* sp ATCC 21145. *N. corallina* JL10 was initially of interest as there was some indication that it was capable of growth with rubber as the sole carbon source and therefore could be a rubber-degrading microorganism (see Appendix 1). *N. corallina* JL10 also produced a bright orange pigment and was therefore a suitable candidate for attempts to clone pigment production. *N. corallina* JL10 was isolated from soil by J. Lomberg, Department of Microbiology, University of Cape Town, and was classified as a *N. corallina* sp according to the scheme of Bergey (Breed *et al.*, 1957).

Nocardia sp ATCC 21145 was investigated because initial screening of squalene degradation by nocardioforms indicated that this species was capable of degrading squalene with concomitant production of novel products. *Nocardia* sp ATCC 21145 produced a buff-coloured pigment and was therefore also of interest as a candidate in attempts to clone pigment production. *Nocardia* sp ATCC 21145 was isolated by Raymond (1971) and is the subject of United States Patent No. 3,592,846 for the microbial oxidation of naphthalene or alkyl-substituted naphthalenes to produce hydroxyphenylketobutyric acids, which are useful as plant growth regulators.

The primary aim of this investigation was to attempt a novel approach to the cloning of genes from nocardioforms. This approach involved the construction of an *Escherichia coli-Streptomyces* positive selection shuttle vector pLR591 (described in Chapter 2) into which DNA from *Nocardia* or *Rhodococcus* sp could readily be inserted. One of the advantages of the vector pLR591 is that the expression of cloned nocardioform genes can be screened in both *E. coli* and the actinomycete *Streptomyces lividans* which is far more closely related to nocardioforms than *E. coli* is, and is therefore more likely to express nocardioform genes. The usefulness of the vector pLR591 is obviously not limited to the cloning of genes from *Nocardia* or *Rhodococcus;* the vector could similarly be used for the cloning of genes from *Mycobacterium* and obviously has application in *Streptomyces* gene cloning. The procedure of the construction of genomic libraries of the two nocardioform *N. corallina* JL10 and

Nocardia sp ATCC 21145 in the vector pLR591 is described in Chapter 3. The function of the vector pLR591 in the cloning of genes from nocardioforms was demonstrated by the successful cloning of pigment producing genes from *N. corallina* JL10 and *Nocardia* sp ATCC 21145 (Chapter 4) and by the screening of the gene library of *Nocardia* sp ATCC 21145 for *E. coli* and *Streptomyces* clones containing DNA from *Nocardia* sp ATCC 21145 coding for the degradation of squalene (Chapter 5).

N. corallina JL10 and *Nocardia* sp ATCC 21145 were both classfied as *Nocardia* sp prior to the clarification of the genera *Nocardia* and *Rhodococcus*, discussed in Section 1.1, and it is possible that according to the criteria currently used to classify *Nocardia* and *Rhodococcus*, these two nocardioforms would be classified in the *Rhodococcus* rather than the *Nocardia* genus. Brownell and Denniston (1984) point out that from a genetic standpoint, grouping of bacteria as nocardioforms may be more significant than their genus descriptions since genetic recombination has been shown to occur between genera, and because assignment of organisms to a genus is subject to change as biochemical and genetic information accumulates. The detailed taxonomic study that would be required to reclassify *N. corallina* JL10 and *Nocardia* sp ATCC 21145 according to present criteria was considered unnecessary in terms of the scope of this investigation and the designation *Nocardia* was therefore retained.

Also included in this study was an assessment of a number of bacterial species, including nocardioforms and other actinomycetes, as potential rubber-degrading organisms (Appendix A). *Nocardia* sp and *Streptomyces* sp have previously been implicated in the biodeterioration of rubber (Hutchinson *et al.*, 1975; Orchard and Goodfellow, 1980; Hookey *et al.*, 1980).

CHAPTER TWO

DEVELOPMENT OF pLR591, A *STREPTOMYCES-E. COLI* POSITIVE SELECTION SHUTTLE VECTOR

CHAPTER TWO

DEVELOPMENT OF pLR591, A *STREPTOMYCES-E. COLI* POSITIVE SELECTION SHUTTLE VECTOR

Summary: The *E. coli* positive selection vector pEcoR251 was ligated with the broad host range high copy number *Streptomyces* plasmid pIJ702 to produce plasmid pLR591, a *Streptomyces-E. coli* positive selection shuttle vector. The *Eco*RI gene of plasmid pLR591 was shown to be functional in *E. coli* and the thiostrepton resistance marker of plasmid pLR591 was expressed in *S. lividans*.

2.1 INTRODUCTION

E. coli shuttle vectors are able to replicate in both *E. coli* and unrelated organisms, and code for selectable markers capable of phenotypic expression in both of the hosts. Bi-functional vectors of this type allow for easy manipulation and characterisation of foreign DNA in the well defined *E. coli* system, and facilitate transfer of DNA back into the unrelated foreign donor. The usual approach to shuttle vector development is to employ a suitable cloning strategy to join well characterised plasmids from *E. coli* and the other organism of interest. The resultant chimeric plasmid, to be suitable for use as a shuttle vector, should (1) be able to replicate in both hosts, (2) code for selectable markers enabling identification of transformed cells in both hosts, and (3) contain suitable restriction endonuclease sites for insertion of host DNA

A range of diverse shuttle vectors have been developed. The plasmid pDP1 mediated gene transfer between *E. coli* and the Gram-negative anaerobe *Bacteroides fragilis* (Guiney *et al.*, 1984). The cosmid cloning vector pAD22 transformed *E. coli* and was mobilised into the Gram-negative bacterium *Vibrio parahaemolyticus* (Datta *et al.*, 1984). A potential shuttle vector for Gram-negative anaerobic methane-producing archaebacteria has also been developed (Meile and Reeve, 1985).

A number of shuttle vectors have been made that can be transferred between *E. coli* and the Gram-positive bacterium *Bacillus subtilis*. The plasmid pOG2165 expressed the penicillinase gene from *Bacillus licheniformis* in *E. coli* and *B. subtilis* (Gray and Chang, 1981). Ishiwa and Tsuchida (1984) used the *E. coli* vector pACYC177, and a *Streptococcus faecalis* plasmid pAMα1, to construct plasmid pHY460, an *E. coli-B. subtilis* shuttle vector with twelve unique cloning sites. The strategy of using a plasmid from a Gram-positive bacterium other than *Bacillus* was also employed in the construction of the *E. coli-B. subtilis* shuttle vectors pMK3 and pMK4, which are hybrids between the *E. coli* plasmid pUC9 and the *Staphycococcus aureus* plasmids pU110 and pC194 (Sullivan *et al.*, 1984).

Shuttle vector development in actinomycetes has been limited to the industrially important antibiotic-producing *Streptomyces* species. A streptomycin resistance plasmid, RSF1010, of *E. coli*, was linked to a pTA2070 *Streptomyces griseus* plasmid to produce pTA2068, a putative *Streptomyces-E. coli* shuttle vector (Horinouchi *et al.*, 1980). Kieser *et al.* (1982) produced the hybrid plasmid pIJ361, which consisted of the entire *E. coli* pBR322 plasmid inserted into a derivative of the *S. lividans* plasmid, pIJ102. A *Streptomyces* viomycin resistance gene on pIJ361 was expressed in both *Streptomyces* and *E. coli*.

A range of low copy number shuttle vectors for gene cloning in *E. coli* and *Streptomyces griseofuscus* were made using the replicon of the *Streptomyces* plasmid SCP2* and *E. coli* pBR322 (Larson and Hershberger, 1984). Low copy number may be an advantage in some applications such as in gene cloning to synthesise antibiotics which may be toxic in high concentrations to the producing organisms. A similar low copy number shuttle vector is plasmid pIJ903, which consists of *E. coli* plasmid pBR327 joined to a derivative of the *Streptomyces coelicolor* plasmid SCP2* (Lydiate *et al.*, 1985).

A number of cosmid vectors have also been developed for use in *Streptomyces* gene cloning. Cosmid vectors contain *cos* sites which enable them to be packaged into lambda phage particles which can be transduced into an appropriate

E. coli strain with high efficiency (Collins and Hohn, 1978). In addition to *cos* sites, cosmids used in *Streptomyces* gene cloning contain both *E. coli* and *Streptomyces* replicons, and resistance genes which function in both organisms. Cosmid vectors allow cloning of large fragments of DNA in *E. coli* and packaging constraints enable selection of clones with inserts (Fayerman, 1986). The cosmid pKC462a was used to clone clustered erythromycin biosynthesis genes from *Streptomyces erythreus*, and to obtain expression of these genes in *S. lividans* (Stanzak *et al.*, 1985). A recently developed bifunctional cosmid vector for *Streptomyces* consists of the *Streptomyces* plasmid pIJ702 inserted into the *E. coli* cosmid pBTI10 to produce cosmid pJP3, which accepts DNA inserts of 23 to 33 kb (Portmore *et al.*, 1987).

Although a wide range of *Streptomyces* plasmid vectors has been developed, there have been no reports of the use of positive selection vectors in gene cloning in this genus or other actinomycetes. This technique was developed in *E. coli* to surmount the problem of non-recombinant transformants in DNA cloning experiments. Biochemical methods are available to minimise non-recombinant transformants. For some applications it is possible to cut the vector to be used with two non-complementary restriction endonucleases to prevent the recircularisation of parental vectors without suitable inserts. Phosphatase treatment of the vector prior to ligation (Ullrich *et al.*, 1977) also achieves this end. The use of positive selection vectors is usually more convenient and reliable than either of these methods (Kuhn *et al.*, 1986).

Positive selection vectors employing various genetic means to eliminate transformants without foreign DNA inserts have been developed. These include inactivation of a lethal gene, inactivation of a dominant function conferring cell sensitivity to metabolites, removal of a lethal site and expression of metabolic resistance function (Kuhn *et al.*, 1986).

Positive selection vectors have been reviewed by Burns and Beacham (1984). Positive selection vectors described since then include pVT25, where positive selection is based on inactivation of the *col*E3 gene, preventing production of the lethal colicin E3 (Vernet *et al.*, 1985). A streptomycin resistant (St^r) positive selection vector pHSG664, an improvement on the St^r vector pNO1523 (Dean, 1981) has recently been developed by Hashimoto-Gotoh *et al.* (1985). Stevis and Ho (1987) have described a positive selection vector, pLX100, based on xylose utilisation suppression.

The positive selection shuttle vector constructed and used in this study employs the 3.36 kb *E. coli* plasmid pEcoR251. This positive selection vector contains the *E. coli Eco*RI gene under the control of the λ rightward promoter p_r and the ampicillin resistance (Ap^r) gene and origin of replication (*ori*) from plasmid pBR322 and was derived from a pCL plasmid (Zabeau and Stanley, 1982) by Botterman (1986). The *Eco*RI gene product is expressed at a high level by the λ promoter and is lethal unless inactivated by the insertion of DNA into the *Eco*RI gene, or regulated by plasmid pCI857^{ts} which contains a temperature sensitive λ repressor gene (Remaut *et al.*, 1983). The *Eco*RI gene contains a single *Bgl*II site, useful for the cloning of DNA fragments generated by partial *Sau*IIIA endonuclease digestions.

The *Streptomyces* plasmid used in the construction of the positive selection shuttle vector plasmid pLR591, was the well characterised plasmid pIJ702 (Katz *et al.*, 1983). This 5.65 kb plasmid contains the gene coding for the enzyme tyrosinase, responsible for melanin synthesis, from *Streptomyces antibioticus*, subcloned into the multicopy plasmid pIJ350, which carries a gene coding for resistance to the antibiotic thiostrepton (Ts) (Katz *et al.*, 1983).

The strategy employed in the construction of plasmid pLR591 was to cleave plasmid pEcoR251 at the single *Bam*HI restriction site and ligate this plasmid with plasmid pIJ702 cleaved at the *Bgl*II restriction site in the melanin gene. The restriction endonucleases *Bam*HI and *Bgl*II generate compatible DNA sequences for subsequent ligation (Kessler *et al.*, 1985). There is a low likelihood of *Bgl*II sites being reconstituted on ligation of these two vectors, so the resultant vector would have a single *Bgl*II site in the *Eco*RI gene (as in pEcoR251) and would be suitable for use as a positive selection vector, with inactivation of the *Eco*RI gene by insertion of DNA into the *Bgl*II site.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and plasmids

Table 2.1 lists bacterial strains and plasmids.

2.2.2 Standard methods, buffers and media

Appendix B lists standard techniques, buffers and media.

2.2.3 Plasmid preparation

The plasmid pEcoR251 was prepared by the method of Ish-Horowicz and Burke (1981) for large-scale preparation of *E. coli* plasmid DNA as described in Appendix B.1.1. Plasmid pEcoR251 was purified by isopycnic caesium chloride-ethidium bromide (CsCl-EtdBr) ultracentrifugation. DNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm and using the relationship $A_{260}=1$ for 50 µg/ml double stranded DNA (Maniatis *et al.*, 1982).

Plasmid pIJ702 was prepared from *S. lividans* TK24 by a method based on that of Hopwood *et al.* (1985) for large-scale preparation of plasmid DNA by neutral lysis as described in Appendix B.1.3. The sample was purified by isopycnic CsCl-EtdBr density gradient ultracentrifugation. DNA concentration was determined spectrophotometrically.

TABLE 2.1 Bacterial strains and plasmids.

Strain or plasmid	Genotype or genetic markers	Reference or source
E. coli K514	*thr*-1 *leu*-6 *thi*-1 *SupE*44 *lac*Y1 *ton*A21 r^-_k m^+_k (C600 derivative)	Wood, 1966
E. coli K514λ	λ lysogen of *E. coli* K514	Zabeau & Stanley, 1983[a]
S. lividans TK24	St^r $SLP2^-$ $SLP3^-$	Hopwood, D A[b]
pEcoR251	Ap^r *Eco*RI	Zabeau, M[a] (Appendix 3)
pIJ702	Ts^r mel^+	Hopwood, D A[b]
pCI587^{ts}	Km^r P_r repressor (ts)	Rameau *et al.*[a]
pLR591	Ap^r Ts^r	This study
pNIL100	Ap^r Ts^r	This study

[a] Gift from M Zabeau, Plant Genetic Systems, Ghent, Belgium.
[b] Gift from D A Hopwood, John Innes Institute, Norwich, UK.

2.2.4 Shuttle vector construction

One μg of pEcoR251 DNA was digested with 4 u *BamHI* restriction endonuclease in 20 μl medium salt restriction buffer (Appendix B.2) for 2 h at 37°C. The *BamHI* endonuclease was inactivated and DNA precipitated and resuspended in 20 μl TE buffer (Appendix B.2) as described by Maniatis *et al*. (1982).

The minimum time to completely digest 1 μg of pIJ702 with 4 u of *BglII* endonuclease in 20 μl low salt restriction buffer (Appendix B.2) at 37°C was determined. Samples of 2 μl were removed at 10 min intervals and electrophoresed in a 0.7% agarose gel in TBE (Appendix B.2). Samples taken after 40 min exhibited complete digestion to one DNA band. A repeat digestion of the plasmid pIJ702 with *BglII* endonuclease was therefore incubated for exactly 40 min at 37°C, and *BglII* endonuclease inactivated by heating the digestion to 68°C for 15 min. DNA was precipitated and resuspended in 20 μl TE buffer as described by Maniatis *et al*. (1982). It was considered necessary to determine the minimum time required for complete *BglII* endonuclease digestion as prolonged digestion with this enzyme frequently reduces the efficiency of subsequent ligations (R Kirby, Dept of Microbiology, University of Cape Town, personal communication).

The two digested plasmids were ligated in the ratios pIJ702:pEcoR251 1:1, 2:1 and 3:1 by mass. Ligations each contained 0.1 μg pEcoR251 DNA, and were done as described in

Appendix B.1.8. Self-ligation controls of pEcoR251 (0.1 μg) and pIJ702 (0.3 μg) were set up in the same way. Ligations were left for 16 h at 20°C.

2.2.5 Preparation and transformation of competent cells

Competent *E. coli* K514λ cells were prepared by the method of Cohen *et al.* (1972), modified according to Dagert and Ehrlich (1979), as described in Appendix B.1.13.

Competent *E. coli* K514λ cells were transformed by the three ligation mixes and two self-ligation controls. Additional controls consisting of ligation mix without added DNA, and 1 μg of pBR322 DNA were processed in the same way. Transformation mixes were plated on LB agar (Appendix B.2) containing 100 μg/ml ampicillin (Ap).

2.2.6 Detection of pEcoR251-pIJ702 recombinants

The Ap resistant (Ap^r) colonies obtained from the pEcoR251-pIJ702 ligations (Section 2.2.5) were screened by "colony hybridisation" studies, according to a method of Maniatis *et al.*, (1982), as described in Appendix B.1.11. A radioactively labelled pIJ702 probe was prepared by nick translation (Rigby *et al.*, 1977), as described in Appendix B.1.10. A specific activity of 3×10^7 counts per minute (cpm)/µg DNA was obtained.

After hybridisation, filters were washed 3 times in 2 x SSC (Appendix B.2), 0.1% (w/v) sodium dodecyl sulphate (SDS) for 10 min per wash and twice in 1 x SSC, 0.1% (w/v) SDS for 1 h per wash. Filters were air-dried and screened in an autoradiation X-ray cassette, using Kodak XAR-5 X-ray film and Fuji X-ray intensifying screens. Film was exposed for 2 d at -70°C and processed using Kodak GBX X-ray developer and fixer. Colonies corresponding to those showing hybridisation to the pIJ702 probe were studied further.

2.2.7 Plasmid preparation, restriction mapping and Southern hybridisation analysis of putative pEcoR251-pIJ702 recombinants

Four Ap^r colonies showed hybridisation to the pIJ702 probe. Plasmids were prepared from each of these colonies (as described for pEcoR251 in Section 2.2.3), and were designated pLR591, pLR592, pLR593 and pLR594.

To confirm the presence of pEcoR251 and pIJ702 DNA in these plasmids, *Pst*I, *Xho*I and *Eco*RI restriction endonuclease digests were done according to the standard procedures described by Maniatis *et al.* (1982). The plasmids pEcoR251 and pIJ702 were similarly digested. All digests were electrophoresed in two 0.7% agarose gels. DNA fragments were transferred from the agarose gels to hybridisation transfer membranes by the method of Smith and Summers

(1980). This method, and pre-hybridisation and hybridisation procedures are described in Appendix B.1.12. Radioactive probes of pEcoR251 and pIJ702 were prepared, as described in Appendix B.1.10. Membranes were washed in 2 x SSC, 0.5% (w/v) SDS for 5 min at 20°C. After a final wash in 0.1 x SSC, 0.5% (w/v) SDS at 68°C for 2 h, membranes were air-dried and placed in X-ray cassettes with intensifying screens. Kodak XAR-5 X-ray film was exposed at -70°C for 16 h.

The plasmids pLR591, pLR592, pLR593 and pLR594 were mapped with a range of additional restriction endonucleases, using single and double digests, according to the standard procedures described by Maniatis *et al.* (1982).

2.2.8 Expression of *Eco*RI gene of pLR591 in *E. coli*

The putative shuttle vector pLR591 and the plasmid pEcoR251 were transformed into *E. coli* K514 containing the plasmid pCI857^{ts}. Transformation mixes were plated on LB agar containing 100 µg/ml Ap and incubated at 28°C. Transformants from the pLR591 and pEcoR251 mixes, and untransformed K514 containing pCI857^{ts} were each suspended in 0.85% (w/v) saline to the same density, and serial dilutions of these suspensions were each plated on two LB agar plates containing 100 µg/ml Ap. One set of plates was incubated at 42°C and the other at 28°C.

2.2.9 Expression of Ts^r of pLR591 in *S. lividans* TK24

Protoplasts of *S. lividans* were prepared and transformed, according to the method of Hopwood *et al.* (1985), with plasmids pIJ702 and pNIL100 (pLR591 with 10 kb *N. corallina* JL10 DNA inserted into the *Bgl*II cloning site). Plasmid pIJ702 was prepared from *S. lividans* TK24 (Section 2.2.3) and pLR591 and pNIL100 were prepared from *E. coli* K514λ as described for pEcoR251 in Section 2.2.3. Exactly 1 μg plasmid DNA was used for each transformation and triplicate transformations were done in each case. Serial dilutions of transformed protoplasts were plated on R2YE plates (Appendix B.2), which were incubated for 18 h at 30oC and overlaid with 2.5 ml sloppy nutrient agar (SNA) (Appendix B.2) containing 500 μg/ml Ts. Ts^r colonies were counted after 3 d.

Plasmid DNA was prepared from cultures inoculated with Ts^r colonies from each of the three transformations, by the neutral lysis method of Hopwood *et al.* (1985) outlined in Appendix B.1.3. This DNA isolated from *S. lividans* TK24 was then retransformed into TK24, following exactly the procedure described above.

Plasmid DNA was prepared from four colonies of each of this second group of transformants, once again by the neutral lysis method of Hopwood *et al.* (1985), and mapped by restriction endonuclease digestion.

2.3 RESULTS AND DISCUSSION

2.3.1. Detection of pEcoR251-pIJ702 recombinants

Approximately 1,500 Ap^r colonies were screened. Four of these showed hybridisation to the pLR591 plasmid probe. Table 2.2 shows results obtained for differing ligation ratios. Increasing the relative concentration of plasmid pIJ702 resulted in increasing numbers of Ap^r transformants and transformants hybridising to pIJ702. However, higher ratios of pIJ702 would result in a greater number of pEcoR251 transformants containing multiple insertions of pIJ702.

2.3.2 Restriction endonuclease mapping and Southern hybridisation analysis of putative pEcoR251-pIJ702 recombinants

Preliminary restriction endonuclease mapping and Southern hybridisation analysis confirmed that the four putative chimeric plasmids contained both pEcoR251 and pIJ702 DNA. Definitive restriction endonuclease maps were then constructed (Fig. 2.1). The plasmids pLR591 and pLR593 were shown to be identical, and therefore pLR593 was not pursued further. The plasmid pLR592 contained a short insert of pIJ702 into pEcoR251, without the gene coding for thiostrepton resistance, and was therefore not studied further. This plasmid could have arisen from carry-over of active *BamHI* restriction endonuclease into the pEcoR251-pIJ702 ligation mix, as pIJ702 had been cleaved at a *BamHI*

TABLE 2.2 *E. coli* transformants obtained at differing pEcoR251 and pIJ702 ligation ratios.

Ligation ratio pEcoR251:pIJ702	Total No. of Ap^r transformants	Total No. of transformants hybridising to pLR591
1:1	224	0
2:1	550	1
3:1	700	3

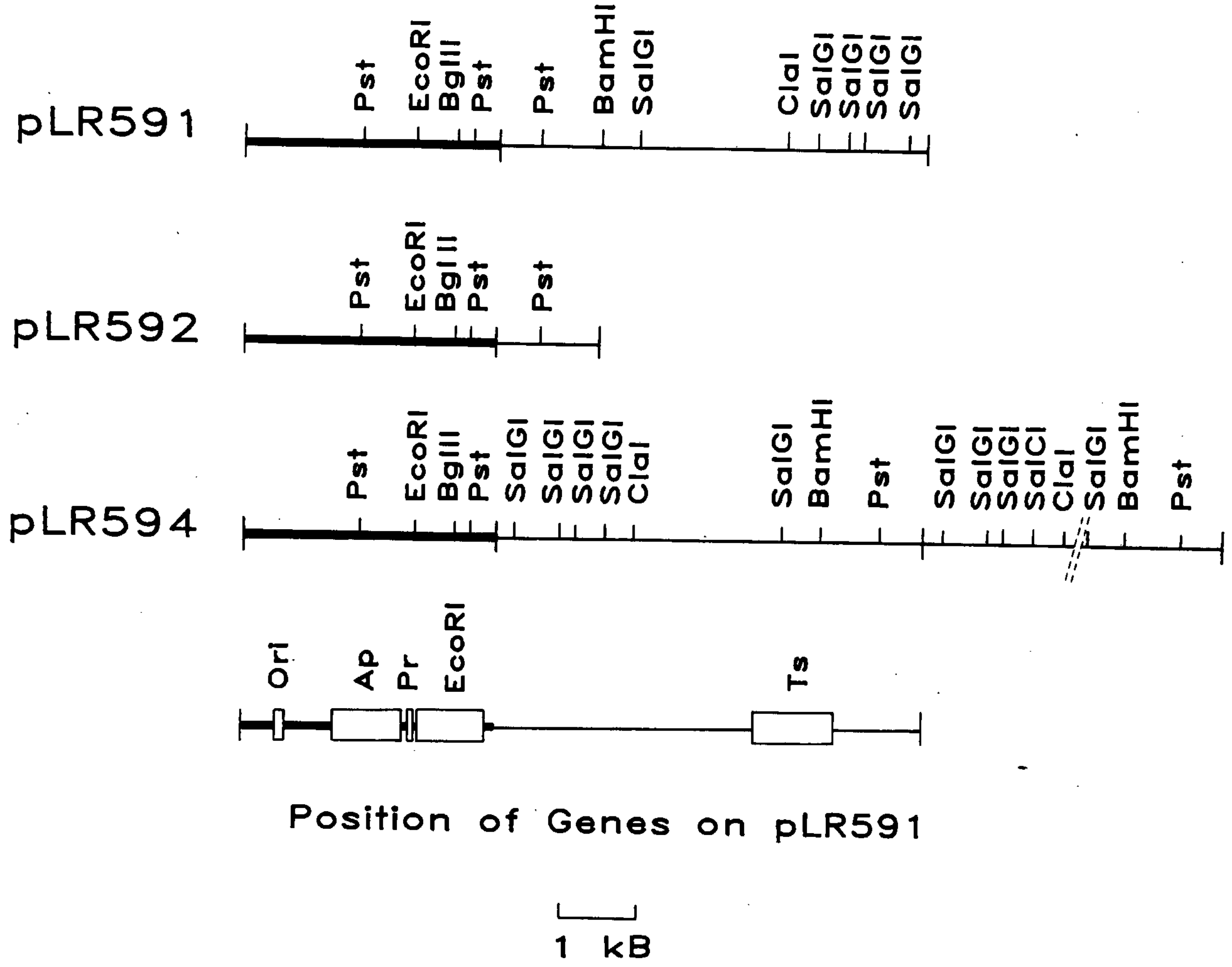

Fig. 2.1. Restriction endonuclease maps of pLR591, pLR592 and pLR594. Bold lines represent DNA derived from pEcoR251 and thin lines represent DNA derived from pIJ702.

site to give this truncated insert. The plasmid pLR594 was shown to contain two molecules of pIJ702, inserted into pEcoR251 in the same orientation as each other, but the opposite orientation from the pIJ702 DNA inserted in pLR591. It would not be easy to excise one of the two pIJ702 inserts in pLR594, as the ligation of *Bgl*II and *Bam*HI endonuclease sites has only a low likelihood of reconstituting either the *Bam*HI or *Bgl*II restriction sites. The larger size of pLR594 makes it less suitable for use as a cloning vector than pLR591. The mode of construction, useful restriction endonuclease sites and positions of genes of pLR591 are shown in Fig. 2.2.

2.3.3 Expression of *Eco*RI gene of pLR591 in *E. coli*

Table 2.3 shows survival of *E. coli* K514, containing pCI857^{ts} carrying the temperature sensitive λ repressor gene, at 28^{o}C and 42^{o}C. Survival of the control K514 strain containing no additional plasmid (ie containing pCI857^{ts} only) was compared with survival of this strain containing the additional plasmids pEcoR251 or pLR591. Control K514 plated on LB agar exhibited similar survival at 28^{o}C and 42^{o}C indicating that the number of colonies produced was not affected by the different temperatures. Survival of *E. coli* K514(pEcoR251) at the permissive temperature of 28^{o}C was of the same order as control K514 cells plated on LB agar. However, *E. coli* K514(pEcoR251) produced far fewer colonies at 42^{o}C compared with 28^{o}C, indicating that cells were being killed at 42^{o}C as a result of the inactivation of the

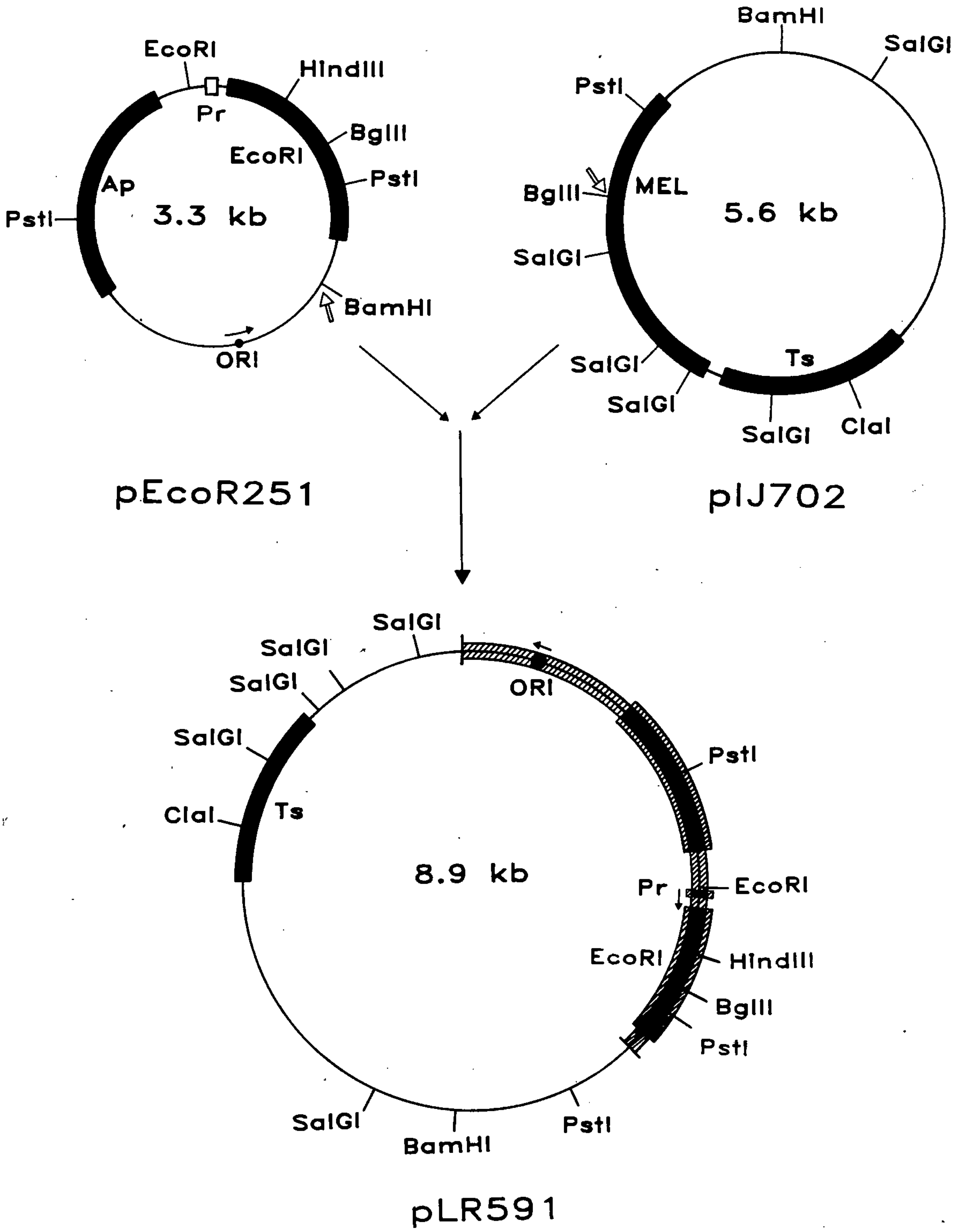

Fig. 2.2. Construction of the *Streptomyces-E. coli* positive selection shuttle vector pLR591. DNA from plasmid pEcoR251 (▨) and plasmid pIJ702 (———) and the position of genes (■) is indicated.

TABLE 2.3 Comparison of the survival of *E. coli* K514 containing pCI857^{ts} and either pEcoR251 or pLR591 at 28°C and 42°C. Cell suspensions were plated on LB agar containing 100 μg/ml Ap.

°C	Viability of *E. coli* K514 cells/ml	
	K514(pEcoR251)	K514(pLR591)
28	7.9×10^{6}	7.4×10^{6}
42	1.2×10^{2}	3.1×10^{2}

TABLE 2.4 Transformation of *S. lividans* TK24 by pLR591 and pNIL100 propagated in *E. coli* K514λ and *S. lividans* TK24. The range of results obtained in three experiments is given in colony forming units (cfu)/μg DNA. Transformation of *S. lividans* TK24 by pIJ702 gave a range of 100 - 400 cfu/μg DNA x 10^{2}.

Host in which plasmids were propagated	Transformation of *S. lividans* TK24 (Ts^{r} cfu/μg DNA x 10^{2})	
	TK24(pLR591)	TK24(pNIL100)
E. coli K514λ	0.02 - 0.07	0.8 - 2.0
S. lividans TK24	90 - 180	90 - 160

temperature sensitive λ repressor coded by $pCI857^{ts}$, and the expression of the *Eco*RI gene.

The plasmid pLR591 produced similar results to pEcoR251, with a reduction of greater than 10^4 in the number of *E. coli* K514 colonies produced at 42°C compared with 28°C. The incorporation of pIJ702 into the *Bam*HI site of pEcoR251 to produce pLR591 therefore had no effect on the function of the *Eco*RI gene in *E. coli*. The functional *Eco*RI gene in pLR591 renders this vector suitable for use as a positive selection vector in *E. coli*.

2.3.4 Expression of the plasmid pLR591 in *S. lividans* TK24

Table 2.4 lists the numbers of transformants from pIJ702, pLR591 and pNIL100 when these plasmids were isolated from *E. coli* K514λ in comparison with the plasmids isolated from *S. lividans* TK24. The protoplast regeneration frequency in this experiment was approximately 1%, with a total number of 3.5×10^9 protoplasts/ml.

The 10^4-fold difference between numbers of transformants produced by pIJ702 and pLR591, when pLR591 was isolated from *E. coli*, could be explained by partial expression of the intact *Eco*RI gene of pLR591 in *S. lividans* TK24 resulting in death of some transformed cells. Expression of some *E. coli* genes in *Streptomyces* sp has been reported, for example by King and Chater (1986) (see Chapter 4 for detailed discussion). However, the number of transformants produced by pNIL100 was also considerably less than pIJ702. In pNIL100, the *Eco*RI gene has been inactivated by a large DNA insert, so partial expression of *Eco*RI cannot occur in this case.

An alternative explanation for transformation frequencies of pLR591 and pNIL100, propagated in *E. coli*, being lower than that of pIJ702, is that these plasmids are unstable in *Streptomyces* due to the segments of *E. coli* DNA contained in them. Instability of a cosmid shuttle vector in *S. lividans* has been reported previously (Stanzak *et al.*, 1986). Schottel *et al.* (1981) working with hybrid plasmids consisting of the *S. lividans* plasmid pSLP111 and *E. coli* plasmids pACYC177 and pACYC184, found a significant amount of restriction of the hybrid plasmids when they were introduced by transformation into *S. lividans* after being propagated in *E. coli*. After surviving the apparent initial extraction barrier, plasmids in this case subsequently transformed *S. lividans* at an efficiency 10^3- to 10^4-fold higher than the initial transformation frequency.

Transformation frequencies of pLR591 and pNIL100 isolated from *S. lividans* were similar to that of pIJ702 as would be expected if modification of the pEcoR251 portion of these vectors by *S. lividans* restriction-modification systems was important to ensure plasmid stability. The large actinomycete DNA insert in the pEcoR251 region of pNIL100 could render this plasmid more stable than pLR591, as observed in the more efficient transformation of pNIL100 isolated from *E. coli* compared with pLR591 isolated from *E. coli*.

Restriction enzyme analyses of pLR591 and pNIL100 after passage through *S. lividans* were identical to restriction enzyme maps of these plasmids isolated from *E. coli*.

The plasmid pLR591 was shown to express Ts^r in *S. lividans* and although transformation frequencies of pLR591 propagated in *E. coli* were about 10^4-fold less than pIJ702, this should not affect the utility of pLR591 as a positive selection *Streptomyces*-*E. coli* shuttle vector. Initial cloning steps would be performed in *E. coli* and large amounts of recombinant DNA could be produced in *E. coli* and subsequently transformed into *Streptomyces*.

2.4 CONCLUSION

The *Streptomyces*-*E. coli* shuttle vector pLR591 was shown to express Ap^r in *E. coli* and to have a functional *Eco*RI gene for positive selection, and expressed Ts^r in *S. lividans* . The vector pLR591 should be useful in the rapid cloning of *Streptomyces* genes in *E. coli*, and will facilitate transfer of cloned genes back to *Streptomyces* sp. Although the high copy number of the vector could be a disadvantage in certain applications, such as the cloning of genes coding for potentially toxic products, pLR591 would be useful in screening for antibiotic resistance genes of *Streptomyces* which have sometimes been found to be linked to antibiotic synthesis genes (Fayerman, 1986).

The vector pLR591 is well suited for investigating the expression of *Streptomyces* genes in *E. coli* and *vice versa*. It is possible to determine whether genes cloned into pLR591 are functioning from the λ p_r promoter or from their own promoters, using the plasmid pCI587 containing a temperature sensitive λ repressor gene.

The broad host range of pIJ702 (Katz *et al.*, 1983) makes it likely that pLR591 would be a useful plasmid for interspecific cloning in *Streptomyces*. In addition, pLR591 could be used in the cloning of genes from other actinomycetes such as *Nocardia* (see Chapters 4 and 5). It has recently been demonstrated (Matsushima *et al.*, 1987) that pIJ702 expresses Ts^r in the actinomycete *A. orientalis* (*"N." orientalis*). The usefulness of pLR591 could extend to gene cloning into actinomycetes such as *Nocardia* sp as transformation systems for these organisms become better understood.

The positive selection *Streptomyces*-*E. coli* shuttle vector pLR591 should be a valuable tool in the field of actinomycete genetics.

CHAPTER THREE

CONSTRUCTION OF GENOMIC LIBRARIES OF *N. CORALLINA* AND *NOCARDIA* SP ATCC 21145 IN THE VECTOR pLR591

CHAPTER THREE

CONSTRUCTION OF GENOMIC LIBRARIES OF *N. CORALLINA* JL10 AND *NOCARDIA* SP ATCC 21145 IN THE VECTOR pLR591

Summary: Complete genomic libraries of *N. corallina* JL10 and *Nocardia* sp ATCC 21145 were constructed in the *Streptomyces-E. coli* positive selection shuttle vector pLR591. Recombinant plasmids comprising the libraries were prepared in *E. coli* and transformed into *S. lividans* TK24.

3.1 INTRODUCTION

Recombinant DNA research often has as an initial goal the isolation of a particular fragment of chromosomal DNA, coding for a characteristic of interest to the researcher. A standard technique used in achieving this goal is the construction of a genomic library of the organism containing the DNA of interest. The library is screened by an appropriate method to isolate the desired DNA sequence.

The choice of vector used to construct a genomic library depends on the genome size of the organism from which the DNA is to be isolated and the expected size of the fragment of DNA of interest. Other factors include the method of screening to be used, and the application for which the DNA fragment is to be used.

Eukaryote genome sizes vary from 1.4 x 10^7 bp for yeast (Watson *et al.*, 1987) to 3.0 x 10^9 bp for mammals (Maniatis *et al.*, 1982). Genome sizes of prokaryotes are usually approximately 2.0 x 10^6 bp (Starr *et al.*, 1981; Mandelstam *et al.*, 1982). Some actinomycete genomes tend to be larger than those of other prokaryotes. The genome of *Streptomyces* has been estimated at 1 x 10^7 bp (Benigni *et al.*, 1975). Determinations of genome size of *Mycobacterium* sp range from 1.5 to 2.0 x 10^6 bp (Clarke-Curtiss *et al.*, 1985).

Vectors commonly used for genomic library construction are bacteriophages, cosmid vectors and plasmids. Derivatives of bacteriophage λ have been widely used in construction of gene banks in *E. coli* and require DNA inserts of approximately 20 kb in order to be packaged into mature bacteriophage λ particles (Williams and Blattner, 1980). Even larger inserts of approximately 40 kb can be stably inserted into cosmid vectors. The number of clones needed to construct a complete gene library is proportional to the average size of insert DNA cloned. Vectors such as bacteriophage λ vectors and cosmids capable of stable insertion of large DNA fragments are therefore useful in limiting the number of clones needed to produce gene libraries of eukaryotes with large genome sizes.

Many naturally occurring plasmids have been adapted for use in genomic library cloning in a wide range of prokaryotic organisms and some eukaryotes (such as yeast). A selection of useful plasmids has been compiled by Pouwels *et al.* (1985). Plasmids are commonly used for cloning DNA fragments up to about 10 kb in size. Very large inserts are sometimes found to be unstable as they tend to slow down the multiplication rate of their host plasmids, which are replaced by smaller derivative plasmids which have lost sections of their DNA inserts (Watson *et al.*, 1987). Plasmids are frequently the cloning vector of choice in prokaryote genomic library construction, as inserts of up to about 10 kb are sufficiently large to cover the entire prokaryotic genome in approximately 5,000 - 10,000 clones.

The number of clones required (N) to give a specific probability of having any DNA sequence represented in the library is given by the formula

$$N = \frac{\ln (1 - P)}{\ln (L - f)}$$

where f is the fraction of the total genome that each insert represents and P is the required probability (Clarke and Carbon, 1976).

In this study, genomic libraries of *N. corallina* JL10 and *Nocardia* sp ATCC 21145 were constructed in the *Streptomyces-E. coli* positive selection vector pLR591. DNA inserts of 5 - 10 kb could readily be cloned into this vector. The number of clones, containing inserts of an average size of 7.5 kb, required to cover the genomes of the *Nocardia* sp to a probability of greater than 95% was approximately 5,000 assuming a genome size of about 1×10^7 bp (Hopwood *et al.*, 1985). This number of clones was manageable in terms of the screening procedures to be used (see Chapters 4 and 5).

The method of choice in preparing fragments of chromosomal DNA of the two *Nocardia* sp was partial digestion of chromosomal DNA with *Sau*IIIA endonuclease. This method was preferred to that of total digestion of chromosomal DNA with restriction enzymes that recognise hexanucleotide sequences. This second method can result in exclusion of complete genes from a library if they happen to contain the sequence recognised by the restriction enzyme used in the complete digestion, or if the fragments on which the genes are located are too large to be readily cloned in the vector being used. Partial *Sau*IIIA digestion results in more random shearing of the chromosomal DNA, giving more representative genomic libraries.

The *Streptomyces*-*E. coli* positive selection vector pLR591 had the advantage of selecting against transformants containing parental plasmids without inserts, thus facilitating efficient genomic library production. In addition, expression of *Nocardia* genes of interest could be readily screened for in both *Streptomyces* and *E. coli*.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and plasmids

Table 3.1 lists bacterial strains and plasmids.

TABLE 3.1 Bacterial strains and plasmids.

Strain or plasmid	Genotype or genetic markers	Reference or source
E. coli K514λ	λ lysogen of *E. coli* K514	Zabeau & Stanley, 1982
E. coli DK1	K-12 derivative. del (*srl-rec*A)	ATCC 35691[a]
E. coli LK111	K514 derivative. *lac*Z M15Δ*lac*Y$^+$	Zabeau & Stanley, 1982
N. corallina JL10	wild type	Microbiology Dept, University of Cape Town
Nocardia sp	wild type	ATCC 21145
S. lividans TK24	St^r SLP2$^-$ SLP3$^-$	Hopwood, D A[b]
pLR591	Ap^r Ts^r	This study

[a] Gift from D Kurnit, The Childrens Hospital, Boston MA, USA

[b] Gift from D A Hopwood, John Innes Institute, Norwich, UK

3.2.2 Standard methods, buffers and media

Appendix B lists standard techniques, buffers and media.

3.2.3 Isolation and digestion of plasmid pLR591

A 400 ml LB broth (Appendix B.2) culture of *E. coli* K514λ containing plasmid pLR591 was grown for 16 h at 37°C with selective pressure (100 µg/ml Ap). Plasmid preparation was according to the method of Ish-Horowicz and Burke (1981). Plasmid pLR591 was purified by isopycnic CsCl-EtdBr density gradient centrifugation, and the plasmid band removed and centrifuged a second time to obtain very pure plasmid. The DNA concentration of purified plasmid in TE buffer was determined spectrophotometrically.

The minimum time to completely digest 1 µg of pLR591 with 4 u of *Bgl*II endonuclease in 20 µl low salt buffer at 37°C was determined as described for pIJ702 in Section 2.2.4. Twenty endonuclease digestions were done using these conditions and *Bgl*II endonuclease was inactivated by heating the digestions to 68°C for 15 min. DNA was precipitated, resuspended in a total volume of 100 µl TE buffer and stored at 4°C.

3.2.4 Isolation, digestion and sizing of *Nocardia* chromosomal DNA

N. corallina JL10 and *Nocardia* sp ATCC 21145 were grown in 400 ml M3 liquid medium (Appendix B.2) for 2 d at 30°C with aeration. The cultures were harvested by centrifugation and 1 g wet weight of each was used in a DNA isolation procedure based on that described by Hopwood *et al.* (1985) for isolation of *Streptomyces* "total" DNA (see Appendix B.1.3 for modified method). The DNA concentrations of both preparations were determined spectrophotometrically.

Partial *Sau*IIIA endonuclease digestions were performed, following the method of Hopwood *et al.* (1985) as described in Appendix B.1.6, on 100 µg of DNA from both preparations. The partially digested DNA from *N. corallina* JL10 and *Nocardia* sp ATCC 21145 was size fractionated by sucrose gradient centrifugation as described by Hopwood *et al.* (1985) (see Appendix B.1.7). Appropriate fractions (containing DNA in the range 5 - 10 kb) were pooled and DNA was recovered by ethanol precipitation, and resuspended in TE buffer.

3.2.5 Construction of genomic libraries

Plasmid pLR591 digested with *Bgl*II endonuclease was ligated with chromosomal DNA (partially digested with *Sau*IIIA endonuclease) from both *Nocardia* sp. In both cases, a number of ligation mixtures were prepared, with varying ratios of vector DNA and chromosomal insert DNA, ranging from 1:1 to 50:1 insert to vector by mass. Ligations were done under the conditions described in Appendix B.1.8. Final total DNA concentration in the ligation mixes varied from 0.1 - 3 µg/µl. A self-ligation control containing only pLR591 DNA (2 µg) was set up in the same way. Ligations were left for 4 h at 20°C.

Competent cells of *E. coli* strains DK1 and LK111 were prepared. *E. coli* DK1 was transformed with the ligation mixtures containing *N. corallina* JL10 and pLR591 DNA and *E. coli* LK111 was transformed with mixtures containing *Nocardia* sp ATCC 21145 and pLR591 DNA. In addition, both *E. coli* strains were transformed with 1 ng pBR322 DNA, as a check on transformation efficiency and on self-ligated pLR591. Transformation mixtures from each ligation were pooled into 100 ml prewarmed LB containing 50 µg/ml Ap and these cultures were shaken vigorously at 42°C for 1 h, to ensure efficient selection of transformants containing pLR591 plasmids with insertionally inactivated *Eco*RI genes. Cultures were harvested by centrifugation, cells resuspended in 2 ml LB broth, and plated on LB agar containing 100 µg/ml Ap.

3.2.6 Preparation, visualisation and storage of recombinant plasmids

Ap^r colonies comprising the genomic libraries of *N. corallina* JL10 and *Nocardia* sp ATCC 21145 were pooled (approximately 750 colonies in 12 pools and 2,400 colonies in 10 pools, respectively) and plasmids were prepared from each pool using the method of Ish-Horowicz and Burke (1981).

The concentration of DNA in each pool of both genomic libraries was determined spectrophotometrically and 5 µg of

DNA from each pool of the *Nocardia* sp ATCC 21145 genomic library was digested with *Not*I endonuclease. The digested DNA was electrophoresed in 0.7% agarose gels according to the method of Maniatis *et al.* (1982). Each plasmid pool was divided and stored at 4°C and -70°C.

3.2.7 Transformation of *Nocardia* genomic libraries into *S. lividans* TK24

Protoplasts of *S. lividans* TK24 were prepared and transformed with 1 µg of DNA from each plasmid pool of the *N. corallina* JL10 and *Nocardia* sp ATCC 21145 genomic libraries, using the methods described in Appendices B.1.14 and B.1.15. Ts^r transformants were selected as described in Section 2.2.9. Once *S. lividans* TK24 transformants had sporulated, spores from each pool of transformants were harvested, filtered through sterile cotton wool, and stored in 15% (v/v) glycerol at -20°C. Plasmid DNA was prepared from 6 Ts^r colonies of each of the genomic libraries, by the neutral lysis method of Hopwood *et al.* (1985). These 12 recombinant plasmids were analysed by agarose gel electrophoresis, to determine the size of the chromosomal DNA insert in each plasmid.

3.3 RESULTS AND DISCUSSION

3.3.1 Construction of genomic libraries in *E. coli*

Table 3.2 lists the number of *E. coli* transformants obtained for each ligation ratio used in the construction of the *N. corallina* JL10 and *Nocardia* sp ATCC 21145 genomic libraries. The total number of clones in the *N. corallina* JL10 genomic library was 8,948 and the total number in the *Nocardia* sp ATCC 21145 library was 25,012. The probability of having any DNA sequence represented in the libraries was determined using the formula of Clarke and Carbon (1976) (see Section 3.1). This probability was 99.88% for the *N. corallina* JL10 library and 99.99% for the *Nocardia* sp ATCC 21145 library, assuming a genome size of 1×10^7 bp and an average insert size of 7.5×10^3 bp.

The number of colonies resulting from transformation with self-ligated pLR591 was less than 1% of the total number of colonies obtained in both genomic libraries. The number of colonies in these libraries resulting from survival of transformants containing pLR591 only (without inserted chromosomal DNA) was therefore regarded as insignificant. This verifies the efficient positive selection function of the vector pLR591. In earlier attempts at genomic library cloning with pLR591 a far higher "background" of transformants containing parental plasmid without detectable inserts was obtained with self-ligated pLR591. This problem was eliminated by double purification of pLR591 by isopycnic CsCl-EtdBr density gradient centrifugation as described in

TABLE 3.2 A and B. *E. coli* transformants obtained at differing pLR591 and *Nocardia* sp chromosomal DNA ligation ratios.

A. *E. coli* transformants harbouring recombinant plasmids containing *N. corallina* JL10 DNA

Ligation ratio	Total No. of Ap^r transformants
pLR591:*N. corallina* JL10 chromosomal DNA	
1:1	234
1:5	322
1:10	187
1:20	1,800
1:30	3,705
1:40	2,700

B. *E. coli* transformants harbouring recombinant plasmids containing *Nocardia* sp ATCC 21145 DNA

Ligation ratio	Total No. of Ap^r transformants
pLR591:*Nocardia* sp ATCC 21145 chromosomal DNA	
1:1	264
1:10	348
1:20	6,120
1:30	6,336
1:40	5,984
1:50	5,960

Section 3.2.3. This suggests that the higher "background" resulted from insertion into the *Eco*RI gene of small fragments of DNA present in the less pure preparations, preventing *Eco*RI expression and causing survival of a relatively high number of transformants, compared with the low numbers obtained for self-ligation with highly purified pLR591.

The number of transformants obtained was found to vary greatly for different ratios of vector to chromosomal DNA insert, in both genomic library cloning experiments. Ratios of greater than 1:20, vector:insert, were the most successful in both experiments. Theoretical optimal conditions for cloning of insert fragments of various sizes in circular vectors have been mathematically derived (Legerski and Robberson, 1985). However, these theoretical conditions may not apply in the case of positive selection vectors where transformation by recircularised parental vectors without inserted DNA is selected against. The analysis of an analagous situation, the treatment of vector DNA with phosphatase to prevent vector self-cyclisation, has proved to be mathematically intractable (Legerski and Robberson, 1985). An empirical approach was therefore adopted in determining ligation ratios, as suggested by Hopwood *et al.* (1985). The number of transformants was used directly to assess the success of ligations, rather than monitoring ligations for the appearance of high molecular mass DNA by gel electrophoresis, as King and Blakesley (1986) have found this latter method to be unreliable in predicting the transformation efficiency of ligation reactions.

3.3.2 Assessment of recombinant plasmids in genomic libraries

The DNA pools comprising the genomic library of *Nocardia* sp ATCC 21145 were digested with *Not*1 endonuclease and visualised by agarose gel electrophoresis (Fig. 3.1). Endonucleases, such as *Not*1 endonuclease, which recognise a sequence of eight nucleotides, will theoretically cut DNA with 50% guanine-cytosine (G-C) content only once every 63,504 bp. *Not*1 endonuclease, however, recognises a sequence rich in guanine and cytosine and therefore cuts actinomycete DNA, which has a high G-C content (Starr *et al.*, 1985), far more frequently than theoretically predicted. There is a recognition site for *Not*1 endonuclease in the vector pLR591. Most recombinant plasmids in the genomic library were cleaved once by *Not*1, and the resultant linearised plasmids were between approximately 13 and 20 kb in size, as expected for chromosomal DNA inserts of approximately 4-11 kb. Recombinant plasmids containing insert DNA with recognition sites for *Not*1 endonuclease would account for the presence of DNA bands smaller than 8.9 kb (ie the size of pLR591). Recombinant plasmids larger than 20 kb would result from insertion of two or more fragments of chromosomal DNA. Very few recombinant plasmids were larger than 20 kb, though the ligation ratios of insert to vector DNA in successful transformations were high, and DNA concentrations in

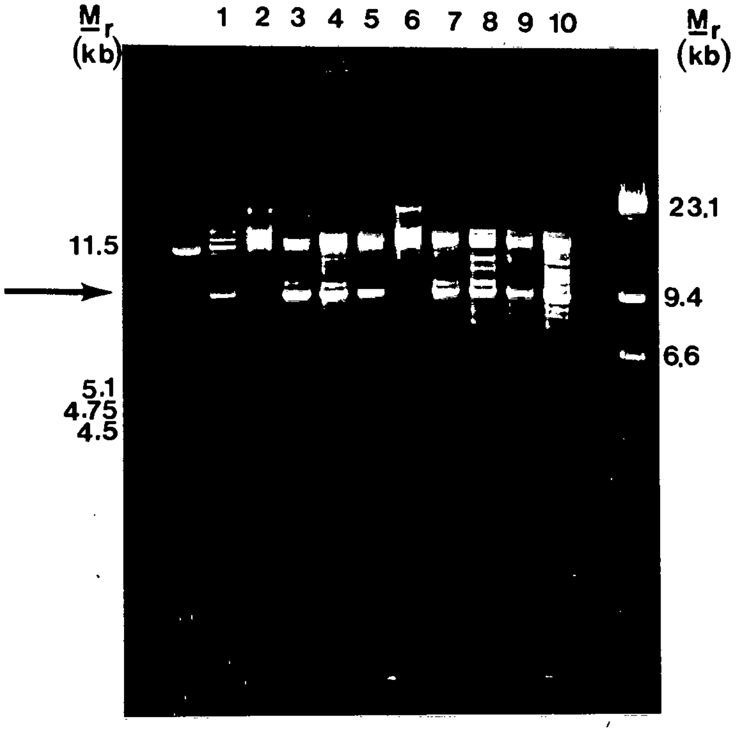

Fig. 3.1. *Not*I restriction endonuclease digested samples from the ten pools of DNA comprising the library of *Nocardia* sp ATCC 21145 in the vector pLR591. DNA samples in lanes 2 and 6 were only partially digested. The arrowed band of approximately 8.9 kb was likely to have resulted from the digestion of pLR591 vector containing no inserted DNA.

ligations were 10- to 200-fold higher than that of 0.015 µg/µl recommended by Hopwood *et al.* (1985).

3.3.3 Transformation of *Nocardia* genomic libraries into *S. lividans* TK24

Total numbers of *S. lividans* TK24 Ts^r transformants were 15,520 and 7,648 for the *N. corallina* JL10 and *Nocardia* sp ATCC 21145 genomic libraries, respectively. Insert sizes in recombinant plasmids of six Ts^r colonies transformed by the *N. corallina* JL10 genomic library ranged from 5.5 to 14.5 kb with an average size of 8.2 kb. A range of insert sizes from 7.0 to 16.0 kb with an average of 10.2 kb was obtained for six recombinant plasmids from the *Nocardia* sp ATCC 21145 library.

The large number of *S. lividans* TK24 transformants obtained and the presence of recombinant plasmids with inserts in the 5.5 to 16.0 kb range in those transformants checked, indicated that the vast majority of clones present in both *Nocardia* sp libraries had been transformed into *S. lividans* TK24.

3.4 CONCLUSION

The *Streptomyces-E. coli* positive selection vector pLR591 was shown to function efficiently in genomic library construction and to facilitate transformation of genomic libraries into *S. lividans* TK24. Representative genomic libraries of *N. corallina* JL10 and *Nocardia* sp ATCC 21145 were constructed in *E. coli* and transformed into *S. lividans* TK24 and these libraries could now be screened for the expression of genes of interest in both these hosts.

CHAPTER FOUR

CLONING STUDIES ON AND CHARACTERISATION OF PIGMENT-PRODUCING GENES FROM *NOCARDIA* SP

CHAPTER FOUR

CLONING STUDIES ON AND CHARACTERISATION OF PIGMENT-PRODUCING GENES FROM *NOCARDIA* SP

Summary: Three recombinant plasmids were isolated from genomic libraries of *N. corallina* JL10 (plasmid designated pNIL100) and *Nocardia* sp ATCC 21145 (plasmids designated pNIL200 and pNIL400), which coded for the production of pigment in *E. coli* transformants. Restriction endonuclease maps of these plasmids were made. The plasmid pNIL200 was shortened to produce a series of smaller plasmids also coding for pigment production. A DNA insert from pNIL200 was subcloned into pUC18 and pUC19 and was found to produce greater amounts of pigment in one orientation than the other, indicating that the *Nocardia* DNA insert contained a promoter region involved in control of pigment production. No pigment was detected in *S. griseus* transformants containing pNIL100, pNIL200 or pNIL400. The pigment produced by *E. coli* transformants containing pNIL200 was extracted, purified and characterised by visible- and UV-light spectroscopy and by infra-red and nuclear magnetic resonance spectroscopy and appeared to be a carotenoid pigment.

4.1 INTRODUCTION

4.1.1 Bacterial pigments

Pigments are produced by a wide variety of microorganisms, including many species of bacteria from diverse genera. The biological functions of pigments include protection against photodynamic damage, involvement in phototaxis and indirect participation in photosynthesis by light harvesting (Liaaen-Jensen and Andrewes, 1985). Some bacterial pigments are produced as secondary metabolites and have been reported to possess antibiotic activity (Rudd and Hopwood, 1980).

Bacterial pigments occur in various genera of both Gram-negative and Gram-positive bacteria. The pigments of Gram-positive cocci are primarily carotenoids; for example, *Staphylococcus aureus* has been found to contain numerous carotenoid pigments (Sobin and Stahly, 1942) and the carotenoid pigment canthaxanthin has been isolated from *Micrococcus roseus* (Cooney *et al.*, 1966). Certain Gram-positive, filamentous actinomycetes have been reported to produce prodiginine (prodigiosin-like) pigments (Gerber and Lechevalier, 1976). The actinomycete *Streptomyces coelicolor* A3(2) produces the pigment actinorhodin which has antibiotic activity (Rudd and Hopwood, 1980) and a red pigment which consists of a mixture of prodigiosin derivatives (Tsao *et al.*, 1985). A novel tyrosine-derived pigment with anti-dermatophyte activity, designated SL-1 has recently been isolated from a mutant of *Streptomyces lavendulae* (Mikani *et al.*, 1987).

Among the Gram-negative bacteria, prodigiosin is produced by *Vibrio psychroerythreus* (D'Aoust and Gerber, 1974) and *Pseudomonas mangesiorubra* (Gandhi *et al.*, 1973). *Pseudomonas* sp are prolific producers; the extracellular, water-soluble yellow-green pigments produced by a range of fluorescent pseudomonads have been reviewed by Loper *et al.* (1984). Other pigment-producing Gram-negative bacteria include the yellow-pigmented epiphyte *Erwinia herbicola* (Billing and Baker, 1963) and the methanotrophic bacterium *Methylosinus trichosporium* which produces the bright red-violet pigment methylosin (Strauss *et al.*, 1983). The genus *Flavobacterium* originally consisted of a heterogenous collection of pigmented bacteria but has subsequently been restricted to seven species producing a range of pigments known as flexirubins (Shewan and McMeekin, 1983).

A particularly well characterised bacterial pigment system is that of prodigiosin production by *Serratia marcescens*. Williams *et al.* (1956) devised a simple procedure for the extraction of the pigment from *S. marcescens* and showed that it consisted of a single blue component and three red fractions which were further characterised by Green *et al.* (1956), and shown to have similar tripyrrole structures and to be prodigiosin-like compounds. Work by Hubert *et al.* (1969) and by Woods *et al.* (1971) on stable orange acriflavine-resistant variants of *S. marcescens* indicated that prodigiosin may be bound to the cell envelope.
A number of studies by Tsang and coworkers (Feng *et al.*, 1982; Mathis *et al.*, 1986; Cho *et al.*, 1987; Lowe and Tsang,

1987) have investigated the effects of various detergents on the biosynthesis of prodigiosin by *S. marcescens* and have confirmed the importance of the outer cell membrane in this process.

Prodigiosin is produced in *S. marcescens* via a bifurcated pathway involving the condensation of 2-methyl-3-amylpyrrole (MAP) and 4-methoxy-2,2'-bipyrrole-5-carboxyaldehyde (MBC), and mutants have been identified which are blocked in either the MAP or MBC pathway (Williams and Qadri, 1980). Qian *et al.* (1982) demonstrated pigment synthesising capability in transconjugants obtained from matings between nonpigmented mutants WF (producing MAP only) and 9-3-3 (producing MBC only). Dauenhauer *et al.* (1984) succeeded in the cloning and expression in *E. coli* of *S. marcescens* genes involved in prodigiosin biosynthesis. Two clones were obtained which could be induced to synthesise prodigiosin when supplied with either one or both of the terminal products of the bifurcated pathway. However, no clones contained the sequences encoding the entire prodigiosin pathway, even though large inserts of *S. marcescens* DNA were introduced into *E. coli* by use of a cosmid vector, the high rate of spontaneous mutation to unpigmented variants in some strains of *S. marsescens* suggests that extrachromosomal elements could be responsible for prodigiosin biosynthesis, but Dauenhauer *et al.* (1984) present data which support the idea that production of prodigiosin is not mediated by a plasmid in the strain *S. marcescens* (Nima) from which the prodigiosin biosynthesis genes were cloned.

The role of plasmids in pigment production has also been investigated in the Gram-negative yellow pigmented bacteria belonging to the herbicola group of the genus *Erwinia*. Chatterjee and Gibbins (1971) demonstrated the induction of nonpigmented variants of *E. herbicola* by incubation at supraoptimal temperatures and suggested that the loss of pigmentation and the concomitant occurrence of thiamine auxotrophy was due to the elimination of a plasmid. Gantotti and Beer (1982) presented evidence that pigmentation and thiamine prototrophy in *E. herbicola* is specified or controlled by genes carried on a large plasmid. Yellow pigmentation and thiamine prototrophy in a closely related strain, *Erwinia uredovora* has also been shown to be dependent on the presence of a large plasmid (Thiry, 1984).

Pigment production by the Gram-negative bacterium *Pseudomonas syringae* pv. *syringae* has also been analysed at the genetic level. A genomic library of this strain was constructed using a broad host range cosmid vector. Individual recombinant plasmids from this library were introduced into nonfluorescent (Flu^-) mutants. Four separate fluorescence restoration groups, each comprising mutants restored to fluorescence by one of four structurally distinct recombinant plasmids were identified, indicating that at least four genes or gene clusters are involved in the production of the fluorescent pigment of *P. s. syringae* (Loper *et al.*, 1984).

Amongst the Gram-positive bacteria, the production of actinorhodin and prodigiosin by *S. coelicolor* has been well characterised. Rudd and Hopwood (1980) found a red pigmented, highly non-polar compound in addition to the previously described (Wright and Hopwood, 1976) actinorhodin pigment in *S. coelicolor* A3(2) and presented evidence that the new pigment did not share the same biosynthetic pathway as actinorhodin. This red pigment was shown to be chemically very similar to prodigiosin from *S. marcescens* and co-synthesis experiments between non-producing mutants of *S. coelicolor* A3(2) and *S. marcescens* demonstrated a similar biosynthetic pathway for the pigments of these two species (Feitelson and Hopwood, 1983). The *S. coelicolor* A3(2) pigment was subsequently shown to be a mixture of prodigiosin derivatives (Tsao *et al.*, 1985). The production of the red pigment undecylprodigiosin in *S. coelicolor* A3(2) has been further studied by DNA cloning and biochemical analysis and a number of the *red* genes involved in this pathway have been characterised (Feitelson *et al.*, 1985). An autoregulatory factor designated A-factor, which was originally found in *S. griseus* and is widely distributed among actinomycetes, including *S. coelicolor* A3(2) and *S. lividans* has been implicated in the control of both actinorhodin and prodigiosin biosynthesis in *S. coelicolor* A3(2) (Horinouchi *et al.*, 1983; Horinouchi and Beppu, 1984).

The pigment of *N. corallina* ATCC 4273 was extracted and separated into a major crystalline red compound and a yellow oil. Initial characterisation indicated that the red pigment did not have a carotenoid structure (Brown and Clark, 1966). Further characterisation of the major red component indicated that a ring structure, a long aliphatic hydrocarbon chain and possible carbonyl groups were present (Stees *et al.*, 1969).

This study reports the cloning in the positive selection vector pLR591 of DNA fragments from *N. corallina* JL10 and *Nocardia* sp ATCC 21145 which code for pigment production in *E. coli*. The pigment producing clones were characterised and their expression in *E. coli* and *S. lividans* was investigated. Pigment was isolated and chemically characterised. One of the pigment producing clones was shortened and extensively mapped with restriction endonucleases with a view to its use in the development of a novel range of chromogenic cloning vectors.

4.1.2 Expression of actinomycete genes in *E. coli*

Gene expression of actinomycetes, including nocardioforms, is of interest because of the capacity of these organisms to produce many important antibiotics and chemicals and because of the medical importance of some actinomycetes. In addition, many actinomycetes undergo extensive morphological differentiation and the control of these processes could be elucidated by an understanding of the regulation of gene expression during cell differentiation. An understanding of gene expression mechanisms such as transcriptional control signals is obviously important in attempts to obtain the expression of actinomycete genes in other microorganisms.

Control of gene expression in actinomycetes has been best studied in *Streptomyces*. Studies on the expression of

Streptomyces genes in *E. coli* and *vice versa*, the isolation and analysis of *Streptomyces* promoters and the nature of RNA polymerases in *Streptomyces* have been important in this regard.

Some attempts to obtain expression of *Streptomyces* genes in *E. coli* have failed. Horinouchi *et al.* (1980) constructed comprehensive genomic libraries of four species of *Streptomyces* in *E. coli* plasmids but could not detect expression in any of the clones of *Streptomyces* DNA which would complement a number of *E. coli* auxotrophic mutants. A neomycin phosphotransferase gene from *Streptomyces fradiae* cloned in the *E. coli* plasmid pBR325 was not expressed in *E. coli*, except when cloned in the correct orientation to the *tet* promoter of pBR325, indicating that the barrier to its expression did not lie within the structural gene, but was rather due to differences in the start signals for transcription (Schupp *et al.*, 1983). Similarly, the chloramphenicol acetyltransferase gene of *Streptomyces acrimycini* cloned in the *E. coli* plasmid pBR322 was only expressed in *E. coli* when transcription was initiated by the *tet* promoter of pBR322 (Gil *et al.*, 1985). Bibb *et al.* (1985a) used promoter probe plasmid vectors to isolate putative promoter-containing DNA fragments of three *Streptomyces* antibiotic resistance genes and found that none of the three DNA fragments which were transcriptionally active in *Streptomyces* could initiate transcription when introduced into *E. coli*, and none of the sequences resembled typical *E. coli* promoters.

In contrast to this, the expression of the *E. coli* genes coding for resistance to the antibiotics chloramphenicol and kanamycin in *S. lividans* has been reported (Schottel *et al.*, 1981), with indications that initiation of transcription is from *E. coli* promoters in both cases. Studies on *Streptomyces* promoters isolated using promoter-probe vectors have helped to elucidate this issue. Bibb and Cohen (1982) investigated the function of a range of heterologous promoters in *S. lividans* using expression of the *E. coli* chloramphenicol acetyltransferase gene as an indicator of promoter activity. The promoter regions from *E. coli*, *S. marcescens* and *B. lichenformis* all appeared to be recognised by the RNA polymerase of *S. lividans*. However, the *Streptomyces* transcriptional control signals isolated did not function in *E. coli*. This led Bibb and Cohen (1982) to speculate that different classes of promoter exist in *Streptomyces*, recognised by different forms of RNA polymerase. Jaurin and Cohen (1984) found that mutations in the promoter region of the *ampC* B-lactamase gene of *E. coli* which led to increased synthesis of *ampC* mRNA in *E. coli* had a similar effect in *S. lividans*, suggesting that *S. lividans* has a RNA polymerase that recognises and uses promoters similar to those found in *E. coli*. Two forms of RNA polymerase were subsequently identified in *S. coelicolor* which recognised different promoter classes, and which may be important in selective expression of different gene sets during differentiation (Westpheling *et al.*, 1985).

Bibb *et al.* (1985b) pointed out that the existence of a form of RNA polymerase in *Streptomyces* capable of recognising typical prokaryote *E. coli*-like promoters predicts the occurrence of some streptomycete promoters resembling the consensus prokaryotic sequence (Hawley and McClure, 1983). Cloning and analysis of the promoter region of the erythromycin resistance gene (*ermE*) of *Streptomyces erythraeus* revealed that tandemly arranged promoters of typical prokaryotic appearance initiate transcription of the coding region of *ermE* (Bibb *et al.*, 1985b). A class of *E. coli*-type promoters was isolated from *S. lividans* by Jaurin and Cohen (1985). These transcriptional control regions, termed *Streptomyces*-*E. coli*-type promoters (SEP) were A+T-rich, digressing dramatically from the 73% G+C composition characteristic of the *Streptomyces* genome (Benigni *et al.*, 1975; Enquist and Bradley, 1971). The SEP contained DNA sequences characteristic of *E. coli* promoters in the '-35' and '-10' regions, but also contained novel structural features, including multiple direct repeats within the promoter region as well as a specific hexameric sequence in the vicinity of the mRNA start-point. The number of SEP in the *S. lividans* genome was estimated to be approximately 200. Therefore, if *S. lividans* contains approximately the same number of promoters as *E. coli* (i.e. approximately 2,000), the SEP constitute a relatively minor fraction of *S. lividans* promoters (Jaurin and Cohen, 1985).

Two recent reports support the idea that one type of RNA polymerase holoenzyme in *Streptomyces* recognises a class of promoters similar to the major consensus promoter of *E. coli*. Deng *et al.*, (1986) have described the expression of a promoter from the *Streptomyces* plasmid pIJ101 in both

S. lividans and *E. coli* and showed that the start-point of the RNA transcribed from this fragment was the same in both organisms. This suggests that the *E. coli* and *S. lividans* RNA polymerases recognise the same sequence determinants and choose the point of initiation of RNA synthesis in the same way. Buttner and Brown (1987) found that a similar situation existed in two promoters also cloned from pIJ101 and expressed in *E. coli*.

Present indications are therefore that *Streptomyces* sp contain different types of promoters, which act as substrates for different forms of RNA polymerase holoenzymes. A small fraction of these promoters which resemble the *E. coli* promoter consensus sequence are expressed in *E. coli*. Expression in *E. coli* of a *Streptomyces* gene with a promoter other than a SEP is dependent on transcription of the structural gene being initiated by an *E. coli* promoter.

The expression of mycobacterial DNA in *E. coli* has also been studied. Labidi *et al*. (1985) found indications that a sequence of DNA from a plasmid of *Mycobacterium fortuitum* may function as a promoter in *E. coli*. Kieser *et al*. (1986) inserted random DNA fragments from *Mycobacterium bovis* BCG into promoter-probe plasmids in *E. coli* and *S. lividans* and found that *S. lividans* efficiently utilised a high proportion of mycobacterial promoters, whereas a smaller fraction was more weakly expressed in *E. coli*.

The expression of actinomycete genes in *E. coli* can be further elucidated by investigations into gene expression of nocardioforms. The expression of pigment production by *E. coli* clones harbouring nocardial DNA, described in this chapter, provide a convenient system for these investigations.

4.1.3 Chromogenic vectors

Chromogenic vectors provide useful tools in molecular genetics for discrimination between clones containing recombinant plasmids (with inserted foreign DNA) and 'parental' clones containing vectors without inserted DNA. The *lac* system of the pUC and M13 vector series is an example of a system of this kind. This system utilises the conversion of the colourless compound 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) to a blue form by the enzyme β-galactosidase. β-galactosidase production is under control of the promoter *lacOP*, which can be induced by isopropylthiogalactosidase (IPTG). Transcription of β-galactosidase can be inactivated by cloning DNA fragments into the multiple cloning site which is situated between the promoter and the structural β-galactosidase gene in vectors of this type. Transcriptional inactivation is detected phenotypically by the growth of white rather than blue *E. coli* colonies on media containing X-gal. White colonies contain plasmids with inserted foreign DNA. Although this system is efficient, certain of the reagents required are expensive.

An example of a chromogenic vector system utilising a DNA fragment which directs synthesis of a pigment has been described by Horinouchi and Beppu (1985). These workers cloned a *S. coelicolor* A3(2) DNA fragment on the plasmid vector pIJ41 which codes for the production in *S. lividans* of a brown pigment, presumably a shunt product in the actinorhodin biosynthetic pathway. By subcloning the fragment, a promoter-probe plasmid vector (pARC1) was constructed which allowed chromogenic identification of transcriptional control signals in *S. lividans* based on the expression of the cloned pigment gene.

The isolation of a pigment gene which expresses phenotypically in *E. coli* would have great potential in the development of a system analogous to the *lac* system used in pUC and M13 vectors, with the advantage that the need for expensive reagents would be eliminated.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and plasmids

Table 4.1 lists bacterial strains and plasmids. Plasmids coding for pigment production were designated pig$^+$.

TABLE 4.1 Bacterial strains and plasmids.

Strain or plasmid	Genotype or genetic markers	Reference or source
N. corallina JL10	wild type	Microbiology Dept, University of Cape Town.
Nocardia sp	wild type	ATCC 21145
E. coli LK111	K514 derivative *lacI*$^-$ *lacZ*ΔM15 *lacY*$^+$	Zabeau & Stanley, 1982
S. lividans TK24	St^r SLP2$^-$ SLP3$^-$	Hopwood, D A[a]
S. griseus	wild type	ISP 5236
pLR591	Ap^r Ts^r EcoRI	This study
pNIL100	Ap^r Ts^r pig$^+$	This study
pNIL200	Ap^r Ts^r pig$^+$	This study
pNIL300	Ap^r Ts^r pig$^+$	This study
pNIL400	Ap^r Ts^r pig$^+$	This study
pNIL240	Ap^r Ts^r pig$^+$	This study
pNIL250	Ap^r Ts^r pig$^+$	This study
pNIL260	Ap^r Ts^r pig$^+$	This study
pNIL270	Ap^r Ts^r pig$^-$	This study
pUC18	Ap^r *lacZ*$^+$	Vieira & Messing 1982
pUC19	Ap^r *lacZ*$^+$	Vieira & Messing 1982
pNC18	Ap^r *lacZ*$^-$ pig$^+$	This study
pNC19	Ap^r *lacZ*$^-$ pig$^+$	This study
pNC181	Ap^r *lacZ*$^-$ pig$^+$	This study
pNC191	Ap^r *lacZ*$^-$ pig$^+$	This study

[a] Gift from D A Hopwood, John Innes Institute, Norwich, UK.

4.2.2 Standard methods, buffers and media

Appendix B lists standard techniques, buffers and media.

4.2.3 Identification of pigment-producing clones

Pigment-producing clones were readily apparent visually amongst the *E. coli* transformants comprising the genomic libraries of both *N. corallina* JL10 and *Nocardia* sp ATCC 21145. These pigmented, Ap^r *E. coli* colonies were subcultured and investigated further.

4.2.4 Plasmid preparation, restriction mapping and transformation studies

Plasmids were prepared from each of the Ap^r pigmented colonies by the method described in Appendix B.1.1. Competent *E. coli* LK111 cells were prepared and re-transformed with these purified plasmids by the procedures described in Appendix B.1.13. Restriction endonuclease maps of the plasmids were deduced after DNA agarose gel electrophoresis of fragments resulting from single, double and partial restriction endonuclease digestions of the purified plasmids with a range of restriction endonucleases. Restriction endonuclease digestion procedures are outlined in Appendix B.1.8.

4.2.5 Southern hybridisation studies

Two of the plasmids, designated pNIL100 (containing DNA from *N. corallina* JL10) and pNIL200 (containing DNA from *Nocardia* sp ATCC 21145), were radioactively labelled as described in Appendix B.1.10. These plasmids were used as probes to detect homologous sequences in chromosomal DNA isolated from *N. corallina* JL10 and *Nocardia* sp ATCC 21145 by Southern hybridisation studies. Chromosomal DNA was digested with *Pst*I endonuclease before agarose gel electrophoresis. The methods used for preparation of chromosomal DNA from *Nocardia* sp and for Southern hybridisations are described in Appendices B.1.5 and B.1.12. Washing of hybridisation membranes was for 15 min in 2 x SSC/0.1% (w/v) SDS at 68^{o}C, followed by 15 min in 1 x SSC/0.1% (w/v) SDS at 68^{o}C and a brief wash in 3 mM Tris base at room temperature.

4.2.6 Shortening of plasmid pNIL200

The plasmid pNIL200 was shortened by a succession of digestions with suitable restriction endonucleases, followed by ligation and selection for Ap^{r}, pigment-producing *E. coli* LK111 transformants (see Appendices B.1.8 and B.1.13 for methods). Suitable transformants were screened by small-scale *E. coli* plasmid DNA isolations (Appendix B.1.2). Shortened derivatives of pNIL200 were selected, and this procedure repeated until a point was reached where subsequent shortening resulted in transformants which were no longer able to produce pigment.

4.2.7 Subcloning of *Nocardia* sp ATCC 21145 DNA insert from pNIL250 into pUC18 and pUC19

The plasmid pNIL250, a shortened derivative of pNIL200, was digested with *Hin*dIII and *Eco*RV endonucleases to excise the *Nocardia* sp ATCC 21145 DNA insert from pNIL250. The plasmids pUC18 and pUC19 were digested with *Hin*dIII and *Sma*I endonucleases and ligated with the digested pNIL250 DNA. Competent *E. coli* LK111 cells were transformed with this ligation mix. Digestion, ligation and transformation procedures are outlined in Appendices B.1.8 and B.1.13. DNA was isolated from Ap^r, pigment-producing recombinants by the small scale *E. coli* DNA isolation procedure described in Appendix B.1.2. Restriction endonuclease mapping confirmed that the *Nocardia* sp ATCC 21145 DNA insert from pNIL250 had been inserted into pUC18 and pUC19; the resultant plasmids were designated pNC18 and pNC19. The plasmids pNC18 and pNC19 were subsequently shortened by digestion with *Bam*HI endonuclease followed by ligation in order to remove a 2.3 kb section of the *Nocardia* sp ATCC 21145 DNA insert. The resultant plasmids were designated pNC181 and pNC191.

Pigment production by *E. coli* LK111 transformants containing the plasmids pNC18, pNC19, pNC181 and pNC191 was assessed after growth at 30°C for 36 h on LB agar and LB broth media. Pigment was extracted from the liquid cultures by the method described in Section 4.2.9, and the absorbances of chloroform extracts of pigment were measured between 450 nm and 650 nm on a Beckman DU-40 spectrophotometer.

4.2.8 Transformation of *S. griseus* by the plasmids pNIL100, pNIL200 and pNIL400

Protoplasts of *S. griseus* were prepared and transformed by the plasmids pNIL100, pNIL200 and pNIL400, using the methods described in Appendices B.1.14 and B.1.15. Transformants were selected by overlaying the R2YE regeneration plates with SNA containing Ts to give a final Ts concentration of 25 µg/ml of agar. Plasmids were isolated from Ts^r transformants by the method described in Appendix B.1.3. The plasmids isolated from Ts^r transformants were confirmed to be intact pNIL100, pNIL200 and pNIL400 plasmids by restriction endonuclease mapping. Pigment production by the *S. griseus* transformants containing pNIL100, pNIL200 and pNIL400 was assessed on R2YE and M3 solid media and in M3 liquid media. The M3 liquid media were extracted with chloroform as described in Section 4.2.9 and the absorbances of the chloroform extracts were measured between 450 nm and 650 nm on a Beckman DU-40 spectrophotometer.

4.2.9 Extraction of pigment from liquid cultures

Extractions were performed on liquid cultures of *N. corallina* JL10 and *Nocardia* sp ATCC 21145 after 5 d growth in LB broth and M3 medium at 30°C, and on *S. griseus* transformants containing plasmids pNIL100, pNIL200 and pNIL400 grown in M3 medium at 30°C for 5 d. Pigment was also extracted from various *E. coli* LK111 transformants containing plasmids coding for pigment production. In all cases, cells were harvested by centrifugation at 16,000 x *g* for 10 min, resuspended in 10 ml water and disrupted by sonication. Suspensions were shaken with an equal volume of chloroform for at least 2 h and centrifuged at 16,000 x *g* for 5 min. Chloroform extraction was repeated and extracts pooled, evaporated to dryness and resuspended in 10 ml chloroform prior to measurement of absorbances on a Beckman DU-40 spectrophotometer.

4.2.10 Purification of pigment

Pigment extracts in chloroform obtained by the procedure described above were purified and separated into various components by preparative partition chromotography performed on a 60 cm x 3.4 cm glass column packed with silica gel 60 (Merck, Darmstadt, West Germany) using chloroform as the eluant.

4.2.11 Chemical characterisation of pigment components

The two components of purified pigment obtained by the above procedure were characterised by visible-UV light spectroscopy on a Beckman DU-40 spectrophotometer, infra-red spectroscopy on a Perkin-Elmer 983 spectrophotometer, and by ^{1}H, ^{13}C- and two-dimensional nuclear magnetic resonance (nmr) experiments on a Varian VXR-200 spectrometer.

The solubilities of the components of the purified pigment were determined in water, ethanol and methanol and components were acidified with 1 M HCl and treated with an alkaline solution (1 M NaOH).

4.3 RESULTS AND DISCUSSION

4.3.1 Identification of pigment producing clones

One pale blue colony of the *N. corallina* JL10 genomic library in *E. coli* DK1 was clearly distinguishable after 18 h incubation at 37°C, from the other *E. coli* transformants comprising the library, which formed translucent pale cream colonies. This colony became more pigmented after a further 24 h at room temperature, appearing dark blue. Two colonies of the *Nocardia* sp ATCC 21145 genomic library in *E. coli* LK111 were blue after 24 h at 37°C and remained this colour after 24 h at room temperature.

4.3.2 Plasmid preparation, restriction mapping and transformation studies.

The plasmid prepared from the pigmented colony of the *N. corallina* JL10 genomic library was designated pNIL100. Plasmids prepared from the dark blue colonies of the *Nocardia* sp ATCC 21145 genomic library were designated pNIL200 and pNIL300 and the plasmid from the pale pink colony of this library was designated pNIL400.

Mapping of these plasmids with a range of restriction endonucleases indicated that pNIL200 and pNIL300 were identical, and pNIL300 was not studied further. Restriction endonuclease maps of pNIL100 and pNIL400 are shown in

Fig. 4.1 and the map of pNIL200 is given in Fig. 4.2. The plasmid pNIL100 contained a DNA fragment of 10 kb inserted into the *bgl*II cloning site of the vector pLR591. Inserts in pNIL200 and pNIL400 were 15.1 kb and 5.0 kb respectively. The DNA inserts in these three plasmids were different since they had different restriction endonuclease maps.

The plasmids pNIL100, pNIL200 and pNIL400 transformed *E. coli* LK111 cells at a high frequency to produce pigmented Ap^r transformants. All transformants were pigmented, indicating that the DNA inserts coded for pigment production and were stably maintained in the pLR591 vector.

4.3.3 Southern hybridisation studies

The plasmid pNIL100 showed homology to four bands of the *N. corallina* JL10/*Pst*I endonuclease-digested chromosomal DNA (Fig. 4.3) and pNIL200 showed homology to six bands of *Nocardia* sp ATCC 21145/*Pst*I endonuclease-digested DNA (results not shown). This confirmed that the DNA inserts in pNIL100 and pNIL200 were derived from *N. corallina* JL10 and *Nocardia* sp ATCC 21145 respectively. Under the stringent washing conditions used, cross-homology between pNIL100 and *Nocardia* sp ATCC 21145 and pNIL200 and *N. corallina* JL10 was not detected.

4.3.4 Shortening of plasmid pNIL200

The plasmid pNIL200 with an insert size of 15.1 kb was shortened by the steps shown in Fig. 4.2 to produce the

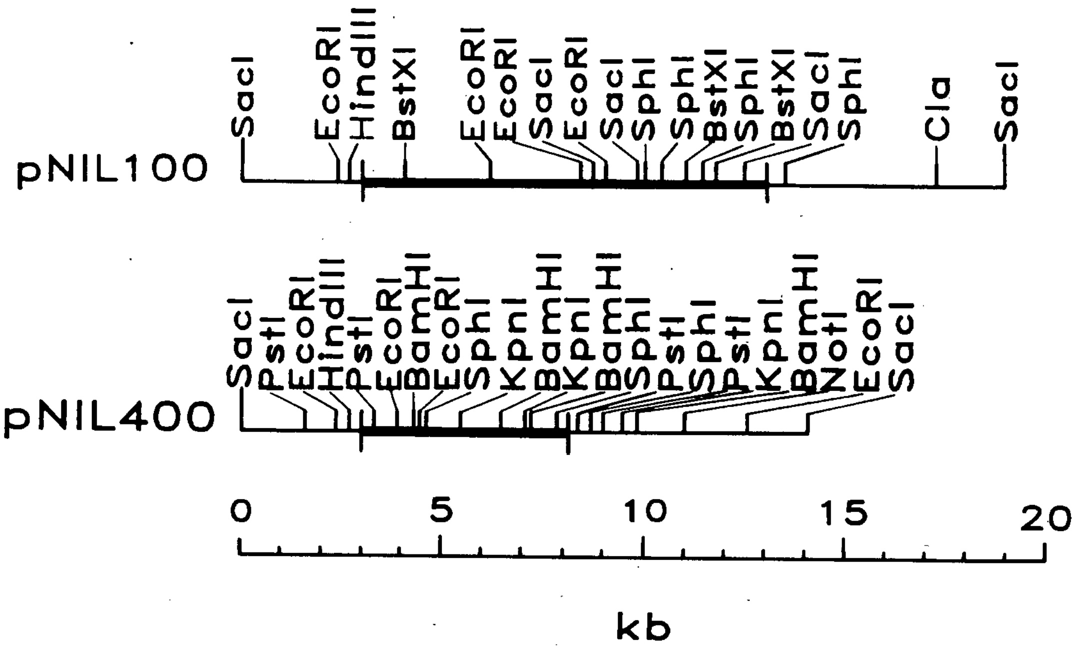

Fig. 4.1. Restriction endonuclease maps of pNIL100 and pNIL400. Bold lines represent chromosomal DNA inserts from *N. corallina* JL10 (in the case of plasmid pNIL100) and *Nocardia* sp ATCC 21145 (in the case of plasmid pNIL400). Thin lines represent DNA derived from the vector pLR591.

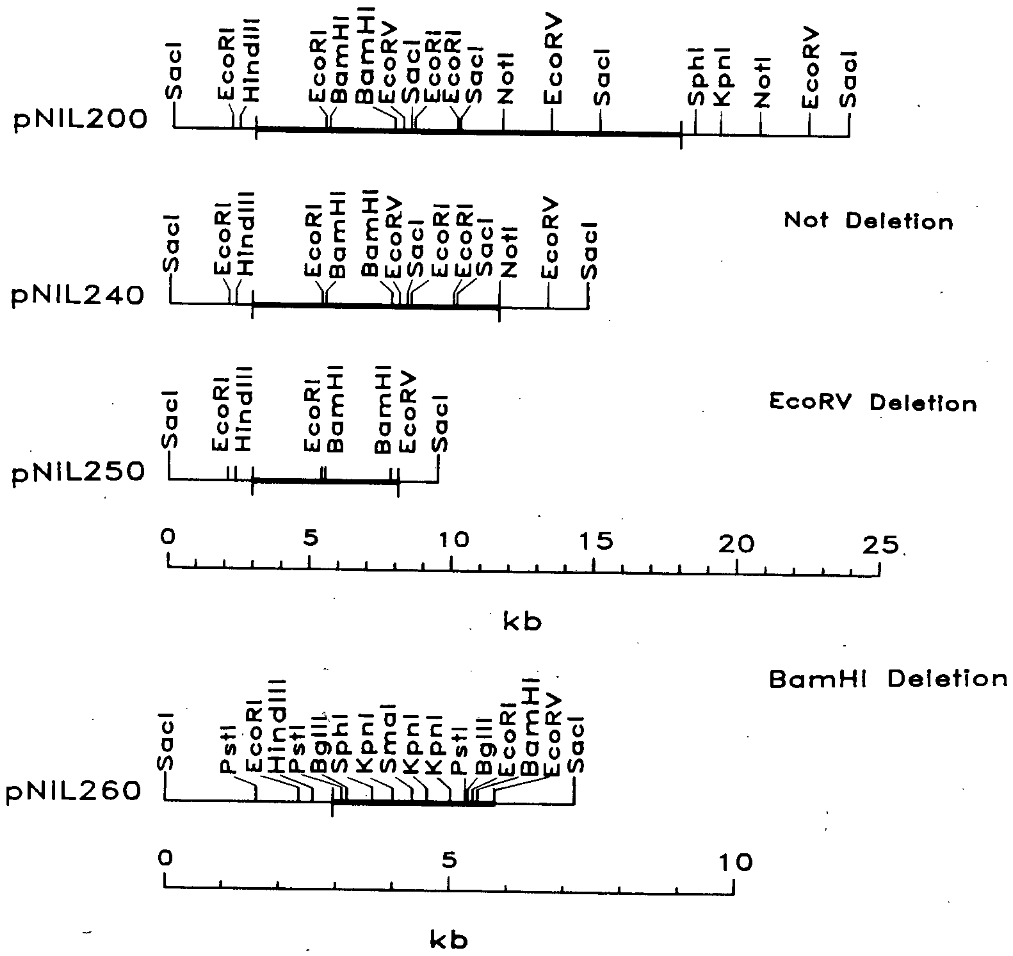

Fig. 4.2. Restriction endonuclease maps of pNIL200 and shortened derivatives of pNIL200. Bold lines represent chromosomal DNA inserts from *Nocardia* sp ATCC 21145 and thin lines represent DNA derived from pLR591.

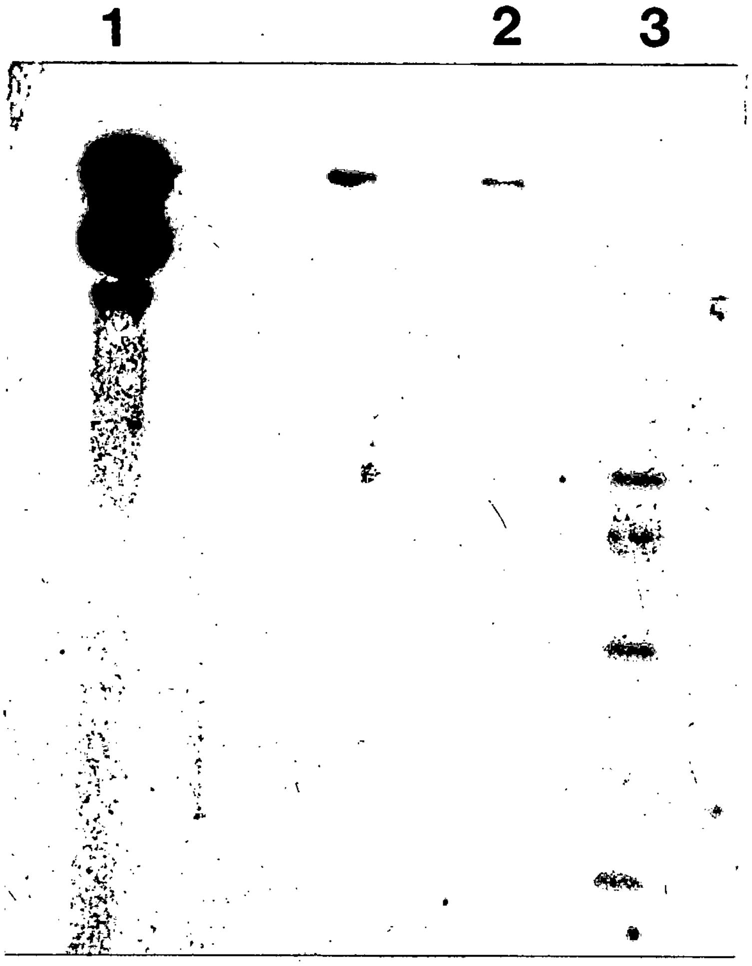

Fig. 4.3 Hybridisation of pNIL100 to *N. corallina* JL10 and *Nocardia* sp ATCC 21145 chromosomal DNA.
Lane 1 contains pNIL100, lane 2 is *Nocardia* sp ATCC 21145 chromosomal DNA digest with *Pst*I endonuclease and lane 3 contains *N. corallina* JL10 chromosomal DNA digested with *Pst*I endonuclease.

plasmids pNIL240, pNIL250 and pNIL260 with insert sizes of 8.7, 5.2 and 2.8 kb respectively. These plasmids all produced pigmented colonies on transformation into *E. coli* LK111. However, a further shortening of pNIL260 (by deletion of the *Sma*I-*Eco*RV section of the insert) gave pNIL270 which no longer coded for pigment production (Fig. 4.4).

The primary objective in the shortening of the plasmid pNIL200 was to isolate as small a fragment of DNA as possible that coded for pigment production in *E. coli* and can therefore be used in the development of a chromogenic vector of the type discussed in section 4.1.3. The reduction of the insert size required for pigment production from 15.1 kb in pNIL200 to 2.8 kb in pNIL260 will greatly facilitate the development of such a vector. The plasmid pNIL200 was selected for shortening rather than pNIL100 or pNIL400 because the insert in pNIL200 coded for the most pronounced, rapid pigment production, resulting in markedly pigmented *E. coli* colonies after 18 h growth at 37^{o}C.

Coding of pigment production by a DNA insert of only 2.8 kb suggests that the pathway resulting in pigment production is not a complex one requiring many enzymes, as an insert of this size could only code for one, two or at most three genes. Work in progress has resulted in further shortening of pNIL260 to produce a plasmid coding for pigment production, which contains a DNA insert of only 1.9 kb (S Hart, Dept of Microbiology, University of Cape Town,

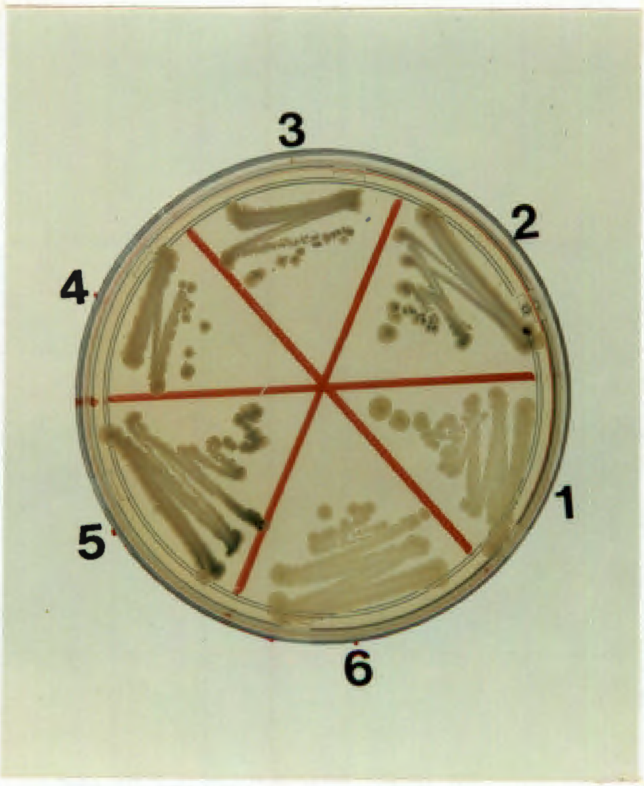

Fig. 4.4. *E. coli* LK111 transformants containing pNIL200 and pNIL200 derivatives, grown at 37°C on LB agar containing 100 µg/ml AP.

1. *E. coli* LK111/pLR591
2. *E. coli* LK111/pNIL200
3. *E. coli* LK111/pNIL240
4. *E. coli* LK111/pNIL250
5. *E. coli* LK111/pNIL260
6. *E. coli* LK111/pNIL270

personal communication). This strongly suggests that pigment production by this plasmid is coded for by a single gene. This further shortening has resulted in a very small fragment of DNA for use in the construction of a chromogenic vector of the type discussed in section 4.1.3.

4.3.5 Subcloning of *Nocardia* sp ATCC 21145 DNA insert from pNIL250 into pUC18 and pUC19

Subcloning of the pigment producing DNA insert from pNIL250 into pUC18 and pUC19 resulted in this insert being present in both orientations relative to the *lacOP* promoter present in pUC18 and pUC19. Pigment production by cells containing the plasmids pNC18 and pNC19 was assessed in liquid medium (Fig. 4.5) and solid medium (Fig. 4.6). Both plasmids coded for pigment production, but cells containing pNC19 produced markedly more pigment in both solid and liquid media than pNC18. The DNA insert in pNC19 is in the same orientation to the *lacOP* promoter that the insert was to the λ P_r promoter of pLR591. The high level of pigment production in this orientation could therefore result from initiation of transcription from the *lacOP* promoter in pNC19 and the λ P_r promoter in pNIL250. However, there is some pigment production when the DNA insert is in the opposite orientation from the *lacOP* promoter (as in pNC18). In this case, initiation of transcription cannot be from the *lacOP* promoter as this promoter is in the reverse orientation from the insert; transcription must be initiated from within the insert. This implies that there is a region within the inserted *Nocardia* DNA which is functioning as a promoter in

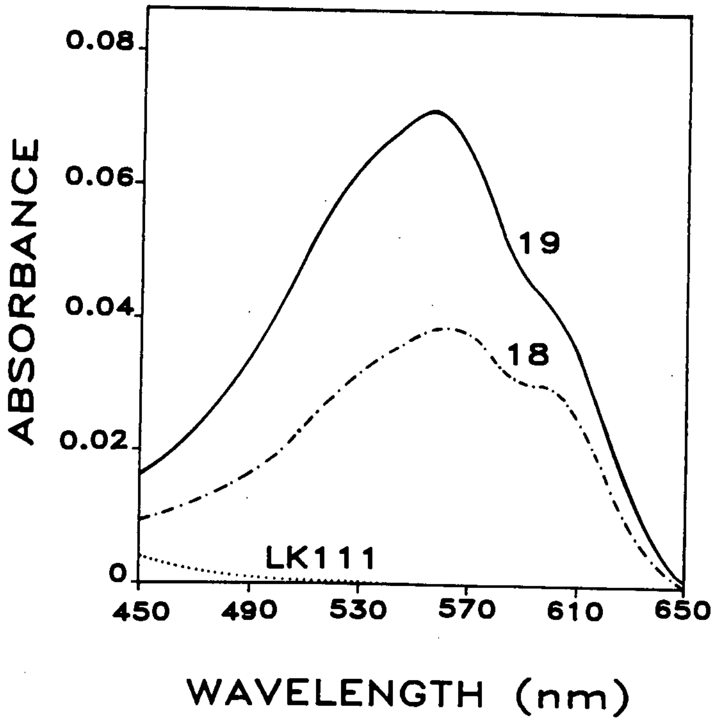

Fig. 4.5 A. Absorbance at wavelengths between 450 and 650 nm of chloroform extracts of *E. coli* LK111 cultures containing the plasmids pNC18 (—·—·—) and pNC19 (————), and untransformed *E. coli* LK111 cells (··················).

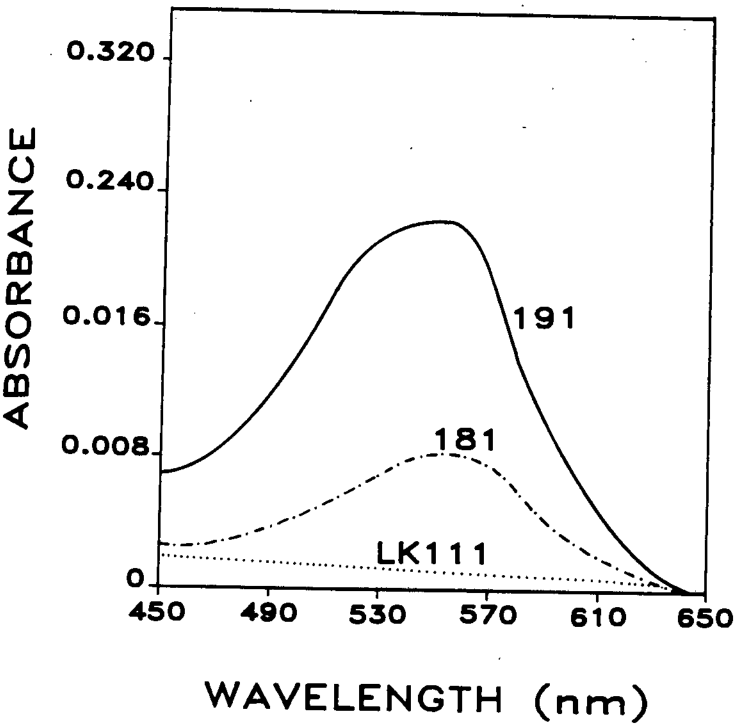

Fig. 4.5 B. Absorbance at wavelengths between 450 and 650 nm of chloroform extracts of *E. coli* LK111 cultures containing the plasmids pNC181 (—·—·—) and pNC191 (————), and untransformed *E. coli* LK111 cells (··················).

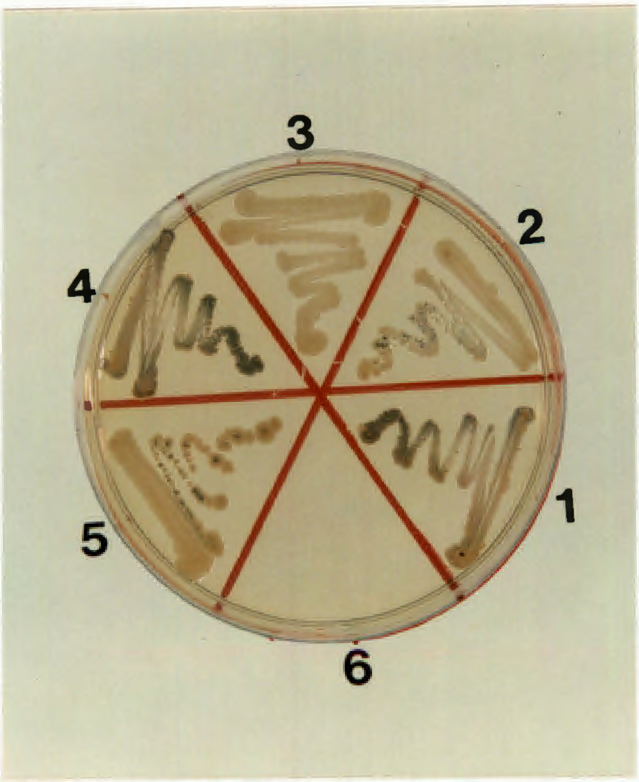

Fig. 4.6. *E. coli* LK111 transformants grown at 37°C on LB agar containing 100 µg/ml Ap and harbouring the following plasmids:
1. pNC19
2. pNC18
3. pUC18
4. pNC191
5. pNC181

E. coli. If pigment production was being controlled solely by this promoter-like region in the *Nocardia* DNA insert, pigment production in cells containing pNC18 would be the same as in those containing pNC19. The difference in levels of pigment production coded by pNC18 and pNC19 is best explained by a low level of pigment production resulting from initiation of transcription from a region within the inserted *Nocardia* DNA functioning as a promoter in *E. coli* in both pNC18 and pNC19, with pigment production being increased to a higher level in pNC19 by transcription initiation from the *lacOP* promoter. Initiation of transcription in pNIL200 is likely to occur in the same way as in pNC19.

The shortened plasmids pNC181 and pNC191 showed the same patterns of pigment production as pNC18 and pNC19 (Figs 4.4 and 4.5). A different pattern would only have resulted if the region of *Nocardia* DNA acting as a promoter in *E. coli* had been present on the fragment of DNA removed by BamHI endonuclease digestion from pNC18 and pNC19 in the construction of pNC181 and pNC191. If this had been the case, pNC181 would not have produced pigment, as there would no longer be a promoter to initiate transcription in the correct orientation to the gene, whereas pNC191 would still have resulted in pigment production (although at a lower level than pNC19) with initiation of transcription from the *lacOP* promoter.

4.3.6 Transformation of *S. griseus* by the plasmids pNIL100, pNIL200 and pNIL400

The plasmids pNIL100, pNIL200 and pNIL400, when transformed into *S. griseus*, did not result in detectable pigment production by the non-pigmented *S. griseus*. The close relationship between nocardioforms and streptomycetes makes it likely that the region of *Nocardia* DNA in pNIL200 that functions as a promoter in *E. coli* (discussed above) would also be likely to initiate transcription in *S. griseus*, even if the λ P_r promoter of pNIL200 does not function in this organism. Even though pNIL200 may be transcribed in *S. griseus*, this organism may have remained unpigmented because the correct metabolic conditions for expression of pigment production are lacking in *S. griseus*. Pigment production by pNIL100 and pNIL400 may not occur in *S. griseus* for this reason or because these plasmids (which may have only the λ P_r promoter and no promoter region from *Nocardia* sp ATCC 21145) are not transcribed in *S. griseus*.

4.3.7 Extraction and purification of pigment from liquid cultures

Pigment was readily extracted from cultures of *E. coli* LK111 containing the plasmids pNIL100, pNIL200 and pNIL400 by the procedure described in section 4.2.8. Purification by preparative column chromatography (section 4.2.9) yielded pink and blue fractions from pNIL100 and pNIL200 and a pink fraction only from pNIL400. Cultures of *E. coli* LK111 containing pNIL200 were used for the preparation of large amounts of pigment for analysis as cultures containing this plasmid yielded the largest amounts of plasmid; 24 mg of the blue fraction and 12 mg of the pink fraction were obtained from a 400 ml culture.

Attempts to extract pigment from cultures of *N. corallina* JL10 and *Nocardia* sp ATCC 21145 were unsuccessful, although these cultures were buff-coloured after 3 d growth at 30°C. Chloroform extracts of these cultures contained no pigment. Extraction procedures used in previous successful isolations of pigments from *N. corallina* (Brown and Clark, 1966; Stees *et al.*, 1969) were far harsher than those used in this study. The pigments may be too tightly bound to cell debris to be extracted by gentle shaking with chloroform.

4.3.8 Chemical characterisation of pigment components

The blue and pink components of pigment described in section 4.3.7 exhibited absorption maxima of 560 nm and 610 nm respectively. Both compounds were soluble at low concentrations in chloroform and insoluble in water, ethanol and methanol. The compounds flocculated at concentrations above 20 mg/ml in chloroform. Addition of acid or alkali to the compounds did not cause any colour change.

Infra-red and nmr spectroscopy was done on the blue compound. The infra-red spectrum indicated the presence of hydroxyl groups (3,776 and 3,682/cm), aliphatic and aromatic C-H groups (3,017 to 2,750/cm), possibly a P-H or C=N group (2,433 and 2,399/cm), carbonyl groups (1,720/cm), C=C or C=N groups (1,622, 1,601 and 1,581/cm) and other signals attributable to C-N, C-C, C-O and C-H groups.

The ^{1}H-nmr spectrum showed 3 main groups of signals. Strong resonances were detected at δ0.8-1.3 due to CH_3 and aliphatic CH_2 groups, and signals at medium field (δ 4.23) and low field (δ7.5) resonances. A similar grouping of signals was found for the ^{13}C resonances. Assignment of these ^{1}H and ^{13}C resonances was aided by use of the HETCOR experiment (Bax, 1983) which established connectivities between the carbon atoms and attached protons and by use of the attached proton test (Patt and Shoolery, 1982) which distinguished between CH, CH_2 and CH_3 ^{13}C resonances. The chemical shift data and assignments are presented in Table 4.2.

These chemical data indicate overall that the pigment obtained from *E. coli* clones containing pNIL200 did not resemble actinorhodin or the prodigiosin-type pigments. Further chemical analyses will be necessary to ascertain a definitive structure but indications are that the cloned pigment may be carotenoid in nature, and consists of long chains of aliphatic CH_2 groups and CH_3 groups, together with olefinic groups in conjunction with carbonyl moieties.

4.4 CONCLUSION

The cloning of pigment genes from *Nocardia* sp and their expression in *E. coli* has resulted in a system whereby large

TABLE 4.2 Nmr data (200 MHz) for the pigment extracted from *E. coli* LK111 harbouring the plasmid pNIL200.

Peak	^{13}C-nmr	^{1}H-nmr			
	δ^{a} (ppm)	δ^{a} (ppm)	Multiplicity^{b}	Integral proton	Assignments
a	14.2	0.82	d or t	12	CH_3-
b	23.9	1.16	s or		
c	25.9	1.23	unresolved	18	$-CH_2-$
d	30.0	1.31	multiplet		
e	28.7	1.62	d or t	6	$-CH_2-$
f	65.8	4.23	t	3	-OCH2-/=CH-CH2-
g	130.2	7.48	m	3	-CH=
h	128.1	7.69	m		

[a] chemical shift in parts per million (ppm)
[b] multiplicity of signals indicated (s = singlet; d = doublet; t = triplet and m = multiplet)

amounts of a possibly novel pigment can be produced for characterisation and identification. In the longer term, this system may also have application in the industrial production of pigment.

Expression of genes from nocardioforms in *E. coli* is of value in the elucidation of control of actinomycete gene expression and this work indicated that some *Nocardia* promoters may function in *E. coli* in the same way as some *Streptomyces* promoters have been found to function in *E. coli*.

An exciting possibility resulting from the cloning of DNA which codes for the production of pigment in *E. coli* is the application of this DNA in the production of a cheap and convenient chromogenic vector. This project is currently being pursued.

CHAPTER FIVE

STUDIES ON SQUALENE UTILISATION BY *NOCARDIA* SP

CHAPTER FIVE

STUDIES ON SQUALENE UTILISATION BY *NOCARDIA* SP

Summary: Three of the ten *Nocardia* sp tested were able to utilise squalene. The degradation products produced by these three strains were investigated by thin layer chromatography. A genomic library of one of the three strains, *Nocardia* sp ATCC 21145 in *E. coli* LK111 and *S. lividans* TK24, was screened for squalene-utilising clones. Induction of proteins by squalene was investigated in *Nocardia* sp ATCC 21145 but no new or increased amounts of cytoplasmic or extracellular proteins were detected.

5.1 INTRODUCTION

Squalene is an acyclic triterpene consisting of six isoprene units, and is ubiquitous as a natural product. Squalene is formed from two molecules of farnesyl pyrophosphate in a reaction catalysed by the enzyme squalene synthetase, recently partially purified by Kuswik-Rabiega and Rilling (1987). Squalene is an important precursor of sterols. In animal and fungal cells, squalene is epoxidised to give 2,3-oxidosqualene which is then cyclicised to form lanosterol, which is converted to cholesterol.

One of the functions of sterols in eukaroytic cells is in the stabilisation of membranes. Sterols occur in only a few prokaryotes; however, many bacteria synthesise a class of pentacyclic triterpenoids, called hopanoids, which may fulfill a role in bacterial membranes similar to that of sterols in eukaryotes (Seckler and Poralla, 1986). Squalene is an intermediate in the biosynthesis of hopanoids.

Squalene has been found to occur in a number of different bacteria. The extremely halophilic bacterium *Halobacterium cutirubrum* contains squalene as a component of its cellular lipids (Tornabene *et al.*, 1969). Amdur *et al.* (1978) report the presence of squalene in 64 strains of Gram-positive bacteria in the genera *Actinomyces, Bacterionema, Corynebacterium, Propionibacterium, Rothia, Bacillus* and *Streptococcus*. Squalene and dehydrosqualene have been found in the neutral lipid fraction of mycelia and membranes of *Streptomyces hygroscopicus, S. griseus* and *Streptomyces noursei* (Gräfe *et al.*, 1985). Ourisson *et al.* (1987) have recently comprehensively reviewed the occurrence and function of bacterial hopanoids.

The bacterial biotransformation of squalene is of interest as it may provide a method for the production of useful starting materials for organic syntheses, and also provides a model system for the degradation of branched olefinic compounds in natural systems (Seo *et al.*, 1983). Yamada *et al.* (1975) isolated an *Arthrobacter* sp which specifically cleaved the squalene molecule at its centre to produce two geranylacetone molecules. Seo *et al.* (1981; 1983) have described the biotransformation of squalene by *Corynebacterium* sp S-401 and *Corynebacterium* sp SY-79 to produce mono-, di-, tri- tetra- and pentahydrated squalene, and squalenedioic acid respectively. *Corynebacterium* sp SY-79 also oxidised terpene alcohol derivatives and a number of squalene analogs such as lycopersene, geranylfarnesyl, digeranyl and 2-hydroxy-2,3-dihydrosqualene to give a range of metabolites (Yamada *et al.*, 1985).

In this investigation, the utilisation of squalene by *Nocardia* sp was studied. Nocardioform bacteria have been shown to interconvert steroid compounds, for example to convert cholesterol to substances which are precursors of steroid hormones or oral contraceptives (Ferreira *et al.*, 1984). This group of bacteria could therefore be of interest in the biotransformation of squalene. In addition a *Nocardia* sp has been shown to be capable of degrading natural rubber (Tsuchii *et al.*, 1985) which, like squalene, is a polymer of isoprene units. The utilisation of squalene by *Nocardia* sp could therefore provide a simple system for the study of the mechanisms of chain scission and the properties of enzymes implicated in rubber degradation by *Nocardia* sp.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains

Table 5.1 lists bacterial strains.

TABLE 5.1. Bacterial strains.

Strain	Source
N. canicruria	ATCC 17896
Nocardia paraffinae	ATCC 21509
N. meditterranei	ATCC 13685
N. globerula	ATCC 21022
N. orientalis	ATCC 19795
N. uniformis subspecies *tsuyamanensis*	ATCC 21806
N. globerula	ATCC 21505
Nocardia autotrophica	ATCC 13181
Nocardia sp	ATCC 21145
N. corallina JL10	Microbiology Dept, University of Cape Town.

5.2.2 Standard methods, buffers and media

Appendix B lists standard techniques, buffers and media.

5.2.3 Utilisation of squalene by *Nocardia* sp

The utilisation of squalene by the ten *Nocardia* sp listed in Table 5.1 was tested in complete and minimal media. Cultures (100 ml) of M3 broth and M9 minimal medium (Appendix B.2) containing 1% (w/v) squalene (Merck) were inoculated from M3 agar plate cultures of the *Nocardia* sp. Cultures were incubated at 30°C with rotary shaking at 150 rpm for 10 d, acidified to pH 2 - 3 and extracted three times with 100 ml dichloromethane (Seo *et al.*, 1981). After evaporation of the dichloromethane at room temperature in a fume hood, samples were dried with anhydrous sodium sulphate and the mass of extract recovered from each culture was determined. Control cultures consisted of sterile M3 broth and M9 minimal medium containing 1% (w/v) squalene, and M3 broth and M9 minimal medium cultures of each *Nocardia* sp without added squalene. Control cultures were extracted in the same manner as the test cultures. The *Nocardia* sp showing utilisation of squalene in this initial screen were re-tested using the same procedure.

5.2.4 Screening for squalene degradation products using thin layer chromatography (TLC)

The fractions obtained by dichloromethane extraction in Section 5.2.3 were resolved by TLC. Each fraction was evaporated down to a volume of 10 ml, and 5 µl samples were applied to precoated silica gel plates (Merck silica gel F_{254} 0.25 mm thick, 20 x 20 cm). Plates were developed using a n-hexane-ether (10:1) solvent system (Seo *et al.*, 1983) in a sealed, glass chromatography tank, lined with filter paper soaked in the solvent. The solvents used were supplied by Merck (LiChrosolv grade). After development, plates were air dried, sprayed with 10% (w/v) molybdatophosphoric acid in ethanol and heated to 120°C for 15 min.

5.2.5 Protein studies on *Nocardia* sp ATCC 21145

M3 medium (100 ml) and M9 minimal medium (100 ml) with and without 1% (w/v) squalene were inoculated with *Nocardia* sp ATCC 21145 and incubated for 5 d with rotary shaking at 30°C. Cells were harvested by centrifugation at 12,000 x *g* for 30 min and resuspended in 5 ml water. Cells were disrupted by sonication with a MSE Ultrasonic Disintegrator set at 25 kHz (3 x 0.5 min bursts, with 1 min intervals between bursts). An equal volume of SDS sample buffer (Appendix B.2) was added and samples were boiled for 2 min. Proteins were resolved by discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970; O'Farrell, 1975) as described in Appendix B.1.16.

Supernatant fluids were concentrated to a volume of 2 ml in an Amicon ultrafiltration cell (Model 202, Amicon Corp., MA, USA) using a 10,000 D $\underline{M}_r$ cut-off ultrafiltration membrane (Amicon Diaflo PM10). After addition of an equal volume of

SDS sample buffer, samples were boiled for 2 min and supernatant fluid proteins were resolved by discontinuous SDS-PAGE.

5.2.6 Studies on binding of *Nocardia* sp ATCC 21145 supernatant fluid proteins to squalene substrate

The supernatant fluid (1 ml) derived from *Nocardia* sp ATCC 21145 grown in M3 medium with 1% (w/v) squalene (described above) was gently shaken with 0.5 ml squalene for 1 h at 30°C. Squalene was separated from the supernatant fluid by low-speed centrifugation. Samples (20 µl) of the supernatant fluid, taken before and after treatment with squalene, were mixed with an equal volume of SDS sample buffer and resolved by discontinuous SDS-PAGE.

5.2.7 Screening of the *Nocardia* sp ATCC 21145 genomic library for squalene-utilising clones

To screen for squalene-utilising *E. coli* transformants, the ten pools of *E. coli* LK111 transformants harbouring recombinant plasmids comprising the genomic library of *Nocardia* sp ATCC 21145 (see Section 3.2.6) were each inoculated into 100 ml of LB broth containing 1% (w/v) squalene and 100 ml of M9 minimal medium (supplemented with 0.1% yeast extract) containing 1% (w/v) squalene. A similar pair of control cultures was inoculated with *E. coli* LK111 containing pLR591. All cultures contained 50 µg/ml Ap. Cultures were incubated at 37°C with rotary shaking for 10 d.

The ten pools of spores harvested from *S. lividans* TK24 transformants representing the genomic library of *Nocardia* sp ATCC 21145 (see Section 3.2.7) were used as inocula to screen for squalene-utilising *Streptomyces* transformants. Each spore pool was inoculated into 100 ml of M3 medium containing 1% (w/v) squalene and 100 ml of M9 minimal medium (supplemented with 0.1% yeast extract) containing 1% (w/v) squalene. A similar pair of control cultures was inoculated with *S. lividans* TK24 transformed with pLR591. All cultures contained 5 µg/ml Ts. Cultures were incubated at 30°C with rotary shaking for 21 d.

The cultures were extracted as described in Section 5.2.3 and the extracts resolved by TLC as described in Section 5.2.4.

5.3 RESULTS AND DISCUSSION

5.3.1 Utilisation of squalene by *Nocardia* sp

Table 5.2 lists masses of product extracted by dichloromethane from complete and minimal medium cultures of ten *Nocardia* sp. The organic solvent dichloromethane would extract unmetabolised squalene and squalene derivatives. This initial screen indicated that *N. canicruria*, *N. globerula* ATCC 21505 and *Nocardia* sp ATCC 21145 were able to utilise squalene during growth in a complete medium. These three strains were therefore re-tested (results are

TABLE 5.2. Amount of product extracted by dichloromethane from complete medium (M3) and M9 minimal medium cultures of *Nocardia* sp after 10 d incubation at 30°C. Initial mass of squalene in all cultures was 1 g.

Strain	Amount of product recovered (g)	
	M3 medium	M9 minimal medium
N. canicruria	0.28	0.59
N. paraffinae	0.67	0.75
N. mediterranei	0.66	0.81
N. globerula ATCC 21022	0.59	0.71
N. orientalis	0.57	0.74
N. uniformis	0.64	0.72
N. globerula ATCC 21505	0.28	0.66
Nocardia sp ATCC 21145	0.30	0.60
N. corallina JL10	0.63	0.79
Sterile control	0.84	0.82

presented in Table 5.3). Utilisation of squalene by *N. canicruria*, *N. globerula* ATCC 21505 and *Nocardia* sp ATCC 21145 in complete medium was confirmed. Squalene also appeared to be utilised to some extent in minimal medium cultures by these three strains (Table 5.2), indicating that squalene may be utilised when present as the sole carbon source.

5.3.2 Screening for squalene degradation products using TLC

Initial screening of the extracts from cultures of the ten *Nocardia* sp grown in M3 medium revealed traces of squalene degradation products from *N. canicruria* and *N. globerula* ATCC 21505 and larger amounts of squalene degradation products from *Nocardia* sp ATCC 21145 (Fig. 5.1). Degradation products were absent in extracts from cultures of the other seven *Nocardia* sp tested. Re-testing of *N. canicruria*, *N. globerula* ATCC 21505 and *Nocardia* sp ATCC 21145 confirmed the presence of squalene degradation products in cultures of these organisms (Fig. 5.2). Three compounds with R_f values of 0.25, 0.21 and 0.06 were present in small amounts in extracts from sterile control cultures. These three compounds were also found in extracts from cultures of the three *Nocardia* sp investigated. The compound with an R_f value of 0.25 was present in larger amounts in extracts from *N. canicruria* and *N. globerula* ATCC 21505 than in the sterile control cultures. The *Nocardia* sp ATCC 21145 cultures contained relatively large amounts of the compound with a R_f value of 0.06, and in

TABLE 5.3. Amount of product extracted by dichloromethane after growth of *N. canicruria*, *N. globerula* ATCC 21505 and *Nocardia* sp ATCC 21145 in complete medium (M3) for 10 d at 30°C. The range of masses of product recovered in three experiments is given. Initial mass of squalene in all cultures was 1 g.

Strain	Amount of product recovered (g)
N. canicruria ATCC 21505	0.43 - 0.54
Nocardia sp ATCC 21145	0.27 - 0.39
Sterile control	0.83 - 0.97

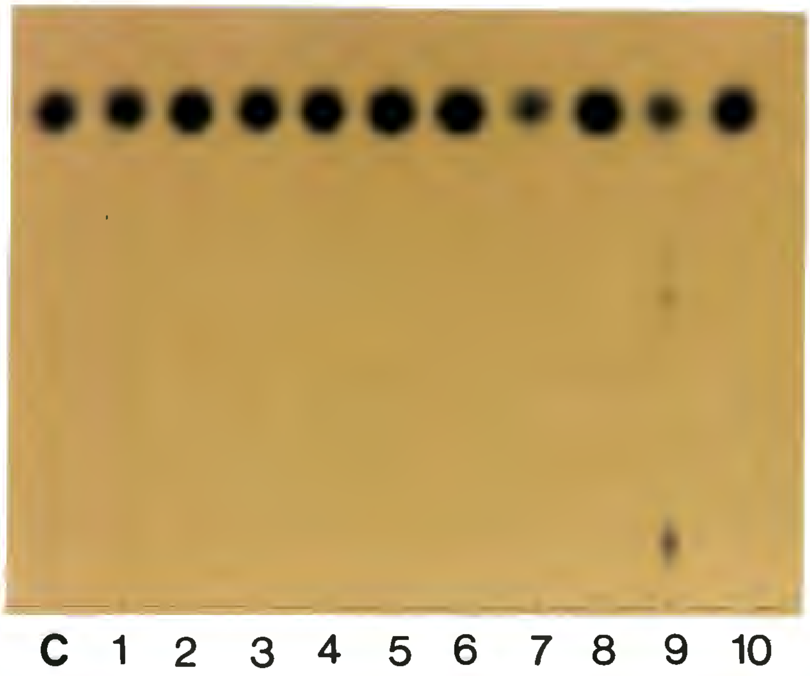

Fig. 5.1. TLC screen of dichloromethane extracts from cultures of ten *Nocardia* sp grown in M3 medium containing 1% (w/v) squalene. Details of TLC procedure are given in Section 5.2.4.

	Lane	Species	*ATCC No. or source*
Key:	1	*N. canicruria*	17896
	2	*N. paraffinae*	21509
	3	*N. meditterranei*	13685
	4	*N. globerula*	21022
	5	*N. orientalis*	19795
	6	*N. uniformis* subsp *tsuyamanensis*	21806
	7	*N. globerula*	21505
	8	*N. autotrophica*	13181
	9	*Nocardia* sp	21145
	10	*N. corallina* JL10	Microbiology Dept. University of Cape Town.

Lane C is an extract from a sterile culture of M3 medium containing 1% (w/v) squalene.

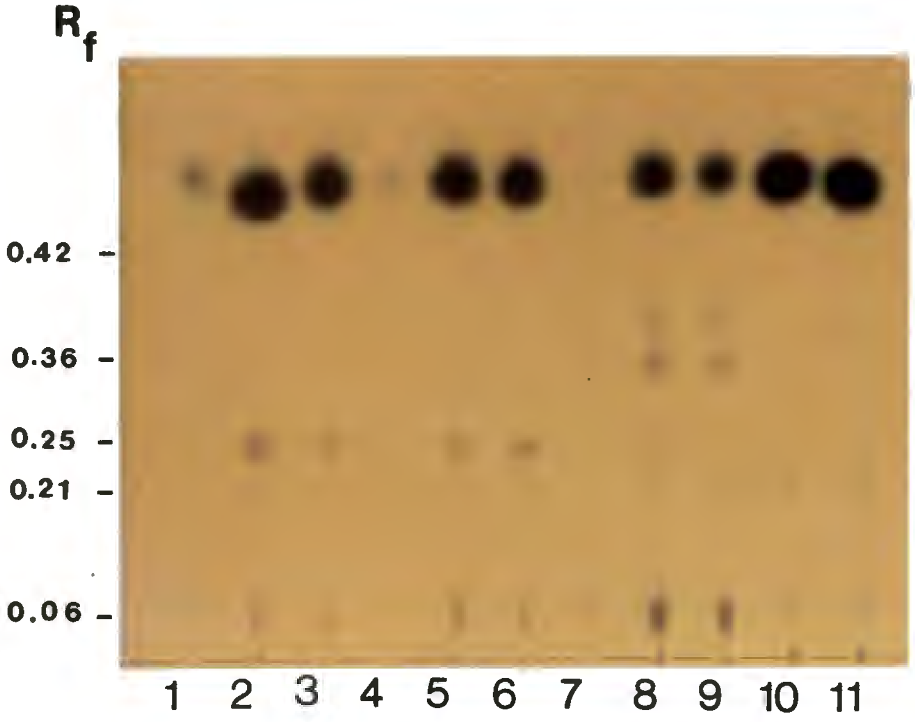

Fig. 5.2. TLC of extracts from cultures of *N. canicruria* ATCC 17896, *N. globerula* ATCC 21505 and *Nocardia* sp ATCC 21145 grown in M3 medium with and without 1% (w/v) squalene. Squalene-containing cultures were done in duplicate. Details of TLC procedure are given in Section 5.2.4.

Key

Lane	Species	Medium
1	*N. canicruria*	M3
2 & 3	*N. canicruria*	M3 + 1% (w/v) squalene
4	*N. globerula*	M3
5 and 6	*N. globerula*	M3 + 1% (w/v) squalene
7	*Nocardia* sp ATCC 21145	M3
8 and 9	*Nocardia* sp ATCC 21145	M3 + 1% (w/v) squalene
10 and 11	Sterile	M3 + 1% (w/v) squalene

addition contained two novel squalene degradation products with R_f values of 0.42 and 0.36.

The amounts of extracts recovered from the cultures of *N. canicruria, N. globerula* ATCC 21505 and *Nocardia* sp ATCC 21145 were significantly less than the initial mass of squalene present in these cultures (Table 5.2). This suggests that the microbial degradation of squalene by these organisms may also lead to other products (besides those detected by TLC) which are metabolised by the bacteria and are therefore not detected as degradation products.

It is interesting to note the presence of trace amounts of a compound with the same R_f value as squalene in extracts from control cultures of the three *Nocardia* sp grown in M3 medium without added squalene (Fig. 5.2). This suggests that squalene may be present in these *Nocardia* sp; Amdur *et al.* (1977) reported the presence of squalene in 64 out of 73 Gram-positive bacteria.

The stain used for visualisation of squalene and squalene degradation products was molybdatophosphoric acid, which specifically stains reducing compounds, lipids, sterols and steroids (Kritchevsky and Kirk, 1952). Compounds running at lower R_f values than squalene (using the same solvent system as in this study) were shown by Seo *et al.* (1983) to be oxidised metabolites of squalene. Compounds of this type are important as precursors for a range of biochemicals including steroids (Yamada *et al.*, 1985). As *Nocardia* sp ATCC 21145 produced the greatest range of squalene degradation products, this strain was selected for further study. The induction of proteins by squalene in this organism was investigated, and a genomic library of this organism was screened for clones able to degrade squalene.

5.3.3 Protein studies on *Nocardia* sp ATCC 21145

No quantitative or qualitative differences were detected in protein profiles of *Nocardia* sp ATCC 21145 cells grown in medium with or without squalene (Fig. 5.3). One-dimensional PAGE may not resolve the large number of intracellular proteins sufficiently to detect any intracellular proteins that may have been induced by squalene, unless these proteins were present in relatively large amounts. However, the induction of any extracellular proteins would have been detected as relatively fewer extracellular proteins were present (ranging between approximately 20 and 50 kD). Initial indications are therefore that proteins implicated in squalene degradation in *Nocardia* sp ATCC 21145 are produced constitutively, or are produced intracellularly in relatively low amounts.

It is interesting to note the presence of a protein of approximately 50 kD which is present in large amounts in the supernatant fluid of the *Nocardia* sp ATCC 21145 culture grown in M9 minimal medium with 1% (w/v) glucose (Fig. 5.3, Lane 7). This could presumably be a loosely-bound outer membrane protein involved in glucose uptake, which is induced in such large amounts in these cultures that it is detectable in the supernatant fluid.

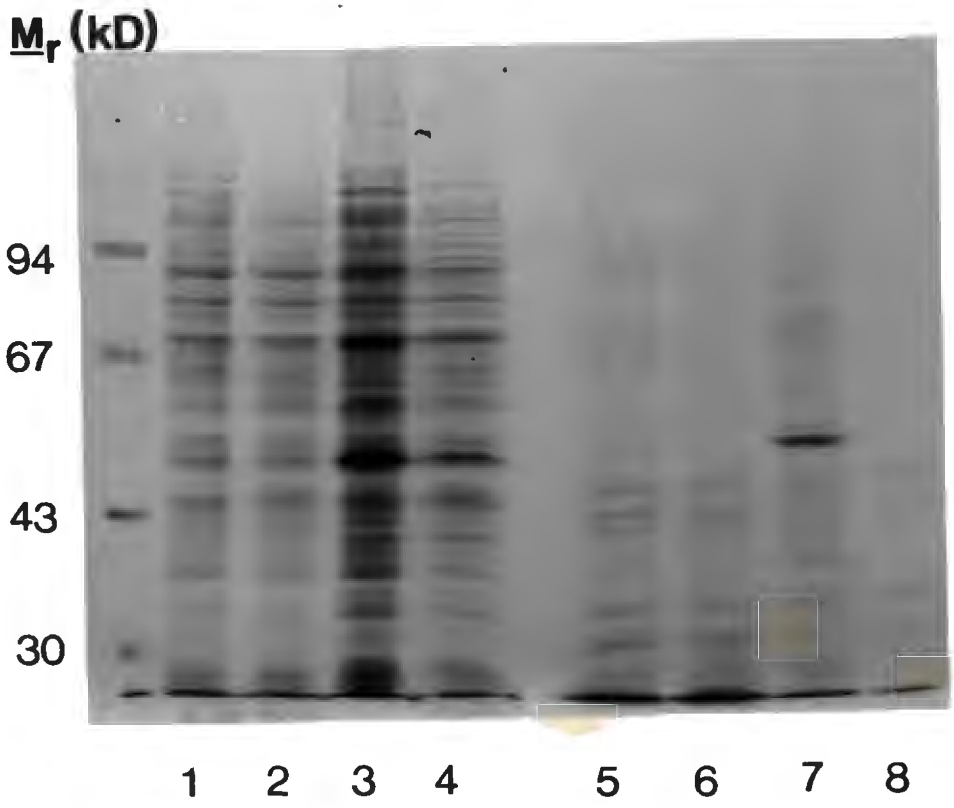

Fig. 5.3 SDS-PAGE protein profiles of intracellular (Lanes 1-4) and supernatant fluid (Lanes 5-8) proteins from *Nocardia* sp ATCC 21145 cells grown in M3 medium and M9 minimal medium with and without 1% (w/v) squalene. Samples of cell lysates (5 µl) and 50-fold concentrated supernatant fluids (20 µl) were boiled with an equal volume of SDS sample buffer. The composition of the media from which samples were derived was as follows:-
Lanes 1 and 5: M3 medium
Lanes 2 and 6: M3 medium + 1% (w/v) squalene
Lanes 3 and 7: M9 minimal medium + 1% (w/v) glucose
Lanes 4 and 8: M9 minimal medium + 1% (w/v) squalene

5.3.4 Studies on binding of *Nocardia* sp ATCC 21145 supernatant fluid proteins to squalene substrate

No differences were detected between protein profiles of supernatant fluids from *Nocardia* sp ATCC 21145 cells grown on M3 medium before and after incubation with squalene (Fig. 5.4). This indicated that none of the proteins present in the supernatant fluid bound to the squalene substrate, and is indirect evidence that none of these proteins is involved in the degradation of squalene in supernatant fluid. If any of the proteins were involved in extracellular degradation of squalene into smaller molecules prior to entry of this substrate into cells, then possibly these proteins would have bound to squalene as an initial step in this degradation. This could have been apparent by a decrease in protein level in supernatant fluids incubated with squalene.

5.3.5 Screening of the *Nocardia* sp ATCC 21145 genomic library for squalene-utilising clones

Cultures containing pools of both *E. coli* LK111 and *S. lividans* TK24 transformants, on extraction, yielded approximately the same mass of product that was obtained on extraction of untransformed *E. coli* LK111 and *S. lividans* TK24 control cultures. Furthermore, TLC of extracts did not reveal any differences between cultures containing transformants and untransformed control cultures.

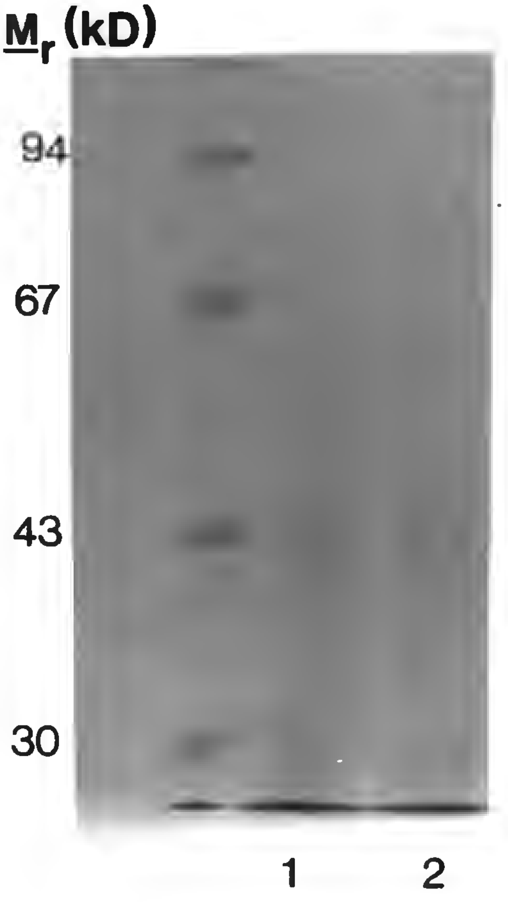

Fig. 5.4. SDS-PAGE protein profiles of supernatant fluid from *Nocardia* sp ATCC 21145 cells grown in M3 medium with 1% (w/v) squalene. Supernatant fluids were concentrated approximately 50-fold. Samples (20 μl) were before (Lane 1) and after (Lane 2) incubation with squalene for 1 h at 30°C, and were boiled with 20 μl SDS squalene buffer.

E. coli LK111 and *S. lividans* TK24 transformants containing genes coding for enzymes implicated in squalene utilisation were therefore not detected by these methods.

It is possible that squalene utilisation is the result of a complex pathway involving many enzymes and was not detected because the genes coding for these enzymes were not all cloned in any of the transformants comprising the *Nocardia* sp ATCC 21145 genomic library. However, some of the metabolites produced by microbial action on squalene are mono-, di-, tri-, tetra- and pentahydrated squalene (Seo *et al.*, 1981) and squalenedioic acid (Seo *et al.*, 1983), which involve only minor modifications of squalene and the production of these compounds therefore seems unlikely to necessitate a complex multi-enzyme pathway.

Another possible reason why squalene-utilising clones were not detected in the *E. coli* LK111 and *S. lividans* TK24 transformants comprising the *Nocardia* sp ATCC 21145 genomic library could be that genes coding for squalene utilisation were present but were not expressed in the *E. coli* LK111 and *S. lividans* TK24 hosts. Actinomycete genes are frequently not expressed in *E. coli*, for reasons discussed in detail in Section 4.1.2. However, it seems likely that *Nocardia* genes would be expressed in the closely-related actinomycete *S. lividans*.

It seems more likely that squalene utilising transformants were not detected because of the relatively crude nature of the screening technique. Screening of pools of a large number of transformants in liquid cultures would only be successful if the desired transformant had a strong selective advantage over other transformants and would come to be the dominant transformant present in a culture. The transformant would then be detectable and could be isolated. As this approach was unsuccessful in this particular case, it would be necessary to develop a more sensitive screening procedure (such as one in which single colonies capable of utilising squalene on plate cultures could be detected) in order to isolate squalene-utilising clones from the *Nocardia* sp ATCC 21145 genomic library.

5.4 CONCLUSION

Three of the ten *Nocardia* sp tested showed the ability to metabolise squalene. This genus therefore shows great potential for biotransformation of squalene to produce potentially useful chemicals. The approach outlined here should prove valuable in the further investigation of squalene-utilising *Nocardia* sp, and the positive selection shuttle vector pLR591 will facilitate the production of genomic libraries of *Nocardia* sp that prove to be important in this regard. Selection of transformants capable of utilising squalene has been shown to be a problem which requires further investigation.

The long term result of research in this field could be industrial-scale biotransformation of squalene to produce other acyclic terpenoids for use in organic syntheses. This biotransformation could be performed by *Nocardia* sp or by transformants containing genes coding for squalene utilisation, derived from *Nocardia* sp.

CHAPTER SIX

GENERAL CONCLUSION

CHAPTER SIX

GENERAL CONCLUSION

The work described in this thesis has opened up a number of avenues for future research.

The positive selection *Streptomyces-E. coli* shuttle vector pLR591, as well as being a useful vector for actinomycete genomic library construction, has potential for further development. This vector has already been used in the construction of a range of integration vectors designed for use in *Streptomyces* strains which will not harbour any known plasmids (R Kirby, Dept of Microbiology, University of Cape Town, personal communication). The vector pLR591 should be particularly useful in the study of expression of actinomycete genes in *E. coli* and *vice versa*.

The genes cloned from *Nocardia* sp ATCC 21145 which code for pigment production in *E. coli* have great potential in the development of chromogenic gene cloning vectors. The sequencing of one of these genes, which is currently in progress (S Hart, Dept of Microbiology, University of Cape Town, personal communication) should yield valuable information on codon useage and control of nocardioform genes. In addition, the cloning of genes coding for pigment production should facilitate characterisation of this pigment and also possibly, the large-scale production of the pigment if it has any useful application.

The studies on squalene utilisation by nocardioforms, although preliminary, have revealed that these organisms could have considerable potential in the biotransformation of squalene to produce useful products.

This is the first report on the cloning of genes from nocardioforms and it is hoped that this study will stimulate further work of this nature and will increase the application of the new technology of genetic engineering to this important group of microorganisms.

APPENDIX A

MICROBIAL DEGRADATION OF RUBBER

APPENDIX A

MICROBIAL DEGRADATION OF RUBBER

A.1 Introduction

Natural rubber is produced from latex collected from the tree *Hevea brasiliensis* and may be susceptible to microbial attack while in the latex form, as raw natural rubber or in its processed, vulcanised state. Synthetic rubbers may also be susceptible to degradation by microorganisms. Vulcanised natural rubber and synthetic rubbers frequently contain a large number of components, such as cross-linking agents, accelerators of cross-linking, anti-oxidants, fillers, colouring materials, fungicides and organic impurities. It therefore is necessary to distinguish between degradation of the rubber polymer and degradation caused by microbial attack of these other ingredients (Heap, 1965).

Latex is a complex mixture of rubber particles in the aqueous phase and contains about 35% rubber, 60% water and 5% proteins, amino acids, lipids and other organic and inorganic compounds. An early study in which sterile latex was prepared and inoculated with strains of actinomycetes demonstrated that the rubber present in latex could be degraded by these microorganisms to less than 30% of its original weight after four weeks (Spence and van Niel, 1936). John and Verstraete (1979) investigated the microorganisms present in latex samples and found many species present, with *Bacillus* sp, *Corynebacterium* sp, *Micrococcus* sp, *Sarcina* sp and *Streptococcus* sp being particularly common.

There have been numerous reports of the loss of weight of raw natural rubber due to microbial deterioration. Nette *et al.* (1959) showed that bacteria could cause up to 55% loss of weight of thin rubber sheets in 70 d, while fungi caused negligible losses in weight. Degradation of raw natural rubber by microorganisms has been reviewed by Zyska (1981).

The deterioration of vulcanised, natural rubber by microbial action has been observed in rubber sealing joints used in sewage and water pipelines. This was investigated by Rook (1955) and by Leeflang (1963) who both isolated *Streptomyces* sp from deteriorated rubber specimens and demonstrated the ability of these actinomycetes to utilise vulcanised natural rubber. Hutchinson *et al.* (1975) reported the isolation of various *Streptomyces* sp from deteriorated rubber rings in contact with water, sewage and soil. Actinomycetes belonging to the *Nocardia asteroides* complex have been isolated from deteriorated rubber rings (Orchard and Goodfellow, 1979; Hookey *et al.*, 1980).

Various fungi have also been implicated in the degradation of natural rubber. The growth of fungi belonging to the genera *Aspergillus*, *Fusarium*, *Mucor*, *Penicillium* and *Ulocladium* on finely divided tyres, and resultant chemical changes in this substrate have been reported (Nickerson and Faber, 1975; Faber and Nickerson, 1979). *Fusarium solani* was found to cause reduction in weight and changes in network density of vulcanised natural rubber (Kwiatkowska *et al.*, 1978). The fungus *F. solani* was the most efficient degrader of rubber of a number of fungi tested in gel permeation chromatography (GPC) studies (Borel *et al.*, 1982).

Various attempts have been made to reduce the susceptibility of vulcanised natural rubber to microbial attack. These include studies on the effects of microbiocides (Cundell and Mulcock, 1973) and the influence of antioxidants and sulphur level (Cundell *et al.*, 1973) on the microbiological deterioration of vulcanised natural rubber.

Synthetic rubbers are not immune to microbiological attack. Heap and Morrell (1968) and Zyska (1981) review reports of microbial degradation of synthetic rubbers and plastics.

Enrichment culture techniques have proved valuable in the isolation of microorganisms able to degrade rubber. ZoBell and Grant (1942) isolated bacteria ascribed to the genera *Actinomyces* and *Proactinomyces* by enrichment cultures containing thin films of purified rubber as a carbon source. The microbial degradation of liquid polybutadiene and *cis*-1,4-polyisoprene by organisms isolated using enrichment culture techniques has been reported by Tsuchii *et al.*, (1978; 1979). More recently, Tsuchii *et al.* (1985) have isolated an actinomycete, *Nocardia* sp strain 835A which grew well on unvulcanised and vulcanised natural rubber and synthetic isoprene rubber, and rapidly degraded thin films of vulcanised natural rubber, with a weight loss of 75% after two weeks.

This study was an attempt to isolate microorganisms capable of degradation of rubber in order to study the mechanism of this degradation and possibly to enhance this process using the recently developed powerful tool of genetic manipulation. An efficient, rubber-degrading microorganism may have potential in the recycling or disposal of waste rubber (such as used car tyres). A number of microorganisms were isolated by enrichment techniques and growth of these organisms on a range of rubber substrates was investigated. The effect of certain of these microorganisms on rubber substrates was assessed using a number of techniques. Protein studies were done on three of these microorganisms grown in cultures containing rubber as the sole carbon source.

A.2 Isolation of microorganisms from enrichment cultures containing rubber as the sole carbon source

A range of enrichment cultures was set up using samples from sewage, soil and deteriorated rubber as inoculi.

Enrichment cultures consisted of buffered minimal salts media containing either raw natural rubber or synthetic 'natural rubber' (Natsyn) as the sole carbon source. The substrate was finely divided in both cases to present a large surface area for possible microbial attack. Natsyn was chosen as one of the substrates as this synthetically produced rubber with the same molecular structure as natural rubber contains none of the organic impurities found in natural rubber.

All enrichment cultures were maintained at 30°C with aeration and inoculi from each culture were transferred to fresh cultures with the same substrate composition at monthly intervals. After six months, the final cultures were examined and the dominant organism in each was isolated in pure culture and identified using standard microbiological procedures. The microorganisms isolated by this enrichment culture technique are listed in Table A.1. It is interesting to note that these microorganisms include *Streptomyces* sp and *Fusarium* sp which have been frequently reported in the literature as important in rubber degradation, and *Pseudomonas* sp which are recognised as a metabolically versatile group of bacteria.

A.3 Assessment of growth of microorganisms in cultures containing rubber as the sole carbon source

In order to select a small number of microorganisms for further investigation, the 14 microorganisms listed in Table A.1 and 14 *Streptomcyces* sp and ten *Nocardia* sp listed in Table A.2 were tested in minimal medium cultures containing natural rubber and Natsyn as sole carbon sources. The *Streptomyces* sp and *Nocardia* sp were tested as these genera have frequently been implicated in rubber degradation. The rate of growth of microorganisms was assessed by spectrophotometric monitoring of absorbance of the cultures

TABLE A.1 Microorganisms isolated from enrichment cultures containing rubber as the sole carbon source.

Type of microorganism	Classification	Strain designation
Gram-negative bacteria	*Alcaligenes* sp	RD1
	Pseudomonas paucimobilis	RD2
	Achromobacter sp	RD3
	Acinetobacter sp	RD4
	Pseudomonas putida	RD5
	Pseudomonas sp	RD6
	Pseudomonas sp	RD7
	Pseudomonas sp	RD8
	Pseudomonas sp	RD9
Gram-positive bacteria	*Streptomyces* sp	RD10
	Streptomyces sp	RD11
	Bacillus sp	RD12
Yeast	*Rhodotorula* sp	RD13
Fungi	*Fusarium oxysporium*	RD14

TABLE A.2 *Streptomyces* and *Nocardia* strains screened for rubber-degradation capabilities.

Microorganism	Strain designation
S. griseus	RD15
S. hydroscopicus	RD16
S. cattleya	RD17
S. aureofaciens	RD18
S. aureus	RD19
S. virginiae	RD20
S. fradiae	RD21
S. venezuela	RD22
S. reticuli	RD23
S. argenteolus	RD24
S. diastaticus	RD25
S. lividans	RD26
S. ambofaciens	RD27
S. sporophytecus	RD28
N. canicruria	ATCC 17896
N. paraffinae	ATCC 21509
N. mediterranei	ATCC 13685
N. globerula	ATCC 21022
N. orientalis	ATCC 19795
N. uniformis subsp *tsuyamanensis*	ATCC 21806
N. globerula	ATCC 21505
N. autotrophica	ATCC 13181
Nocardia sp	ATCC 21145
N. corallina JL10	JL10

and by direct counting of cfu present by plate counts of dilution series of the cultures. The mass of rubber remaining in the cultures was determined after 30 d. The *Alcaligenes* sp RD1, *Pseudomonas paucimobilis* RD2, *Pseudomonas putida* RD5 and *Streptomyces* sp RD10 from the enrichment cultures described in A.2, showed good growth, with cell numbers increasing from approximately 1×10^4 cells/ml to between 1×10^7 and 5.0×10^7 cells/ml in the case of *Alcaligenes* RD1 and *Pseudomonas* sp RD2 and RD5, and to approximately 6.0×10^7 cfu/ml in the case of *Streptomyces* sp RD10. Cell numbers in cultures of the other microorganisms typically increased from approximately 1×10^4 cfu/ml to between 1×10^5 and 1×10^6 cfu/ml during the first week and then declined. There was no significant decrease in the mass of rubber present in any of the cultures.

In order to assess whether any of these four microorganisms showing promising growth characteristics in minimal medium cultures containing rubber as the sole carbon source were implicated in rubber degradation, protein profiles of these bacteria grown in the presence and absence of rubber were examined (Appendix A.4) and viscometry and GPC analyses were used to attempt to detect changes in the rubber substrate (Appendices A.5 and A.6).

A.4 Protein studies on four possible rubber-degrading microorganisms

The protein profiles of whole cell lysates and supernatant fluids of *Alcaligenes* RD1, *P. paucimobilis* RD2, *P. putida* RD5 and *Streptomyces* sp RD10 grown in cultures with and without Natsyn were visualised using discontinuous SDS-PAGE. Proteins in supernatant fluids were concentrated approximately 50-fold by ammonium sulphate precipitation prior to electrophoresis. No differences were detected between protein profiles of organisms grown with and without rubber. It was concluded that enzymes necessary for rubber degradation, if produced, must be present constitutively rather than being induced by the presence of the rubber substrate. These protein studies therefore did not help to resolve whether any of the microorganisms studied possessed rubber-degrading characteristics.

A.5 Viscometric analysis of rubber substrate

Microbial degradation of a rubber substrate could result in changes in the average length of the rubber molecules which would be reflected by changes in viscosity of the rubber when dissolved in a solvent. Natsyn incubated in minimal salts medium with cultures of *Alcaligenes* RD1, *P. paucimobilis* RD2, *P. putida* RD5 and *Streptomyces* sp RD10 was dissolved in toluene and viscosities of solutions were determined using a Cannon-Ubbelode No 75 flow viscometer.

Values obtained were compared with that of a sterile Natsyn control sample. No significant changes in viscosity were detected.

A.6 GPC analysis of rubber substrate

GPC analysis is a sensitive, accurate technique which may be used to determine the $\underline{M}_r$ distribution of the molecules in a rubber sample.

GPC was used in order to detect any small changes in the rubber substrate which may be caused by microbial degradation too slight to be detected by the less sensitive technique of viscometry.

In order to maximise any effect that microorganisms may be having on the rubber substrate, a solvent system was developed to dissolve the rubber and disperse it in aqueous culture. Of 16 solvents tested, Natsyn was soluble in di-isobutylketone, tetrahydrofuran, cyclohexanone, hexane, heptane and octane. The toxicity of these solvents to the four microorganisms of interest was tested and octane was found to be non-toxic at a concentration of 1% (v/v).

Alcaligenes RD1, *P. paucimobilis* RD2, *P. putida* RD5 and *Streptomyces* sp RD10 were grown in minimal salts medium containing 1% (v/v) of a 1 g/ml solution of Natsyn in octane. After 30 d, Natsyn was precipitated from these cultures and from a similar sterile control culture by addition of 50% (v/v) ethanol. The precipitated rubber was analysed by GPC. No differences in $\underline{M}_r$ distribution were detected between Natsyn substrate incubated with microorganisms present and the sterile, control sample of Natsyn. It was concluded that microbial degradation of Natsyn substrate by these microorganisms was not detectable by GPC.

A.7 Growth of microorganisms on thin films of rubber substrate

In a further attempt to identify rubber-degrading microorganisms, all the microorganisms listed in Tables A.1 and A.2 were tested in a culture system similar to that described by Tsuchii *et al.* (1985).

A variety of sources of thin rubber sheets was assessed as potential substrates in these cultures. The quantitative detection of β-sitosterol by TLC (Davies, 1967) was used to determine the percentage of natural rubber present in the substrates under consideration. Thin rubber sheet derived from Durex 'Gossamer' contraceptive sheaths was selected as the most suitable substrate as it was a readily available source of very thin, 100% natural rubber. This substrate was boiled in acetone prior to addition to minimal salts media in order to remove lubricants and other impurities.

Cultures were incubated at 30°C and growth was monitored by spectrophotometric and plate count methods. The mass of rubber present in cultures (initially 1 g/100 ml) was determined after 90 d. Although some cultures had grown considerably in the initial stages of this experiment, no significant reduction in the mass of rubber was detected in any of the cultures.

A.8 Conclusions

None of the microorganisms tested degraded rubber to any significant extent as this would have been detected by the methods used to assess changes in the rubber substrate. The growth of some of these microorganisms in minimal medium cultures containing rubber as the only added source of carbon must have reflected a very efficient ability to scavenge trace amounts of carbon present as impurities in the salts used to formulate the minimal media, as well as airborne sources of contamination. Geller (1983) has reported significant growth of microorganisms in minimal medium cultures as a result of airborne contamination. The results obtained in this study illustrate the importance of using changes in substrate rather than growth of a microorganism as the criterion for the successful isolation of a microorganism with a particular degradative ability from minimal medium cultures.

A promising possibility for further research in rubber degradation would be to use the positive-selection vector pLR591 and the methods outlined in this thesis to isolate rubber-degrading genes from the very efficient rubber-degrading *Nocardia* strain isolated by Tsuchii *et al.* (1985). It would however be necessary to develop a method for screening of clones for rubber-degrading characteristics.

The isolation of a rubber-degrading clone from a genomic library of the *Nocardia* strain of Tsuchii *et al.* (1985) would greatly facilitate studies on the mechanism of this rubber degradation and would open up the possibility of enhancement of this degradation by genetic manipulation.

APPENDIX B

STANDARD METHODS, BUFFERS AND MEDIA

APPENDIX B

STANDARD METHODS, BUFFERS AND MEDIA

B.1 Standard methods

B.1.1	Large-scale *E. coli* plasmid DNA isolation.
B.1.2	Small-scale *E. coli* plasmid DNA isolation.
B.1.3	Large-scale isolation of *Streptomyces* plasmid DNA by neutral lysis.
B.1.4	Plasmid purification by isopycnic CsCl-EtdBr density gradient ultracentrifugation.
B.1.5	Isolation of *Nocardia* chromosomal DNA.
B.1.6	Partial *Sau*IIIA endonuclease digestion of chromosomal DNA.
B.1.7	Size fractionation of chromosomal DNA by sucrose gradient centrifugation.
B.1.8	Restriction endonuclease digestion and DNA ligation reactions.
B.1.9	DNA agarose gel electrophoresis.
B.1.10	Preparation of radioactively-labelled DNA probes.
B.1.11	Transfer of DNA from *E. coli* colonies to nitrocellulose filters for 'colony hybridisation' studies.
B.1.12	Transfer of DNA from agarose gels to membranes and hybridisation with radioactive DNA probes.
B.1.13	Preparation and transformation of competent *E. coli* cells.

B.1.14 Preparation of *Streptomyces* protoplasts and determination of protoplast regeneration frequency.
B.1.15 Transformation of *Streptomyces* protoplasts.
B.1.16 Discontinuous SDS-PAGE.

B.2 **Buffers and Media**

B.1 STANDARD METHODS

B.1.1 Large-scale *E. coli* plasmid DNA isolation.

A 400 ml LB broth culture, grown overnight at 37°C in the presence of the appropriate antibiotic, was harvested by centrifugation at 16,000 x *g* for 10 min. Cells were resuspended in 8 ml of Solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA) and held at room temperature for 5 min. Solution II (16 ml of 0.2 M NaOH, 1% (w/v) SDS, prepared fresh weekly) was added, mixed gently, and the sample put on ice for exactly 5 min. Precooled Solution III (12 ml of 5 M potassium acetate, pH 4.8) was added, mixed gently and the mixture left on ice for 5 min. Precipitated protein, SDS and chromosomal DNA was removed by centrifugation at 12,000 x *g* for 10 min. Two volumes of 95% ethanol were added to the supernatant fluid. The sample was kept for 2 min at room temperature and the precipitated nucleic acid was pelleted by centrifugation at 27,000 x *g* for 15 min. The pellet was washed with 70% ethanol, resuspended in 5 ml TE buffer and purified by isopycnic CsCl-EtdBr ultracentrifugation.

B.1.2 Small-scale *E. coli* plasmid DNA isolation

A LB broth culture was grown overnight at 37°C in the presence of the appropriate antibiotic. Cells were harvested from 1.5 ml of the culture by centrifugation in an Eppendorf microfuge for 1 min. All subsequent centrifugation steps in this preparation were also performed in an Eppendorf microfuge. The pellet was drained, resuspended in 100 µl Solution I (Solutions I, II and III are described in Section B.1.1), and the sample was held at room temperature for 5 min and on ice for 1 min. Solution II (200 µl) was added and mixed briefly on a vortex mixer. The sample was held for 5 min on ice. Solution III (150 µl) was added, the sample mixed as before, and held for 5 min on ice. The sample was centrifuged for 5 min and the supernatant fluid was transferred to a fresh tube. Two volumes of 95% ethanol were added and the sample was centrifuged for 15 min. The pellet was dried and resuspended in 150 µl TE buffer. Sodium acetate (15 µl of a 3 M solution) and two volumes of 95% ethanol were added. The sample was held at -20°C for 15 min and centrifuged for 15 min. The pellet was washed in 70% ethanol, dried and resuspended in 20 µl TE buffer.

B.1.3 Large scale isolation of *Streptomyces* plasmid DNA

The plasmids pIJ702, pLR591 and pLR591 derivatives were prepared from cultures of *Streptomyces* sp grown in 500 ml YEME medium (Appendix B.2) with 5 µg/ml thiostrepton for

48 h at 30°C and shaken at 150 rpm on a rotary shaker. Mycelia were harvested by centrifugation at 16,000 x *g* for 30 min and resuspended in 50 ml 34% sucrose in TE buffer. Ten ml of 0.25 M EDTA was added, followed by 5 ml lysozyme solution (50 mg/ml lysozyme in 0.01 M TRIS, 0.25 M EDTA, pH 8.0). The suspension was incubated at 30°C for approximately 30 min, until the beginning of lysis was detected by increased viscosity. Ice cold 34% sucrose in TE buffer (50 ml) was added and the sample was mixed and transferred to an ice bath. Ice cold 0.25 M EDTA, pH 8.0 (30 ml) and 0.01 M TRIS, pH 8.0 (0.5 ml) were added. The sample was divided into 25 ml aliquots in pre-cooled centrifuge tubes. To each tube, 3.6 ml 10% (w/v) SDS pre-warmed to 50°C was added and mixed gently by inversion. Ice cold 5 M NaCl (7.2 ml) was added to each tube and mixed. Samples were left at 4°C for 2 h and the precipitated protein, SDS and chromosomal DNA was removed by centrifugation at 29,000 x *g* for 30 min. Two volumes of 95% ethanol were added to the supernatant fluid, and, after 2 min at room temperature, precipitated nucleic acid was pelleted by centrifugation at 27,000 x *g* for 15 min. Pellets were washed with 70% ethanol, pooled in 5 ml TE buffer and purified by isopycnic CsCl-EtdBr ultracentrifugation.

B.1.4 Plasmid purification by isopycnic CsCl-EtdBr density gradient ultracentrifugation

Crude plasmid preparations were prepared for isopycnic CsCl-EtdBr density gradient ultracentrifugation by the addition of CsCl (final conc 1 g/ml) and EtdBr (final conc 250 µg/ml). The refractive index was adjusted to 1.396, the sample sealed in a Beckman Quickseal ultracentrifuge tube, and centrifuged at 340,000 x *g* for 12 h in a Beckman VTi rotor. Plasmid and chromosomal DNA bands were visualised under UV light (350 nm). The Quickseal tube was pierced and the lower (plasmid) band was collected in as small a volume as possible. The EtdBr was removed from the sample by repeated extraction with an equal volume of NaCl-saturated isopropanol. DNA was precipitated from the CsCl solution by the addition of two volumes of water and one (new) volume of isopropanol. DNA was pelleted by 15 min centrifugation in an Eppendorf microfuge. The pellet was washed in 70% ethanol and resuspended in TE buffer.

B.1.5 Isolation of *Nocardia* chromosomal DNA

Nocardia mycelia (1 g wet weight) were resuspended in 5 ml TE buffer and lysozyme was added to a final concentration of 2 mg/ml. The sample was incubated at 30°C for 10 min and 1.2 ml 0.5 M EDTA was added. Pronase solution (10 mg/ml Boehringer Mannheim lyophilised pronase in water; 'predigested' at 30°C for 10 min) was added to a final concentration of 0.2 mg/ml and the sample was mixed gently. The sample was held at 30°C for 5 min and 0.7 ml 10% (w/v) SDS was added. Phenol (6 ml of a solution containing 500 g melted phenol, 0.5 g 8-hydroxyquinoline and 65 ml TE buffer containing 0.1 M NaCl) was added and the sample was shaken thoroughly by hand for 10 min. Chloroform (6 ml) was added and the sample shaken for a further 5 min. After centrifugation for 10 min at 2,000 x *g*, the aqueous phase was transferred to a fresh tube and the phenol extraction was repeated. The resultant aqueous phase was mixed with 6 ml chloroform, shaken for 5 min, and then was separated by centrifugation at 2,000 x *g* for 5 min. The aqueous phase was removed and treated with RNase (40 µg/g Sigma Bovine Pancreas RNase-A) at 37°C for 1 h. NaCl (0.25 volumes of 5 M solution) was added, followed by 2 volumes of 95% ethanol. The sample was held at room temperature for 5 min and the DNA precipitated by centrifugation at 27,000 x *g* for 15 min. The pellet was washed with 70% ethanol and resuspended in 2 ml TE buffer.

B.1.6 Partial *Sau*IIIA endonuclease digestion of chromosomal DNA

Pilot digestions were performed with differing *Sau*IIIA-endonuclease concentrations to establish the optimal enzyme concentration to obtain DNA fragments in the range 5 - 10 kb. Large-scale digestions were done on 100 µg chromosomal DNA in a total volume of 0.5 ml medium salt restriction buffer and the appropriate *Sau*IIIA endonuclease concentration (determined by a pilot digestion) at 37°C for 1 h. *Sau*IIIA endonuclease was inactivated by heating at 68°C for 15 min. A sample of each large scale digestion was checked by agarose gel electrophoresis.

B.1.7 Size fractionation of chromosomal DNA by sucrose gradient centrifugation

A sucrose gradient (10 - 40% (w/v) sucrose in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 M NaCl) was prepared, and *Sau*IIIA endonuclease digested DNA was loaded on the gradient. After centrifugation at 130,000 x *g* for 24 h in a Beckman SW27 rotor, 0.5 ml fractions were collected. A 10 µl sample of every third fraction was checked by agarose gel electrophoresis and the appropriate fractions were pooled, diluted with a half volume TE buffer, and an equal volume of isopropanol was added. DNA was precipitated by centrifugation at 27,000 x *g* for 15 min, washed with 70% ethanol and resuspended in 200 µl TE buffer.

B.1.8 Restriction endonuclease digestion and DNA ligation reactions

The procedures described by Maniatis *et al.* (1982) were followed for restriction endonuclease digestions. The digestion volumes were routinely 20 µl, and 4 u of restriction enzyme was used per 1 µg of DNA. Digestions were incubated at 37°C, except *Bcl*I which was incubated at 65°C, for 1 h in low, medium or high salt restriction buffer, according to the salt concentration specified by the supplier. Restriction endonucleases were obtained from Boehringer Mannheim GmBH-Biochemica, West Germany, Anglian Biotechnology Limited, UK, and New England Biolabs, Inc., MA, USA.

Restriction endonuclease digestions were stopped by heating to 65°C or by one of the methods recommended by Maniatis *et al.* (1982). Ligations were done in 20 µl ligation buffer (Appendix B.2) at 20°C for at least 4 h.

B.1.9 DNA agarose gel electrophoresis

DNA samples in TE buffer were mixed with a half volume of DNA sample loading buffer (Appendix B.2). Electrophoresis of DNA was carried out using a horizontal gel system with TBE buffer, following methods described by Maniatis *et al.* (1982). Agarose concentrations varied from 0.5% for resolution of large DNA fragments to 1.2% for smaller DNA fragments. The DNA was visualised using a 254 nm wavelength Transilluminator (Chromato-Vue Model TS-15, UV Products Inc., San Gabriel, CA, USA) and photographed with a Polaroid CU-5 Land camera using Polaroid 667 film. Sizes of DNA fragments were deduced from standard curves, made by plotting the distance travelled by DNA fragments of known mass against the log of their molecular masses. The standard DNA fragments used were obtained from *Pst*I or *Eco*RI endonuclease digests of λ DNA.

B.1.10 Preparation of radioactively-labelled DNA probes

Radioactively-labelled DNA probes were prepared by nick translation using kit PB5025 supplied by Amersham International, UK. The manufacturers instructions were followed. Unincorporated [^{32}P]-labelled nucleotides were separated from the labelled probe using a Sephadex G-50 spin column (Maniatis *et al.*, 1982).

B.1.11 Transfer of DNA from *E. coli* colonies to nitrocellulose filters for 'Southern hybridisation' studies

Colonies to be screened were plated in a grid pattern onto duplicate LB agar plates. One set of these plates (master plates) was stored at 4°C, and the other set (test plates) was used for 'Southern hybridisation' studies. A sterile nitrocellulose filter (HATF 085 50 Millipore Co., Bedford, MA, USA) was placed on each test plate, removed with adherent colonies and placed, colonies upwards, on 0.75 ml 0.5 M NaOH in a Petri dish. After 2 min, the filter was removed, blotted, and this step was repeated. The filter was transferred to 0.75 ml of 1 M Tris-HCl (pH 7.4) for 5 min. This step was repeated, followed by transfer to 0.75 M NaCl and 0.5 M Tris-HCl (pH 7.4). After 5 min, the filter was removed, blotted and dried at room temperature for 60 min. Filters were baked at 80°C for 2 h in a vacuum oven. Filters were gently shaken in 100 ml prehybridisation solution (Appendix B.2) for 2 h at 60°C. The radioactively-labelled probe was denatured by boiling for 10 min and added to the prehybridisation solution. Hybridisation was left to continue overnight at 60°C.

B.1.12 Transfer of DNA from agarose gels to membranes and hybridisation with radioactive DNA probes

DNA fragments were transferred from agarose gels to GeneScreen hybridisation transfer membranes (New England Nuclear Corp., Boston, MA, USA). Agarose gels were washed in 2 volumes of 0.25 M HCl with gentle agitation for 15 min and rinsed in distilled water. The DNA fragments were denatured by two 15 min washes in 2 volumes 0.5 M NaOH, 1.5 M NaCl and gels neutralised by two 30 min washes in 2 volumes of 1 M ammonium acetate, 0.02 M NaOH. The transfer membrane was placed over the gel. Three sheets of Whatman 3 MM filter paper were laid on the membrane, followed by a 4 cm thick layer of absorbent paper. A light weight was placed on top of this, and transfer left to continue for 2 h. The membrane was air dried and baked in a vacuum oven at $80^{o}C$ for 2 h.

Membranes were gently shaken in 100 ml of prehybridisation solution for 2 h at $60^{o}C$. The radioactively-labelled probe to be used was denatured by boiling for 10 min and added to the prehybridisation solution. Hybridisation was left to continue overnight at $60^{o}C$.

B.1.13 Preparation and transformation of competent *E. coli* cells

A 1/100 dilution of an overnight LB broth culture was inoculated into 50 ml of prewarmed LB broth, and incubated at $37^{o}C$, with vigorous shaking, until the culture had reached early exponential phase (OD_{600} = 0.2). A 1/50 dilution of this culture was then inoculated into a 400 ml prewarmed LB broth main culture which was grown at 37°C, with vigorous shaking to ensure good aeration, to an OD_{600} of 0.2. The culture was cooled on ice for 5 min and cells harvested by centrifugation at 4,000 x *g* for 5 min at 4°C. Cells were resuspended in 200 ml ice cold 0.1 M $CaCl_2$ and left for 20 min on ice. Cells were harvested from this $CaCl_2$ solution by centrifugation at 3,000 x *g* for 5 min at 4°C and resuspended in 4 ml 0.1 M CaCl.

DNA in TE buffer was added to competent cells (2 µl DNA solution/100 µl competent cells) and the sample held on ice for 10 min. Cells were heat shocked at 42°C for 2 min and returned to ice for 10 min. Transformation mixes were routinely diluted with 1 ml LB broth and incubated at 37°C for 1 h to allow expression of transferred DNA.

B.1.14 Preparation of *Streptomyces* protoplasts and determination of protoplast regeneration frequency

A dense spore suspension of the *Streptomyces* sp to be protoplasted was prepared. Spores from a well-sporulated agar plate culture were harvested in 10 ml sterile water, which was filtered through sterile cotton wool to remove mycelial fragments. Spores were pelleted by centrifugation at 3,000 x *g* for 10 min, resuspended in 1 ml sterile water, and inoculated into 50 ml YEME medium containing 0.5% (w/v) glycine. The culture was incubated for 40 h at 30°C with rotary shaking, and harvested by centrifugation at 3,000 x *g* for 10 min. The pellet was resuspended in 15 ml of 10.3% sucrose and harvested by centrifugation at 3,000 x *g* for 10 min. The pellet was resuspended in 4 ml lysozyme solution (1 mg/ml lysozyme in P buffer). The suspension was incubated at 30°C for approximately 30 min, until phase contrast microscope monitoring showed that most mycelia had formed protoplasts. P buffer (5 ml) was added and the suspension filtered through sterile cotton wool, and transferred to a disposable plastic tube. Protoplasts were sedimented by centrifugation at 3,000 x *g* for 7 min and resuspended in 2 ml P buffer.

To determine the protoplast regeneration frequency, protoplasts were serially diluted in P buffer and in 0.01% (w/v) SDS and kept at room temperature for 1 h. Subsequently, 100 µl of each dilution was plated on R2YE plates and incubated at 30°C. Dilution in SDS was to determine the total number of regenerants resulting from unprotoplasted mycelia, as all protoplasted units would lyse in this mild detergent solution. The total number of protoplasts was determined microscopically using a counting chamber. Regeneration frequency was calculated by the formula:

Protoplast regeneration frequency = 100 x (b - c)/a where 'a' is the total number of protoplasts, 'b' is the number of cfu after dilution in P buffer, and 'c' is the number of cfu after dilution in 0.01% (w/v) SDS.

B.1.15 Transformation of *Streptomyces* protoplasts

DNA in TE buffer was added to *Streptomyces* protoplasts (5 µl DNA solution/50 µl protoplasts) and 200 µl T buffer (Appendix B.2) was well mixed into the solution within 0.5 min. The protoplast suspension was plated on R2YE plates.

B.1.16 Discontinuous SDS-PAGE

Discontinuous SDS-PAGE was done according to the method of Laemmli (1970), using a Hoefer SE600 vertical slab electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA). Protein samples were boiled in an equal volume of SDS sample buffer.

Proteins were electrophoresed at 150 V in the stacking gel and 25 mA in the resolution gel. The following buffers and solutions were used for discontinuous SDS-PAGE:

<u>Acrylamide Solution</u>

Acrylamide	29.2 g
Bis-acrylamide	0.8 g

<u>Resolution Gel Buffer</u>

Tris (1.5 M)	18.17% (w/v)
SDS	0.4% (w/v)
pH 8.8	

Reservoir Buffer

Tris (0.067 M)	4.07% (w/v)
Glycine (0.238 M)	17.87% (w/v)

SDS Sample Buffer

Tris (pH 6.8)	0.125 M
SDS	4% (w/v)
Glycerol	20% (v/v)
2-mercaptoethanol	10% (v/v)

Stacking Gel Buffer

Tris (0.5 M)	6.06% (w/v)
SDS	0.4% (w/v)
pH 6.8	

Stain and Destain Solutions

Destain:

acetic acid	270 ml
distilled water	2,430 ml
methanol	900 ml

Stain:

PAGE blue 83	0.2% in destain solution
(BDH Chemicals, Poole, UK)	

Acrylamide gels (10%) were prepared as follows:

Solution	Resolution gel	Stacking gel
Acrylamide solution	12 ml	2 ml
Resolution gel buffer	8.2 ml	-
Stacking gel buffer	-	3 ml
Distilled water	13.65 ml	7 ml
Ammonium persulphate (10%)	160 µl	64 µl
TEMED	18 µl	13 µl

B.2 BUFFERS AND MEDIA

DNA sample loading buffer

Bromophenol blue	0.25 % (w/v)
Glycerol	50% (v/v)
EDTA	100 mM

Denatured herring sperm DNA (Maniatis *et al.*, 1982)

Herring sperm DNA (lyophilised sodium salt, Boehringer Mannheim) was dissolved in distilled water (10 mg/ml) and sheared by passing the solution several times through an 18-gauge hypodermic needle. The DNA was denatured by boiling for 10 min, immediately placed on ice and stored in 1 ml aliquots at -20°C. The DNA was boiled for 5 min and placed on ice prior to use.

Denhardt's solution (50x) (Maniatis *et al.*, 1982)

Ficoll	1% (w/v)
Polyvinylpyrrolidone	1% (w/v)
BSA (Fraction V, Sigma)	1% (w/v)

Filter sterilised and stored at -20$_{o}$C.

Ligation buffer (10x) (King and Blakesley, 1986)

Tris-HCl, pH 7.0	500 mM
Magnesium chloride	100 mM
Polyethylene glycol 8000	50% (w/v)
Adenosine triphosphate	10 mM

LB agar

LB broth with 1.5% agar added.

LB broth

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Distilled water	1 000 ml

M3 agar

M3 broth with 2.2% agar added.

M3 broth

Malt extract	24 g
Yeast extract	5 g
Distilled water	1 000 ml

M9 Minimal Medium

Na_2HPO_4	6 g
KH_2PO_4	3 g
NH_4Cl	1 g
NaCl	0.5 g
Distilled water	1 000 ml

P Buffer (Hopwood *et al.*, 1985)

The following solution was made and dispensed in 80 ml aliquots:-

Sucrose	103 g
K_2SO_4	0.25 g
$MgCl_2.6H_2O$	2.02 g
Trace element solution	2 ml
Distilled water	800 ml

Before use, the following solutions were added to each aliquot, in order:-

KH_2PO_4 (0.5% w/v)	1 ml
$CaCl_2.2H_2O$ (3.68% w/v)	10 ml
TES buffer (5.73% w/v, pH 7.2)	10 ml

Prehybridisation solution

SSC	3 x
SDS	0.1%
Denatured Herring sperm DNA	100 µg/ml

Restriction buffer - low salt (10 x)

Tris-HCl (pH 7.5)	100 mM
$MgCl_2$	100 mM
Dithioerythritol	10 mM

Restriction buffer - medium salt (10 x)

Tris-HCl (pH 7.5)	100 mM
$MgCl_2$	100 mM
NaCl	500 mM
Dithioerythritol	10 mM

Restriction buffer - high salt (10 x)

Tris-HCl (pH 7.5)	500 mM
$MgCl_2$	100 mM
NaCl	1 M
Dithioerythritol	10 mM

R2YE (Hopwood *et al.*, 1985)

The following solution was prepared:-

Sucrose	103 g
K_2SO_4	0.25 g
$MgCl_2.6H_2O$	10.12 g
Glucose	10 g
Casamino acids (Difco)	0.1 g
Distilled water	800 ml

The solution was divided into 80 ml aliquots, 2.2 g of agar was added to each, and the aliquots were autoclaved. Prior to use, the medium was remelted and the following solutions were added to each aliquot:-

KH_2PO_4(0.5% w/v)	1 ml
$CaCl_2.2H_2O$ (3.68% w/v)	8 ml
L-proline (20% w/v)	1.5 ml
TES buffer (5.73% w/v, pH 7.2)	10 ml
Trace element solution	0.2 ml
NaOH (1 M)	0.5 ml
Yeast extract	5 ml

SNA medium

Nutrient broth powder (Difco)	8 g
Agar	3 g
Distilled water	1 000 ml

SSC buffer (pH 7.0)

NaCl	0.15 M
Sodium citrate	0.015 M

T buffer (Hopwood *et al.*, 1985)

The following sterile solutions were mixed:-

Sucrose (10.3% w/v)	25 ml
Trace element solution	
K_2SO_4 (2.5 w/v)	1 ml
Distilled water	75 ml

The following solutions were added to 9.3 ml of the above solution:-

$CaCl_2$ (5 M)	0.2 ml
Tris maleic acid buffer	0.5 ml

(made by adjusting a 1 M Tris solution to pH 8.0 with maleic acid.)

Prior to use, 3 parts by volume of the above solution was added to 1 part by mass of polyethylene glycol 1,000.

TBE buffer (pH 8.0)

Tris	89 mM
Boric acid	89 mM
EDTA	2.5 mM

TE buffer (pH 8.0)

Tris	10 mM
EDTA	1 mM

Trace element solution (Hopwood *et al.*, 1985)

$ZnCl_2$	40 mg
$FeCl_3.6H_2O$	200 mg
$CuCl_2.2H_2O$	10 mg
$MnCl_2.4H_2O$	10 mg

$Na_2B_4O_7.10H_2O$	10 mg
$(NH_4)_6MO_7.4H_2O$	10 mg
Distilled water	1 000 ml

YEME medium

Yeast extract (Difco)	3 g
Bacto-peptone (Difco)	5 g
Malt extract	3 g
Glucose	10 g
Sucrose	340 g
Distilled water	1 000 ml

After autoclaving, the following solution was added:-

$MgCl_2.6H_2O$ (2.5 M)	2 ml/1 000 ml

For protoplast preparation, the following solution was added:-

Glycine (20% w/v)	25 ml/1 000 ml

LITERATURE CITED

Adams J.N. and Brownell G.H. 1976. Genetic studies in *Nocardia erythropolis*. In: Goodfellow M., Brownell G.H. and Serrano J.A. (Eds). The biology of the nocardiae. Academic Press, London, UK.

Adams J.N. and Bradley S.G. 1963. Recombination events in the bacterial genus *Nocardia*. Science 140, 1392-1394.

Aiso K., Arai T., Shidara I. and Ogi K. 1954. Nocardorubin, an antibiotic produced by *Nocardia* sp. J. Antibiot. 7, 1-6.

Amdur B.H., Szabo E.I. and Socransky S.S. 1978. Presence of squalene in Gram-positive bacteria. J. Bacteriol. 135, 161-163.

Aoki H., Sakai H-I., Kohsaka M., Konomi T., Hosoda J., Kubochi Y.L., Iguchi E. and Imanaka H. 1976. Nocardicin A, a new monocyclic β-lactam antibiotic I. Discovery, isolation and characterization. J. Antibiot. 29, 492-500.

Apajalahti J.H.A. and Salkinoja-Salonen M.S. 1986. Degradation of polychlorinated phenols by *Rhodococcus chlorophenolicus*. Appl. Microbiol. Biotechnol. 25, 62-67.

Apajalahti J.H.A. and Salkinohja-Salonen M.S. 1987. Dechlorination and *para*-hydroxylation of polychlorinated phenols by *Rhodococcus chlorophenolicus*. J. Bacteriol. 169, 675-681.

Barna J.C.J. and Williams D.H. 1984. The structure and mode of action of glycopeptide antibiotics of the vancomycin group. Ann. Rev. Microbiol. 38, 339-359.

Bax A. 1983. Broadband homonuclear decoupling in heteronuclear shift correlation NMR spectroscopy. J. Magn. Reson. 53, 517-520.

Beaman B.L. 1976. Possible mechanisms of nocardial pathogenesis. In: Goodfellow M., Brownell G.H. and Serrano J.A. (Eds). The biology of the nocardiae. Academic Press, London, UK.

Beaman B.L., Burnside J., Edweards B. and Causey W. 1976. Nocardial infections in the United States, 1972-1974. J. Infect. Dis. 134, 286-289.

Benigni R., Antonov P.P. and Carere A. 1975. Estimate of the genome size by renaturation studies in *Streptomyces*. Appl. Microbiol. 30, 324-326.

Bibb M.J. and Cohen S.N. 1982. Gene expression in *Streptomyces*: Construction and application of promoter-probe plasmid vectors in *Streptomyces lividans*. Mol. Gen. Genet. 187, 265-277.

Bibb M.J., Bibb M.J., Ward J.M. and Cohen S.N. 1985a. Nucleotide sequences encoding and promoting expression of three antibiotic resistance genes indigenous to *Streptomyces*. Mol. Gen. Genet. 199, 26-36.

Bibb M.J., Janssen G.R. and Ward J.M. 1985b. Cloning and analysis of the promoter region of the erythromycin resistance gene (*ermE*) of *Streptomyces erythraeus*. Gene 38, 215-226.

Billing E. and Baker L.A.E. 1963. Characteristics of *Erwinia*-like organisms found in plant material. J. Appl. Bacteriol. 26, 58-65.

Borel M., Kergomard A. and Renard M.F. 1982. Degradation of natural rubber by *Fungi imperfecti*. Agric. Biol. Chem. 46, 877-881.

Botterman J. 1986. Expression vector systems and fermentation technology for the production of specific proteins in *Escherichia coli*. PhD Thesis. State University of Ghent, Belgium.

Bousfield I.J. and Goodfellow M. 1976. The '*rhodochrous*' complex and its relationships with allied taxa. In: Goodfellow M., Brownell G.H. and Serrano J.A. The biology of the nocardiae. Academic Press, London, UK.

Breed R.S., Murray E.G.D. and Smith N.R. (Eds.) 1957. Bergey's manual of determinative bacteriology, 7th Ed. The Williams and Wilkins Company, Baltimore, USA.

Brown O.R. and Clark J.B. 1966. Pigment of *Nocardia corallina*. J. Bacteriol. 92, 1844-1845.

Brownell G.H. 1981. A protoplasting and transfecting procedure for *Nocardia* (*Rhodococcus*) species. Actinomycetes 16, 54-56.

Brownell G.H., Adams J.N. and Bradley S.G. 1967. Growth and characterization of nocardiophages for *Nocardia canicruria* and *Nocardia erythropolis* mating types. J. Gen. Microbiol. 47, 247-256.

Brownell G.H. and Denniston K. 1984. Genetics of the nocardioform bacteria. In: Goodfellow M., Mordarski M. and Williams S.T. (Eds). The biology of the actinomycetes. Academic Press, London, UK.

Brownell G.H., Enquist L.W. and Denniston-Thompson K. 1980. An analysis of the genome of actinophage ΦEC. Gene 12, 311-314.

Brownell G.H., Saba J.A., Denniston K. and Enquist L.W. 1982. The development of a *Rhodococcus*-Actinophage gene cloning system. Dev. Ind. Microbiol. 23, 287-298.

Burns D.M. and Beacham I.R. 1984. Positive selection vectors: a small plasmid vector useful for the direct selection of *Sau*3A-generated overlapping DNA fragments. Gene 27, 323-325.

Buttner M.J. and Brown N.L. 1987. Two promoters from the *Streptomyces* plasmid pIJ101 and their expression in *Escherichia coli*. Gene 51, 179-186.

Campagna R. and Bückmann A.F. 1987. Comparison of the production of intracellular L-phenylalanine dehydrogenase by *Rhodococcus* species M4 and *Sporosarcina ureae* at 50 litre scale. Appl. Microbiol. Biotechnol. 26, 417-421.

Chacko C.I., Lockwood J.I. and Zabik M. 1966. Chlorinated hydrocarbon pesticides, degradation by microbes. Science 154, 893-895.

Chatterjee A.K. and Gibbins L.N. 1971. Induction of nonpigmented variants of *Erwinia herbicola* by incubation at supraoptimal temperatures. J. Bacteriol. 105, 107-112.

Cho L.K.N., Wong P.C.L. and Tsang J.C. 1987. Effects of polyamines on pigment formation in *Serratia marcescens*. Microbios Lett. 35, 33-38.

Clark-Curtiss J.E., Jacobs W.R., Docherty M.A., Ritchie L.R. and Curtiss R. 1985. Molecular analysis of DNA and construction of genomic libraries of *Mycobacterium leprae*. J. Bacteriol. 161, 1093-1102.

Clarke L. and Carbon J. 1976. A colony bank containing synthetic ColEI hybrid plasmids representative of the entire *Escherichia coli* genome. Cell 9, 91-99.

Cohen S.N., Chang A.C.Y. and Hsu L. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA. 69, 2110-2114.

Collins J. and Hohn B. 1978. Cosmids: A type of plasmid gene cloning vector that is packageable *in vitro* in bacteriophage λ heads. Proc. Natl. Acad. Sci. USA. 75, 4242-4246.

Cook A.M. and Hütter R. 1986. Ring dechlorination of deethylsimazine by hydrolases from *Rhodococcus corallinus*. FEMS Microbiol. Lett. 34, 335-338.

Cooney J.J., Marks H.W. and Smith A.M. 1966. Isolation and identification of canthaxanthin from *Micrococcus roseus*. J. Bacteriol. 92, 342-345.

Crawford R., Robinson L. and Chet M. 1981. ^{14}C labelled lignins as substrates for the study of lignin biodegradation. In: Kirk T.K., Miguchi T. and Chang M. (Eds). Lignin Biodegradation, Vol. 1. CRC Press, Boca Raton, USA.

Cundell A.M. and Mulcock A.P. 1973. Effects of microbiocides on the microbiological deterioration of vulcanised natural rubber. Dev. Ind. Microbiol. 14, 253-257.

Cundell A.M., Mulcock A.P. and Hills D.A. 1973. The influence of antioxidants and sulphur level on the microbiological deterioration of vulcanised NR. Rubber J. 22-35.

Dabbs E.R. 1987. A generalised transducing bacteriophage for *Rhodococcus erythropolis*. Mol. Gen. Genet. 206, 116-120.

Dabbs E.R. and Sole G.J. 1988. Plasmid-borne resistance to arsenate, arsenite, cadmium, and chloramphenicol in a *Rhodococcus* sp. In preparation.

Daggert M. and Ehrlich S.D. 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene 6, 23-28.

D'Aoust J.Y. and Gerber N.N. 1974. Isolation and purification of prodigiosin from *Vibrio psychroerythreus*. J. Bacteriol. 118, 756-757.

Datta A.R., Kaper J.B. and MacQuillan A.M. 1984. Shuttle cloning vectors for the marine bacterium *Vibrio parahaemolyticus*. J. Bacteriol. 160, 808-811.

Dauenhauer S., Hull R.A. and Williams R.P. 1984. Cloning and expression in *Escherichia coli* of *Serratia marcescens* genes encoding prodigiosin biosynthesis. J. Bacteriol. 158, 1128-1132.

Davies J.R. 1967. A thin-layer chromatographic method for distinguishing between natural rubber and synthetic polyisoprene. J. Chromatog. 28, 451-455.

Dean D. 1981. A plasmid cloning vector for the direct selection of strains carrying recombinant plasmids. Gene 15, 99-102.

Deng Z., Kieser T. and Hopwood D.A. 1986. Expression of a *Streptomyces* plasmid promoter in *Escherichia coli*. Gene 43, 295-300.

Enquist L.W. and Bradley S.G. 1971. Characterisation of deoxyribonucleic acid from *Streptomyces venezuelae* spores. Dev. Ind. Microbiol. 12, 225-236.

Faber M.D. and Nickerson W.J. 1979. Growth of microorganisms on insoluble polymers: transformation of automobile tires. Biotechnol. Lett. 1: 121-126.

Fayerman J.T. 1986. New developments in gene cloning in antibiotic producing organisms. Bio/Technology 4, 786-789.

Feitelson J.S. and Hopwood D.A. 1983. Cloning of a *Streptomyces* gene for an O-methyltransferase involved in antibiotic biosynthesis. Mol. Gen. Genet. 190, 394-398.

Feitelson J.S., Malpartida F. and Hopwood D.A. 1985. Genetic and biochemical characterization of the *red* gene cluster of *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. 131, 2431-2441.

Feng, J.S., Webb J.W. and Tsang J.C. 1982. Enhancement by sodium dodecyl sulphate of pigment formation in *Serratia marcescens*. Appl. Environ. Microbiol. 43, 850-853.

Ferreira N.P., Robson P.M., Bull J.R. and van der Walt W.H. 1984. The microbial production of 3aα-H-4α-(3'-propionic acid)5α-hydroxy-7aβ-methylhexahydro-indan-1-one-δ-lactone from cholestrol. Biotechnol. Lett. 6, 517-522.

Furuhashi K. and Takagi M. 1984. Optimization of a medium for the production of 1,2-epoxytetradecane by *Nocardia corallina* B-276. Appl. Microbiol. Biotechnol. 20, 6-9.

Furuhashi K., Taoka A., Uchida S., Karube I. and Suzuki S. 1981. Production of 1,2-epoxyalkanes from 1-alkanes by *Nocardia corallina* B-276. Eur. J. Appl. Microbiol. Biotechnol. 12, 39-45.

Gandhi N.M., Nazareth J., Divekar P.V., Kohl H. and de Souza N.J. 1973. Magnesidin, a novel magnesium-containing antibiotic. J. Antibiot. 26, 797-798.

Gantotti B.V. and Beer S.V. 1982. Plasmid-bourne determinants of pigmentation and thiamine prototrophy in *Erwinia herbicola*. J. Bacteriol. 151, 1627-1629.

Geller A. 1983. Growth of bacteria in inorganic medium at different levels of airborne organic substances. Appl. Environ. Microbiol. 46, 1258-1262.

Gerber N.N. and Lechevalier M.P. 1976. Prodiginine (prodigiosin-like) pigments from *Streptomyces* and other aerobic actinomycetes. Can. J. Microbiol. 22, 658-667.

Gil J.A., Kieser H.M. and Hopwood D.A. 1985. Cloning of a chloramphenicol acetyltransferase gene of *Streptomyces acrimycini* and its expression in *Streptomyces* and *Escherichia coli*. Gene 38, 1-8.

Goodfellow M. and Alderson G. 1977. The actinomycete-genus *Rhodococcus*: a home for the '*rhodochrous*' complex. J. Gen. Microbiol. 100, 99-122.

Goodfellow M. and Cross T. 1984. Classification In: Goodfellow M., Mordarski M. and Williams S.T. The biology of the actinomycetes. Academic Press, London, UK.

Goodfellow M. and Minnikin D.E. 1981. The Genera *Nocardia* and *Rhodococcus* In: Starr M.P., Stolp H., Trüper H.G., Balows, A. and Schlegel H.G. (Eds). The Prokaryotes. Springer-Verlag, Berlin, West Germany.

Goodfellow M. and Williams S.T. 1983. Ecology of actinomycetes. Ann. Rev. Microbiol. 37, 189-216.

Goodfellow M., Beckham A.R. and Barton M.D. 1982a. Numerical classification of *Rhodococcus equi* and related actinomycetes. J. Appl. Bacteriol. 53, 199-207.

Goodfellow M., Weaver C.R. and Minnikin D.E. 1982b. Numerical classification of some rhodococci, corynebacteria and related organisms. J. Gen. Microbiol. 128, 731-745.

Gräfe U., Reinhardt G., Hänel F., Schade W. and Gumpert J. 1985. Occurrence of squalene and dehydrosqualene in streptomycetes. J. Basic Microbiol. 25, 503-507.

Gray O. and Chang S. 1981. Molecular cloning and expression of *Bacillus licheniformis* β-lactamase gene in *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. 145, 422-428.

Green J.A., Rappoport D.A. and Williams R.P. 1956. Studies on pigmentation of *Serratia marcescens*. II. Characterization of the blue and the combined red pigments of prodigiosin. J. Bacteriol. 72, 483-487.

Guiney D.G., Hasegawa P. and Davis C.E. 1984. Plasmid transfer from *Escherichia coli* to *Bacteroides fragilis*: Differential expression of antibiotic resistance phenotypes. Proc. Natl. Acad. Sci. USA. 81, 7203-7206.

Häggblom M., Apajalahti J. and Salkinoja-Salonen M. 1986. Metabolism of chloroguaiacols by *Rhodococcus chlorophenolicus*. Appl. Microbiol. Biotechnol. 24, 397-404.

Hashimoto-Gotoh T., Kume A., Masahashi W., Takeshita S. and Fukuda A. 1986. Improved vector, pHSG664, for direct streptomycin-resistance selection:cDNA cloning with G:C-tailing procedure and sub-cloning of double-digest DNA fragments. Gene 41, 125-128.

Hawley D.K. and McClure W.R. 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucl. Acids Res. 11, 2237-2255.

Heap W.M. 1965. Microbiological deterioration of rubbers and plastics. Rubber and Plastics Research Association of Great Britain. Information Circular No. 476.

Heap W.M. and Morrell S.H. 1968. Microbiological deterioration of rubbers and plastics. J. Appl. Chem. 18, 189-194.

Hirsch P. and Alexander M. 1960. Microbial decomposition of halogenated propionic and acetic acid. Can. J. Microbiol. 6, 241-249.

Hookey J.V., Goodfellow M. and Ridgeway J.W. 1980. Selective isolation, enumeration and identification of *Nocardia* strains associated with rubber in contact with water and sewage. J. Appl. Bacteriol. 49, 14-15.

Hopwood D.A., Bibb M.J., Chater K.F., Kieser T., Bruton C.J., Kieser H.M., Lydiate D.T., Smith C.P. and Ward J.M. 1985. Genetic manipulation of *Streptomyces*. A Laboratory Manual. The John Innes Foundation, Norwich, UK.

Hori M., Ito E., Takita T., Koyama G., Takeuchi T. and Umezawa H. 1964. A new antibiotic, formycin. J. Antibiot. 17, 96-99.

Horinouchi S. and Beppu T. 1984. Production of large quantities of actinorhodin and undecylprodigiosin induced by *afsB* in *Streptomyces lividans*. Agric. Biol. Chem. 48, 2131-2133.

Horinouchi S., Hara O. and Beppu T. 1983. Cloning of a pleiotrophic gene that positively controls biosynthesis of A-factor, actinorhodin and prodigiosin in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. J. Bacteriol. 155, 1238-1248.

Horinouchi S., Uozumi T. and Beppu T. 1980. Cloning of *Streptomyces* DNA into *Escherichia coli*: absence of heterospecific gene expression of *Streptomyces* genes in *E. coli*. Agric. Biol. Chem. 44, 367-381.

Hosoda J., Konomi T., Tani N., Aoki H. and Imanaka H. 1977. Isolation of new nocardicins from *Nocardia uniformis* subsp. *tsuyamanensis*. Agric. Biol. Chem. 41, 2013-2020.

Hubert E.G., Potter C.S., Kalmanson G.M. and Guze L.B. 1969. Pigment formation in L-forms of *Serratia marcescens*. J. Gen. Microbiol. 55, 165-167.

Hummel W., Schütte H., Schmidt E., Wandrey C. and Kula M-R. 1987. Isolation of L-phenylalanine dehydrogenase from *Rhodococcus* sp. M4 and its application for the production of L-phenylaline. Appl. Microbiol. Biotechnol. 26, 409-416.

Hutchinson M., Ridgeway J.W. and Cross T. 1975. Biodeterioration of rubber in contact with water, sewage and soil. In: Lovelock D.W. and Gilbert R.A. (Eds). Microbial aspects of the deterioration of materials. Academic Press, London, UK.

Ish-Horowicz D. and Burke J.F. 1981. Rapid and efficient cosmid cloning. Nucl. Acids Res. 9, 2989-2998.

Ishiwa H. and Tsuchida N. 1984. New shuttle vectors for *Escherichia coli* and *Bacillus subtilis*. I. Construction and characterization of plasmid pHY460 with twelve unique cloning sites. Gene 32, 129-134.

Jaurin B. and Cohen S.N. 1984. *Streptomyces lividans* RNA polymerase recognizes and uses *Escherichia coli* transcriptional signals. Gene 28, 83-91.

Jaurin B. and Cohen S.N. 1985. *Streptomyces* contain *Escherichia coli*-type A+T-rich promoters having novel structural features. Gene 39, 191-201.

John C.K. and Verstraete W. 1979. Microbial degradation and preservation of *Hevea* latex. Proc. Int. Biodeterior. Symp. 4th. 127-134.

Kasweck K.L. and Little M.L. 1982. Genetic recombination in *Nocardia asteroides*. J. Bacteriol. 149, 403-406.

Kasweck K.L., Little M.L. and Bradley S.G. 1982. Plasmids in mating strains of *Nocardia asteroides*. Dev. Ind. Microbiol. 23, 279-286.

Katz E., Thompson C.J. and Hopwood D.A. 1983. Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. 129, 2703-2714.

Kessler C., Neumaier P.S. and Wolf W. 1985. Recognition sequences of restriction endonucleases and methylases - a review. Gene 33, 1-102.

Kieser T., Hopwood D.A., Wright H.M. and Thompson C.J. 1982. pIJ101, a multi-copy broad host range *Streptomyces* plasmid: functional analysis and development of DNA cloning vectors. Mol. Gen. Genet. 185, 223-238.

Kieser T., Moss M.T., Dale J.W. and Hopwood D.A. 1986. Cloning and expression of *Mycobacterium bovis* BCG DNA in *Streptomyces lividans*. J. Bacteriol. 168, 72-80.

King A.A. and Chater K.F. 1986. The expression of the *Escherichia coli lacZ* gene in *Streptomyces*. J. Gen. Microbiol. 132, 1739-1752.

King P.V. and Blakesley R.W. 1986. Optimizing DNA ligations for transformation. Focus 8, 1-3.

Kirby R. and Usdin K. 1985. The isolation and restriction mapping of a miniplasmid from the Actinomycete *Nocardia corallina*. FEMS Microbiol. Lett. 27, 57-59.

Koyama G. and Umezawa H. 1965. Formycin B and its relation to formycin. J. Antibiot. 18, 175-177.

Kritchevsky D. and Kirk M.R. 1952. Detection of steroids in paper chromatography. Arch. Biochem. Biophys. 35, 346-351.

Kuhn I., Stephenson F.H., Boyer H.W. and Greene P.J. 1986. Postive selection vectors utilizing lethality of the *Eco*RI endonuclease. Gene 44, 253-263.

Kurane R., Suzuki T. and Takahara Y. 1979. Removal of phthalate esters by activated sludge inoculated with a strain of *Nocardia erythropolis*. Agric. Biol. Chem. 43, 421-427.

Kurane R., Suzuki T. and Takahara Y. 1980. Metabolic pathway of phthalate esters by *Nocardia erythropolis*. Agric. Biol. Chem. 44, 523-527.

Kurane R., Suzuki T. and Fukuoka S. 1984. Purification and some properties of a phthalate ester hydrolyzing enzyme from *Nocardia erythropolis*. Appl. Microbiol. Biotechnol. 20, 378-383.

Kuswik-Rabiega G. and Rilling H.C. 1987. Solubilization and partial purification of squalene synthetase, copurification of presqualene pyrophosphate and squalene synthetase activities. J. Biol. Chem. 262, 1505-1509.

Kwiatkowska D., Zyska B.J. and Zankowicz L.P. 1978. Microbiological deterioration of natural rubber sheet by soil microorganisms. Proc. Int. Biodeterior. Symp. 4th. 135-141.

Labidi A., David H.L. and Roulland-Dussoix D. 1985. Cloning and expression of mycobacterial plasmid DNA in *Escherichia coli*. FEMS Microbiol. Lett. 30, 221-225.

Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Larson J.L. and Hershberger C.L. 1984. Shuttle vectors for cloning recombinant DNA in *Escherichia coli* and *Streptomyces griseofuscus* C581. J. Bacteriol. 157, 314-317.

Lechevalier M.P. 1976. The taxonomy of the genus *Nocardia*: some light at the end of the tunnel? *In*: Goodfellow M., Brownell G.H. and Serrano J.A. (Eds). The biology of the nocardiae. Academic Press, London, UK.

Lechevalier M.P. and Lechevalier H.A. 1970. Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Bacteriol. 20, 435-444.

Lechevalier M.P., Prauser H., Labeda D.P. and Ruan J-S. 1986. Two new genera of nocardioform actinomycetes: *Amycolata* gen. nov. and *Amycolatopsis* gen. nov. Int. J. Syst. Bacteriol. 36, 29-37.

Leeflang K.W.H. 1963. Microbiologic degradation of rubber. J. Am. Wat. Wks Ass. 55, 1523-1535.

Legerski R.J. and Robberson D.L. 1985. Analysis and optimization of recombinant DNA joining reactions. J. Mol. Biol. 181, 297-312.

Liaaen-Jensen S. and Andrewes A.G. 1985. Analysis of carotenoids and related polyene pigments. *In*: Gottschalk G. (Ed). Methods in Microbiology Vol 18. Academic Press, London, UK.

Loper J.E., Orser C.S., Panopoulos N.J. and Schroth M.N. 1984. Genetic analysis of fluorescent pigment production in *Pseudomonas syringae* pv. *syringae*. J. Gen. Microbiol. 130, 1507-1515.

Lowe J.A. and Tsang J.C. 1987. Investigation of biosynthesis of prodigiosin and its condensing enzyme in *Serratia marcescens*. 1. Charge group effects of various detergents. Microbios 49, 139-150.

Lydiate D.J., Malpartida F. and Hopwood D.A. 1985. The *Streptomyces* plasmid SCP2*: its functional analysis and development into useful cloning vectors. Gene 35, 223-235.

Mandelstam J., McQuillen K. and Dawes I. 1982. Biochemistry of bacterial growth. Blackwell Scientific Publications, Oxford, UK.

Maniatis T., Fritsch E.F. and Sambrook J. 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Mathis J., Wong W.W. and Tsang J.C. 1986. Susceptibility of *Serratia marcescens* to various polyamines. Microbios Lett. 32, 35-40.

Matsushima P., McHenney M.A. and Baltz R.H. 1987. Efficient transformation of *Amycolatopsis orientalis* (*Nocardia orientalis*) protoplasts by *Streptomyces* plasmids. J. Bacteriol. 169, 2298-2300.

Meile L. and Reeve J.N. 1985. Potential shuttle vectors based on the methanogen plasmid pME2001. Bio/Technology 3, 69-72.

Mikami Y., Takahashi K., Fukushima K., Yazawa K. and Arai T. 1987. A new pigment from *Streptomyces lavendulae*. J. Antibiot. 40, 385-387.

Mordarski M., Goodfellow M., Tkacz A., Pulverer G. and Schaal K.P. 1980. Ribosomal ribonucleic acid similarities in the classification of *Rhodococcus* and related taxa. J. Gen. Microbiol. 118, 313-319.

Mordarski M., Schaal K.P., Szyba K., Pulverer G. and Tkacz A. 1977. Classification of *Nocardia asteroides* and allied taxa based upon deoxyribonucleic acid reassociation. Int. J. Syst. Bacteriol. 27, 66-70.

Nette I.T., Pomortseva N.V. and Koslova E.I. 1959. Destruction of caoutchouc by microorganisms. Mikrobiologiya 28, 881-886.

Nickerson W.J. and Faber M.D. 1975. Microbial degradation and transformation of natural and synthetic insoluble polymeric substances. Dev. Ind. Microbiol. 16, 111-118.

O'Farrell P.H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250, 4007-4021.

Orchard V.A. and Goodfellow M. 1980. Numerical classification of some named strains of *Nocardia asteroides* and related isolates from soil. J. Gen. Microbiol. 118, 295-312.

Ourisson G., Rohmer M. and Poralla K. 1987. Prokaryotic hopanoids and other polyterpenoid sterol surrogates. Ann. Rev. Microbiol. 41, 301-333.

Patt S.L. and Shoolery J.N. 1982. Attached proton test for carbon-13 NMR. J. Magn. Reson. 46, 535-539.

Peczyńska-Czoch W. and Mordarski M. 1984. Transformation of xenobiotics. In: Goodfellow M., Mordarski M. and Williams S.T. (Eds). The biology of the actinomycetes. Academic Press, London, UK.

Portmore J.J., Burnett W.V. and Chiang S. 1987. Bifunctional cosmid vector for *Streptomyces*. Biotechnol. Lett. 9, 7-12.

Pouwels P.H., Enger-Valk B.E. and Brammar W.J. 1985. Cloning Vectors. Elsevier Publishers, Amsterdam, The Netherlands.

Qian H.L., Feng J.S. and Tsang J.C. 1982. Pigments and antibiogram of transconjugants from nonpigmented mutants of *Serratia marcescens*. Microbios 33, 101-110.

Rast H.G., Engelhardt G., Diegler W. and Wallhoffer P.R. 1980. Bacterial degradation of model compounds for lignin and chlorophenol derived lignin bound residues. FEMS Microbiol. Lett. 8, 259-263.

Raymond R.L. 1971. Hydroxy-phenyl- α -ketobutyric acids. United States Patent Office. Patent No. 3,592,846.

Raymond R.L. and Jamison V.W. 1971. Biochemical activities of *Nocardia*. Adv. Appl. Microbiol. 14, 93-122.

Reh M. and Schlegel H.G. 1981. Hydrogen autotrophy as a transferable genetic character of *Nocardia opaca* 1b. J. Gen. Microbiol. 126, 327-336.

Remaut E., Tsao H. and Fiers W. 1983. Improved plasmid vectors with thermo-inducible expression and temperature regulated runaway replication. Gene 22, 103-113.

Rigby P.W.J., Dieckmann M., Rhodes G. and Berg P. 1977. Labelling DNA to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. 113, 237-251.

Rook J.J. 1955. Microbiological deterioration of vulcanized rubber. Appl. Microbiol. 3, 302.

Rudd B.A.M. and Hopwood D.A. 1980. A pigmented mycelial antibiotic in *Streptomyces coelicolor*: control by a chromosomal gene cluster. J. Gen. Microbiol. 119, 333-340.

Schaal K.P. and Beaman B.L. 1984. Clinical significance of actinomycetes. In: Goodfellow M., Mordarski M. and Williams S.T. (Eds). The biology of the actinomycetes. Academic Press, London, UK.

Schottel J.L., Bibb M.J. and Cohen S.N. 1981. Cloning and expression in *Streptomyces lividans* of antibiotic resistance genes derived from *Escherichia coli*. J. Bacteriol. 146, 360-368.

Schupp T. and Divers M. 1986. Protoplast preparation and regeneration in *Nocardia mediterranea*. FEMS Microbiol. Lett. 36, 159-162.

Schupp T., Toupet C., Stålhammar-Carlemalm M. and Meyer J. 1983. Expression of a neomycin phosphotransferase gene from *Streptomyces fradiae* in *Escherichia coli* after interplasmidic recombination. Mol. Gen. Genet. 189, 27-33.

Seckler B. and Poralla K. 1986. Characterization and partial purification of squalene-hopene cyclase from *Bacillus acidocaldarius*. Biochim. Biophys. Acta. 881, 356-363.

Sensfuss C., Reh M. and Schlegel H.G. 1986. No correlation exists between the conjugative transfer of the autotrophic character and that of plasmids in *Nocardia opaca* strains. J. Gen. Microbiol. 132, 997-1007.

Seo C.W., Yamada Y., Takada N. and Okada H. 1981. Hydration of squalene and oleic acid by *Corynebacterium* sp. S-401. Agric. Biol. Chem. 45, 2025-2030.

Seo C.W., Yamada Y., Takada N. and Okada H. 1983. Microbial transformation of squalene: terminal methyl group oxidation by *Corynebacterium* sp. S-401. Appl. Environ. Microbiol. 45, 522-525.

Setchell C.H., Bonner J.F., Wright S.J., Caunt P. and Baker P.B. 1985. Microbial transformation of squalene: formation of a novel ketone from squalene by a *Rhodococcus* sp. Appl. Microbiol. Biotechnol. 21, 255-257.

Shewan J.M. and McMeekin T.A. 1983. Taxonomy (and ecology) of *Flavobacterium* and related genera. Ann. Rev. Microbiol. 37, 233-252.

Smith G.E. and Summers M.D. 1980. The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzylomethyl-paper. Anal. Biochem. 109, 123-129.

Sobin B. and Stahly G.L. 1942. Chromogenesis by variants of *Staphylococcus aureus*. J. Bacteriol. 92, 1578-1579.

Spence D. and van Niel C.B. 1936. Bacterial decomposition of the rubber in *Hevea* latex. Ind. Eng. Chem. 28, 847-850.

Stanzak R., Matsushima P., Baltz R.H. and Rao R.N. 1985. Cloning and expression in *Streptomyces lividans* of clustered erythromycin biosynthesis genes from *Streptomyces erythreus*. Bio/Technology 4, 229-232.

Starr M.P., Stolp H., Truper H.G., Balows A. and Schlegel H.G. 1981. The Prokaryotes. Vol. 1. Springer-Verlag, Heidelberg, Germany.

Stees J.L., Brown O.R. and Grigsby R.D. 1969. Structural features of the major pigment component of *Nocardia corallina*. Microbios 2, 199-200.

Stevis P.E. and Ho N.W.Y. 1987. Positive selection vectors based on xylose utilization suppression. Gene 55, 67-74.

Strauss D.G., Berger U. and Fleck W.F. 1983. Pigments from microorganisms. Chemistry and Biotechnology of Biologically Active Natural Products, 2nd Int. Conf. Budapest, Hungary.

Sullivan M.A., Yasbin R.E. and Young F.E. 1984. New shuttle vectors for *Bacillus subtilis* and *Escherichia coli* which allow rapid detection of inserted fragments. Gene 29, 21-26.

Tárnok I. 1976. Metabolism in nocardiae. In: Goodfellow M., Brownell G.H. and Serrano J.A. (Eds). The biology of the nocardiae. Academic Press, London, UK.

Thiry G. 1984. Plasmids of the epiphytic bacterium *Erwinia uredovora*. J. Gen. Microbiol. 130, 1623-1631.

Tornabene T.G., Kates M., Gelpi E., and Oro J. 1969. Occurrence of squalene, di- and tetrahydrosqualenes and vitamen MK_8 in an extremely halophilic bacterium, *Halobacterium cutirubrum*. J. Lip. Res. 10, 294-303.

Trojanowski J., Haider K. and Sundman V. 1977. Decomposition of ^{14}C-labelled lignin and phenols by a *Nocardia* sp. Archiv. fur Mikrobiologie 114, 149-153.

Tsao S-W., Rudd B.A.M., He X-G., Chang C-J. and Floss H.G. 1985. Identification of a red pigment from *Streptomyces coelicolor* A3(2) as a mixture of prodigiosin derivatives. J. Antibiot. 38, 128-131.

Tsuchii A., Suzuki T. and Takahara Y. 1978. Microbial degradation of liquid polybutadiene. Agric. Biol. Chem. 42, 1217-1222.

Tsuchii A., Suzuki T. and Takahara Y. 1979. Microbial degradation of *cis*-1,4-polyisoprene. Agric. Biol. Chem. 43, 2441-2446.

Tsuchii A., Suzuki T. and Takeda K. 1985. Microbial degradation of natural rubber vulcanizates. Appl. Environ. Microbiol. 50, 965-970.

Ullrich A., Shine J., Chirgwin J., Pictet R., Tischer E., Rutter W.J. and Goodman H.M. 1977. Rat insulin genes: construction of plasmids containing the coding sequences. Science 196, 1313-1319.

Vernet T., Lau P.C.K., Narang S.A. and Visentin L.P. 1985. A direct selection vector derived from pColE3-CA38 and adapted for foreign gene expression. Gene 34, 87-93.

Vieira J. and Messing J. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19, 259-268.

Wakisuki Y., Koizumi K, Nishimoto Y., Kobayashi M. and Tsuji N. 1980. Hygromycin and epihygromycin from a bacterium *Corynebacterium equi*. J. Antibiot. 33, 695-704.

Watson J.D., Hopkins N.H., Roberts J.W., Steitz J.A. and Weiner A.M. 1987. Molecular biology of the gene. 4th Ed. Benjamin/Cummings Publishing Co. Inc., Menlo Park, CA, USA.

Westpheling J., Ranes M. and Losick R. 1985. RNA polymerase heterogeneitty in *Streptomyces coelicolor*. Nature 313, 22-27.

Williams B.G. and Blattner F.R. 1980. Bacteriophage vectors for DNA cloning. In: Genetic Engineering. Setlow J.K. and Hollaender A. (Eds). Vol. 2. Plenum Press, New York, USA.

Williams R.P. and Qadri S.M.H. 1980. The pigment of *Serratia* In: Von Graevenitz A. and Rubin S.J. (Eds) The genus *Serratia*. CRC Press, Inc., Boca Raton, USA.

Williams R.P., Green J.A. and Rappoport D.A. 1956. Studies on pigmentation of *Serratia marcescens*. I. Spectral and paper chromatographic properties of prodigiosin. J. Bacteriol. 71, 115-120.

Williams S.T., Sharples G.P., Serrano J.A., Serrano A.A. and Lacey J. 1976. The micromorphology and fine structure of nocardioform organisms. In: Goodfellow M., Brownell G.H. and Serrano J.A. (Eds). The biology of the nocardiae. Academic Press, London, UK.

Wood W.B. 1966. Host specificity of DNA produced by *Escherichia coli;* bacterial mutations affecting the restriction and modification of DNA. J. Mol. Biol. 16, 118-133.

Woods D.R., Mosmann T.R., Hanson S. and Hendry D.A. 1971. Pigmentation and acriflavine resistance in *Serratia marcescens*. J. Bacteriol. 108, 765-769.

Wright L.F. and Hopwood D.A. 1976. Actinorhodin is a chromosomally determined antibiotic in *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. 96, 289-297.

Yamada Y., Motoi H., Kinoshita S., Takada N. and Okada H. 1975. Oxidative degradation of squalene by *Arthrobacter* species. Appl. Microbiol. 29, 400-404.

Yamada Y., Seo C.W. and Okada H. 1985. Oxidation of acycylic terpenoids by *Corynebacterium* sp. Appl. Environ. Microbiol. 49, 960-963.

Zabeau M. and Stanley K. 1982. Enhanced expression of cro--galactosidase fusion proteins under the control of the P_R promoter of bacteriophage . EMBO J. 1, 1217-1224.

ZoBell C.E. and Grant C.W. 1942. The bacterial oxidation of rubber. Science. 96, 379-380.

Zyska B.J. 1981. Rubber. In: Rose A.H. Economic Microbiology. Academic Press, London, UK.