Characterization of rubber degrading isolates and the cloning of DNA conferring an apparent latex degrading ability

Chapter III abstract

Four rubber degrading strains designated BA1, Est, Chiba and Yeo were isolated from soil samples collected in Buenos Aires, Estonia, Chiba and South Africa respectively. From 16S rRNA sequencing BA1 was identified as *S. tendae*, Est as a member of the species *Pseudonocardia*, Chiba as *S. flavogriseus* and Yeo as *S. griseus*. On latex agar plates colonies of all strains formed translucent halos. Scanning electron microscopy revealed that these isolates were able to colonize and penetrate vulcanized glove rubber. Genomic libraries were created and using latex enrichment cultures a potential rubber degrading segment of DNA approximately 4kbp in size was identified in *Pseudonocardia* spp. Preliminary analysis of part of the fragment revealed the highest sequence similarity corresponded to the TetR transcriptional regulator family. Additionally, a homologue of the rubber degrading gene *(latex clearing protein)* was amplified from *S. coelicolor* and heterologously expressed in three nocardioform actinomycetes. This gene facilitated efficient colonization of solid latex rubber, however did not lead to fragmentation of the substrate.

1. Introduction

1.1 Microbial rubber biodegradation

Latex is an important commercial polymer with approximately 10 million tons harvested annually to produce over 40 thousand products (Mooibroek and Cornish, 2000, Jendrossek *et al*., 1997a). Its versatility ranges from use in latex gloves to rubber seals, tubing and tyres (Bode *et al*., 2001). Although more than 2500 plant species produce this polymer its main source comes from the Brazilian rubber tree *Hevea brasiliensis.* Latex consists of rubber particles (*cis*-1,4 polyisoprene) and a small percentage of non-rubber constituents (protein, carbohydrates and salts) suspended in an aqueous serum (Othmer, 1997). For commercial purposes this polymer normally undergoes a process of vulcanization, altering its molecular structure through the cross-linking of isoprene chains. Vulcanization is achieved in tyres by heating in the presence of sulfur [Fig. 1.1] or in latex gloves by peroxidation and irradiation (Berekaa *et al.,* 2000). This process yields rubber with which we are familiar, the type used to make wire covering, gloves and footwear. Additionally, various chemical components are also added to raw latex to achieve a degree of stiffening, elasticity and durability.

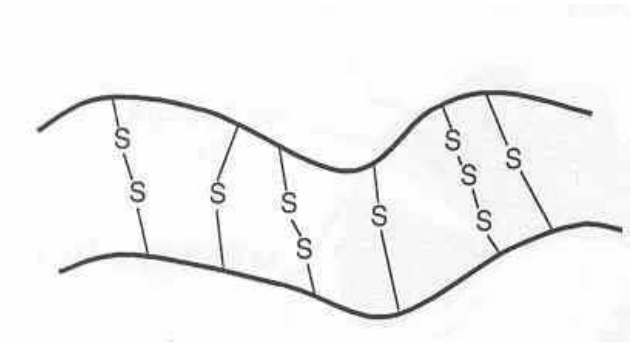


Fig. 1.1: Schematic illustration of sulfur bond cross-links formed in tyres

Although rubber biodegradation has been investigated for over 90 years, it is only in the last ten that substantial progress has been made in this biotechnological field (Braaz *et al*., 2005). The difficult isolation of relevant bacteria, long culturing periods in addition to paucity of genetic tools due to inefficient transformation systems has

hindered advancement in this area of research (Rose and Steinbuchel, 2005). Earlier studies were focussed on preventing microbial degradation of polyisoprene. However, in recent years the abundant use and consequent extensive waste generation of this material has enhanced interest in this area for the purpose of bioremediation.

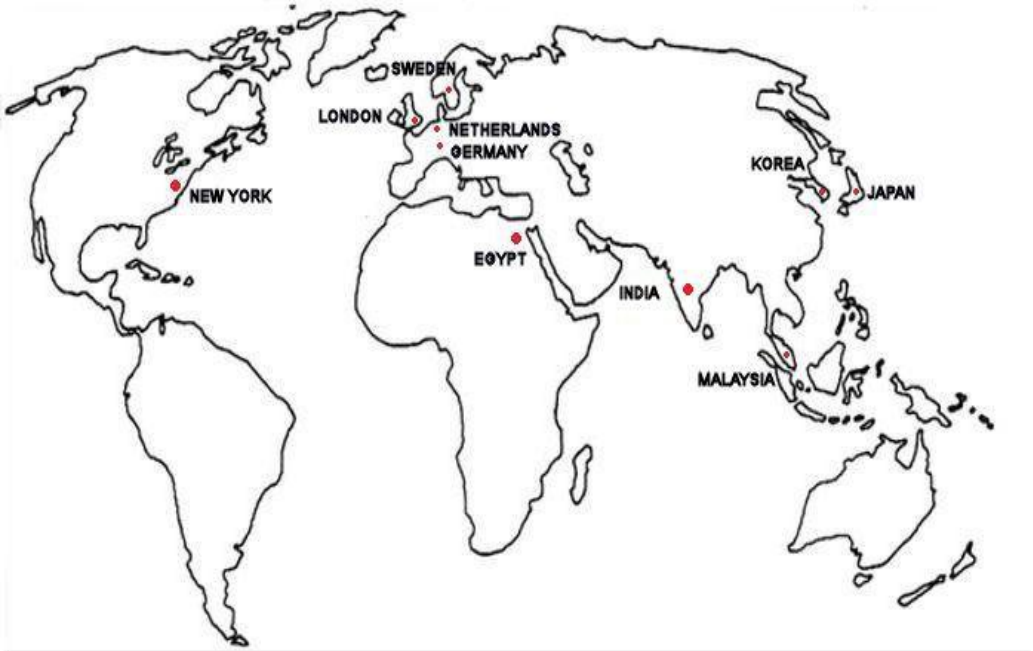


Fig 1.2: Parts of the world which have undertaken studies concerning rubber biodegradation

From the widespread research carried out in numerous regions it is evident that much interest revolves around the subject of rubber degradation [Fig. 1.2]. The most substantial work has been conducted in Germany as it is here that the first rubber degrading genes were identified, latex degrading enzyme characterized and biodegradative mechanism uncovered. Both American and Swedish studies are primarily directed towards the issue of rubber recycling. Since strong sulfur cross-links within tyres prevent them from being reused, these studies have tested the effectiveness of desulfurization by both bacteria and fungi (Christiansson *et al*., 1998; Sato *et al*., 2004; Kim and Park, 1999). The latest Japanese research has adopted a different approach, employing the use of free radical chain reactions to cleave *cis*-1,4 polyisoprene. Unexpectedly, research has not progressed to the stage of bioreactor testing. To date just a single study concerning biodegradation in a bioreactor system has been published. Substrate degradation was monitored in a continuous system with

respect to changes in temperature, pH and aeration rate. With regard to the actinomycete used the researchers were able to establish the optimal parameters allowing for rubber breakdown (Azhari *et al.*, 2002).

1.2 Rubber degrading bacteria

Actinomycetales have dominated the literature with regard to *cis*-1,4 polyisoprene degradation, with all rubber degrading isolates except three identified as members of this order [Table 1.1]. *Streptomyces, Nocardia* and *Gordonia* are the most prominent genera. Contrastingly, rubber degradation among Gram negatives is rare. Thus far, just three Gram negative strains namely, *Xanthomonas* spp. 35Y, *Pseudomonas citronellolis,* and *Actinobacter calcoaceticus* possess this ability (Bode *et al*., 2000, Bode *et al.,* 2001). However, it has been suggested that Gram negative isoprene degraders are infrequently isolated due to the absence of growth factors in the media used for screening purposes (Jendrossek *et al*., 1997).

1.3 Decomposition strategies

Rubber degrading bacteria are divided into two classes along the basis of the biodegradative strategy adopted. Accordingly, two isolation techniques exist for the detection of these bacteria.

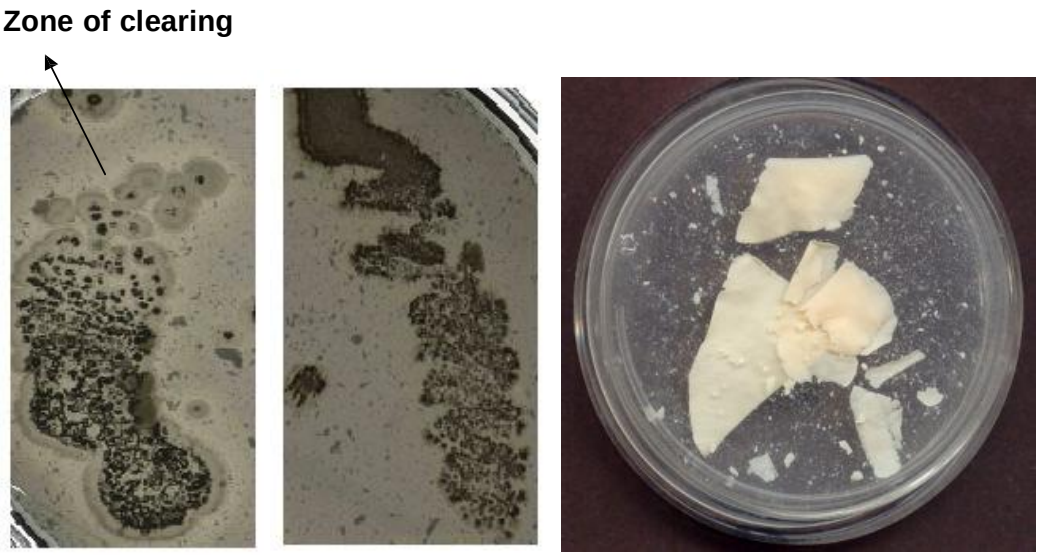


Fig. 1.3: The clear zone formed around bacterial colonies is indicative of rubber degradation by this strain (left). A non-latex utilizing strain on latex agar (centre) and direct attack of the rubber substrate by adhesive bacteria showing visible glove fragmentation (right) (Chengalroyen, 2005)

The clear zone isolation technique developed by Spence and Niel is used to detect bacteria which secrete extracellular enzymes during *cis*-1,4 isoprene degradation (Jendrossek *et al*., 1997a). This method selects for latex-utilizing strains on the basis of the formation of translucent halos (clear zones) around colonies on opaque latex agar [Fig. 1.3]. *Streptomyces, Actinoplanes* and *Micromonospora* fall into this category. Solid pieces of rubber are used to select for adhesive rubber degraders. These bacteria attack the rubber substrate directly, forming a biofilm and merging into the polymer initiating degradation at the cell surface (Linos *et al*., 2000) [Fig. 1.3]. This encompasses *Mycobacterium, Gordonia* and *Nocardia* species. Compared to strains which secrete enzymes the adhesive bacterial group have been implicated as far more effective degraders of this material, shifting focus towards them in recent years (Arenskotter *et al*., 2001).

1.4 Rate of rubber degradation

The rate of polyisoprene degradation is variable among strains and dependent on both the biodegradative strategy of the bacteria and nature of the substrate, as shown in

Table 1.1. Depending on the rubber product required, the polymer is exposed to different treatments through the addition of accelerators, antioxidants, anti-abrasives and fillers, making the substrate nature inconsistent (Rook, 1955).

Studies carried out on the degradation of rubber deal with latex, natural rubber bands or vulcanized rubber. As mentioned before, latex is a milky sap containing rubber particles, since it is devoid of chemicals it is more easily degraded and many microbes capable of breaking down this compound have been isolated. Rubber bands are vulcanized but unlike gloves are exposed to fewer chemicals and resultantly more easily degradable. Relevant cultivation conditions also play a role, a semi-continuous system i.e. supplementation of the culture with fresh media after 6 weeks of incubation led to complete break down of the polymer within a week by *Gordonia* spp. VH2 (Berekaa *et al.,* 2000).

Interesting organisms isolated include *Nocardia* 835A strain Rc and *Gordonia* strains, which were found to be strong rubber decomposers. *Nocardia* spp. 835A was able to degrade unvulcanized, vulcanized and synthetic isoprene. This strain led to the mineralization of more than 90% of a latex glove piece in just over 2 weeks (Tsuchii *et al.,* 1985). A mutant of strain 835A was isolated. Its ability to break down natural rubber was similar to that of the parent strain; however this isolate named Rc was also discovered to be potent degrader of tyre tread. It penetrated into the material and was responsible for an 81% weight loss of the polymer in 8 weeks, of which 47% was completely mineralized. An interesting aspect examined in this study dealt with the influence of rubber content of the product on microbial degradation. They found a rubber content of over 70 parts per hundred (phr) supported decomposition while < 50 phr showed little degradation. This is a significant factor when considering recycling since different portions of tyre for instance have varying amounts of natural rubber (Tsuchii and Tokiwa, 1999). Strain Rc is the only published isolate found to degrade tyre tread which is not promising when considering tyre recycling.

Many members of *Gordonia* are capable of the biodegradation of recalcitrant compounds, and it seems to possess an ability to break down rubber as well. Four adhesive strains capable of mineralizing natural and synthetic isoprene were isolated and characterized, namely *G. westfalica* Kb2 and *G. polyisoprenivorans* strains VH2,

Kd2 and Y2K (Linos *et al.,* 2002; Linos *et al.,* 1999; Arenskötter *et al.,* 2001). All isolates reportedly fragmented the material.

There is evidence that the chemicals added to rubber products such as gloves inhibit microbial decomposition. The extraction of antimicrobial agents (antioxidants), using organic solvents demonstrated the enhanced colonization and break down of the polymer by *Gordonia* spp. Kb2 and *Micromonospora* spp. W2b, although this did not positively influence *Gordonia* spp. VH2 and *Mycobacteria* spp. NF4. This study revealed that the chemical pre-treatment of rubber could vastly improve microbial degradation (Berekaa *et al.,* 2000). However, the use of large quantities of chemical solvents to pretreat rubber is environmentally unsafe. Hence, the degradation of antioxidants by microbial means has also been examined. Wood rotting fungi, which are well documented with regard to their lignin-degrading capacities, were tested. In view of the resemblance between rubber additives and fungi degradable compounds, these they were considered ideal candidates. The white-rot fungus *R. bicolor* reduced rubber chemical toxicity, allowing a desulfurization strain to effectively grow in the presence of the polymer (Bredberg *et al.,* 2002). Similarly, Kim and Park (1999) compared the use of chemical and microbial means to desulfurize rubber. They found that the sulfur content was reduced by 30% and 8% for *T. peromatabolis* and chemically treated tyre rubber respectively. Additionally, the improved mechanical properties of microbe treated rubber were similar to that of natural rubber.

To reiterate, the main problems in recycling rubber revolve around the difficulty of breaking cross-linked chains induced by vulcanization and the presence of additives which inhibit microbe growth thus effecting break down of the polymer (Bredberg *et al.,* 2002; Roy *et al.,* 2006a).

Table 1.1: Rate of rubber degradation by various rubber degrading strains

Organism	**Degradative strategy**	**Rubber reduction (%)**	**Incubation (week/s)**	**Reference**
Nocardia spp. 835A strain Rc	B	80 (tyre)	8	Tsuchii and Tokiwa, 1999
Nocardia spp. 835A	B	> 90	3	Tsuchii and Tokiwa, 1999
Gordonia spp. VH2	B	>50	4	Linos *et al*., 2000
Pseudomonas citronellolis	B	13	10	Bode *et al*., 2000
Acinetobacter calcoaceticus	B	12	10	Bode *et al*., 2000
Pseudomonas spp.	B	10.38 and 43.11 (natural rubber)	6	Roy *et al*., 2006
Xanthomonas spp. 35Y	A	60 (natural latex rubber)	1	Tsuchii and Takeda, 1990
Xanthomonas spp.	A	12	10	Bode *et al*., 2001
Streptomyces griseus 1D	A	18	10	Bode *et al*., 2001
Streptomyces coelicolor 1A	A	10-18	6	Bode *et al*., 2001
Streptomyces spp. K30	A	13.4	12	Rose *et al*., 2005
Streptomyces spp. S1G, S3D, S4C, S4E, S4F, S4G	A	>10	6	Heisey and Papadatos, 1995

Unless otherwise stated rubber reduction was calculated using glove rubber

Table 1.2: Rubber degrading bacteria identified in current literature

Bacterial Strain	**Type of rubber degraded**	**References**
Gordonia polyisoprenivorans	Natural and synthetic rubber (after removal of antioxidants), natural latex *	Linos *et al*., 1999
Gordonia westfalica	Natural and synthetic rubber, natural latex *	Linos *et al*., 2002
Gordonia polyisoprenivorans VH2 and Y2K	Natural and synthetic rubber (after removal of antioxidants), natural latex *	Arenskotter *et al*., 2001
Streptomyces coelicolor and *griseus* 18a, *Nocardia* DSMZ43191, ***Actinobacter calcoaceticus***, ***Xanthomonas* spp.**	Vulcanized rubber (glove)	Bode *et al*., 2001
Streptomyces spp. La7	Latex, unvulcanized natural rubber	Gallert, 2000
Nocardia spp. 835A	Unvulcanized natural and synthetic rubber, vulcanized natural rubber (latex gloves, bands, tubing)	Tsuchii *et al*., 1985
***Xanthomonas* spp.**	Purified natural latex, synthetic rubber	Jendrossek and Reinhardt, 2003
Gordonia spp. Kb2, Kd2 and VH2*; Micromonospora aurantiaca* W2b*; Mycobacterium fortuitum* NF4	Natural and synthetic rubber (following antioxidant removal), natural latex *	Berekaa *et al*., 2000
Streptomyces spp. S1G, S3D, S4C, S4E, S4F and S4G*; Amycolatopsis* spp. S1A and S1D*; Nocardia* spp. SF3	Vulcanized rubber (glove)	Heisey and Papadatos, 1995
Nocardia 835A mutant strains Wh, Rw and Rc	Vulcanized rubber (tyre)	Tsuchii and Tokiwa, 1999
Streptomyces spp.*; Micromonospora* spp.*; Microtetraspora* spp.; *Actinoplanes* spp.*; Nocardia* spp.*; Actinomadura* spp.; *Dactylssporangium* spp.	Natural rubber latex	Jendrossek *et al*., 1997b
Streptomyces coelicolor 1A*;* ***Pseudomonas citronellolis***	Synthetic rubber	Bode *et al*., 2000
Gordonia spp. VH2 and Kb2, *Mycobacterium fortuitum* NF4	Vulcanized rubber (glove)	Linos *et al*., 2000b
Micromonospora aurantiaca W2b	Latex dispersed in agar	Linos *et al*., 2000a
Actinomadura spp. E6, *Nocardia farcinica* E1, *Thermomonospora curvata* E4 and E5	Latex dispersed in agar, synthetic rubber	Ibrahim *et al.*, 2006
***Pseudomonas* spp.**	Natural and vulcanized rubber (glove)	Roy *et al*., 2006

*- Grew on latex spread directly onto plates as thin layer but not when dispersed in agar
Gram negatives are represented in bold print

1.5 Mechanism of rubber biodegradation

Due to its high molecular weight, rubber can not be taken up by bacterial cells and must be extracellularly cleaved (Jendrossek *et al*., 1997a). Tsuchii and co-workers (1985) were the first to elucidate a mechanism for rubber degradation, hypothesizing dioxygenase endocleavage of the double bond as the initial step. With studies done on *Nocardia* and *Xanthomonas*, an analysis of the low molecular weight degradation products using nuclear magnetic resonance confirmed oxidative cleaving, as did Fourier transform infrared (FTIR) spectroscopy on *Gordonia* VH2 [Fig. 1.4] (Linos *et al*, 2000b). Studies done on *Streptomyces coelicolor* 1A enabled the biochemical pathway of rubber metabolism to be proposed (Bode *et al*., 2000) [Fig. 1.5]. The polymer is broken down initially by oxidative cleaving at the double bond, resulting in acetonyl diprenyl acetoaldehyde. Aldehyde groups are oxidized to carboxylic acid. Following β-oxidation carboxylic acid is activated as a coenzyme A ester. The β-keto acid decarboxylates, forming $C_{12}H_{20}O_2$ and $C_{17}H_{28}O_2$ and the lower molecular weight compounds are taken up by bacteria. Labelling experiments with $^{18}O_2$ and $H_2{}^{16}O$ conducted by Braaz and co-workers (2005) definitively determined that polyisoprene was cleaved by a dioxygenase (as opposed to a monooxygenase) mechanism.

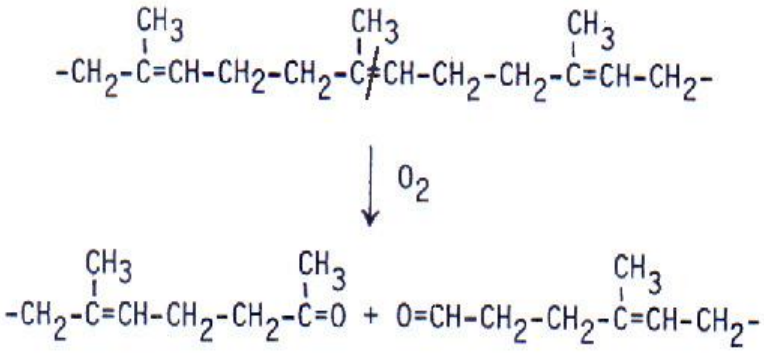


Fig. 1.4: Diagram of products generated through rubber degradation by *Nocardia* 835A and *Xanthomonas* 35Y. The black line represents oxidative endocleavage of the double bond within the rubber polymer (Tsuchii *et al*., 1985; Tsuchii and Takeda, 1990)

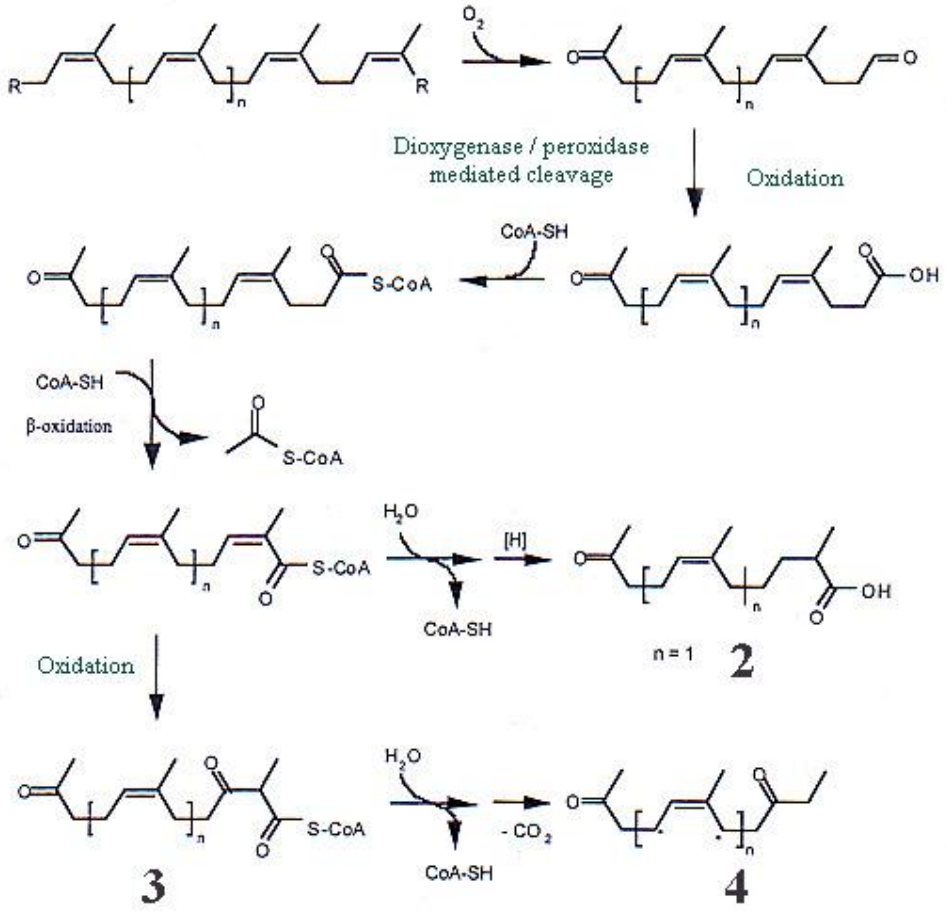


Fig. 1.5: Mechanism for rubber metabolism proposed in *Streptomyces coelicolor* 1A (Bode *et al*., 2000). Compounds: **2** – $C_{13}H_{22}O_3$, **3** – $C_{12}H_{20}O_2$, **4** - $C_{17}H_{28}O_2$.

1.6 *In vitro* rubber degradation

Japanese workers have introduced an innovative means of dealing with rubber biodegradation, employing the use of enzyme-mediator systems. Three systems were investigated, namely lipoxygenase/ linoleic acid, horseradish peroxidase/ 1-hydroxybenzotriazole and Fenton reagent/ linoleic acid, all of which were effective against both *trans*- and *cis*-1,4 polyisoprene. The basis of these systems involves the generation of radicals which oxidatively attack the polymer by β-scission. The results yielded from these treatments were promising, revealing hole formation in the substrate after just 2-7 days (Enoki *et al*., 2003; Sato *et al*., 2003)

1.7 Genes responsible for rubber biodegradation

Rubber degrading genes were identified in a Gram negative *Xanthomonas* strain and Gram positive Streptomycete, both of which release extracellular enzymes specific for rubber degradation. Back translation from the amino acid sequence of the *Xanthomonas* polyisoprene enzyme allowed primers to be constructed and the gene assigned the name *Rubber oxygenase A* (*roxA*) to be cloned. Amino acid sequence analysis revealed two heme binding motifs (Braaz *et al.*, 2004). It has been suggested that this novel heme oxidase is a member of a new family of proteins (Jendrossek and Reinhardt, 2003).

In *Streptomyces* spp. strain K30, three genes involved in rubber degradation and catabolism were identified using complementation (Rose *et al.*, 2005). The latex clearing protein, product of the *lcp* gene, is speculated to bind to and cleave the polymer while *oxiA* and *oxiB* located downstream of *lcp* catabolize the degraded products. These enzymes are all members of the xanthine oxidase family [Fig. 1.6].

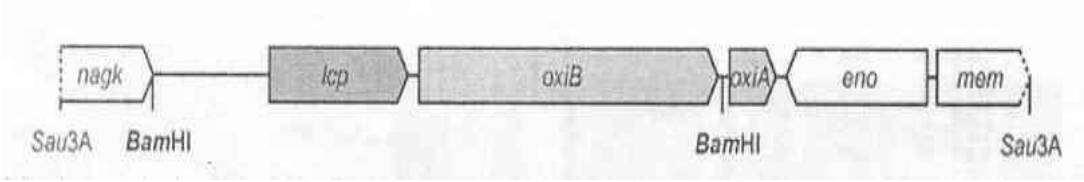


Fig. 1.6: Schematic representation of the *lcp*, *oxiA* and *oxiB* genes (Rose *et al*., 2005)

The entire genome of *S. coelicolor* A3 (2) was sequenced. Aligning the rubber degrading gene complex from *Streptomyces* K30 to the genome of *S. coelicolor* (also an extracellular rubber decomposer) showed a clear homologue of the *lcp* gene. However, no *oxiA* or *oxiB* homologues were found. Notably, there are over 100 putative oxidoreductases believed to be present in *S. coelicolor*. Thus it is possible that further catabolism of rubber is controlled by oxidoreductases elsewhere on the chromosome.

Broker and coworkers (2004) performed the first study which attempted to identify the rubber degrading gene from an adhesive degrading strain. The plasmid pKB1 was isolated from *Gordonia westfalica* under the assumption that it may confer a rubber degrading ability to the bacterium. Curing of the plasmid confirmed an inability to utilize *cis*-1,4 polyisoprene as a carbon source. The sequencing of the 101 kb plasmid revealed 105 open reading frames of which three showed sequence similarity to cytochrome C genes. These served as candidates for a rubber degrading gene; however no definite gene was assigned [Fig. 1.7].

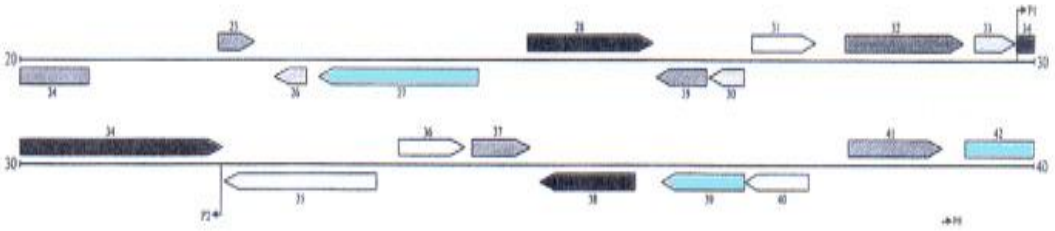


Fig. 1.7: Part of the sequenced plasmid pKB1. The blue ORFs (27, 39 and 42) represent the proposed rubber degrading genes (Broker *et al*, 2004).

In a more successful study, a rubber degradative gene was isolated from *Nocardia farcinica* E1 by means of southern hybridization using *lcp* as a probe (Ibrahim *et al*., 2006). Notably, the degradative strategies differ between *Nocardia* and *Streptomyces*, suggesting again that the *lcp* homologue might be responsible for the initial cleaving in both adhesive and extracellular enzyme producing rubber degrading bacteria

Possible rubber degrading genes were also identified in the adhesive degrader, *G. polyisoprenivorans* VH2 utilizing insertional mutagenesis. Twenty five thousand mutants were screened and 6 clones displaying an inability to degrade rubber were isolated, in addition to a further 2 exhibiting a rubber leaky phenotype. The rubber leaky phenotype referred to clones which initially displayed an inability to utilize the substrate, however reverted back to the wildtype phenotype. Their findings are summarized in Table 1.3 and brief descriptions of each mutant are given below.

Table 1.3: Characterization of rubber-deficient mutants of *G. polyisoprenivorans* obtained by insertional mutagenesis

Mutant	**Pheno -type**	**Insertion locus**	**Accession no.**	**Identical amino acids (%)**	**E value**
A1-1-44	+/-	Hypothetical protein, *C. efficiens* YS-314	BAB99514	15/ 32 (46)	0.023
A32-S	-	iscA, HesB-like protein, *M. avium* subsp. *paratuberculosis* k10	AAS04261	35/ 48 (72)	1e-09
A46-51-33	+/-	*mmsA*, putative methylmalonate semialdehyde dehydrogenase, *M. avium* subsp. *paratuberculosis* k10	BAC75042	220/ 288 (76)	e-116
B9-27-27	+/-	Putative Lux-R family transcriptional regulator, *S. avermitilis* MA-4680 Putative integral membrane protein, *S. coelicolor* A3(2)	CAB88464 AAS02633	42/ 129 (36) 29/ 63 (46)	0.009 1e-04
B31-72-50	+/-	Putative recR, *M. avium* subsp. *paratuberculosis* k10	AAS02633	130/ 203 (64)	2e-36
D21-94-19	-	*mcr*, putative α-methylacyl-CoA racemase, *N. farcinica* IFM10152	BAD60217	44/ 69 (63)	3e-16
K8-77-41	-	Putative oxidoreductase *M. tuberculosis* CDC1551	NP_334806	159/ 384 (41)	7e-67
J38-58-40	-	Putative Na+/H+ antiporter, *S. coelicolor* A3(2)	SCO5246	29/ 104 (27)	0.007

+/- = leaky (Bahn *et al.,* 2005)

In mutant D21-94-19 the transposon was located upstream of a gene encoding a α-methyl-acyl coenzyme A racemase. It is believed that this enzyme is involved in the polyisoprene degradative pathway, converting (R) isomers to (S) isomers, allowing the acyl-coenzyme A dehydrogenase to act upon the substrate.

In mutant A46-51-33 the transposon replaced a gene with high similarity to methylmalonate semialdeyde dehydrogenase. This enzyme is believed to play a role in rubber catabolism. In mutant K8-77-41, transposition was mapped to a gene encoding an oxidoreductase. However, it did not show sequence similarity to that of the degradative polyisoprene complex identified in *Streptomyces* spp. K30.

In mutant A1-1-44, the transposon was inserted in a gene displaying similarities to hypothetical proteins, including one in *Corynebacterium efficiens*. In this bacterium the protein is in close proximity to a *NifS* gene, a cofactor of the iron-sulfur (Fe-S) cluster. By analogy, the *oxiA* gene contains a Fe-S cluster and thus its inactivation would lead to a rubber negative phenotype. Similarly, in mutant A32-5 transposition disrupted homologous HesB/IscA proteins which are responsible for iron delivery are involved in Fe-S cluster assembly.

In mutant B31-72-50, transposon insertion occurred in a gene encoding a RecR homologue. Downstream of RecR was a putative cobyric acid synthase which was hypothesized to play a role in degradation, possibly through methyl group rearrangement.

In mutant B9-27-27 the insertion occurred in a region encoding a putative LuxR transcriptional regulator, whose disruption was believed to have prevented relevant gene inductions.

1.8 Motivation for research

i) Rubber is a relatively recalcitrant compound as pointed out in a publication by Keursten and Groenvelt (1996) which determined the biodegradative rate of rubber particles in soil. Carbon dioxide measurements in conjunction with statistics calculated that just 22.2 g of styrene-butadiene rubber would take more than 27 years to be completely degraded.

ii) Natural rubber is used for the production of adhesives, latex gloves, tubing and tyres. This widespread use is accompanied with an extensive generation of waste rubber material. In many parts of the world, especially industrialized countries, this has prompted legislation to be passed to govern the proper disposal of rubber waste (Othmer, 1997). Even so, the recycling of this polymer is not widely practised. Reclaiming rubber material through physical or chemical treatments and its reuse is unfeasible due to the significant reduction in polymer properties. Scrap tyres in particular have become a major concern. Since the burning of scrap tyres for fuel is more expensive than the burning of natural gasses this is seen as an economically unviable recycling route. A study in the US has shown that of the 242 million used tyres generated in 1990, 11% were used as a fuel source, 7% were recycled, 5% exported and 77% (about 186 million) discarded in landfills (Smith *et al*., 1995). Similarly, 9 million tyres were disposed of in landfills in the UK (Collins *et al*., 2002). In addition to an environmental hazard this poses a health risk (Bredberg *et al.*, 2001). Continued inadequate rubber recycling remains a global problem which will certainly impact the environment in the future.

The isolation and characterization of useful organisms able to degrade natural rubber have the potential for use in biotechnological applications. Furthermore, the identification of rubber degrading genes would permit genetic manipulation of these strains, optimizing the production of biodegrading enzymes. Moreover, from an evolutionary perspective gene identification would allow the level of divergence of rubber degrading genes among actinomycetes to be evaluated.

In the past ten years considerable genetic work has been conducted on latex biodegraders. The identification of the *lcp* gene in both weak extracellular and potent

adhesive rubber degraders was unexpected, since there is a startling difference in the outcome of this gene in both types of degraders. Reasonably, the morphological and metabolic differences as well as genetic interactions could play a role in this differential expression. Thus the introduction of this gene from a weak mycelia rubber degrader into non-mycelial bacteria was an interesting facet to investigate.

1.9 Objectives of project

Main project:

Primary objective:

The identification and characterization of rubber degrading strains and isolation of potential rubber degrading gene

Secondary objectives:

i. Isolation and identification of extracellular enzyme rubber degraders

ii. Characterization of isolates with regard to degradative capacity

iii. Creation of genomic libraries

iv. Screening of libraries

v. Cloning and sequencing of potential rubber degrading gene/s

vi. Generation of a mutant strain incapable of degrading rubber

Sub-project:

Primary objective:

To establish whether the *lcp* homologue from an extracellular rubber degrader would be expressed in nocardioform actinomycete strains

Secondary objectives:

i. Amplification of the *lcp* homologue from *S. coelicolor* A3(2)

ii. Transformation of the gene into members of nocardioform actinomycetes, specifically *Rhodococcus* spp., *Gordonia* spp. and *Mycobacterium* spp.

iii. Incubation of the transformed nocardioform actinomycetes with latex glove pieces and visual monitoring for signs of colonization and substrate alteration

2. Materials and methods

2.1 Polymers

Two rubber polymers were used: Liquid LATZ (low ammonia latex containing tetramethylthiuram disulfide and zinc oxide) and ExamTex Plus powdered latex gloves.

2.1.1 *Latex and rubber glove preparation*

LATZ was prepared by adding the liquid latex to an equal volume of 0.05% Tween 80. After gentle inversion, the suspension was centrifuged (10 000 rpm; 10 min.) and the upper cream layer extracted. This was used to make latex agar plates. Latex glove pieces were sterilized in 70% methanol, rinsed in sterile water and added to enrichment cultures. Additionally, these were also sterilized by means of a chloroform-acetone treatment. Glove pieces were placed in chloroform for 5 h, removed and placed directly into acetone for a further 5 h. This was transferred to a beaker of sterile water and left overnight, allowing the organic solvents to diffuse out. The rubber was then placed onto a foil lined Petri dish and allowed to dry under the fume hood.

2.2 Culturing conditions

2.2.1 *Culturing of mixed soil samples*

One gram of soil was added to 25 ml of X1 stock III solution, a minimal liquid media supplemented with ammonium chloride (0.1g/100 ml). Methanol and chloroform-acetone treated latex glove pieces, 2-5cm in diameter were added to Erlenmeyer flasks containing minimal media. This was placed on a 30 rpm rotating shaker at 30^{o}C. Sub-culturing was done after the first month. Thereafter, stock III solution was routinely added to the cultures.

2.2.2 *Culturing and isolation of latex utilizing strains*

Mixed cultures were streaked onto latex agar. Colonies which exhibited clearing zones on opaque latex agar plates were purified by streaking until a pure strain was obtained.

For scanning electron microscopy and Schiff's reagent staining, latex glove pieces were sterilized as described previously and added to minimal media liquid inoculated with bacterial cultures. These were incubated on a 30 rpm shaker at 30^{o}C for a month. For protein content experiments the same procedure as above was followed, however the incubation period was extended to 3 months. Liquid minimal media was routinely replenished. When testing for viable cell count, natural latex (0.1%) was added to 10 ml of liquid minimal media inoculated with a dense bacterial pre-culture and incubated at 30^{o}C.

2.2.3 *Enrichment cultures*

To enrich for the clone carrying the rubber degrading gene, 10-20 µl of the pooled genomic library clones were transferred to 10 ml minimal media supplemented with latex as the sole carbon source and incubated at 30^{o}C on a rotating shaker. 30 µl of the culture was extracted weekly and spread onto latex agar plates. This was checked for clones displaying clearing zones.

2.3 Amplification of the *lcp* gene from *S. coelicolor* A3(2)

The entire 1194 bp *lcp* homologue in the genome of *S. coelicolor* A3(2) was amplified using the following forward primer - ATGGAGAATCTCAGCAGGCGA and reverse primer – GGTCAGCCCGGCCTGTTG. The following was added to a PCR reaction tube: 4 µl of 25mM $MgCl_2$, 5 µl 10X Taq buffer + $(NH_4)_2SO_4$, 5 µl 2mM dNTP, 1 µl DMSO, 2 µl 25 µM forward primer, 2µl 25 µM reverse primer, 1 µl 200 ng/ul genomic DNA, 29.5 µl sterile Milli-Q water and 0.5 µl Taq polymerase (5U/µl), giving a total reaction volume of 50 µl. The PCR conditions were as follows: initial denaturation at 95^{o}C for 3 minutes (a single cycle) and denaturation at 95^{o}C for 30 seconds, annealing at 60^{o}C for 1.30 minutes and extension at 72^{o}C for 3 minutes

set at 30 cycles and final extention of 72^{o}C for 10 minutes, using a BioRad MJ Mini TM Gradient Thermal Cycler PCR machine.

2.4 Genomic libraries

2.4.1 *Construction and screening of genomic libraries*

See section 3.5 page 62 for construction of genomic library. *S. lividans* transformants were patched onto latex agar plates containing 0.1% glucose and 30 µg/ml thiostrepton. These plates were incubated at 30^{o}C and routinely analyzed for clear zone formation around colonies.

2.5 Induced Mutagenesis

2.5.1 *Ultraviolet (UV) mutagenesis in the presence of 8-methoxypsoralen (8-MOP)*

One volume of the DNA sensitizing agent, 8-methoxypsoralen (1mg/ml) was added to 9 volumes of the spore suspension (resuspended in 15% glycerol w/v). The suspension was poured into a Petri dish and exposed to near ultra violet light (260 nm; 6 cm) in 1 min. intervals. This was diluted, plated onto LA plates and the colony forming units/ml determined. Consequently, this was used to calculate the time at which a 1% survival rate was achieved. The plate yielding a 99% inactivation rate was patched onto latex agar plates for screening purposes.

2.5.2 *NTG (N-methyl-N′-nitro-N-nitrosguanidine) mutagenesis*

NTG was prepared by resuspending 1mg NTG powder in 1 ml 0.02M Tris-HCl (pH8.5) and heating briefly till fully dissolved. *S. tendae* BA1 was grown in 10 ml of LB at 37^{o}C for 2 days. 50-100 µl of the dense culture was transferred into 10 ml of fresh LB and grown for 13-14 h at 37^{o}C. 1 ml of the culture was transferred to an Eppendorf tube and pelleted by microfuging at room temperature for 1 min. The pellet was washed twice in 0.02M Tris-HCl (pH 8.5) and resuspended in NTG (1 mg/ml) solution. This was incubated at 37^{o}C for 30 min. and frequently inverted.

Cells were pelleted by microfuging for 1 min. and washed twice in phosphate buffer (pH 7.0). Cells were then washed twice in LB and added to 5 ml of fresh LB. This was incubated for 1-2 days with 50- 100 µl spread onto non-selective media and allowed to grow at 37^{o}C for 1 day. Individual colonies were patched onto latex agar plates.

2.5.3 *Mutant analysis*

The non-latex utilizing mutant was spotted onto starch agar prepared as described by Mac Faddin (1980) and incubated at 37^{o}C for 2 days. The plate was flooded with Gram's iodine and allowed to stand for 5 min. Excess iodine was poured off and the plate analyzed for the formation of clearing zones around the colonies.

2.6 Strain characterization

2.6.1 *Staining with Schiff's reagent*

Colonized latex glove pieces were harvested from minimal media liquid cultures following 3 months of incubation. Samples were rinsed in sterile water and placed into bottles containing 5 ml of Schiff's reagent and allowed to stand for 10 min. Once the reagent was discarded sulfite solution was immediately added and the glove pieces analyzed.

2.6.2 *Scanning electron microscopy (SEM)*

Colonized latex glove pieces were removed from liquid minimal media cultures following 3 months of incubation for use in SEM. To observe colonization the glove pieces were fixed directly without any pretreatment. To examine rubber alteration glove pieces were vigorously vortexed to dislodge cells before fixing. All samples were fixed in 3% gluteraldehyde and left overnight. Once the fixative was drawn off using a Pasteur pipette the treated samples were dehydrated in a graded ethanol series (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% and 100%). Consequently, these were subject to critical point drying, mounted onto aluminum stubs by means of carbon discs and lined with graphite. Additionally, these were sputter coated with a

thin layer of gold and palladium and viewed under a scanning electron microscope with an electron acceleration setting of 20kV. Images were taken on negative film.

2.6.3 *Additional carbon source added to latex agar*

Individual carbon sources (1%) which include glucose, succinate, fructose, tween 80, mannitol, sucrose, arabinose, xylose, maltose and inositol were added to latex agar plates and the formation of clear zones monitored.

2.7 Cell growth determined by optical density

Three Erlenmeyer flasks containing 10 ml liquid minimal media were prepared for each bacterial strain as follows: (i) no carbon source supplemented media, (ii) glucose (1%) supplemented media and (iii) latex (0.1%) supplemented media. These were incubated at 30^{o}C on a rotating shaker (30 rpm) and harvested at 1 week intervals. Accordingly, 1ml of the culture was extracted and an optical density reading taken at 450 nm.

2.8 Substrates used as carbon sources

Carbon substrates were added directly to minimal media plates. Cells were washed in sterile water before being spotted onto relevant plates and analyzed routinely for growth.

3. Results

3.1 Isolation and identification of extracellular rubber degrading strains

Sixteen soil samples collected from regions in America, Europe and Africa were screened for the presence of rubber degrading strains. One gram of soil was added to liquid minimal media and incubated for 3 months [Fig. 3.1].

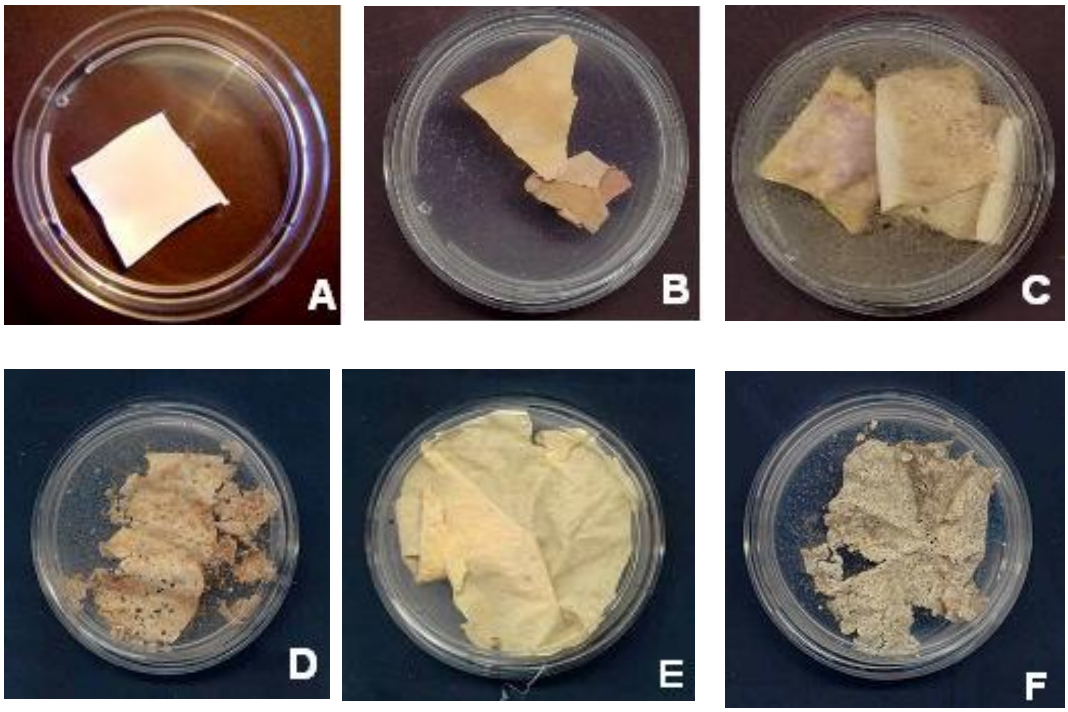


Fig. 3.1: Soil sample enrichment cultures, from which the following strains were isolated (A) Uninoculated control, (B) Est, (C) BA1, (D) Yeo, (E) Chiba and (F) SY3

Twenty-five strains displaying clear zone formation on latex agar plates were purified. All isolates were broadly characterized on the basis of rubber degradation. However, four of the bacterial strains were chosen for library construction. These include strain BA1 isolated from a soil sample collected in Buenos Aires, strain Est isolated from soil collected in Estonia, strain Yeo from South Africa and strain Chiba from Japan. Below are pictures of a few of the twenty-five extracellular rubber degraders isolated [Fig. 3.2].

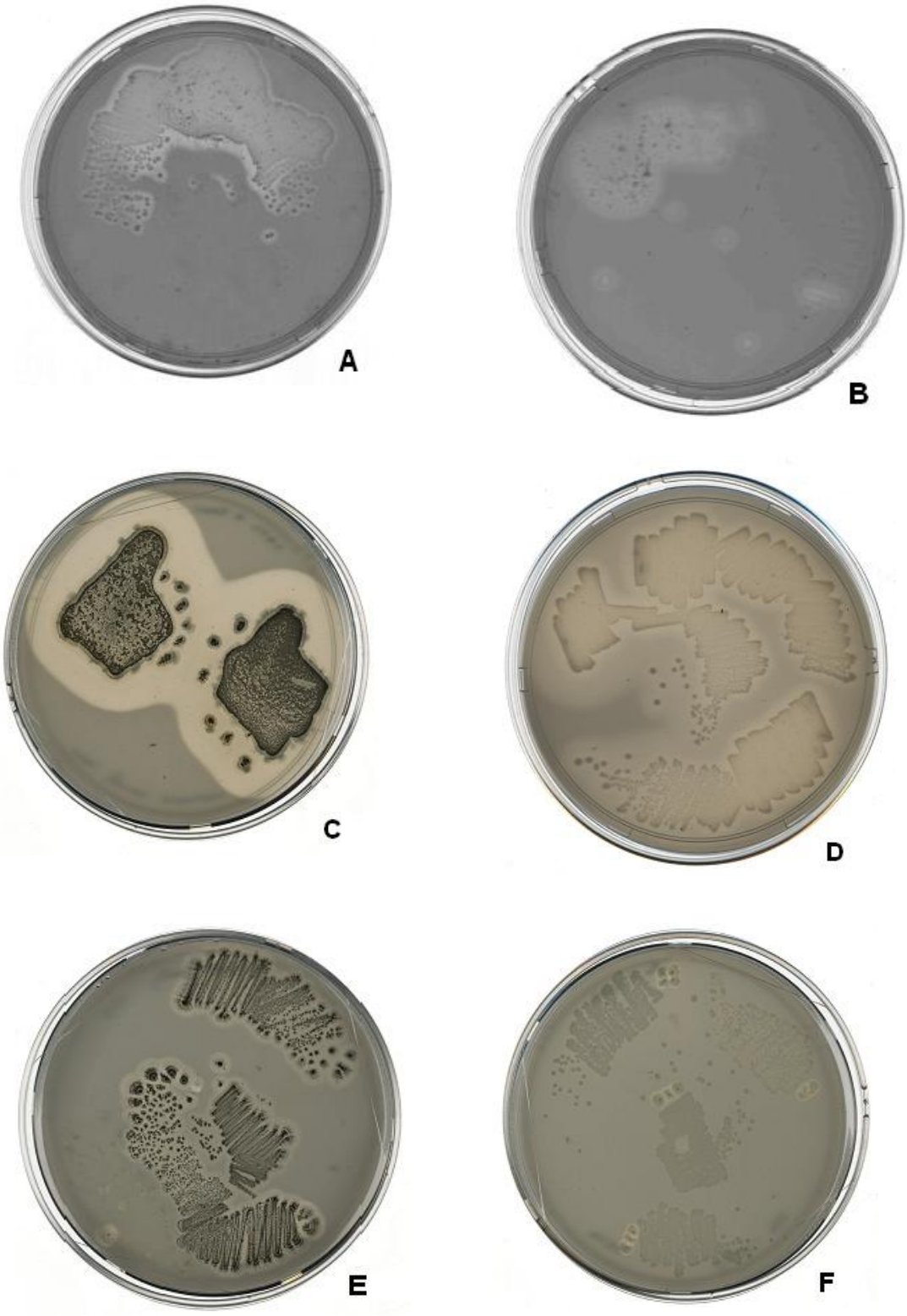


Fig. 3.2: Purified strains streaked onto latex agar plates, strains (A) Est, (B) BA1, (C) Chiba, (D) HZ, (E) Yeo and (F) SY6

Noticeably, strains Chiba and HZ were strong extracellular degraders, with the zones of clearing extending far beyond the periphery of the cells. *Methylibium* spp. HZ was the only Gram negative isolated. Strain SY6 was the weakest degrader, with a zone of less than 0.5 mm surrounding the colony. The other isolates (not shown) produced moderate sized zones similar to strains BA1 and Est.

3.1.1 *Tests used to differentiate species*

Alignment of the 16S rRNA sequence from strain BA1 to those in the Entrez PubMed Blast Database revealed the following percentage identity to Streptomycete species: *S. tendae* (99.8%), *S. tritolerans* DAS 165 (99.8%) and *S. coelicolor* (99.0%). Further tests showed BA1 was most similar to *S. tendae*. Consequently this strain was referred to as *S. tendae* strain BA1. The 16S rRNA sequence alignment of isolate Est showed sequence similarities to *Pseudonocardiacea* Gsoil 857 (98.4%), *Streptomyces* spp. SL1 (95.5%) and *Streptomyces* spp. SM4 (95.0%). Phenotypic and colonial characteristics from Bergey's manual were used to identify *Pseudonocardiacea* strain Est as a member of the species *Pseudonocardia*. Accordingly, Est was referred to as *Pseudonocardia* spp. strain Est.

S. tritolerans is able to endure harsh conditions, a feature separating it from *S. tendae*. Thus, BA1 was streaked onto media plates and its ability to grow at a high temperature, saline and pH conditions monitored [Table 3.1].

Table 3.1: Exposure of isolate BA1 to varying conditions

Conditions	**Strain BA1**
Temperature (37^{o}C)	+
Temperature (45^{o}C)	-
NaCl (0.5%)	+
NaCl (7%)	-
pH (7.0)	+
pH (10)	-

- = no growth; + = growth

One well known feature possessed by *S. tendae* is related to its antifungal ability [Fig. 3.3]. Inhibition of the growth of the fungus *A. niger* was observed.

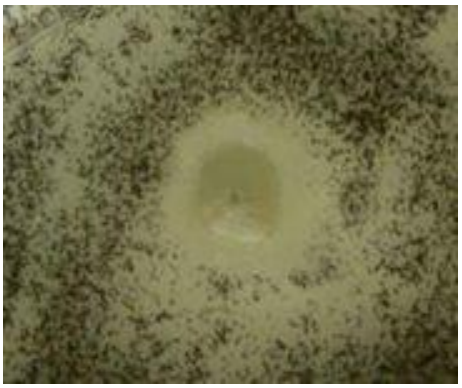


Fig. 3.3: Zone of inhibition of fungus *Aspergillus niger* by *S. tendae* BA1

Strain Chiba was identified as *Streptomyces flavogriseus* and strain Yeo as *Streptomyces griseus* subsp. *griseus*. These strains were tested for their ability to utilize varied energy sources by scoring the growth of the isolates on plates supplemented with these compounds in relation to the non-supplemented control [Table 3.2]. All isolates were inhibited by the presence of heavy metals. None could efficiently utilize alcohols or lignin and nylon components.

Table 3.2: Carbon source utilization by listed strains detected quantitatively by growth on supplemented media compared to non-supplemented media

Carbon source	**Concen-tration (g/L)**	***S. lividans*** TK23	***S. tendae*** **BA1**	***Pseudo-nocardia*** **spp. Est**	***S. griseus Yeo***	***S. flavo-griseus Chiba***
None	-	++	++	++	++	++
Glucose	0.1	+++	+++	++	+++	+++
Sucrose	0.1	++	++	++	++	++
Methanol	0.1	++	++	++	++	++
Ethanol	0.1	++	++	++	++	++
2-propanol	0.1	++	++	++	++	++
Lignin	0.02	++	++	-	++	+++
Nylon	0.02	++	++	++	++	++
$CuCl_2$	0.01	-	-	-	-	-

Zn	0.01	-	-	-	-	-
$CoCl_2$	0.01	-	-	-	-	-

- = no growth, + = poor growth, ++ = moderate growth, +++ = good growth

3.1.2) *Effect of carbon sources on clear-zone formation*

Supplementary carbon compounds were added to latex agar media and its effect on the formation of clear-zones examined [Table 3.3]. As seen in the table below, with the exception of Tween 80, other carbon sources in general did not affect enzyme activity. The only exceptions were *A. orientalis* SY6 and *Streptomyces* spp. Hunt's activity which was repressed in the presence of most carbon sources.

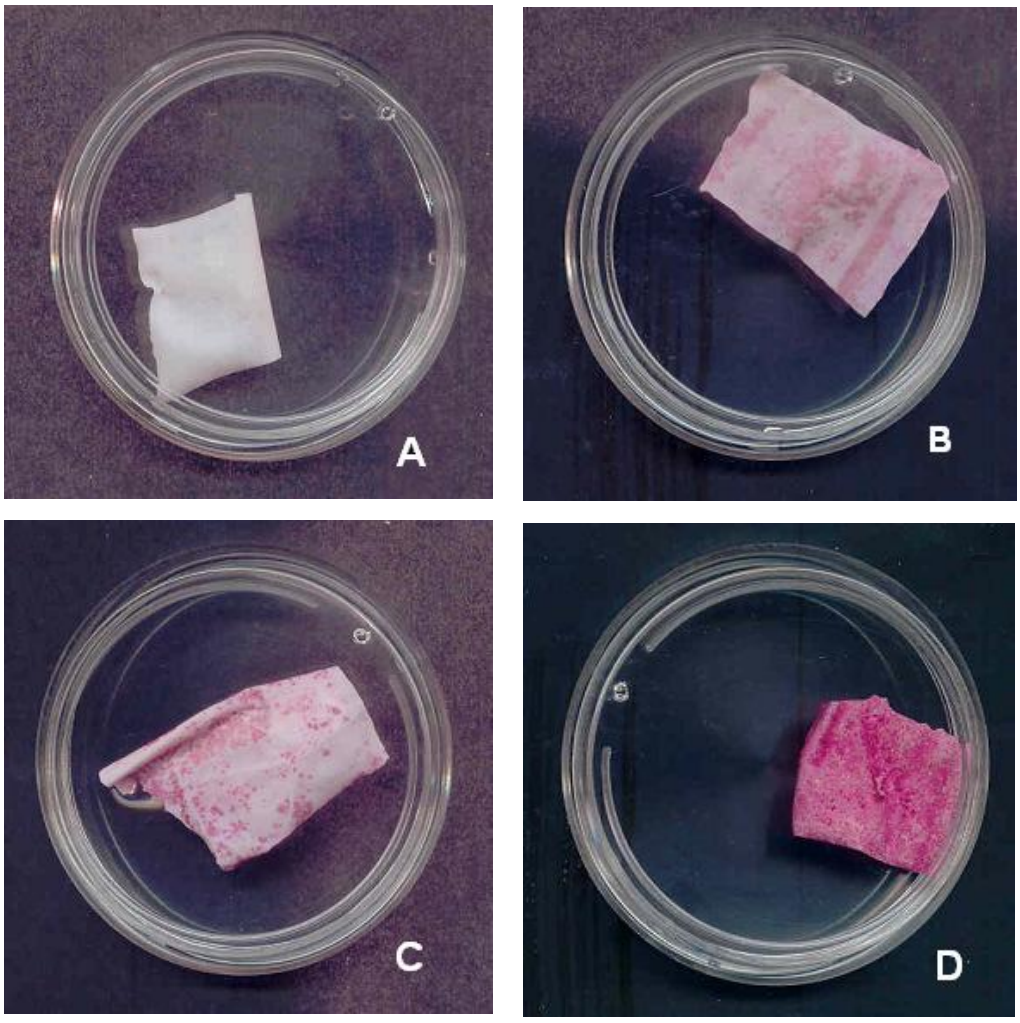
Table 3.3: Consequence of additional carbon compounds on rubber biodegradation. + = zone of clearing and - = no zone.

	Carbon source (1% w/v)									
	No carbon source	**Sucrose**	**Mannitol**	**Arabinose**	**Xylose**	**Glucose**	**Maltose**	**Inositol**	**Fructose**	**Tween 80**
Strains										
BA1	+	+	+	+	-	+	+	+	+	-
Gam	+	+	+	+	+	+	+	+	+	-
Reu	+	+	+	+	+	+	+	+	+	-
Berlin	+	+	+	+	+	+	+	+	-	-
Hak	+	+	+	+	+	+	+	+	+	-
Cal	+	+	+	+	+	+	+	+	+	-
Pasa	+	+	+	+	+	+	+	+	+	-
Bot1	+	+	+	+	+	+	+	-	+	-
FHome	+	+	+	+	+	+	+	+	+	-
WitsP	+	+	+	+	+	+	+	+	+	-
SY3	+	+	+	+	+	+	+	+	+	-
SY5	+	+	+	+	+	+	+	+	+	-
SY6	+	-	-	-	-	-	-	-	-	-
HY	+	+	+	+	+	+	+	+	+	-
WITS	+	+	-	-	-	-	+	+	+	-
Bedd	+	+	+	+	+	+	+	+	+	-
H2	+	+	+	+	+	+	+	+	+	-
H3	+	+	-	+	+	+	-	+	+	-
BotY	+	+	-	-	+	+	+	+	+	-
Chiba	+	+	+	+	+	+	+	+	+	-
Yeo	+	+	-	+	+	+	-	+	+	-
Hunt	+	+	-	-	-	-	-	+	-	-
HZ	+	+	+	+	+	+	+	+	+	-

3.2 Characterization of rubber biodegradation

3.2.1 *Staining with Schiff's reagent*

Strains were all grown in liquid media containing rubber glove pieces. Colonized rubber pieces were stained with Schiff's reagent following nine weeks of incubation. The purple coloration indicative of the presence of aldehyde groups due to microbial decomposition of the polymer extended across the whole surface. This clearly showed that both strains Est and BA1 had formed biofilms which covered the substrate surface [Fig. 3.4].



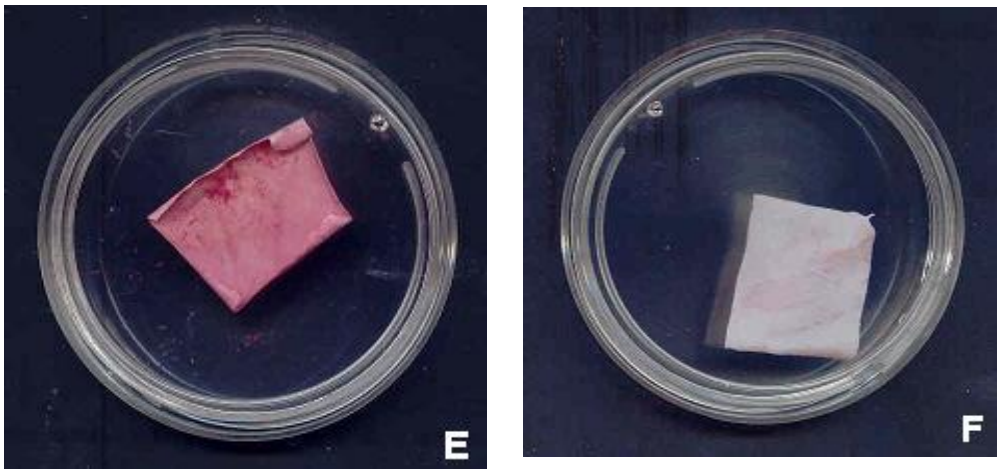


Fig. 3.4: Staining of latex glove pieces with Schiff's reagent following inoculation with the following strains: (A) Uninoculated control, (B) Chiba, (C) Yeo and (D) BA1, (E) Est and (F) non-rubber degrading BA1 mutant

The *S. tendae* BA1 non-rubber degrading mutant cultured rubber piece, similar to the uninoculated control was not stained, exhibiting that no degradation had taken place. Noticeably however, the mutant colonized the polymer. Strains Chiba and Yeo also showed signs of polyisprene break down.

3.2.2 *Scanning electron microscopy (SEM)*

A more detailed analysis into rubber degradation involved the use of SEM. This was investigated following three months incubation of bacterial inoculated liquid media in the presence of latex glove pieces. Samples were routinely examined and biofilm formation detectable by eye was recorded. Colonization was slow, the *S. tendae* mutant colonized the glove piece after 4 weeks, the wildtype strain took 5 weeks and *Pseudonocardia* spp. Est colonization was detectable following 7 weeks. When the glove pieces were harvested for SEM after 9 weeks, the strains had all formed dense biofilms covering the entire surface and no free cells were detected in the liquid media. *S. flavogriseus* and *S. griseus* colonized the glove pieces within the first 3 weeks.

SEM imaging was used to investigate colonization and surface modification. The uninoculated control glove remained uncolonized [Fig.3.5 (A)]. As expected, *S. lividans* 66 showed no signs of colonization or the ability to exert an effect on the

polymer [Fig. 3.5 (G)]. The BA1 mutant strain formed such a dense biofilm that not even the individual hyphae could be detected [Fig. 3.5 (C)]. The *S. tendae* wildtype biofilm abundantly covered the rubber and merged into the polymer [Fig. 3.5 (D)]. Likewise, the *Pseudonocardia* spp. biofilm spread across the substrate and was embedded in the material as visualized by the uneven surface [Fig. 3.5 (H)]. *S. prasinus* strain Berlin formed a loose mycelial mat which covered the substrate surface [Fig. 3.5 (B)]. *A. orientalis* SY6 and *S. griseus* Yeo formed similar loose biofilms over the surface of the rubber piece [Fig. 3.5 (E) and (F)].

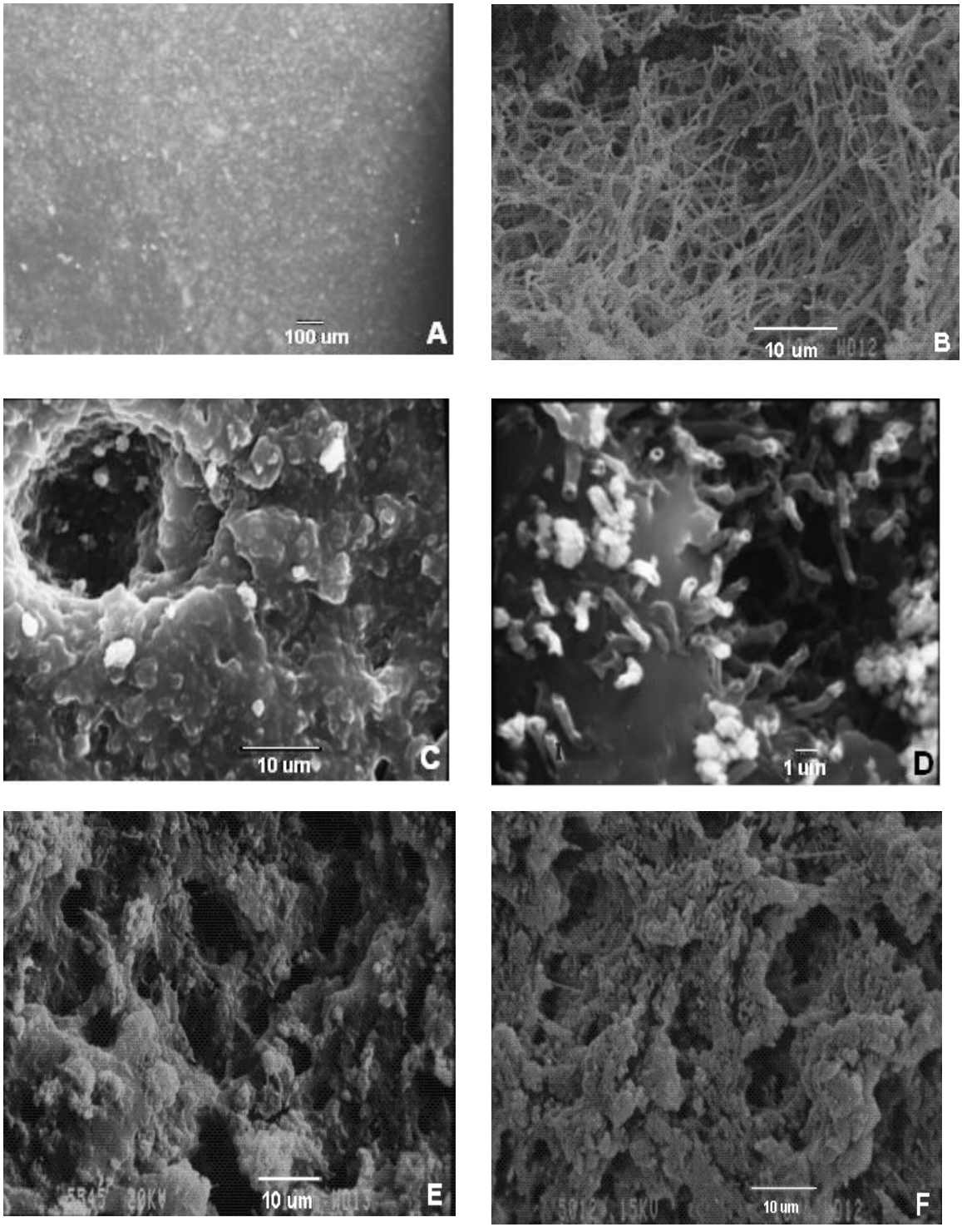


Fig. 3.5 A-F: SEM of natural latex glove pieces following microbial inoculation after 9 weeks. (A) uninoculated control, (B) biofilm formation by *S. prasinus* Berlin, (C) compact biofilm formed by *S. tendae* BA1 mutant, (D) *S. tendae* BA1 penetration into the rubber substrate (E) Colonization of glove piece by *S. griseus* Yeo and (F) *A. orientalis* SY6 colonization

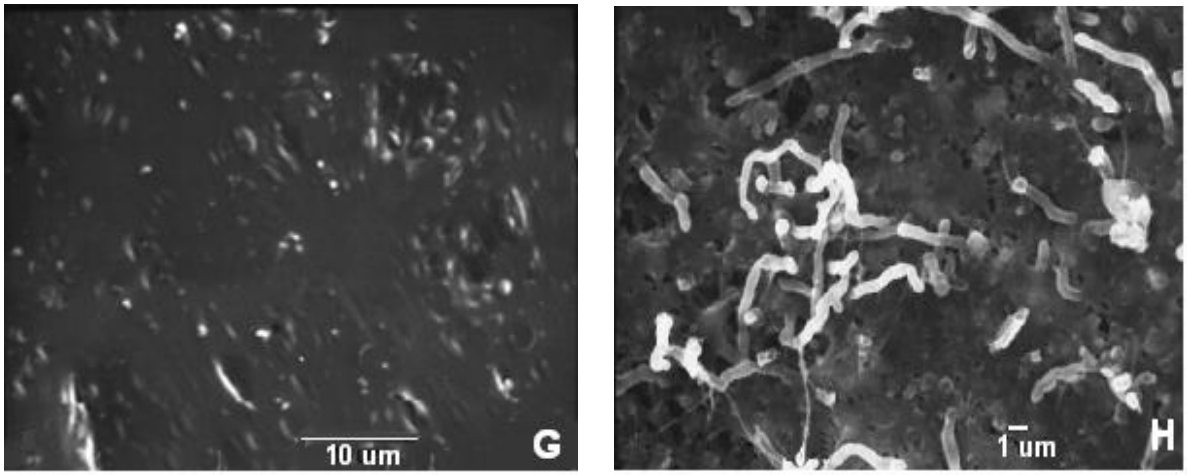


Fig. 3.5 G-H: (G) Inability of *S. lividans* 66 to colonize latex, (H) Rubber surface modification by *Pseudonocardia* spp. Est

3.3 Mutagenesis

Mutagenesis was carried out to generate a non-rubber degrading mutant. Strain BA1 cells were treated with UV in the presence of 8-methoxypsoralen to induce mutations in the DNA. Ultraviolet mutagenesis proved ineffective, following the patching of 901 cells, no mutants were obtained. As a result chemical mutagenesis was used instead. After patching 326 NTG treated *S. tendae* cells onto latex plates, one mutant incapable of forming clear zones on latex was obtained. To ensure that the latex gene was inactivated and that the phenotype was instead not as a result of the extracellular enzyme releasing mechanism being affected the mutant was spotted onto starch agar (3 % w/v) [Fig. 3.6]. The ability of the mutant to degrade starch showed that the secretion mechanism was not affected. No *Pseudonocardia* mutant could be generated as the strain was extremely susceptible to NTG treatment, resulting in a low survival rate.

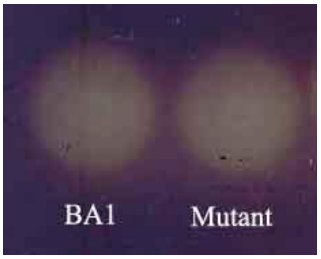


Fig. 3.6: (Left) Zone surrounding colonies of wildtype *S. tendae* BA1 and (right) *S. tendae* BA1 mutant, on starch agar treated with iodine

3.4 Attempted transformation of the non-rubber degrading mutant

An attempt was made to introduce vector pIJ702 (Streptomycete replicon vector) into the *S. tendae* BA1 mutant using PEG-mediated transformation. Unfortunately, this was not successful, yielding no transformants. In this regard the mutant could not be considered further for use in complementation of the rubber degrading gene. Instead it was adopted for use as a control in characterization studies.

3.5 Creation of a genomic library

Two of the libraries were selected for screening in a *Rhodococcus* spp. strain due to ease of the screening process and the other libraries screened in a Streptomycete host. The digestion of *S. tendae* BA1 DNA with *Bsp*1191 yielded an average DNA fragment size of 4700 bp. Calculations estimated that 5000 clones were needed to ensure a high probability of containing the gene of interest. A partial *Pst*I library of *Pseudonocardia* spp. Est was constructed. From an average insert size of 2400 bp, calculations estimated that 10 000 clones were needed to ensure a high probability of containing the gene of interest. These libraries were constructed in pDA71 for the purpose of screening in *R. erythropolis*. A complete *Bgl*II restriction was performed on *S. flavogriseus* Chiba and *S. griseus* Yeo DNA. These were ligated to the *Streptomyces* spp. vector pLR591. Calculations estimated over 7000 clones were required for the strain Chiba library on the basis of an average insert size of 3400 and approximately 10 000 colonies for strain Yeo library from an average insert size of 2500 bp.

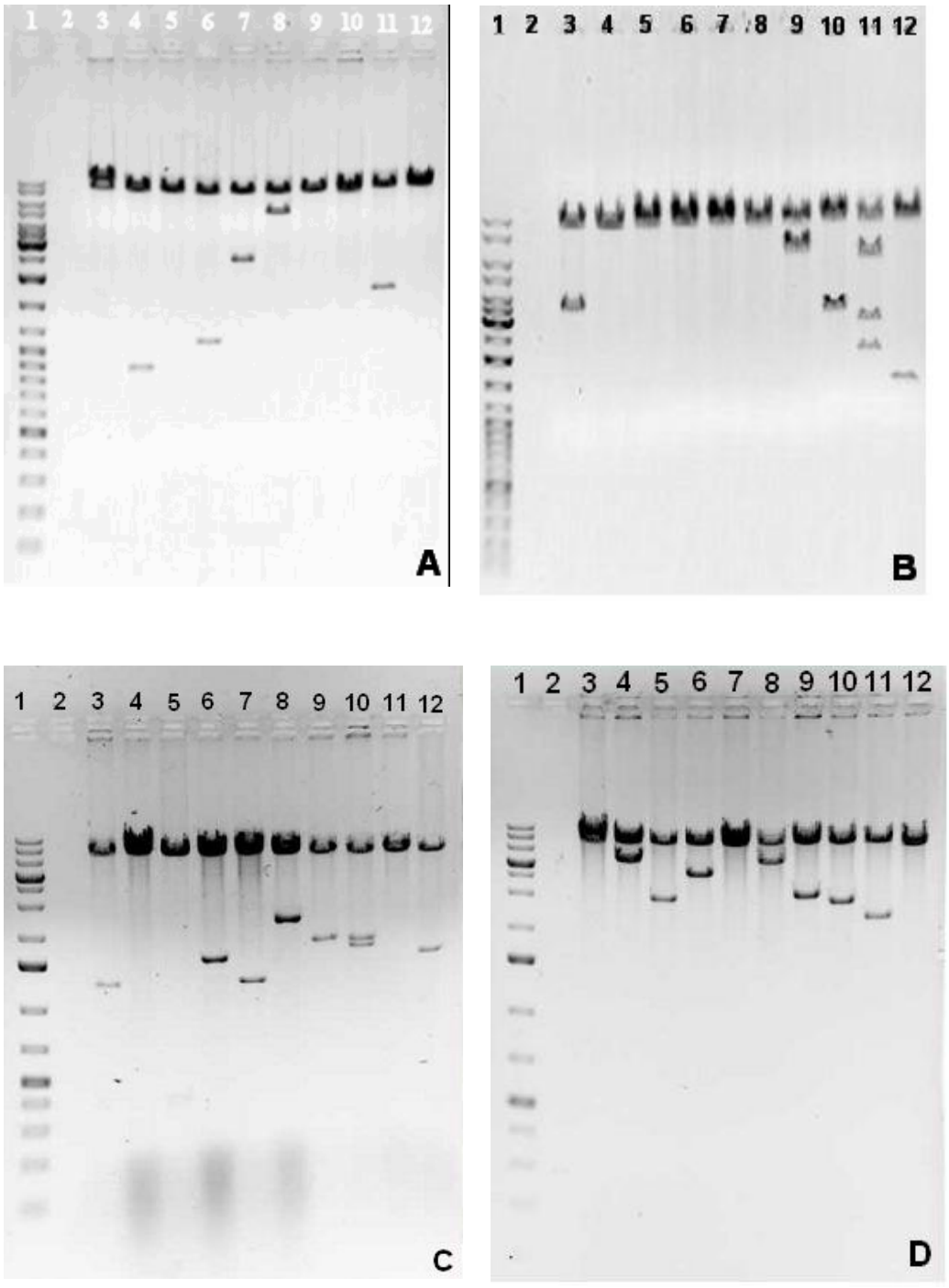


Fig. 3.7: Inserts released from clones of (A) *S. tendae* library, (B) *Pseudonocardia* spp. library, (C) *S. griseus* Yeo library and (D) *S. flavogriseus* Chiba library. DNA ladder markers (bp): 20 000, 10 000, 7000, **5000**, 4000, 3000, 2000, **1500**, 1000, 700, **500**, 400, 300, 200, 75

Once the actinomycete DNA was digested with the appropriate restriction enzyme and ligated to vector pDA71 (*Rhodococcus* replicon vector) [Fig. 6.6.3 page 320], these DNA libraries were transformed into an intermediate *E. coli* host using calcium chloride mediated transformation, to concentrate and purify the DNA.

Recombinant vector DNA was re-isolated from the *E. coli* pooled library by means of a large scale maxi-prep and transformed into *R. erythropolis* SQ1 using PEG mediated transformation, for the purpose of screening. Approximately 10 000 clones carrying *S. tendae* BA1 DNA were pooled while an estimated 10 000 clones of *Pseudonocardia* spp. Est DNA were pooled as well.

3.6 Screening for the clone carrying the rubber degrading gene on latex agar

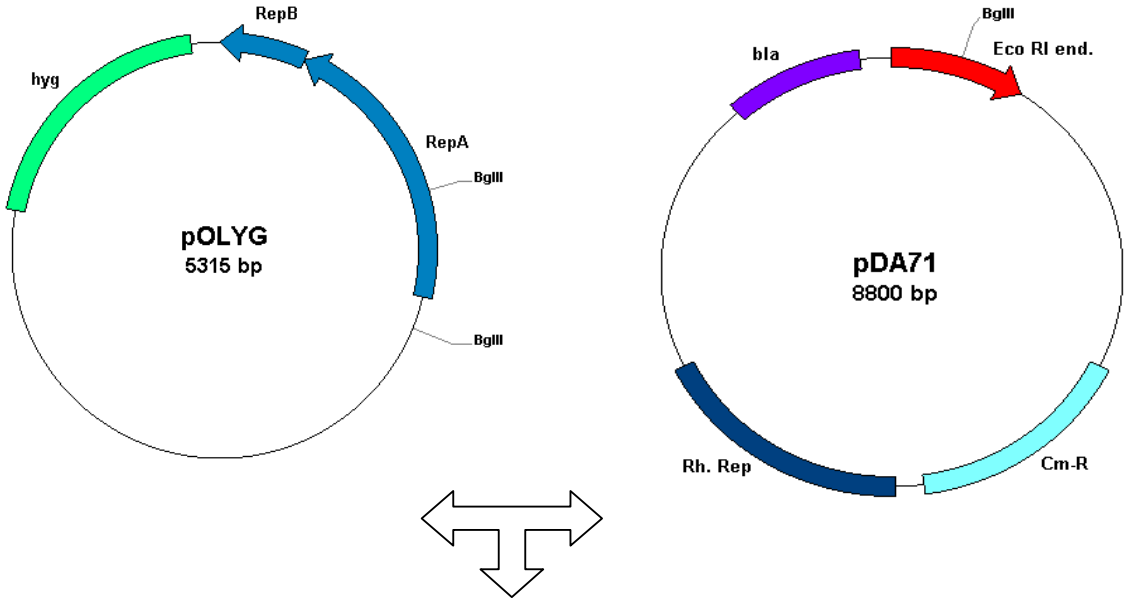
Screening of the library clones directly onto latex agar was ineffective due to the small colony sizes. It was apparent that this would make it difficult to detect a clearing zone around the colonies. To overcome this, the medium was supplemented with glucose which positively led to the formation of larger colonies. Streaking of the original BA1 and Est isolates onto latex agar supplemented with an additional carbon source verified that the rubber degrading gene was not repressed in the presence of glucose and actually led to the formation of larger clearing zones (data not shown). Subsequently, pooled *Rhodococcus* libraries were appropriately diluted and spread onto latex agar plates containing 0.1% glucose [Fig. 3.8].

Fig. 3.8: (Left) Positive control – *S. tendae* BA1 spotted onto latex agar in which the clearing zone is evident and (right) *Rhodococcus* spp. library colonies on latex agar

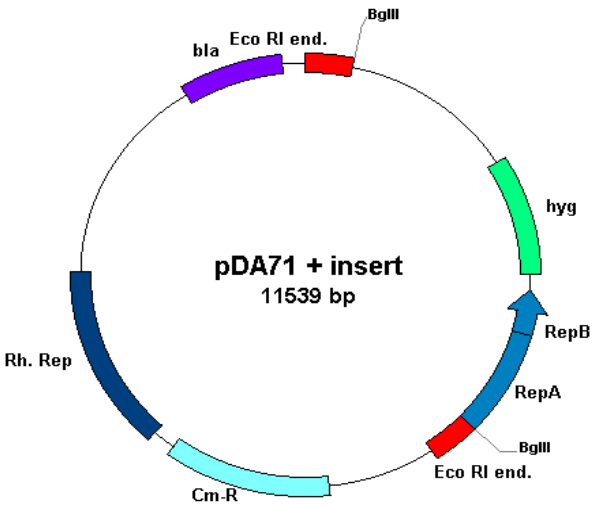
Plates were routinely analyzed for the presence of a clearing zone around colonies. About 20 000 BA1 DNA carrying clones were screened in addition to around 15 000 *Pseudonocardia* spp. DNA carrying clones. However, no colonies displaying a clearing zone on latex agar were detected. Since screening was unsuccessful, the ability of *Rhodococcus* strain SQ1 and pDA71 to express a Streptomycete gene was tested.

3.7 Expression of a streptomycete gene by *R. erythropolis* SQ1

To establish whether *R. erythropolis* SQ1 would be capable of expressing a mycelial actinomycete gene, the hygromycin resistance antibiotic gene originating from *Streptomyces hygroscopicus* on vector pOLYG was excised and ligated to pDA71. This was transformed into strain SQ1 and plated onto hygromycin [Fig. 3.9].



Digested with *Bgl*II and ligated



Transformation into *R. erythropolis* SQ1

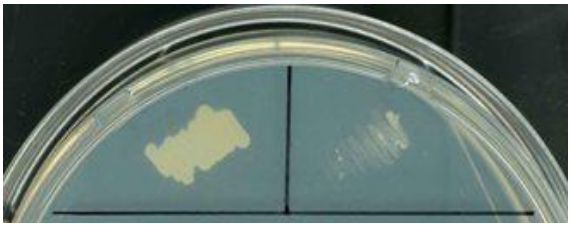


Fig. 3.9: Above: Ligation of hygromycin encoded gene fragment into pDA71. Below: Transformation of the recombinant vector into *R. erythropolis* SQ1. (Left) Growth of *R. erythropolis* SQ1 carrying pDA71 with the hygromycin gene and (right) negative control of *R. erythropolis* SQ1, both spread onto a hygromycin plate

Growth of *R. erythropolis* SQ1 carrying the recombinant vector clearly showed that the Streptomycete gene was expressed. Furthermore, to establish how closely related *R. erythropolis* was to both mycelial rubber degrading strains, 16S rRNA sequences of these isolates were aligned and a tree constructed [Fig. 3.10]. For the purpose of

accuracy full 16S rRNA sequences which most closely matched the original isolates BA1 and Est were used instead of the partial 500 bp PCR amplified sequences.

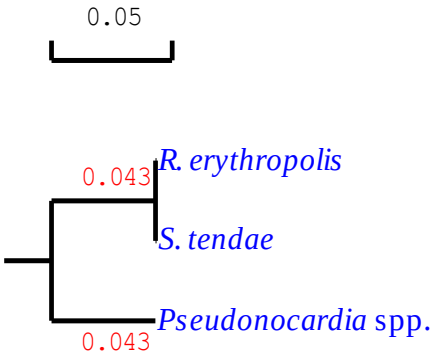


Fig. 3.10: Phylogenetic distance between *S. tendae, Pseudonocardia* spp. and *R. erythropolis* (constructed using DNAMAN applying default parameters)

The low values indicated on the branch length of the tree represent low divergence (high homology) among these strains.

Likewise, the Streptomycete host libraries were screened. Around 5000 clones were screened by patching both *S. griseus* Yeo and *S. flavogriseus* libraries. Again no clones displaying a clear zone were detected.

3.8 Enrichment cultures set up for isolating the clone carrying the rubber degrading gene

Since no latex-clearing clones were obtained by directly screening for the gene of interest on latex agar plates, liquid cultures were set up. This involved adding an aliquot of the library into latex media. With rubber serving as the sole carbon source the intention was to selectively enrich for the clone/s within the libraries which would be able to degrade polyisoprene. In practice, the appropriate clone/s able to breakdown the substrate and utilize it as an energy source should increase, out-competing other irrelevant clones. Following 3 weeks of incubation, a distinct difference with the library inoculated cultures was noticeable. While the latex rubber merely coagulated in the control flask, a murky suspension was evident in the streptomycete library and the liquid latex clearly remained in suspension in the flask containing the *Pseudonocardia* spp. library [Fig. 3.11]. In particular the milky

suspension detected in the presence of the Est library is the same results observed when the original rubber degrading isolates are inoculated in liquid latex media.

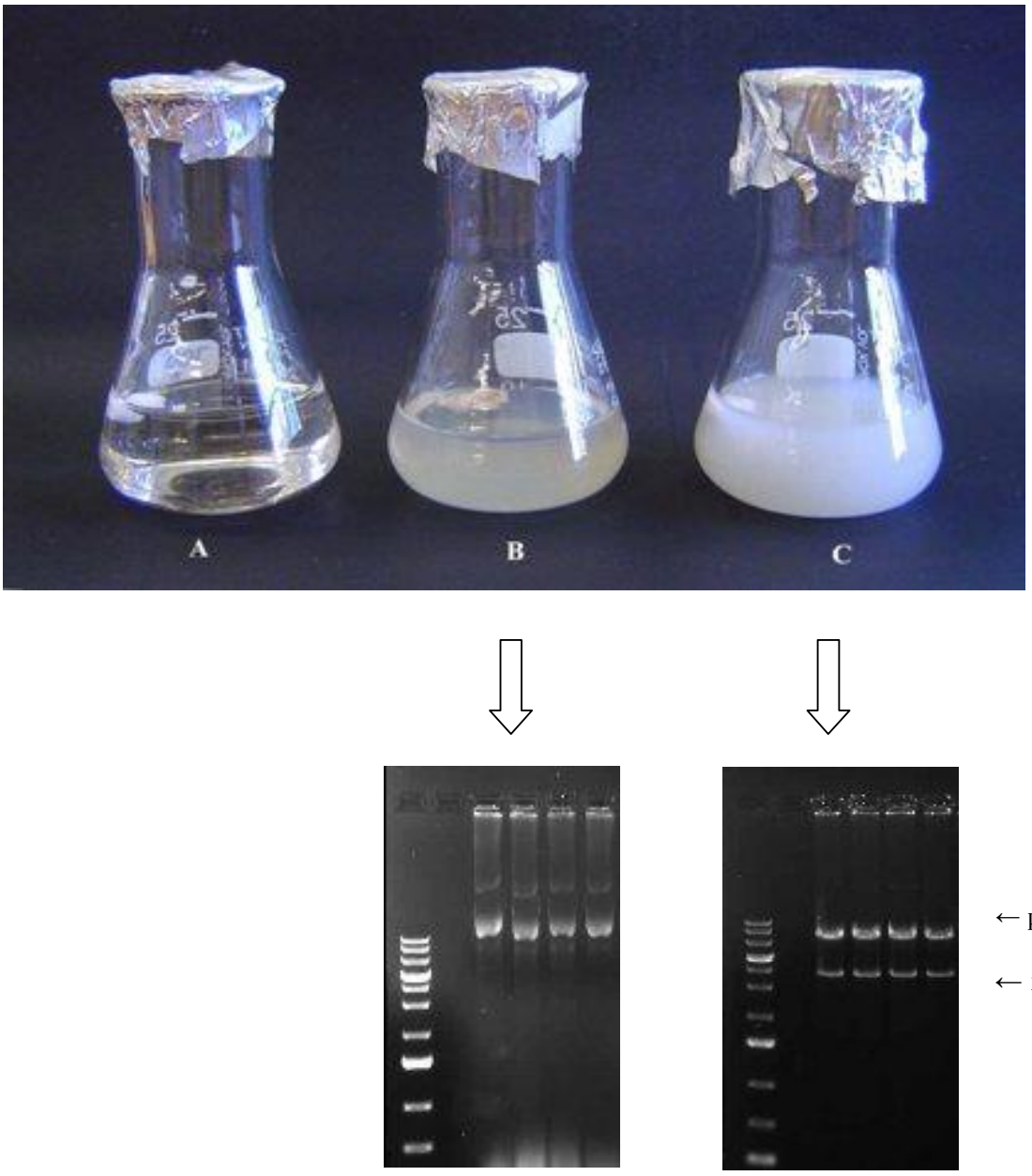


Fig. 3.11: (Above) Enrichment cultures of latex liquid minimal media containing A: *R. erythropolis* SQ1, B: *S. tendae* BA1 library and C: *Pseudonocardia* spp. Est library. (Below) Miniprep of four clones selected from each culture run on a 0.8% agarose gel (w/v)

A small volume of the each culture was removed and plated [Fig. 3.12]. Resulting colonies were prepped and checked for the presence of an insert. DNA of clones from the BA1 library were not cut by *Bsp*1191, suggesting that the vector was not present in these clones. Clones from the Est library all carried a 4 kbp insert. However, when these were streaked onto latex agar plates they did not display a clearing zone, suggesting that if this fragment did contain the rubber degrading gene complex, only a partial *lcp* homologue would be present or it could be completely absent.

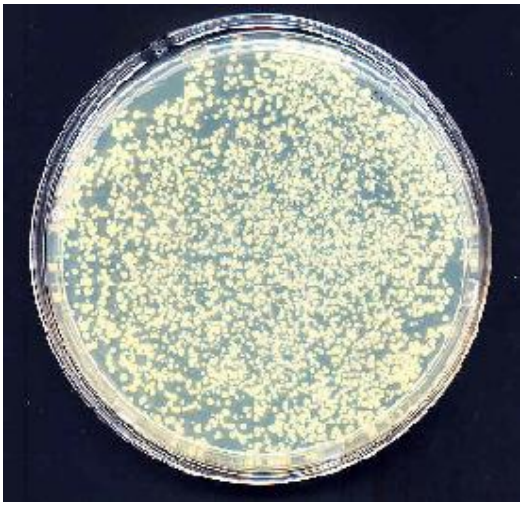


Fig. 3.12: *Pseudonocardia* spp. library clones aliquoted from the enrichment culture

The examination of a further 20 random colonies all displayed the presence of a 4kbp insert. This recombinant plasmid will be referred to as pMDC10. Retransformation of pMDC10 into *E.coli* for the purpose of sub-cloning, led to the unexpected result of < 1 transformant /µg DNA [Fig. 3.13]. It is possible that overexpression of the gene product is lethal.

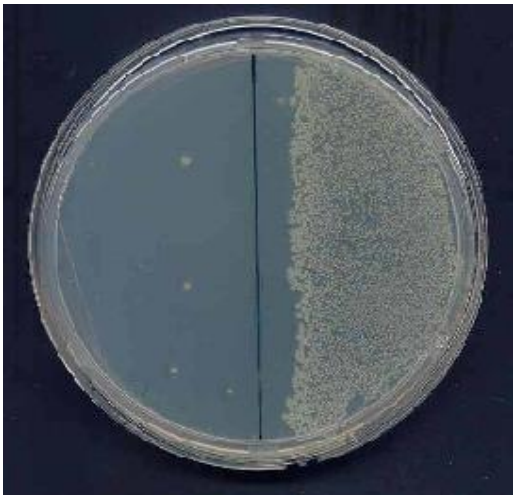


Fig. 3.13: (Left) Retransformation of pMDC10 and (right) pDA71 carrying a genomic DNA insert transformed into *E. coli* MM294-4

3.9 Restriction of pMDC10 insert

The insert was separated from the vector and purified via gel extraction.

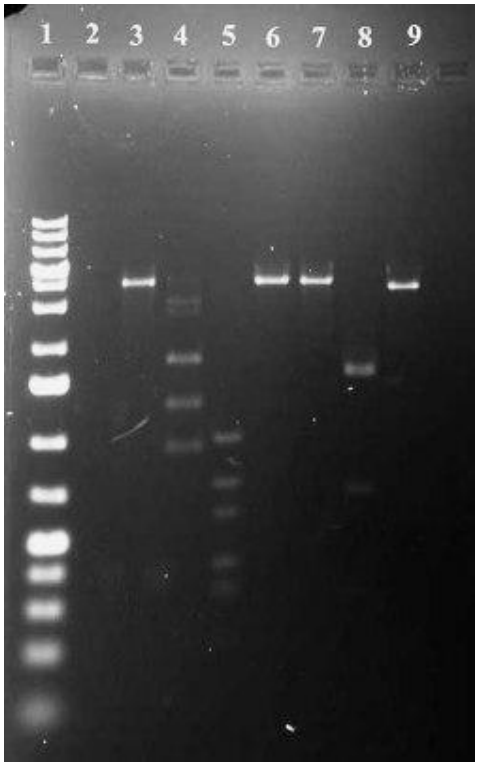


Fig. 3.14: Restriction of the 4kbp insert with various enzymes, run on a 0.8% agarose gel. Lanes **1**: DNA molecular weight marker, **3**: *Sac*I, **4**: *Sac*II, **5**: *Sal*I, **6**: *Apa*I, **7**: *Pma*CI, **8**: *Pin*AI and **9**: *Mlu*I.

A basic restriction analysis was conducted to establish if the fragment was related to that of the rubber degrading gene isolated from *Streptomyces* K30. Since the sequence was available, enzymes with known restriction sites within the gene complex were chosen [Fig. 3.14]. In fact the digestions did not follow the same restriction pattern as the gene found in strain K30 [Table 3.4].

Table 3.4: Restriction of the 4kbp insert in comparison to the rubber degrading gene complex (*lcp*, *oxiA* and *oxiB*)

Restriction enzymes	**Number of times restriction enzymes cut the fragment**	**Number of times restriction enzymes cut in *lcp, oxiA* and *oxiB* genes**
*Hind*III	0	**0**
*Pst*I	0	**0**
*Bgl*II	0	**0**
*Bam*HI	0	**0**
*Xba*I	0	**0**
*Eco*RI	0	**0**
*Sac*I	0	2
*Sac*II	4	8
*Sal*I	> 2	1
*Sph*I	0	1
*Bgl*I	> 2	4
*Apa*I	0	1
*Sfu*I	1	1[a]
*Xho*I	0	2
*Mlu*I	0	1
*Pin*AI	> 1	2
*Pma*CI	0	2

0 – did not cut; in bold represents the digestions corresponding to what would be expected in the rubber degrading gene; [a] - fragment sizes are variable

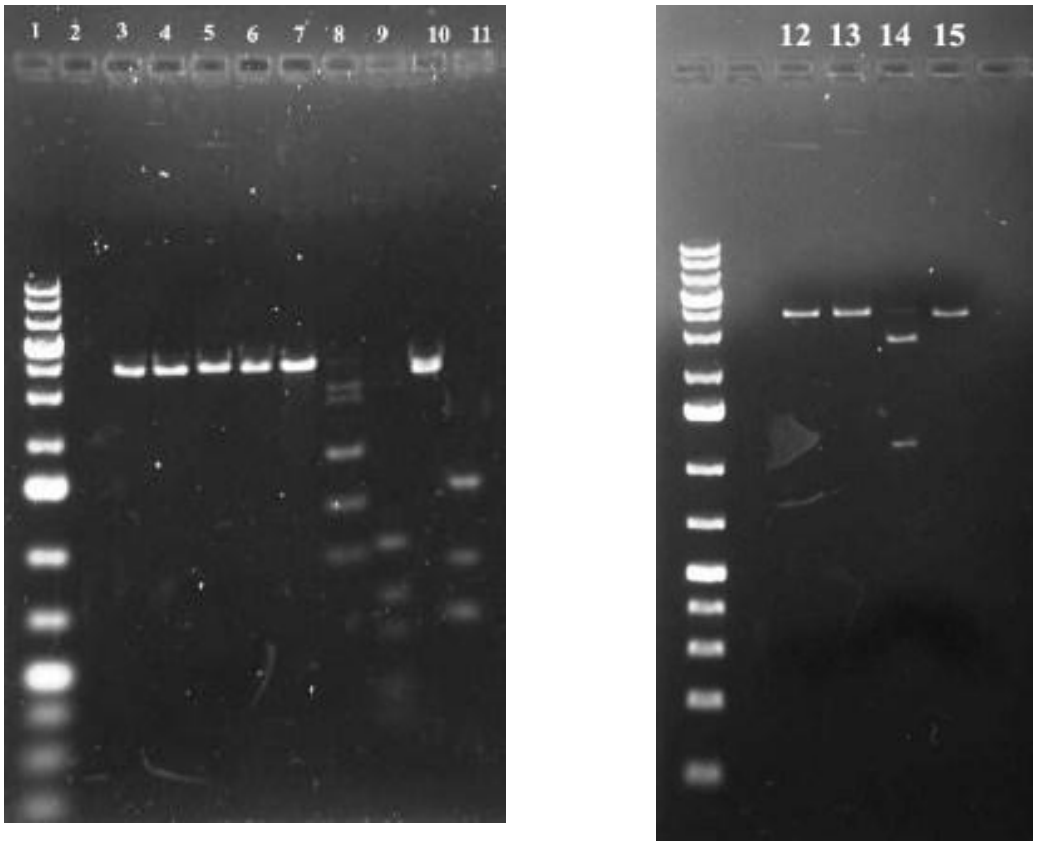


Fig. 3.15: Restriction of the *Pseudonocardia* spp. insert with the following enzymes: Lanes **3**: uncut, **4**: *Bam*HI, **5**: *Xba*I, **6**: *Eco*RI, **7**: *Sac*I, **8**: *Sac*II, **9**: *SalI*, **10**: *Sph*I, **11**: *Bgl*I, **12**: uncut, **13**: *Apa*I, **14**: *Sfu*I, **15**: *Xho*I. DNA ladder markers (in bp): 20 000, 10 000, 7000, **5000**, 4000, 3000, 2000, **1500**, 1000, 700, **500**, 400, 300, 200, 75.

The insert was then digested with restriction enzymes to assess which would yield fragment sizes, preferably less than 1kbp appropriate for sequencing [Fig. 3.15]. From the digestions it was apparent that the *Sfu*I digestion (lane 14) would be suitable, since it generated fragments of approximately 3kbp and 1kbp.

3.10 Partial sequencing of 4kbp fragment and analysis utilizing bioinformatics software

The 1kbp fragment was subsequently purified, cloned into pUC18 and sent for sequencing to a genomics company. An analysis of the sequence using PubMed Blast revealed no significant similarity to the rubber degrading gene isolated from *Streptomyces* spp. K30 (Altschul *et al.,* 1997). Nucleotide blast results revealed the

three highest identities were to genes in *Caulobacter crescentus* CB15, *Mycobacterium avium* 104 and *Mycobacterium avium* subsp. *paratuberculosis*. These included: a putative succinylornithine transaminase and hypothetical protein match to *Caulobacter crescentus* and molybdopterin biosynthesis protein moeA and hypothetical protein match to *Mycobacterium avium*. Noticeably, regions of sequence similarity to these genes were short, less than 70 nucleotides and were not related in any way to the mechanism of rubber cleaving. As mentioned previously rubber is broken down by means of an oxidative reaction, thus sequences associated with this feature were searched for. Relevant functional sequences included a *Nocardia farcinica* (putative monooxygenase) 33/39 nucleotides match and *Frankia alni* str. ACN14A (oxidoreductase) 44/55 nucleotide similarity. Unfortunately, the nucleotide blast did not yield positive results; clearly these similarities were minor and not useful. The software program FramePlot 2.3.2 was then used to predict the protein coding regions within the 1kbp fragment (Ishikawa and Hotta, 1999). This software is based on the gene analysis of *Streptomyces* spp., which are high GC content bacteria. Their results indicated that the chances of finding either a G or C at the third letter of a codon were unusually high. For *Streptomyces* spp. this was calculated to be 92%. It is this occurrence that allows the reading frame to be predicted.

Fig. 3.16: Open reading frame of 1kbp fragment (predicted using New England Biolabs NEBCutter). GC = 70%, AT = 30%

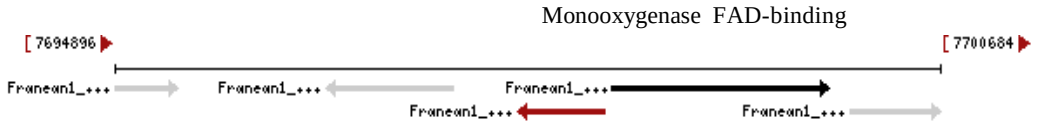
Using FramePlot several open reading frames (ORF's) were predicted. Further analysis utilizing PubMed Protein Blast showed that just one was relevant. The 205 amino acid reading frame showed homology to the TetR transcriptional regulator family in several strains [Table 3.5].

Table 3.5: The four closest protein matches to the predicted ORF

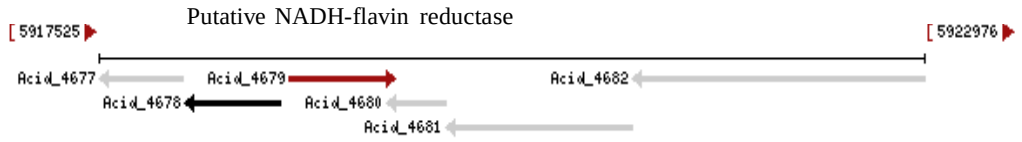
Protein match	**Accession no.**	**Identical amino acid (%)**	**Positives (%)**	**E-value**
Frankia spp. EAN1pec, TetR transcriptional regulator	YP_001510584	66/ 197 (33%)	102/ 197 (51%)	4e-23
Saccharopolyspora erythraea, TetR transcriptional regulator	YP_001102658	58/ 183 (31%)	89/ 183 (48%)	2e-18
Solibacter usitatus Ellin6076, TetR transcriptional regulator	YP_825923	62/ 203 (30%)	96/ 203 (47%)	2e-16
Mycobacteria spp. MCS, TetR transcriptional regulator	YP_639907	71/ 206 (34%)	96/ 206 (46%)	2e-16

In all the above bacteria, the locations of these TetR proteins within their genomes were in close proximity to genes with oxidative functions, as illustrated in Fig 3.17.

i) *Frankia* spp. EAN1pec



ii) *Solibacter usitatus* Ellin6076



iii) *Mycobacterium* spp. MCS

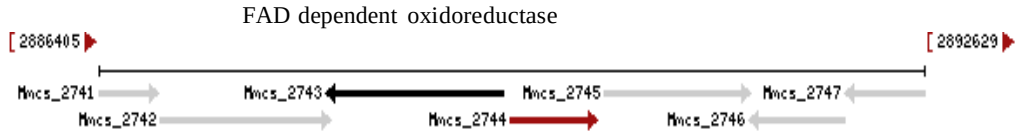


Fig. 3.17: Locations of *TetR* genes within bacterial genomes. Arrows in red represent similarities to TetR *Pseudonocardia* spp. fragment in these bacterial genomes and arrows in black are labelled with the relevant gene with an associated oxidative or reduction function.

A phylogenetic tree was constructed to visulalize the closest members of this family in relation to the hypothesized TetR fragment isolated from *Pseudonocardia* spp. Est [Fig. 3.18].

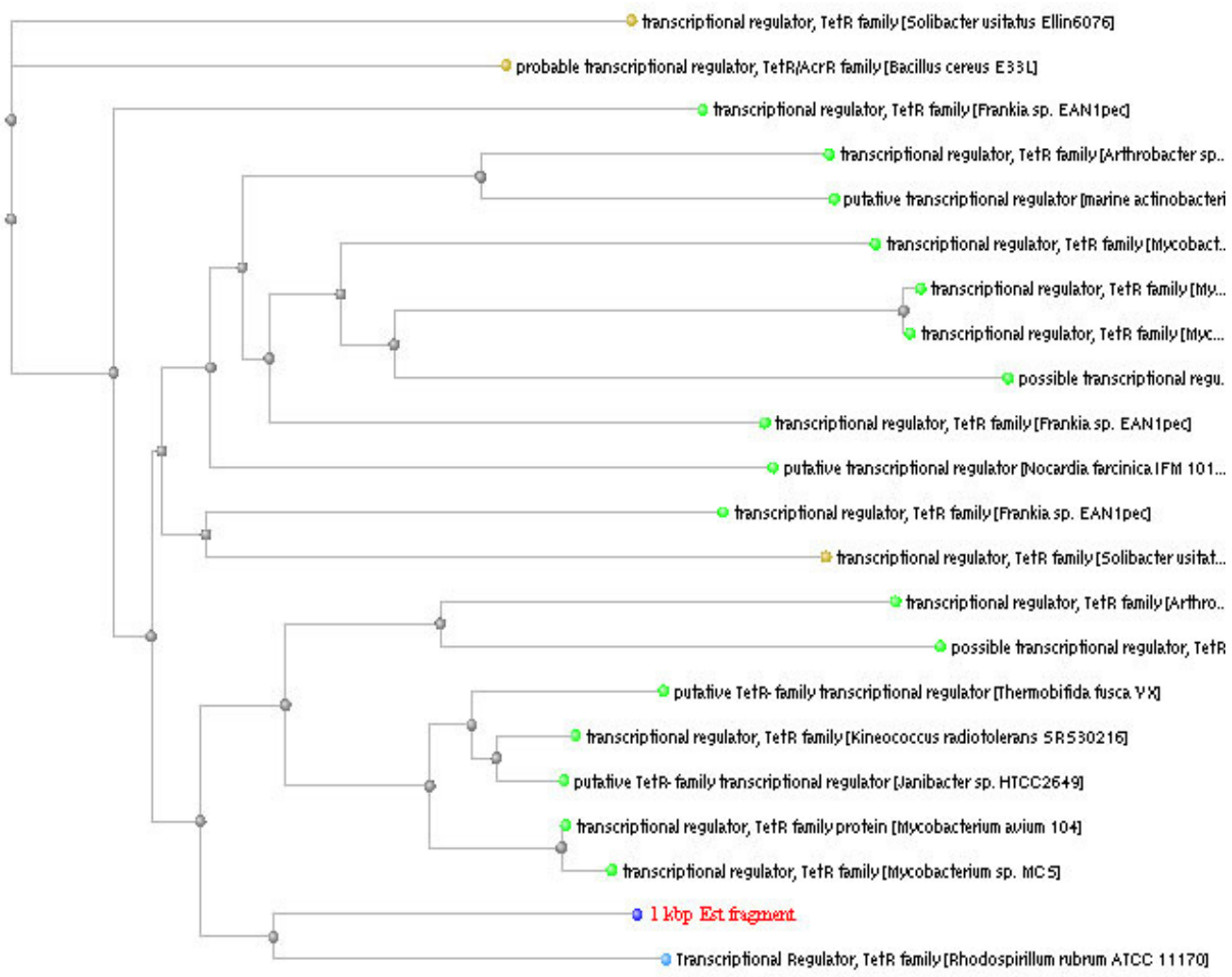


Fig. 3.18: A neighbour joining tree showing the phylogenetic distance between TetR members in relation to the *Pseudonocardia* spp. TetR fragment (constructed with PubMed utilizing default parameters). The *Pseudonocardia* spp. TetR fragment is represented in red.

Since this protein family has a highly conserved structure, additional structural features of the TetR *Pseudonocardia* spp. homolog were also examined in order to try and identify the gene of interest from the genetic context of other bacteria.

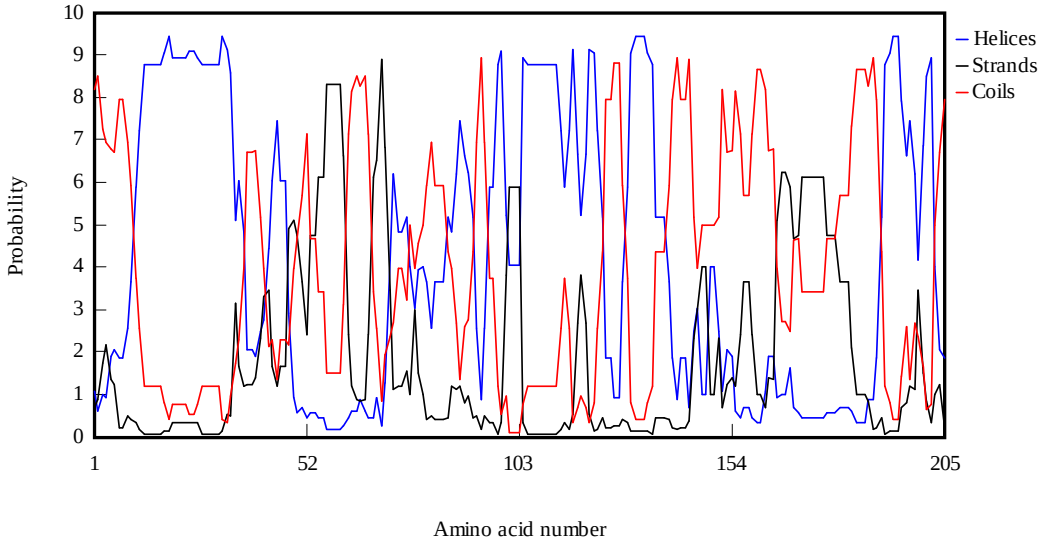


Fig. 3.19: Secondary structure prediction, illustrating the location of helices and coils (constructed using DNAMAN).

From the sequence 7 helices were detected, 2 large helices and 4 smaller ones [Fig. 3.19]. Approximately 95% of the transcriptional factors bind the appropriate DNA sequence by means of the helix-turn-helix (HTH) motif. Conservation is limited to this domain; it is believed that structural differences outside this region are the reason for diverse associated actions of this family.

Expression of the lcp homolog from an extracellular rubber degrader in nocardioform actinomycete strains

3.11 Amplification of the *lcp* gene from *S. coelicolor* A3(2)

Since the *lcp* gene has been been identified in both extracellular and adhesive rubber degraders I evaluated whether the *lcp* homologue from an extracellular rubber degrader would be expressed in nocardioform actinomycete strains and which strategy would be adopted. Primers were designed to amplify the *lcp* homologue identified in the genome of *S. coelicolor* A3(2). These were designed to attach to the predicted start site and end of the gene, amplifying the predicted 1194 bp gene [Fig. 3.20].

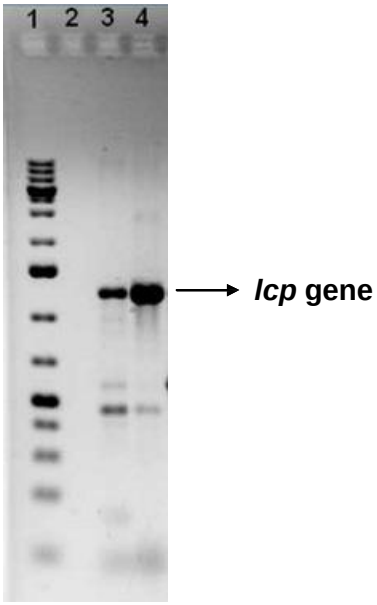


Fig. 3.20: Amplification of *lcp* homologue from *S. coelicolor* A3 (2). Lanes **1**: 1kb GeneRuler DNA ladder, **3**: lcp amplified using 1.5 mM $MgCl_2$ and **4**: lcp amplified using 2 mM $MgCl_2$

3. 12 Heterologous expression of *lcp* in *Rhodococcus* spp., *Mycobacterium* spp. and *Gordonia* spp.

The gene was ligated into the appropriate vectors and established to be in the correct orientation. Thereafter, these were transformed into the respective hosts which were inoculated with latex glove pieces for three weeks and visually monitored in relation to the control [Fig. 3.21]. Although not as clearly visible as with strain 25593, all strains carrying the *lcp* homologue colonized the rubber more efficiently.

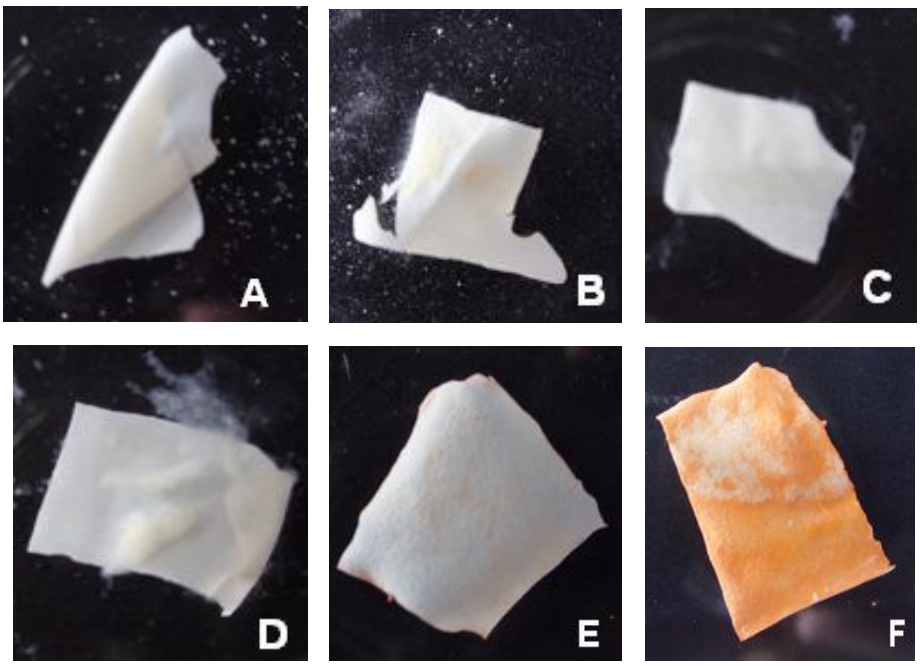


Fig 3.21: Strains colonizung latex rubber substrate: (A) *R. erythropolis* SQ1, (B) *R. erythropolis* SQ1 + lcp, (C) *M. smegmatis* mc^2 155, (D) *M. smegmatis* mc^2 155 + lcp, (E) *G. rubripertincta* 25593, (F) *G. rubropertincta* 25593 + lcp

In parallel, these strains were also spotted onto latex agar plates and monitored for the presence of clear zones. No zones were detected, suggesting that extracellular secretion was not the mode of action adopted in these strains.

4. Discussion

Rubber is a fairly recalcitrant hydrocarbon compound (Roy *et al*., 2006b). The mixture of chemicals added to enhance its properties and cross-linking contributes further to its resistant nature. Thus, not only must microbes be able to degrade vulcanized bonds, but also resist a plethora of additives. Yet bacteria have evolved pathways to catabolize this compound. Rubber degrading bacteria are abundant and have been isolated from diverse environments in many countries. These include both soil and water samples collected in parts of Asia, Europe and Africa (Jendrossek *et al.*, 1997; Rifaat and Yosery, 2004).

In this study latex agar plates were used to strictly select for extracellular enzyme releasing rubber decomposers, identified by the formation of translucent halos on an opaque background. Resultantly, twenty-five strains were isolated and four chosen for detailed characterization.

16S rRNA similarities of < 96% are considered to indicate that an isolate belongs to a separate genera (Janssen, 2006). The partial sequencing of the 16S rRNA gene of one strain showed a 99.8% sequence similarity to both species *S. tendae* and *S. tritolerans*. Subsequently, research was conducted on both strains to identify any distinguishing phenotypic features. A detailed study conducted by Syed and coworkers (2007) using phenetic properties and genetic techniques showed that despite a 99.6% similarity of the complete 16S rRNA gene (three nucleotide differences) between *S. tendae* and *S. tritolerans* there remained clear disparity between each strain. Three clearly discernible and easily testable traits found in *S. tritolerans* and not shared by *S. tendae* were tolerance towards salinity, alkalinity and temperature. Notably, it possessed the ability to grow at a temperature of 45^{o}C, tolerate a pH of 10 and sodium chloride concentration of 7%. When the streptomycete strain BA1 was tested it displayed no tolerance to any of these factors, supporting its classification as *S. tendae*.

The 16S rRNA sequence linked strain Est to the family *Pseudonocardiacea.* The isolate to which it was matched was however not characterized further to the species level. Thus, Bergey's manual was used to classify the strain. Phenotypic

characteristics were matched to the species *Pseudonocardia*. The zigzag shaped hypha is a characteristic feature of this species. In this work just one Gram negative rubber degrader was isolated; as observed in many studies, isolation is rare (Rifaat and Yosery, 2004). The strain identified as *Methylibium fulvum* HZ displayed a strong extracellular activity, much like the only well characterized Gram negative rubber degrader, *Xanthamonas* spp. 35Y (Tsuchii and Takeda, 1990). Unfortunately, with no appropriate vector available screening for this gene was not possible.

The rubber degrading potential of *Pseudonocardia* spp. has not previously been reported, although members of this genus have been tested. Of 37 *Pseudonocardia* strains analyzed by Jendrossek and coworkers (1997) none displayed a polyisoprene degradative ability. It was not surprising that the majority of the rubber degrading isolates from this study were identified as members of the species *Streptomyces*. Previous studies have shown that this species tends to be the most commonly isolated with regard to rubber decomposition. This was observed in a study conducted by Jendrossek and co-workers (1997) whereby the screening of 1220 bacteria on latex agar led to the isolation of 46 rubber degrading isolates of which 31 were Streptomycetes.

The strain *S. tendae* has been studied previously and is of interest as it produces nikkomycin, a fungicide and insecticide (Evans *et al.,* 1995). It also secretes streptofactin, a biosurfactant which induces aerial mycelia (Richter *et al.,* 1998). Members of the species *Pseudonocardia* have been linked with varied features such as fatty acid catabolism, biodegradation of tetrahydrofuran and cellulose production (Malfait *et al.,* 1984; Kohlweyer *et al.,* 2000; Chen *et al.,* 2005). Both isolates were tested for their ability to utilize diverse carbon compounds. Growth of these isolates on media plates supplemented with no additional carbon source reveals a capacity to use micronutrients from the agar and possibly gaseous elements from the air to sufficiently support their development, suggesting a chemoautotrophic lifestyle.

Actinomycetes exhibit tremendous metabolic diversity, with an ability to degrade a vast array of both natural and xenobiotic compounds and have been implicated in the degradation of polycyclic aromatic hydrocarbons, pesticides and recalcitrant plastics (Lee *et al.,* 1991; Miller *et al.,* 2004; Harada *et al.,* 2006). However, none of these

strains displayed an ability to efficiently utilize any of the diverse carbon elements as sole carbon sources. From the substrates tested, lignin and nylon were of particular interest. Since other Streptomycetes capable of lignin degradation have been isolated it was presumed that these strains might possess those abilities (Ramachandra *et al*., 1987), nevertheless screening revealed they did not. Similarly, these isolates were incapable of utilizing alcohols as a carbon source and were strongly inhibited by heavy metals.

To test whether an alteration in the nutritional composition of the latex media would affect clear zone formation, one extra carbon source was added. Glucose and succinate were tested since these were reported previously as repressing rubber degrading enzymes in most strains. Investigations concerning the regulation of enzyme activity were conducted by Jendrossek and coworkers (1997) and Rifaat and Yosery (2004) on latex degraders. The authors stated that from 47 *Streptomyces* spp. examined, 35 were inhibited by succinate and 45 inhibited by glucose. Also, fructose and mannitol were the only carbon sources which had no effect on enzyme expression. Results recorded here did not show a similar pattern. Apparently, none of the strains enzyme production was affected by the addition of glucose, succinate or fructose. However, Tween 80 repressed clear zone formation. These results were peculiar since many bacteria exhibit catabolite repression, as it is more energy efficient to metabolize simpler carbon sources than a complex hydrocarbon. Yet supplementation of latex agar with glucose resulted in an enhanced clearing zone, suggesting instead that these isolates were utilizing both carbon sources.

Colonization of rubber pieces by strains BA1, Est, Chiba and Yeo was evident due to the intense purple color of Schiff's. As discussed at length by Heisey and Papadatos (1995) the colonization of the hydrocarbon does not definitively constitute an ability to utilize the substrate as an energy source. The presence of non-rubber constituents is enough to sustain the growth of organisms (Rook, 1955). Hence, it is necessary to either demonstrate a weight loss or microscopic modification of the material. Accordingly, SEM was used to monitor colonization, penetration and surface modification.

All four rubber degrading strains formed dense biofilms, penetrating into the polymer and altering the surface. This is similar to observations made by Heisey and Papadatos (1995), who examined *Streptomyces* spp. modification of the material using SEM. Contrary to what was reported with respect to *Streptomyces* spp. K30, when glucose was added to cultures of strains BA1, Est, Chiba and Yeo containing latex glove pieces, none of the strains colonized the rubber. This demonstrated that minimal nutrient conditions triggered colonization.

Notably, the glove pieces colonized by all four strains retained the same shape and composition (no additional stickiness occurred). Although fully colonized these isolates failed to mineralize the glove rubber. This is in accordance with other studies concerning extracellular rubber degraders such as *Xanthomonas* spp., *Streptomyces coelicolor* 1A, and *Streptomyces* spp. S1G which induced small weight losses of vulcanized rubber by approximately 10 %. Since the polymer remained intact this suggested as other studies have that these strains are either incapable or inefficient at breaking vulcanized bonds or effected by antimicrobial chemicals (Linos *et al.,* 2000). The inhibition of rubber degrading isolates by antioxidants was well characterized by Berekaa and coworkers (2000) who found the removal of these compounds enhanced both colonization and disintegration of latex gloves. Taking the case of *Pyrococcus furiosus*, it was able to efficiently utilize sulfur thus weakening the vulcanized bonds. Nonetheless, this strain was sensitive to rubber additives, reducing its applicability (Bredberg *et al.,* 2001).

It should be noted that while enzyme-releasing rubber degraders are weak decomposers this does not imply that they are conclusively of no use. It might be possible to employ these bacteria in biotechnological recycling at a later stage. For instance, it is possible to break the vulcanized cross-links using adhesive degraders and use enzyme releasers to further degrade and catabolize the resulting by-products. Alternately, detoxifying bacteria may be used to pretreat the material in preparation for decomposition by isoprene degrading bacteria.

In an attempt to screen by means of complementation, UV and NTG mutagenesis was used in order to generate a latex negative phenotypic strain. Since UV mutagenesis seems to be the method of choice in *Streptomyces* spp., this was attempted first

(Hopwood *et al.*, 1985). UV induces the formation of thymine dimers which in the presence of 8-methoxypsoralen sensitizes DNA by inducing mono-adducts and strand crosslinks (Bridges and Stannard, 1982). The action of the mutagen, NTG modifies guanine residues. UV mutagenesis on *S. coelicolor* 1A and *S. griseus* 1D conducted by Bode *et al.* (2001) to induce non-rubber degrading mutants was a lengthy task. Out of ~ 10 700 strain 1A mutagenized bacteria, one true mutant was obtained. Similarly, following the screening of ~ 27 000 UV treated strain 1D cells, four mutants were obtained. The calculated frequency of finding a mutant ranged between 0.0009-0.015 %. Another study described the use of NTG to generate a *Mycobacterium aurantiaca* latex negative isolate. Mutants were obtained at a frequency of 0.04 %, notably higher than that of UV mutagenesis (Rose and Steinbüchel, 2002). In this study the frequency of inducing a non-rubber degrading mutant using NTG was calculated at 0.31 % while UV mutagenesis proved ineffective.

To confirm that the latex breakdown gene was disrupted and that the extracellular enzyme releasing mechanism was not affected, the induced mutant was spotted onto starch agar. The formation of a translucent halo indicative of starch degradation proved that the general enzyme secretion pathway was not affected. The ability to form clear zones in the presence of starch and not latex suggested that the mutant carried a mutation specific for latex use. Further tests showed that this mutant was still able to colonize and penetrate rubber, although unable to degrade the substrate. Distinct differences between the wildtype and mutant strains were the enhanced growth rate in liquid media and earlier sporulation of the mutant strain on latex agar. This implied that other mutations were present in the genome. Although concerning at first, it appears that differences in mutant strains is not unusual. In particular, Tsuchii and Tokiwa (1999) published a study of 3 spontaneous *Nocardia* 835A mutants with significantly different colonial phenotypes compared to the parent strain. Colonies of Rw, Rc and Wh were pale orange, cream and powdery white respectively.

A problem encountered in the transformation of *S. tendae* is its restriction system. PEG-mediated transformation using the standard *Streptomyces* spp. transformation procedure of Hopwood *et al.* (1995) is not possible. To overcome this, the protoplasts must be subjected to heat treatment (50^{o}C for 30 min.) which further reduces the transformation efficiency. According to Engel (1987) less than 0.1% of the

protoplasts survives heat shock and are transformed. He found that transformation with pIJ702 into *S. tendae* ATCC 31160 resulted in just 10^2 transformants/µg DNA. Attempted transformation of the *S. tendae* BA1 induced mutant with pIJ702 following the procedure described by Engel (1987) was unsuccessful. It was thought that the induced mutant could be used to identify the relevant gene by means of complementation. However, the inability to transform the strain did not allow this avenue to be explored.

Among *Streptomyces* spp., *S. lividans* is often used as a host cell for the expression of DNA. It is favored since it lacks a restriction system and is easily transformable (Nakashima *et al.,* 2005). I decided to screen two libraries in a *S. lividans* host and the other two in *Rhodococcus* spp. host. In the latter case, *R. erythropolis* was used as a host cell and an *E.coli-Rhodococcus* shuttle vector to express mycelial actinomycete DNA. To paraphrase, Nakashima and colleagues (2005) mentioned that "it is often recommended to use host cells that are phylogenetically closely related to the origin of the protein of interest. This is due to similarity in frequency of codon usage, compatibility with machineries of translation and molecular chaperones and/or redox states of the cells." The phylogenetic distance between *Streptomyces* and *Pseudonocardia* to *Rhodococcus* spp. showed that there was a low divergence among these strains. Moreover, the expression of the hygromycin gene by strain SQ1 was a positive result. The expression of Streptomycete genes by a *Rhodococcus* replicon is not uncommon. One merely has to look at vectors to realize this. pMVS301, a *Rhodococcus* spp. H13-A replicon is capable of expressing the Streptomycete thiostrepton resistance gene (Singer and Finnerty, 1988). Similarly, pNC9501, a *R. ruber* P-II-123-1 replicon is able to express the thiostrepton gene (Matsui *et al.,* 2006).

Notably, a chief advantage of using *Rhodococcus* is the screening process. Due to the morphological nature of *Streptomyces*, this species grows beneath the agar, thus screening involves the tedious task of individually patching each colony to test for the presence of the trait of interest. Thus, it is not uncommon to patch thousands of transformants. In contrast, *Rhodococcus* clones can be pooled, diluted appropriately and spread directly onto the respective media.

Unfortunately, the screening in *S. lividans* did not yield positive results. With regard to screening of the latex enrichment cultures, no vector DNA carrying clones were isolated from the *S. tendae* genomic library. It is uncertain what the reasons for this are. Perhaps there exists an inability to recognize Streptomycete regulatory elements. The latex liquid enrichment however identified *Rhodococcus* clones carrying a common *Pseudonocardia* genomic fragment which led to latex remaining in suspension. Once the *Pseudonocardia* Est fragment of interest was cloned into pUC18 and transformed into *E.coli*, a dramatic decrease in the number of transformants was noticeable. This suggested that the gene product induced cytotoxicity. This was not witnessed in *Rhodococcus* spp. Since pDA71 has a copy number ≤ 5 while pUC18 has a copy number ranging from 500-700. Analysis of the unsequenced 3kbp region should clarify the reason for this toxicity.

Since the restriction analysis clearly demonstrated no relation of the fragment to *Streptomyces* K30 rubber degradative gene it was not unexpected that the sequence analysis would reveal similarity instead to an unrelated element, a TetR transcriptional regulator.

Ramos and coworkers (2005) published an extensive and detailed review on the family of TetR proteins. Much of the literature revolving around TetR deals with the most prominent member of the family, the protein involved in tetracycline resistance. In fact regulators within this family are involved in many diverse activities. They control gene products which have been linked to resistance, catabolic pathways, biosynthesis and pathogenicity. Bacteria must be capable of dealing with sudden changes in their surroundings, be it nutritional or environmental. When faced with a harmful condition they must be able to make appropriate cellular adjustments to withstand this or alternately if it is beneficial, take advantage of this. These "rapid, adaptive responses" are controlled by regulators which react to specific cues, adjusting gene expression in response (Ramos *et al.*, 2005). *TetR* genes have been located on both chromosomal and plasmid DNA and are commonly found to be widespread in microbes encountering environmental changes such as *Nocardia* spp., *Streptomyces* spp., *Mycobacterium* spp. and *E. coli.* Just 85 members of the 2353 TetR regulator sequences are associated with a known function. From the results of the phylogenetic tree it is clear that the TetR sequence of strain Est is variable

compared to other sequenced regulators in this family. The highest sequence similarity was matched to *Frankia* spp, *Saccharopolyspora erythraea*, *Solibacter usitatus* and *Mycobacterium* spp. Interestingly, in all these bacteria the locations of the TetR proteins within their genomes were in close vicinity of genes with oxidative functions. With relevance, in *Bacillus megaterium* ATCC 14581, a TetR regulator controls the expression of the oxidative gene p4508M-1, an inducible P450 monooxygenase which catalyzes the hydroxylation of fatty acids. These proteins regulate various biodegradative pathways (Ramos *et al*., 2005). For instance in *R. erythropolis* SQ1, it acts as a repressor of *kstD* influencing the phytosterol degradative pathway. Additionally, these genes regulate *p*-cumate degradation. By analogy, this gene could be responsible for the regulation of isoprene break down in *Pseudonocardia* spp. Est.

Notably, the investigation of this fragment is still in the preliminary stage. As such it should be stressed that there could be several possible reasons for the results obtained. These possibilities include a mutation within the *Rhodococcus* clone or the vector pMDC10, inducing an activity to degrade the rubber. Otherwise, part of the 4kbp fragment could encode for a biosurfactant. This would reduce the surface tension, keeping the latex in suspension. A further possibility includes the gene regulating an unrelated gene with an oxidative function.

To recap, rubber degrading genes or possibilities have been identified in 6 strains, these include *Xanthomonas* 35Y, *Streptomyces* spp. K30, *Nocardia farcinica* E1, *S. coelicolor* A3 (2) and tentatively from *Gordonia polyisoprenivorans* and *Gordonia westfalica*. Unfortunately further genomic work was not carried out on *Pseudomonas citronellolis*, the Gram negative adhesive degrader. It would be interesting to know if the genes playing a role in polyisoprene mineralization are related to that of *Xanthomonas* (since it is also Gram negative), to the Gram positive adhesive degraders (since they share the same decomposition strategy) or whether it is novel.

As previously mentioned *lcp*, *oxiA* and *oxiB* involved in rubber decomposition were found in *Streptomyces* spp. K30. *Lcp* homologues were found in the extracellular degrader *S. coelicolor* A3 (2) and unexpectedly in the adhesive degrader *N. farcinica* E1. Although no oxidoreductases bearing sequence similarity to the *oxiAB* genes were

found in either strain. This is in view of the fact that the entire genome of both *N. farcinica* IFM 10152 and *S. coelicolor* A3 (2) have been sequenced. Notably, *roxA* and *lcp* share no sequence homology, suggesting independent evolution of these systems (Tsuchii and Takeda, 1990). The identification of *lcp* homologues in other rubber degraders strongly suggests that this gene is the main element in isoprene biodegradation. However, taking into account studies conducted on *Gordonia* does not reflect this. Recalling that none of the 105 ORF's on the plasmid of *G. westfalica* showed similarity to *lcp* or to any of the genes identified in rubber biodegradation from *G. polyisoprenivorans*. Following the screening of such a vast number of clones of *G. polyisoprenivorans*, researchers stated that it would have been difficult for them to have overlooked a rubber negative degrading phenotype. In this regard they have proposed that this species might use a different rubber cleaving enzyme.

Concerning the expression of the *lcp* in nocardioform actinomycetes, it is believed that the *lcp* gene amplified from *S. coelicolor* A3(2) was expressed in all the strains since they were able to effectively colonize the rubber pieces. However, I expected these strains to adopt potent rubber degrading abilities yet no fragmentation of the substrate was observed. It seems possible that additional genetic elements contribute towards the adhesive degradative ability displayed by nocardioform actinomycetes harbouring native *lcp* homologues.

4.1 Concluding remarks

This work was conducted with the purpose of characterizing all four rubber degrading strains and isolating the relevant rubber degrading genes. As shown in other studies regarding extracellular degraders, the characterization revealed weak rubber biodegraders. While no clone related to rubber degradation could be isolated from the three Streptomycete strains, encouragingly a potential genomic fragment was isolated from *Pseudonocardia* spp. Est. However, more work must be done to determine the functional aspect associated with the entire 4kbp fragment. Also, this is the first report of a *Methylibium* spp. possessing the ability to degrade rubber.

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Preliminary characterization of *A. orientalis* phytase activity

Chapter IV abstract

A modified plate staining technique involving the use of Taussky-Shoor was developed for the identification of phytase positive strains. Twenty-seven actinomycete strains were screened for phytase activity; a strong zone was detected around *A. orientalis* SY6. Basic characterization studies revealed that the enzyme worked optimally at 30^{o}C and at a pH of 6.5 suggesting that this could be a β-propeller phytase. A moderate activity of 50 U/ml was estimated.

1. Introduction

1.1 Phytic acid

Phytic acid (phytate) is the form in which phosphorus is stored in plants and seeds (Greiner *et al.*, 1993). Monogastric animals are incapable of utilizing phosphorus in this form causing it to be excreted in their manure (Pandey *et al.*, 2001). In areas where there is a high concentration of farm animals, this excess phosphorus enters the environment leading to eutrophication (Hussin *et al.*, 2007). The most problematic factor associated with phytate is its ability to bind vital elements such as calcium, magnesium, iron, zinc, copper, manganese and cobalt, causing it to be labeled an anti-nutrient (Greiner *et al.*, 1997) [Fig. 1.1]. Additionally, it is also believed to inhibit digestive enzyme activity. Hence, the use of phytase to hydrolyse phytate makes phosphorus as well as micro and macro elements available to animals. Harland and Harland (1980) showed that an increase in phytase producing yeast and fermentation time reduced the level of phytate thus increasing the quantity of minerals available in bread.

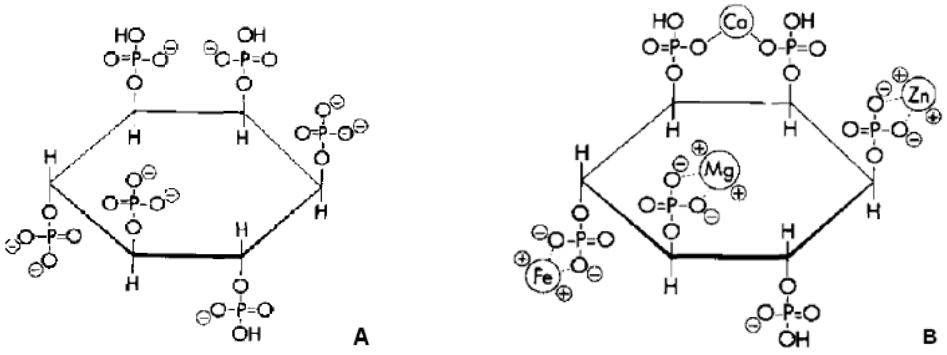

Fig. 1.1: (A) Structure of phytic acid and (B) phytic acid chelate, binding metal elements (Erdman, 1979).

1.2 Types of phytase

Four classes of phytases are known, these are the histidine acid phosphatases, purple acid phosphatases, β-propeller phytases and cysteine phosphatases (Turner *et al.*, 2007). Recently, a phytase sharing no similarity with any of the other phytase classes was isolated from *S. ruminantium* (Yanke *et al.*, 1999). Histidine acid phosphatases can be identified on the basis of a conserved motif, RHGXRXP (Cheng and Lim, 2006). Purple acid phosphatases are metalloenzymes. The β-propeller phytase as the name states is made up of six β-propeller structures with two phosphate and six calcium highly conserved binding sites. Apart from their use in animal feed, these enzymes are also believed to play an important role in the environment. Of the four classes, only the β-propeller phytase class has been found to occur in the aquatic environment and are believed to be linked to the recycling of phosphorus (Cheng and Lim, 2006).

1.3 Phytase sources

These enzymes are present in plants, bacteria, fungi, yeast and certain animal tissue (Pandey *et al.*, 2001; Greiner *et al.*, 1993). However, the commercial production of phytase is dominated by *Aspergillus* spp. Below are some examples of microbial phytases [Table 1.1].

Table 1.1: Bacterial strains which produce phytase

Bacterial strain	**Name and type of phytase**	**Reference**
Yersinia kristeensenii	appA; histidine acid phosphatase	Fu et al. (2008)
Shewanella oneidensis	phyS; β-propeller phytase	Cheng and Lim (2006)
Enterobacter spp.	β-propeller phytase	Yoon et al. (1996)
B. subtilis VTT E-68013	phyC; β-propeller phytase	Kerovuo et al. (1998)
Klebsiella oxytoca MO-3	-	Jareonkitmongkol et al. (1997)

Bacterial strain	Name and type of phytase	Reference
Pseudomonas syringae MOK1	-	Cho et al. (2003)
Klebsiella terrigena	-	Greiner et al. (1997)
Escherichia coli	P1 and P2; acid phosphatase	Greiner et al. (1993)

1.4 Mechanism of phytate biodegradation

Phytase (myo-inositol hexakisphosphate phosphohydrolase) hydrolyses phytate producing myo-inositol and inorganic phosphate. In most cases phytase is induced by phosphate limiting conditions, other cases of carbon limitation and an anoxic environment has also been reported as positive contributors to phytase production (Greiner *et al.*, 1997).

1.5 Detection of phytase activity

Generally, phytase activity is identified by the formation of a halo around colonies on opaque calcium phytate supplemented plates [Fig. 1.2]. In liquid media it is determined indirectly using a color reagent relying on the detection of inorganic phosphorus. When phytase hydrolyzes phytate the inorganic phosphorus released can be detected by the interaction of molybdate with the element, forming a phosphomolybdate complex. This complex is reduced to molybdenum blue whereby the color intensity is an indication of phosphorus present (Bhattacharya *et al.*, 2005).

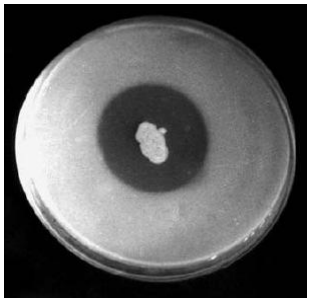

Fig. 1.2: Zone of clearance around colony of *Staphylococcus* spp. phytate degrader on calcium phytate agar (Mukesh *et al.*, 2004)

1.6 Significance of phytase

It has been estimated the terrestrial environment holds fifty one million metric tons of phytate stored in the form of seeds, grain and fruit (Cheng and Lim, 2006). Phosphate is a non-sustainable mineral and its increased use results in an unpleasant chain reaction. This starts off with excess rock phosphate being added to animal feed to counteract the effects of undernourishment due to limited intake of this compound. This is excreted into the environment whereby the 'nutrient runoff' from manure can lead to algal blooms which results in the release of large amounts of toxins into water bodies. Resultantly, marine fauna and flora are adversely affected (Mullaney *et al.*, 2000). The use of phytase would prevent the misuse of natural phosphate reserves and avert the release of surplus phosphate in animal manure consequently preventing the mineral from entering the environment.

1.7 Phytase market trends

The discovery of phytase in 1907 and realization of its commercial importance led to the current five hundred million dollar market for its use as an animal feed additive (Mullaney *et al.*, 2000). Clearly, the use of enzymes in animal feed is a prominent market, particularly the use of phytase. The industrial production of this enzyme is currently sourced from a recombinant *Aspergillus niger* or *Aspergillus oryzae* strain through overexpression of the *phyA* gene (Pandey *et al.*, 2001). BASF together with a Dutch based company are the most successful marketers of phytase, supplying regions in Asia, Europe, the USA and Canada. Marketed under the name Natuphos, it is said to increase phosphate availability by 30 % and subsequently reduce phosphorus addition by 17 % (Pandey *et al.*, 2001). To put the significance of phytase into perspective; if this enzyme was to be included in the diets of all farm animals in the US this would release phosphorus with a value of $\$1.68 \times 10^8$ per annum (Wodzinski and Ullah, 1996). In terms of pollution 8.23×10^7 kg of phosphorus would be prevented from entering the environment (Wodzinski and Ullah, 1996).

1.8 Sterol degradation

Many members of the family actinomycetes such as *Mycobacterium, Nocardia, Rhodococcus* and *Streptomyces* species are able to degrade cholesterol (Sojo *et al.*, 1997). This is broken down by oxidation of the OH group at the C^3 position in addition to the isomerization of the C^4-C^5 bond to generate 3-keto-4-ene cholestenone (Sojo *et al.*, 1997). This activity is mediated by cholesterol oxidases. These enzymes are of commercial importance in the determination of cholesterol in food and serum (Sojo *et al.*, 1997). Stigmasterol is a plant sterol, chemically similar to cholesterol (Jones *et al.*, 1997). Consumption of this sterol has been linked to lower cholesterol levels (Jones *et al.*, 1997).

1.9 Objectives of project

Main objective:

To identify phytase producing actinomycetes and characterize the phytase produced

Specific objectives:

i. Identify phytase producing strains using a modified Taussky-Shoor staining technique

ii. Partial characterization of the enzyme by conducting a temperature and pH profile

iii. Screening of isolates capable of degrading cholesterol and stigmasterol

2. Materials and methods

2.1 Phytate stock solutions

Sodium phytate and calcium phytate were dissolved in sterile distilled water prepared as 0.25 % stock solutions. The media was allowed to cool to approximately 40^{o}C after being autoclaved and dissolved phytate added directly to this.

2.2 Replica plating

Bacterial cells were grown in 1 ml of LB for 2-3 days. This was washed three times with sterile distilled water. 100 µl of this was transferred into the replicator well. The metal replicator was heat sterilized and allowed to cool. This was dipped into the replicator wells and replicated onto selective phytase media and a non-selective LA plate. The plates were incubated for 2 days.

2.3 Screening for phytase activity

2.3.1. *Taussky-Shoor staining*

Following the two day incubation, the plates were gently flooded with Taussky-Shoor reagent and allowed to stand for 5-10 min. The excess regent was discarded and the plates left for 30-60 min. to allow for zone development. Positive phytase activity was detected as a clear zone surrounding the periphery of the colony against a dark blue background.

2.4 Determination of phytase activity

2.4.1. *Enzyme assay*

Phytase activity was determined as described by Harland and Harland (1980). The following calculation was used:

$$\text{Phytase activity (U/ml)} = \frac{\text{OD organism}}{\text{OD control}} \times \frac{100}{\text{incubation time (min.)}}$$

Absorbance was taken at 680 nm. The OD organism was calculated by blanking the experimental tube against the control tube (see section 2.4.3) and OD control was calculated by blanking the standard phosphate solution against Taussky-Shoor reagent (see section 2.4.4). All experiments were done in duplicate (inter-experimental duplication) and the averages plotted onto a graph.

2.4.2. *Crude enzyme preparation*

Flasks containing 20 ml of dextrose broth supplemented with 0.25 % sodium phytate were inoculated with dense bacterial precultures and incubated at 30^{o}C for 2 days. The cells were pelleted by centrifuging (10 000 rpm; 10 min.) and the supernatant used as as a source of extracellular phytases.

2.4.3. *Determination of the effect of temperature and pH on phytase activity*

500 µl of the crude enzyme preparation was added to 100 µl of 0.05M Tris-HCl pH 7.0 and 300 µl H_2O. To start the reaction 100 µl of 10mM sodium phytate was added and the test tubes incubated for 1 h. at the appropriate temperature. The control test tubes against which the experimental tubes were blanked were prepared as above with sterile distilled water replacing the crude enzyme. The tubes were incubated at either 25^{o}C, 30^{o}C, 37^{o}C or 60^{o}C.

For pH determination the tubes were prepared as above and incubated with different buffer solutions. For pH 3.0 and pH 5.5. 0.05M citrate buffer was used, for pH 7.0 0.05M Tris-HCl was used and for pH9.0 0.05M HEPES buffer was used. These were incubated at 37^{o}C for 1 h. Following the incubation, 2.5 ml of Taussky-Schoor was added. The contents were vortexed briefly and left to stand for 1 min. at room temperature after which the absorbance was recorded.

2.4.4. *Standard phosphate solution*

500 µl of a 10 nmol standard phosphate solution was added to a test tube with 500 µl of sterile distilled water. This was incubated along with the experimental tubes at different temperatures (25^{o}C, 30^{o}C, 37^{o}C and 60^{o}C). After incubation 2.5 ml Taussky-Shoor solution was added and the absorbance recorded. The solution was blanked against 2.5 ml Taussky-Shoor solution and 1 ml sterile distilled water.

3. Results and discussion

3.1 Preliminary characterization of phytase from *A. orientalis* SY6

Twenty-six strains isolated from the environment were tested for phytase activity. These were spotted onto conventional phytate screening media supplemented with and without phytate. It was found that on media containing no phytate all the actinomycete strains exhibited either extremely poor growth or were completely inhibited. It is believed that the presence of the metal elements, manganese and iron might be responsible for this. A literature search showed that this is a commonly used media for bacterial phytase screening purposes (Vats and Banerjee, 2002). The inability of the media to support microbial growth was problematic and thus a richer screening media used by Mukesh and coworkers (2004) was adopted. This media contained dextrose, tryptone, salt and potassium chloride (Mukesh *et al.*, 2004). In order to optimize growth of the strains, a pH of 5.5 and pH 7.0 was tested and supplemented with either calcium or sodium phytate. The addition of calcium phytate at both pH 5.5 and pH 7.0 inhibited microbial growth. Replacement of calcium phytate with sodium phytate at pH 5.5 led to improved growth, inhibiting just seven of the isolates. The pH 7.0 sodium phytate media supported the growth of all strains tested. Conventionally, strains are tested on solid media by streaking onto opaque calcium phytate supplemented plates and monitored for the presence of surrounding clear zones, an indication of phytate hydrolysis (Quan *et al.*, 2001). The use of sodium phytate prohibited the generation of an opaque media, thus the media was stained directly with Taussky-Schoor [Fig. 3.1].

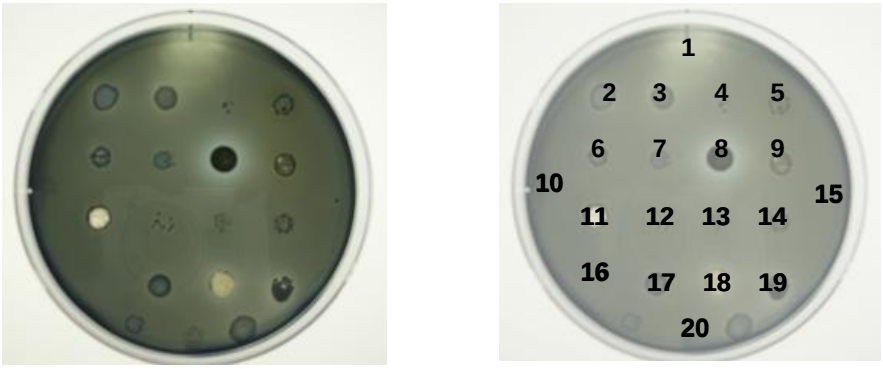

Fig. 3.1: Phytate media flooded with Taussky-Schoor reagent (left). Strains tested include 1: *T. tyrosinosolvens* YeoE, 2: strain Bot2, 3: strain FHome, 4: *S. prasinus* Berlin, 5: strain Hak, 6: *S. tendae* BA1, 7: *S. prasinus* HZWS, 8: *A. orientalis* SY6, 9: *S. flavogriseus* Chiba, 10: *S. griseus* Yeo, 11; *S. lividans* TK23, 12: strain WitsP, 13: strain SY3, 14: strain Cal, 15: strain HY, 16: *Streptomyces pseudogriseolus* Reu, 17: strain H3, 18: strain SY5, 19: strain WITS, 20: strain BotY

The results of the above experiment are represented in the table below [Table 3.1].

Table 3.1: Preliminary phytase screening results using Taussky-Shoor reagent

	Na-phytate (pH5.5)	**Na-phytate (pH7.0)**
Strains		
BA1	-	-
Gam	-	-
Reu	-	-
Berlin	-	-
Hak	-	-
Cal	-	-
Pasa	-	-
Bot1	-	-
FHome	-	-
WitsP	-	-
SY3	-	-
SY5	-	+
SY6	+	+
HY	-	-
WITS	-	-
Bedd	-	+
H2	-	-
H3	-	-
BotY	-	-
Bot2	-	-

	Na-phytate (pH5.5)	**Na-phytate (pH7.0)**
Est	-	-
Chiba	-	-
Yeo	-	-
S. lividans TK23	-	+
YeoE	-	-
HZ	-	-
HZBS	-	+
HZWS	-	-
S. coelicolor A3(2)	-	+

I identified four strains that exhibited strong zones of clearing around the colonies. These were *A. orientalis* SY6, *Streptomyces* spp. SY5, *Streptomyces* spp. Bedd and *Streptomyces* spp. HZBS. *S. coelicolor* A3 (2) was carried as a positive control, identified by computational analysis of the genome as a potential producer of a β-propeller phytase (Cheng and Lim, 2002). Further testing i.e. liquid assay work revealed Streptomycete strains Bedd, SY5 and HZBS were possibly false positives. The values when plotted onto a graph were erratic with sudden increases and decreases. The use of this reagent is said to be incapable of distinguishing between phytase and acid producing bacteria (Turner *et al.*, 2007). This technique of course is only capable of detecting extracellular phytase activity and thus it cannot be discounted that these strains possess intracellular phytase activity.

A. orientalis SY6 showed optimal activity at 30^{o}C at a pH of 6.5 [Fig. 3.2]. Since its optimal activity is closest to a neutral pH it is likely that this phytase is a β-propeller. The histidine acid phosphatase isolated from *Y. kristeensenii* also exhibited a deviation from the normal optimal pH of this class of phytases. It exhibited its highest activity at pH 4.5 rather than the more commonly observed pH of 5.5 (Fu *et al.*, 2008). At 60^{o}C the enzyme activity dropped by half demonstrating weak thermostability. Many phytases show optimal activity at the higher temperature range. For instance, *Enterobacter* spp. phytase displayed optimal activity at pH 7.5 and 50^{o}C (Yoon *et al.*, 1996). *B. subtilis* was found to work best at pH 7.0 and 55^{o}C (Kerovuo *et al.*, 1998). Similarly, *K. terrigena* phytase was active at pH 5.0 and 58^{o}C (Greiner *et al.*, 1997). An exception is the phytase isolated from *P. syringae* which worked optimally at 40^{o}C (Cho *et al.*, 2003).

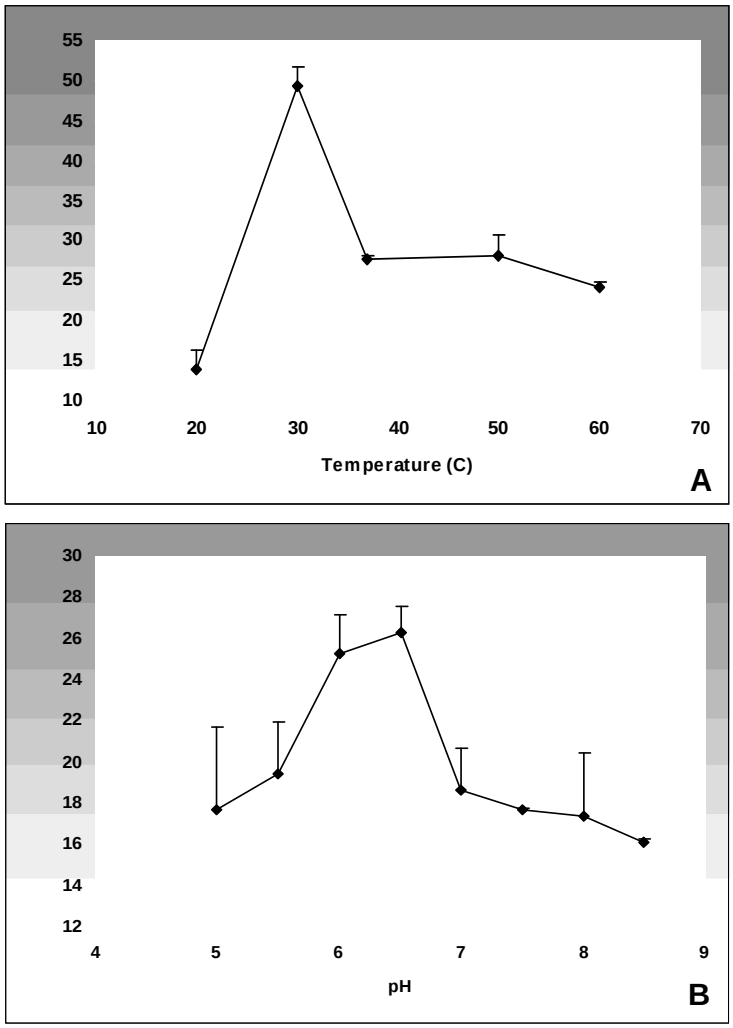

Fig. 3.2: Influence of (A) temperature and (B) pH on phytase activity

A. orientalis SY6 was insignificantly affected by ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT) and the presence of calcium ions [Fig. 3.3]. Since EDTA acts as a chelating agent, phytase tolerance shows that it does not require metal ions for activity. DTT is a strong disulfide reducing agent, its ineffectiveness against the enzyme suggests the sulfide bonds were not accessible to the mineral. The *P. syringae* enzyme was inhibited by Cu^{2+}, Cd^{2+}, Mn^{2+} and EDTA (Cho *et al.*, 2003). *K. terrigena* phytase showed tolerance towards several metal chelators such as EDTA, oxalate, citrate and tartrate, though was sensitive to phosphate, molybdate, vanadate and fluoride (Greiner *et al.*, 1997).

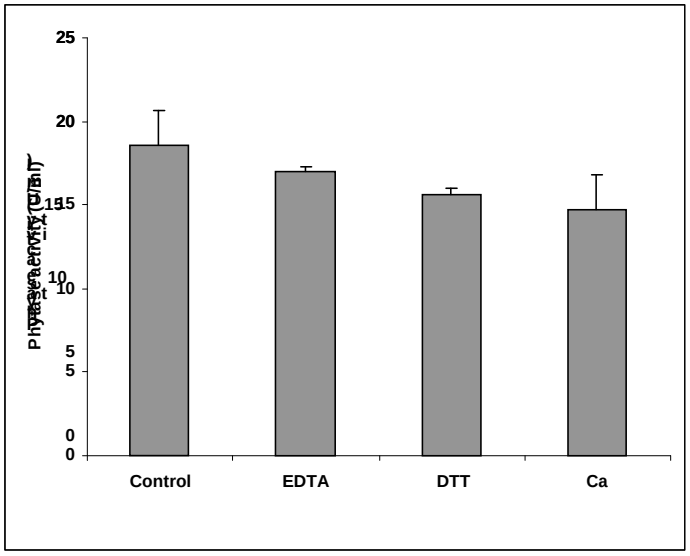

Fig. 3.3: Influence of factors on phytase activity

At its highest level strain SY6 phytase activity was just less than 50 U/ml. Hussin *et al.* (2007) screened 249 isolates from a maize plantation for phytase activity. They found that just 1.6 % of these strains showed an activity above 1 U/ml and the highest activity detected was 1.9 U/ml. The bacteria displaying the highest activities included *Staphylococcus* spp., *Bacillus* spp., *Brevibacillus* spp. and *Kocuria* spp. In comparison to other bacteria: *S. ruminantium* (0.0703 U/ml), *B. subtilis* (0.044 U/ml) and *E. coli* (5.6 U/ml), *A. orientalis* SY6 activity is relatively good (Hussin *et al.*, 2007).

3.2 Degradation of cholesterol and stigmasterol by *Tsukamurella* spp. YeoE

The growth of *Tsukamurella* spp. YeoE increased in the presence of stigmasterol and cholesterol, suggesting an ability to degrade and utilize these compounds, although not as efficiently as glucose [Fig. 3.4]. The growth of strain YeoE was also monitored in the absence of any supplements; the optical density remained uniform throughout the 15 days (data not shown). Several *Rhodococcus* spp. to which *Tsukamurella* spp. is closely related, synthesize cholesterol oxidase and results suggest that this enzyme is present in strain YeoE (Sojo *et al.*, 1997).

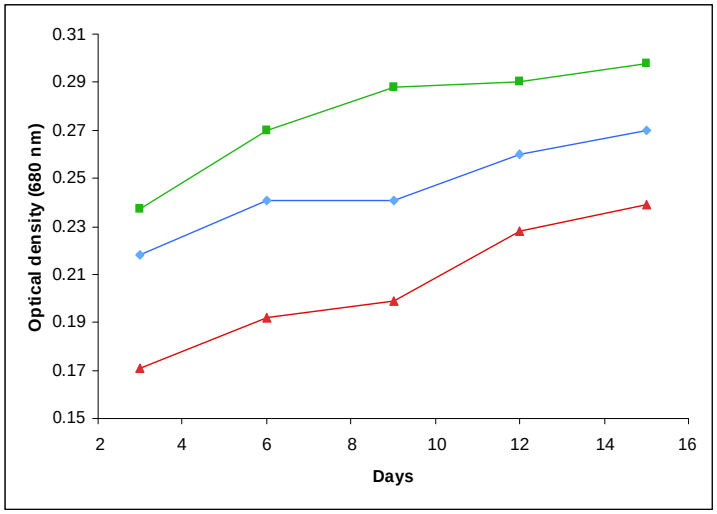

Fig. 3.4: Growth of *Tsukamurella* spp. YeoE in the presence of (green) glucose (0.5%), (blue) stigmasterol (0.5%) and (red) cholesterol (0.5%)

3.3 Concluding remarks

The phytase characterized from *Amycolatopsis* spp. SY6 exhibited poor properties for use as a feed additive. Appropriate phytases need to be thermostable between 65 – 95^{o}C and to survive within the digestive tract tolerate an acidic pH (Hussin *et al.*, 2007). Nevertheless, this is the first report on a phytase identified in this particular species.

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Screening for antimicrobial compounds from soil isolates

Chapter V abstract

Twenty-eight strains isolated from soil samples originating from diverse regions were tested for antibacterial activity alongside fifteen known strains. Three isolates displayed a strong antagonism towards *S. aureus, B. subtilis* and *R. erythropolis*. Sequencing of the 16S rRNA revealed that two of the strains were closely related to *S. prasinus* and the third was identified as *A.orientalis.* Basic characterization tests showed that the antimicrobial compounds were heat stable and tolerated a pH range of 6-9. The inhibitory activity extended to several other species, namely *Micrococcus* spp., *Gordonia* spp., *Thiobacillus* spp., *Agrobacterium* spp. and *Arthrobacter* spp. However, in general Gram negative bacteria were not susceptible to the activity of these compounds. It was hypothesized that inhibition was most likely due to the antibiotic prasinomycin from the two *S. prasinus* strains and the antibiotics vancomycin and chloroeremomycin from *A. orientalis.* Two Streptomycete strains with potential bacteriocin-like activity were also isolated. However, due to exceptionally weak activity these compounds were not characterized.

1. Introduction

Bacteria produce a host of inhibitory products which include toxins, lytic agents, bacteriophages, metabolic by products, bacteriocins and antibiotics (Jack *et al.*, 1995; Tagg *et al.*, 1976).

1.1 *Properties of bacteriocins*

Most research on bacteriocins centred on research done on colicins from the family *Enterobacteriaceae*. Although highly restricted, an in-depth knowledge of the characteristics, mode of action and associated genes were obtained from this work (Tagg *et al.*, 1976). Interest in these peptides in Gram positive bacteria caught on just recently yet has already overtaken the superfluous literature originally based just on Gram negative bacteria. In 1953 researchers first coined the term 'bacteriocin'. The definition of a bacteriocin is still somewhat obscure. The conventional definition describes several criteria in order for a compound to be labeled as a colicin bacteriocin. This includes (i) a narrow inhibitory host range, (ii) the presence of a peptide moiety, (iii) bactericidal mechanism of action, (iv) recognition and interaction with specific cell receptors, (v) synthesis mediated by plasmid borne genes and (vi) the induction of bacteriocin production when exposed to SOS-inducing agents (Jack *et al.*, 1995). These documented criteria however are not followed by many of the Gram positive bacteriocin producers. Hence, many researchers have adopted the term 'bacteriocin-like inhibitory substance' to describe bacteriocins produced by Gram positives which fit most but not all of the criteria.

The mechanism of action by colicins tends to be through either nuclease activity on entering the cell or the formation of pores, causing leakage to occur (Jack *et al.*, 1995). Additionally, the resistant producer strains are believed to produce proteins capable of preventing pore formation or alternately interacting with the bacteriocin preventing its activity (Jack *et al.*, 1995). Observations suggest that the adsorption of bacteriocins tends to be pH dependent, showing maximal activity at lower pH ranges (pH6 - pH2).

1.2 *Properties of antibiotics*

Antibiotics inhibit microbial growth through bacteriostatic or bactericidal activity (Walsh, 2003). Many antibiotic classes exist, these include penicillins, cephalosporins, tetracyclines, rifamycins and glycopeptides (Walsh, 2003). The mode of action of these antibiotics targets cell wall biosynthesis, DNA replication, protein biosynthesis or the folic acid biosynthesis pathway specific for prokaryotes (Walsh, 2003). These compounds are generally synthesized by multiple enzyme complexes (Jack *et al.*, 1995).

1.3 *Strategies for detecting antagonism*

Two common approaches for detecting inhibition exist. The first, known as the simultaneous or direct procedure entails spreading the indicator strain onto the agar plate and spotting the test organism directly onto this. Hence, the potential antagonist must release the inhibitory compound at the early stages of its growth. The second method, which is more widely used is known as the deferred procedure [Fig. 1.1]. The test strain is allowed to fully grow on the media after which it is killed by exposure to either heat or chloroform. The plate is then overlayed with the indicator strain (Tagg *et al.*, 1976).

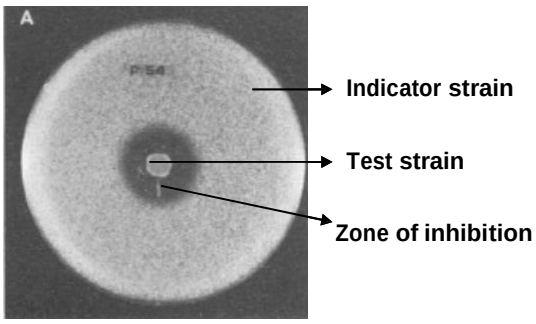

Fig. 1.1: Inhibition indicator strain, *Propionibacteria* by the test organisms, *P. jensenii* (Grinstead and Barefoot, 1992)

1.4 Objectives

i. Identification of isolates possessing antimicrobial activity.

ii. Performing a basic characterization based on heat stability, pH range of activity, UV tolerance, resistance to proteolytic enzymes and the effect of phosphate and glucose supplementation.

iii. Determine the host range of activity.

1.5 Project significance

The purpose of this study was to investigate the antimicrobial activity of environmental isolates. A basic characterization was performed in an attempt to determine if the antimicrobial compounds were antibiotic or bacteriocin-like. The strains assayed against include *S. aureus* 64-1 and *B. subtilis* IA3, closely related to the human pathogens, methicillin resistant *S. aureus* (MRSA) and *B. anthracis*. Identification of antagonism towards the assayed species offers the potential of possible inhibitory compounds against related infectious bacteria.

2. Materials and methods

2.1 Detection of antimicrobial activity

2.1.1 *Deferred procedure*

i) This was done using a modification of the deferred procedure. The strains were replica plated onto Luria agar plates and incubated for less than two days.

ii) 500-1000 µl of sterile distilled water was added to an Eppendorf tube along with 5 µl of a stationary phase indicator culture. This was added onto the plates and spread by gently tilting the plates. The flooded plates were then placed in a 42^{o}C incubator till the water evaporated. This was incubated at 37^{o}C and monitored after 11 h, checking for zones of inhibition.

2.2 Characteristics of antimicrobial compounds

The deferred procedure (i) was followed as above. Following the appropriate treatment, the second half of the procedure (ii) was conducted. Unless, otherwise stated all tests were done on pH 7.0 ½ LA plates (these plates are made as conventional LA is made, however the agar percentage is halved to 0.75%).

2.2.1 *Heat tolerance*

The plates were secured with parafilm and incubated over a 90^{o}C water bath for 1 h.

2.2.2 *Ultraviolet tolerance (UV)*

The plates were exposed to long wavelength UV for 1 h.

2.2.3 *pH range of activity*

Strains were replica plated onto LA plates in which the pH was adjusted to 6, 7 and 9 using either HCl or NaOH.

2.2.4 *Detection of resistance/ sensitivity to trypsin*

Plates were flooded with trypsin (200 µg/ml) and left overnight to absorb into the media.

2.2.5 *Activity on glucose supplemented media*

Strains were replica plated onto LA plates supplemented with glucose (0.1%).

2.2.6 *Activity on phosphate supplemented media*

Strains were replica plated onto phosphate supplemented LA plates.

2.2.7 Live cell and cell extract assay

Plates were first prepared by flooding with 500 µl water containing 2.5 µl of indicator strain. This was gently spread with a glass pipette till the entire plate was covered. Once dry, the broad side of a sterile Pasteur pipette was used to puncture holes into the agar.

2.2.7.1 *Live cell assay*

Test strains were grown to stationary phase in LB broth. Between 60-80 µl of the strain in liquid broth was added into the well of the prepared plate. This was incubated in the cold room for 4 h to allow for diffusion and incubated at 37^{o}C overnight.

2.2.7.2 *Cell extract assay*

Test strains were grown to stationary phase in LB broth. The cells were washed once in TE buffer and resuspended in the same buffer. The culture was sonicated (20 kHz; 1 min.) three times. The cofactor NADH (10 µg/ml) was added and 60-80 µl of this

added to the well of the prepared plate. This was incubated in the cold room for 4 h to allow for diffusion and incubated at 37^{o}C overnight.

2.3 Range of antimicrobial activity

The deferred procedure was followed, however several additional indicator strains were tested. These include: *R. erythropolis, M. smegmatis, M. luteus, G. rubripertincta, Thiobacillus* spp., *Agrobacterium* spp., *S. marcescens, E. cloacae, E. coli, P. aeruginosa, S. lactis, Lactobacillus* spp., *Arthrobacter* spp. and *S. cerevisiae*.

3. Results and discussion

The widely used deferred method was adopted for the detection of antagonistic activity [Fig. 3.1]. The indicator strains used included *E. coli* and *B. subtilis,* both of which have short doubling times and the slower growing R. erythropolis strain. Due to the high likelihood that the slower growing actinomycete strains would be outcompeted by the indicator strains the simultaneous method was not an option (section 1.3 page 230).

Fig. 3.1: Zones of inhibition surrounding antimicrobial producing strains using *R. erythropolis* SQ1 as the indicator strain (left). Depiction of strain numbering: 1: *T. tyrosinosolvens* YeoE, 2: strain Bot2, 3: strain FHome, 4: *S. prasinus* Berlin, 5: strain Hak, 6: *S. tendae* BA1, 7: *S. prasinus* HZWS, 8: *A. orientalis* SY6, 9: *S. flavogriseus* Chiba, 10: *S. griseus* Yeo, 11; *S. lividans* TK23, 12: strain WitsP, 13: strain SY3, 14: strain Cal, 15: strain HY, 16: *Streptomyces pseudogriseolus* Reu, 17: strain H3, 18: strain SY5, 19: strain WITS, 20: strain BotY

Thirty-five strains tested were members of the order Actinomycetales. Other bacteria included *S. aureus, S. marcescens, M. luteus, M. flavus, Agrobacterium* spp. and *B. subtilis*. Just two Gram negative isolates were tested, namely *M. fulvum* and *P. aeruginosa*. Of these strains, three possessed a remarkably potent antimicrobial activity against all three indicator strains. These isolates were identified as *S. prasinus* Berlin [Fig. 3.1 no. 4], *S. prasinus* HZWS [Fig. 3.1 no. 7] and *A. orientalis* SY6 [Fig. 3.1 no. 8]. [Please see table 6.1 page 294 for details of which specific strains showed activity against the indicator strains]. Strains Berlin and HZWS were reported to be the same species and reacted similarly although not identically when conducting the

basic characterization experiments. Thus, I propose that these isolates could be different strains of the same species. Notably, strain HZWS was isolated from soil in Johannesburg while strain Berlin was isolated from soil in Germany. Other strains showed moderate to weak activity evident by small zones of inhibition. Of the forty-three strains tested, eleven *Streptomyces* spp. and one *Amycolatopsis* spp. displayed antimicrobial inhibitory activity.

Table 3.1: Zone of inhibition against tested strains conducted on pH 7.0 media

Strain	**Zone radius (mm) against *S. aureus***	**Zone radius (mm) against *B. subtilis***	**Zone radius (mm) against *R. erythropolis***
Berlin	12±2	4±1	13±1
Pasa	1±0.5	5±2	<0.5
Bot1	6±1	1±0.5	10±1
WitsP	-	5±2	-
SY3	1.5±0.5	6±1	1.5±0.5
SY5	1±0.5	4±1	1±0.5
SY6	11±1	5±1	7±1
Bedd	-	2±1	-
H3	2±1	7±2	1±0.5
BotY	-	2±1	-
HZWS	8±2	5±1	10±1
HZBS	1±0.5	3±1	-

From the above table it was evident that the sensitivities of the indicator strains differed in response to the antibacterial compound [Table 3.1]. Additionally, within the inhibitory zones it was not uncommon to find a small number of bacteria that were resistant.

With preliminary antibacterial plating experiments, zones of inhibition can be attributed to factors other than bacteriocin or antibiotic production. In order to rule out bacteriophage production, the UV tolerance and heat stability of the antibacterial substance were tested. In general it was found that the zones of inhibition were not significantly affected by either of these treatments to which phages would be susceptible (Tagg *et al.*, 1976). Observations showed that most compounds were UV resistant while seven strains produced heat-labile compounds [Table 3.2]. The majority of bacteriocins are heat stable, examples being Jenseniin G (100^{o}C for 15 min.), and better known Staphylococcin 1262a (100^{o}C ; 60 min.) and Streptococcin FF22 (100^{o}C; 60 min.) (Grinstead and Barefoot, 1992; Tagg *et al.*, 1976). Notably, with the UV treatment the zones were clear and

exceptionally distinct compared to non-treated plates, however since the radius was not significantly enhanced, I do not believe UV stimulated furtherinduction of thecompound, however can not rule out that the induction of prophage caused lysis of the surrounding cells hence the clearer zones [Table 3.2].

Table 3.2: Characteristics of antimicrobial compounds

	Heat tolerance		**UV tolerance**		**pH range of activity**		**Sensitivity to trypsin (200µg/ml)**		**Activity on glucose supplemented media (0.1%)**		**Activity on phosphate supplemented media**	
Strain	64-1	IA3	64-1	IA3	64-1	IA3	64-1	IA3	64-1	IA3	64-1	IA3
Berlin	+	+	+	+	6-9	7-9	±	±	-	-	+	+
Pasa	-	-	-	+	7	7-9	-	-	-	-	+	+
Bot1	+	+	+	+	6-9	6-9	+	+	-	+	+	+
WitsP	N	+	N	+	N	7	N	+	N	+	N	-
SY3	-	+	+	+	7-9	6-7	+	-	-	+	+	+
SY5	-	-	+	+	7-9	7-9	+	+	-	+	-	+
SY6	+	+	nd	+	nd	6-7	nd	±	nd	+	nd	±
Bedd	N	-	N	-	N	7-9	N	-	N	-	N	±
H3	-	+	+	+	6-7	6-9	+	+	-	+	+	±
BotY	N	-	N	+	N	7-9	N	-	N	+	N	-
HZWS	+	+	-	+	6-9	6-9	+	-	-	-	+	+
HZBS	-	-	-	+	7-9	7-9	+	+	-	-	-	+

The non-treated control showed an inhibitory zone in all cases
+ - inhibitory zone present
- - no inhibitory zone present
± - weak zone
* - strain did not grow at pH 6.0
N – no antimicrobial activity against strain
nd – not determined
64-1 – *S, aureus*
IA3 – *B. subtilis*

When conducting live cell assays I observed no antimicrobial activity in liquid broth. There was one exception however, strain HZWS was the only isolate which showed a greatly reduced activity in liquid media. This is apparently in accordance with results observed in several other studies (Tagg *et al.*, 1976). It was found that an increase in viscosity of the media through the addition of agar (0.1 %), glycerol or starch (0.5 %) led to increased production of antimicrobials (Kelstrup and Gibbons, 1969). Within Gram positives antimicrobial activity can normally be detected both inside and outside the cell. Through cell extract assays we were unable to detect any intracellular activity. However, it can not be discounted that the use of sonication to break open the cells did not adversely affect the compound.

Glucose was found to decrease the production of streptococcin B-74628 (Tagg and Wannamaker, 1978). Similarly, we checked whether glucose or phosphate supplementation would have an effect on inhibitory activity. Only strains Berlin and HZWS were completely inhibited in the presence of glucose, the other ten strains showed slight reductions (1-3 mm) in the presence of glucose. Strains Bot1 and Berlin reacted positively to the addition of phosphate with Berlin exhibiting a two times increase in zone radius, although the reason for this is not known. Other isolates showed either minor reductions in zones of inhibition or were not affected at all.

pH is also a major factor known to affect antimicrobial production (Tagg *et al.*, 1976). In general I found pH 7.0 was the optimal activity while pH 6.0 and pH 9.0 led to slight reductions in inhibition activity. In general, the isolates showed more tolerance for acidic media. Jack and colleagues (1995) reported that many bacteriocins are cationic at pH 7.0 exhibiting the highest activity at pH 6.0 or above.

It is well known that *S. lividans* produces several antibiotics, namely actinorhodin, γ-actinorhodin, undecylprodigiosin and three related prodiginines and calcium-dependent antibiotic, however it was not found to inhibit any strains. This is explained by the nutritional composition of the media since it is known that these antibiotics are only released under specific nutrient conditions (Kieser *et al.*, 2000).

Table 3.3: Range of antimicrobial activity against various strains

Strain	*R. erythropolis* 1069	*M. smegmatis* mc^2 155	*M. luteus*	*G. rubropertincta* 25593	***Thiobacillus* spp.**	*Agrobacterium* pE 1913 spp.	*S. marcescens*	***E. cloacae***	***E. coli* MM294-4**	***P. aeruginosa***	*S. lactis*	*Lactobacillus* spp.	*Arthrobacter* spp.	*S. cerevisae* Ray 3A-D
Berlin	+	-	+	-	+	+	-	-	-	-	-	-	+	-
Pasa	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Bot1	-	-	+	-	+	+	-	-	-	-	+	-	+	-
WitsP	+	-	-	+	-	-	-	-	-	-	-	-	-	-
SY3	+	-	+	-	+	-	-	-	-	-	-	-	+	-
SY5	+	-	+	+	-	-	-	-	-	-	-	-	+	-
SY6	+	-	+	+	-	-	-	-	-	-	-	-	+	-
Bedd	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	+	+	+	-	-	-	-	-	-	-	+	-
BotY	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HZWS	+	-	+	+	+	+	-	-	-	-	-	-	+	-
HZBS	-	-	+	-	+	-	-	-	-	-	-	-	+	-

Gram negative bacteria represented in bold print

With the exception of *Thiobacillus* spp. none of the tested strains showed activity against the other Gram negative bacteria. It has been reported that in general Gram negative bacteria show little resistance to Gram positive bacteriocins and is believed to be related to the exterior membrane associated with this group. Exceptions do exist however, such as the viridins from *Streptococcus* spp. (Dajani *et al.*, 1976). From the literature Gram positive bacteria demonstrate inhibitory activity mainly against other Gram positive bacteria particularly *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp., *Clostridia* spp. and *Mycobacterium* spp. (Jack *et al*., 1995). Media composition may also play a role in the response of the indicator strain to the inhibitory compound. For instance, in *S. mutans* the addition of sucrose to the media induces the formation of an extracellular polysaccharide making it resistant (Tagg *et al.*, 1976). Strain HZWS displayed the widest spectrum of inhibitory activity against six of fourteen strains. Noticeably, the Actinomycetales (*Micrococcus* spp., *Gordonia* spp., *Rhodococcus* spp. and *Arthrobacter* spp.) most closely related to the antagonistic strains were most susceptible. An exception was *Mycobacterium* spp., however it is possible that the atypical composition of the bacterial cell wall is the reason for this. Unlike other bacteria, this genus possesses a cell wall with an extremely low permeability due to an unusually high lipid content with low fluidity (Jarlier and Nikaido, 1994). Also, no inhibition against *S. cerevisiae* was observed. Arndt and coauthors (1999) reported on the inhibition of *S. cerevisiae* by *Streptomyces* spp. by- products, FK506/FK520 and rapamycin. Otherwise little documentation exists on this occurrence. It seems that several strains produce more than one antimicrobial compound, evident by differences in heat and UV tolerance in addition to pH activity.

This study aimed to detect bacteriocins or antibiotic production from strains isolated from soil. However, the primary stages of this research were concerned more with bacteriocins from *Streptomyces* spp. opposed to antibiotics. The identification of bacteriocin-like compounds is rare amongst this genera and this was done with the realization that difficulty would be met on attempting to distinguish between bacteriocin and antibiotic activity. The advantages of bacteriocins over antibiotics include that antibiotic synthesis involves complex pathways controlled by multiple enzyme complexes which are not easily cloned (Okanishi *et al.*, 1983). On the other hand since bacteriocins are mainly plasmid encoded they can be easily transferred to

other bacterial strains, facilitating genetic engineering. Additionally, their narrow host range of activity makes them safer for use in humans.

The only strains with potential bacteriocin-like activity are from strains Pasa and Bedd since they reacted negatively to treatment with trypsin and displayed very restrictive host range inhibitions. Still this is based strictly on two criteria, thus I cannot say with confidence that these are bacteriocin in nature. Antibiotics containing peptide moieties do exist and can also be inhibited by trypsin. Nevertheless, the activity displayed by these strains was disappointingly weak (zones in the range of < 0.5-5 mm) and thus did not demand further attention.

The potent antibacterial inhibitor strains Berlin, SY6 and HZWS are more likely to be antibiotic related, due their tolerance of trypsin. With the high likelihood of these being antibiotic in nature it is unlikely that these are unknown antibiotics and thus further characterization was abandoned. However, knowing which species these isolates are most closely related to I referred to the literature in an attempt to identify the inhibitory compounds. From the literature it was determined that *S. prasinus* produces a phosphorus containing antibiotic, prasinomycin which inhibits cell wall synthesis and *A. orientalis* produces two glycopeptide antibiotics, namely vancomycin and chloroeremomycin.

Experimentation done by Meyers and workers (1969) on the antimicrobial spectrum of prasinomycin revealed an inhibition of both Gram negatives and Gram positives. A closer look at the inhibitory concentrations showed that the Gram negatives were inhibited at relatively high concentrations while Gram positives were susceptible to low concentrations of the antibiotic. For instance, *S. aureus* and *B. subtilis* were inhibited by 0.15 and 0.06 µg/ml respectively while *E. coli* and *P. aeruginosa* were susceptible to 37.5 and 50 µg/ml respectively. The yeast *C. albicans* was resistant up to a concentration of 100 µg/ml. It can be assumed that in this study a low concentration of the antibiotic is secreted into the surrounding region which prevented us from witnessing Gram negative and yeast inhibition. It was noted earlier that a few resistant cells appeared within the inhibition zone. Meyers and coworkers (1969) established that the resistance by *S. aureus* to this antibiotic developed after just five

subcultures with increased exposure to prasinomycin. Additionally, the resistance was stably maintained over twenty subcultures under no antibiotic exposure.

Paradoxically, vancomycin, discovered by Eli Lilly in 1956 and approved by the FDA two years later was the antibiotic of choice used to treat *S. aureus* infections (Nicolaou *et al.*, 1999). *S. aureus* is resistant to erythromycin, penicillin and tetracycline and in 1997 independent cases of vancomycin resistance started to emerge (Nicolaou *et al.*,1999). MRSA inherited its resistance due to a 21-67 kb island, which evolved giving rise to multiple antibiotic resistance (Walsh, 2003). The vancomycin resistant *S. aureus* strain first identified in Japan possesses a thicker than usual cell wall and provides binding sites in excess for the drug (Walsh, 2003). Vancomycin was the last line of treatment for *S. aureus* infections and currently no adequate treatment exists for these antibiotic resistant strains (Walsh, 2003). Chloroeremomycin is a vancomycin type antibiotic and differs to vancomycin only with respect to it glycosylation pattern. Fig. 3.2 below shows the similarity between the two antibiotics.

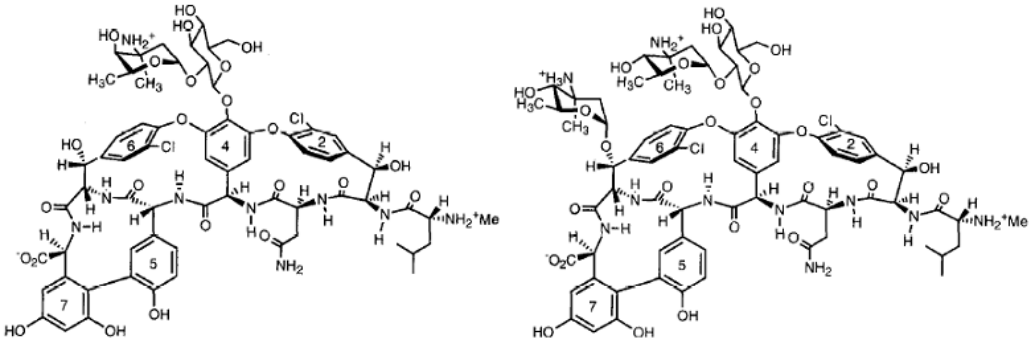

Fig. 3.2: Structures of (left) vancomycin and (right) chloroeremomycin (van Wageningen *et al.*, 1998).

Both antibiotics have a heptapeptide backbone which clarifies the reason for weak inhibitory zones in the presence of proteolytic treatment. Otherwise, it is documented

as possessing a potent ability against *Staphylococcus* spp. and several other Gram positive bacteria (Nicolaou *et al.*, 1999).

3.1 Concluding remarks

Unfortunately, the strongest bacterial inhibitors produced antibiotics which have previously been identified. It was found that 43 % of the soil isolates tested possessed antimicrobial activity. Of the fifteen strains selected from the Genetics culture collection, none showed inhibitory activity. Two potentially weak bacteriocin-like producers were isolated.

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METHOD DEVELOPMENT

Construction of broad host range positive selection vectors

Chapter VI abstract

Vector pMDC3 was constructed by joining the suicide vector pEcoR251 to *Nocardia-E. coli* shuttle vector pNV18. This vector contains a unique *Hind*III site for cloning. It also possesses a kanamycin antibiotic resistance gene for selection in both Gram negative and Gram positive bacteria. Similarly, pCCC2 was constructed by joining pEcoR251 to shuttle vector pOLYG. It holds two unique restriction sites, specifically *Hind*III and *Pst*I within the suicide gene and a *Cla*I site outside the gene. pMDC3 could not be re-extracted from its Gram positive host and was considered unstable. In contrast, pCCC2 was maintained as a stable construct in both *R. erythropolis* SQ1 and *E. coli*. Additionally, once re-extracted from *R. erythropolis* the vector maintained its suicide function. The plasmid host range extended to three species, namely *Rhodococcus* spp., *Mycobacterium* spp. and *Gordonia* spp.

1. Introduction

1.1 Nocardioform actinomycetes

Nocardioform actinomycetes possess a broad metabolic diversity. They are capable of the biosynthesis of an array of substrates and decomposition and utilization of harmful compounds. Their capacity to degrade recalcitrant compounds can be demonstrated by numerous studies. *R. rhodochrous* strains were implicated in the degradation of crude oil, herbicides and pesticides (Harada *et al.*, 2006; Sorkhoh *et al.*, 1989). Mycobacterial isolates were capable of breaking down polycyclic aromatic hydrocarbons and morpholine, an industrial pollutant (Miller C.D. *et al.*, 2004; Poupin P. *et al.*, 1998). A *Nocardia* spp. strain proficient in the mineralization of tyre tread was also isolated (Tsuchii and Tokiwa, 1999).

In view of the benefits offered by this group of prokaryotes it is useful to construct suitable vectors for the investigation of these valuable traits. Cloning vectors have undoubtedly played a tremendous role in the understanding of prokaryotic genetics. To be brief, they make possible the determination of gene function and the expression of gene products while contributing valuable information pertaining to metabolic pathways.

1.2 Reasons for choosing pAL5000 based cloning vectors

The *Mycobacterium* spp. cryptic plasmid, pAL5000 extracted from *M. fortuitum* is one of the most commonly used replicons in Mycobacterial derived vectors. The regions within this plasmid have been studied at length and the roles and interactions of the genes are well understood.

pAL5000 replication genes share homology with the replicons of many other microbial species, suggesting an extended host range beyond *Mycobacteria*. Homologues of pAL5000 rep genes have thus far been found in *B. longum* (pMB1*)*, *R. erythropolis* (pFAJ2600), *B. linens* (pBLA8), *N. gonorrheae* (pJD1) and *C.*

glutamicum (pXZ10142) (Hatfull and Jacobs, 2000). Furthermore, many pAL5000 based vectors exist, though few positive selection cloning vectors are available.

1.3 Features of pAL5000

pAL5000 comprises 4837 bp with a GC content of 65%. It carries 5 open reading frames (ORF's), namely *repA (orf1), repB (orf2), orf3, orf4* and *rap (orf5).* Both *repA* and *repB* genes are required for plasmid replication while rap is believed to play a role in stability. The *rep* genes overlap by a single base pair and are transcribed as one RNA molecule.

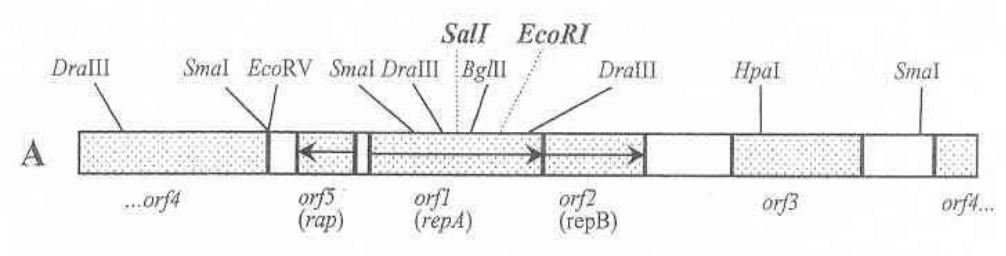


Fig. 1.1: Schematic diagram of pAL5000 (Hatfull and Jacobs, 2000)

pAL5000 derived vectors are commonly used to transform Mycobacterial strains. These low copy number plasmid derivatives (≤ 5 copies per cell) have been transformed into *M. fortuitum, M. bovis* BCG, *M. aurum, M. tuberculosis* and *Mycobacteria* w (Hatfull and Jacobs, 2000).

1.4 Features of the main vectors used in the study

The basis of pMDC3 was vectors pEcoR251 and pNV18, while pCCC2 is based on pOLYG and pEcoR251. pNV18 is a pAL5000 based shuttle vector consisting of the *repA* and *repB* Mycobacterial replicon, pMB1 *E.coli* replicon, kanamycin selective antibiotic resistance gene and multiple cloning site. Likewise, pOLYG carries the same mycobacterial and *E.coli* replicon and a hygromycin antibiotic selectable marker. pEcoR251 contains the *Eco*RI *endonuclease* suicide gene, pMB1 replicon and ampicillin selectable marker.

1.5 Advantage of positive selection cloning vectors

pMDC3 and pCCC2 both made use of the *Eco*RI endonuclease suicide gene. Other positive selection systems also exist such as the *sacB* gene and *rpsL* genes. Vector DNA is commonly treated with phosphatase to reduce the number of transformants carrying religated or uncut vector. While this does work, it is less effective than the use of positive selection vectors. Consequently, its use reduces the number of clones required, making it convenient particularly in the application of library construction. It has the added advantage of preventing background growth. The basic mechanism of positive selection is illustrated in Fig. 1.2.

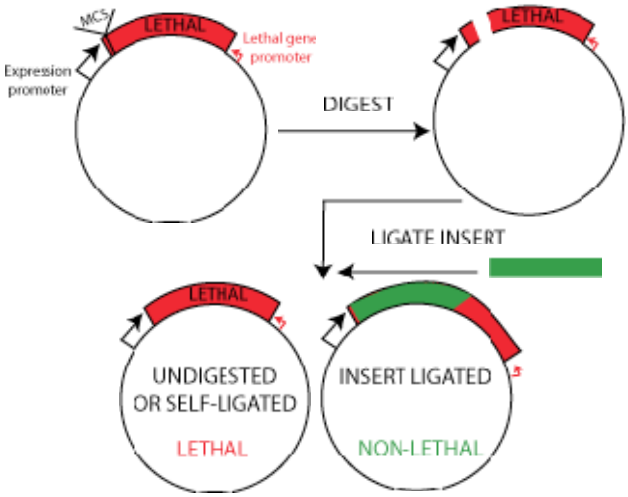


Fig. 1.2: Simplified illustration of the functioning of a suicide gene (BitesizeBio, 2006)

From the above diagram it is clear that the eradication of the suicide function relies on insertional inactivation, strictly selecting for recombinant vectors.

1.6 Objectives

i. Host range determination of pNV18 and pOLYG

ii. Manipulation of pNV18 and pOLYG

a) Maintenance of unique restriction site

b) Introduction of the suicide gene into pNV18 and pOLYG

iii. Assessing structural stability of vectors

iv. Establishing the maintenance of the vector suicide function

2. Materials and methods

2.1 *Electroporation*

Cultures were grown in 5 ml LBSG (with the appropriate glycine concentration) overnight. These were transferred to Eppendorf tubes and the cells pelleted (13 000 rpm; 2 min.) at 4^{o}C. The cells were washed three times in sterile distilled water and resuspended in 1 ml cold sterile distilled water. 100 µl of the culture was transferred into an Eppendorf tube with 1-10 µl of DNA. This was mixed by bubbling air through the mixture and transferred into a prechilled sterile electroporation cuvette. The electroporation parameters were set as follows: capacitance 25 uF, voltage 2.5 kV and resistance 400 Ω. The cuvette was electroporated and the time constant recorded after which LB was immediately added. A no DNA control was included. The cells were incubated on a shaker at 30^{o}C for 2-5 h. Following the incubation the cells were added onto the appropriate antibiotic plates and gently spread. This was incubated at 30^{o}C till growth was seen.

3. Results

3.1 Removal of *Hind*III sites on pNV18 and pOLYG

The *Hind*III site present on pNV18 and pOLYG were filled in using the DNA polymerase I large Klenow fragment, effectively removing the site [Fig. 3.1]. This enzyme possesses a 5'-3' polymerase and 3'-5' exonuclease activity, allowing it to synthesize DNA complementary to the DNA template. Since a *Hind*III site is present on vector pEcoR251, this allowed unique cloning sites to be maintained once the suicide gene was introduced. These vectors were then used for all subsequent manipulations. Also the modification of this sequence did not introduce any unfavorable restriction sites [Table 3.1]. Klenow treatment of both *Bgl*II sites was also attempted, however since one site was present in the *repA* gene, this inactivated the Mycobacterial replicon preventing its replication in any gram positive host.

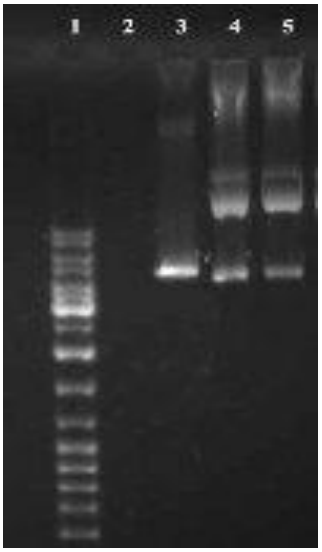


Fig. 3.1: Digestion of pNV18. Lanes **1**: DNA marker, **3**: negative control – pNV18 linearized with *Bam*HI, **4**: positive control – uncut pNV18 and **5**: pNV18 digested with *Hind*III.

Table 3.1: Modified DNA sequence following Klenow treatment

Restriction sites	**Recognition sequence**	**Newly generated sequence**	**Restriction sites introduced**
*Hind*III	A↓AGCTT	AAGCTAGCTT	*Alu*I, *Bmt*I, *Cac*81, *Cvi*JI, *Fsp*BI, *Nhe*I

3.2 Vector host range

3.2.1 *Host range of various vectors*

The host range of vectors pNV18, pNV19, pOLYG, pCY104 and pK4 were tested in the bacterial species *Rhodococcus*, *Mycobacteria* and *Gordonia* utilizing electroporation [Table 3.2]. The time constant of each transformation was recorded [Table 3.3]. This procedure relies on the exposure of cells to an electric field, causing the pores on the cell membrane to open, allowing extracellular complexes to enter (MacNeil, 1987). Transformation was confirmed by re-extracting the vector and transforming into *E. coli*. Afterwards, the vector was digested (to linearize the plasmid) and examined on an agarose gel. The detection of a band of similar size to the original vector in relation to the molecular weight marker was used to verify the presence of the vector.

Table 3.2: Host range of selected vectors

Organism	**Strain**	**pNV18**	**pNV19**	**pCY104**	**pK4**	**pOLYG**
Rhodococcus erythropolis	SQ1	+	+	+	+	+
Rhodococcus erythropolis	ATCC 4277	+	+	+	+	+
Rhodococcus erythropolis	DSM 1069	+	+	+	+	+
Rhodococcus rhodochrous	RI8	+	+	+	+	+

Organism	Strain	pNV18	pNV19	pCY104	pK4	pOLYG
Rhodococcus opacus	HL-PA1	+	+	+	+	+
Mycobacterium smegmatis	mc^2 155	+	+	+	+	+
Mycobacterium parafortuitum	490	+	+	-	-	+
Gordonia rubropertincta	ATCC 25593	+	+	+	+	+
Gordonia spp.	NB4	+	+	-	-	+
Gordonia spp.	NB13	+	+	-	-	+
Gordonia australis	A554	+	+	-	-	+

- no transformants on plate

3.2.2 *Transformation efficiency*

The transformation efficiency of members of the species *Rhodococcus*, *Mycobacteria* and *Gordonia* with vectors pNV18, pNV19, pOLYG, pCY104 and pK4 was determined [Table 3.4].

Table 3.3: Number of transformants/µg vector DNA

	pNV18	pNV19	pCY104	pK4	pOLYG
Rhodococcus erythropolis SQ1	4.0×10^5	3.8×10^5	4.9×10^5	2.5×10^3	2.8×10^6
Rhodococcus erythropolis 1069	3.4×10^4	3.9×10^4	2.4×10^5	8.4×10^5	3.5×10^4
Gordonia rubropertincta ATCC 25593	4.8×10^4	4.9×10^4	1.8×10^2	1.6×10^1	4.5×10^3
Mycobacterium smegmatis mc^2 155	6.8×10^3	7.6×10^3	5.1×10^3	4.3×10^3	2.1×10^4

The resultant broad host range and high transformation efficiencies of the pNV vectors and pOLYG led to their selection for further manipulation experiments.

Hence, the suicide gene originating from pEcoR251 was introduced into both of these vectors [Fig. 3.2-3.5].

3.3 Construction of pMDC vectors

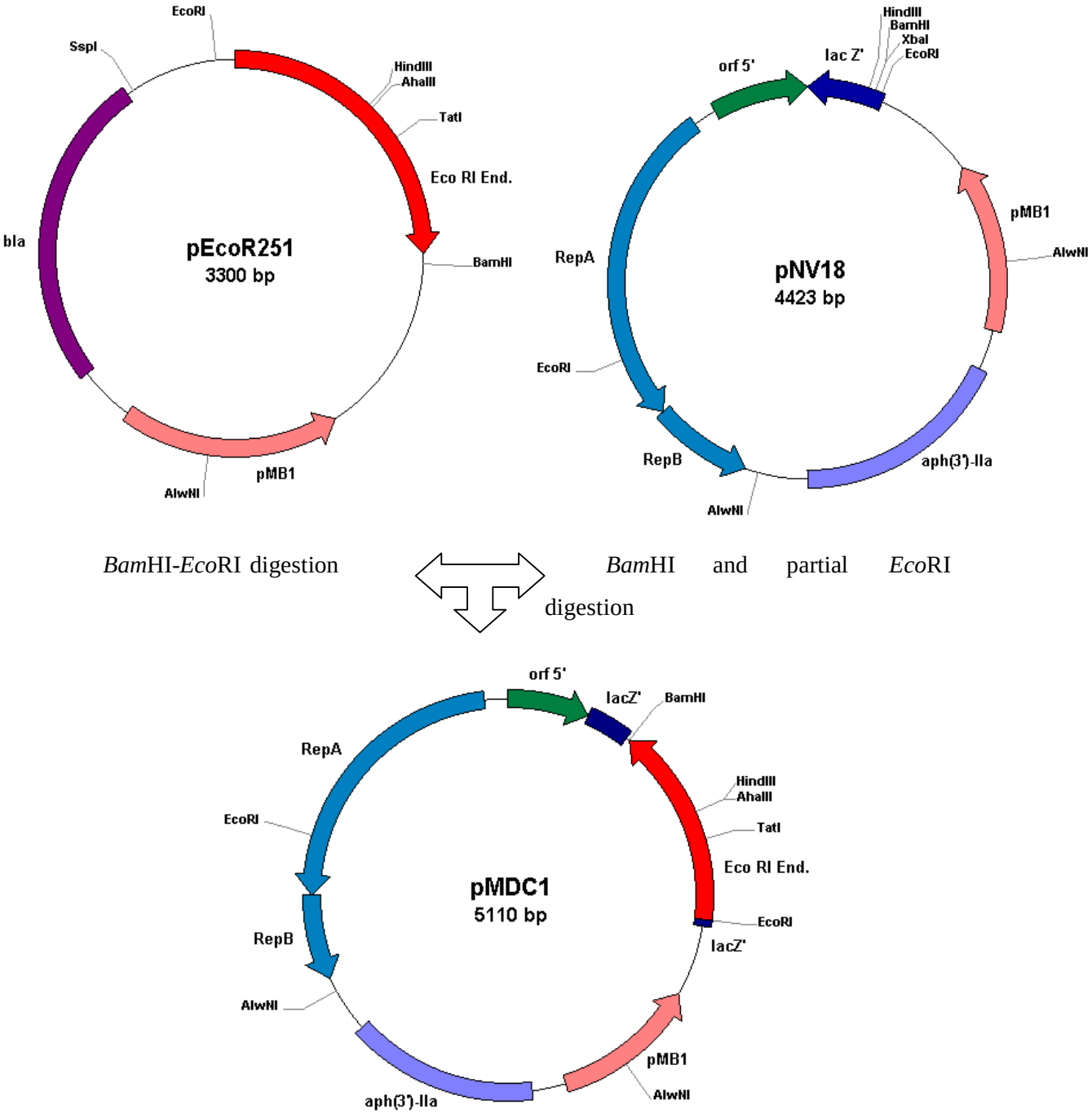
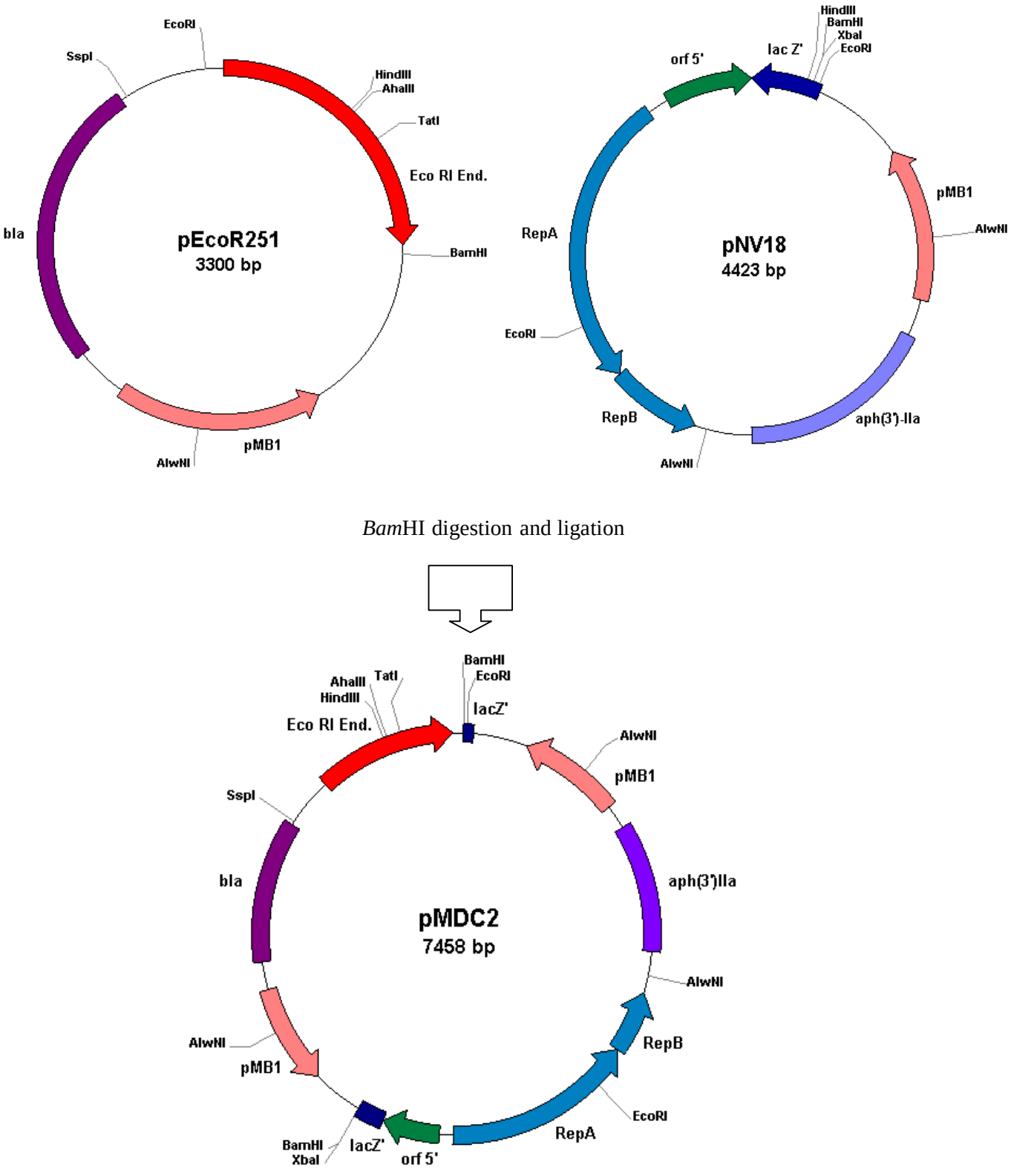


Fig. 3.2: Construction of vector pMDC1

Only relevant restriction sites are shown for all illustrated vectors. pNV18 was partially digested at the *Eco*RI site and completely digested at *Bam*HI. pEcoR251 was

restricted with *Eco*RI and *Bam*HI. Vector pMDC1 was formed following the ligation of the *Bam*HI-*Eco*RI fragment encoding the suicide gene (*Eco RI end*.) to the digested vector pNV18. The resulting vector yielded no transformants, thus a second approach was adopted. It should be noted that the suicide gene in pMDC1 is in the opposite orientation compared to pEcoR251.



*Bam*HI digestion and ligation

Fig. 3.3: Construction of vector pMDC2

pMDC2 was constructed by ligating pNV18 and pEcoR251 together once restricted at their unique *Bam*HI sites. Like pMDC1, the resulting construct did not yield transformants. Once again a different strategy was attempted.

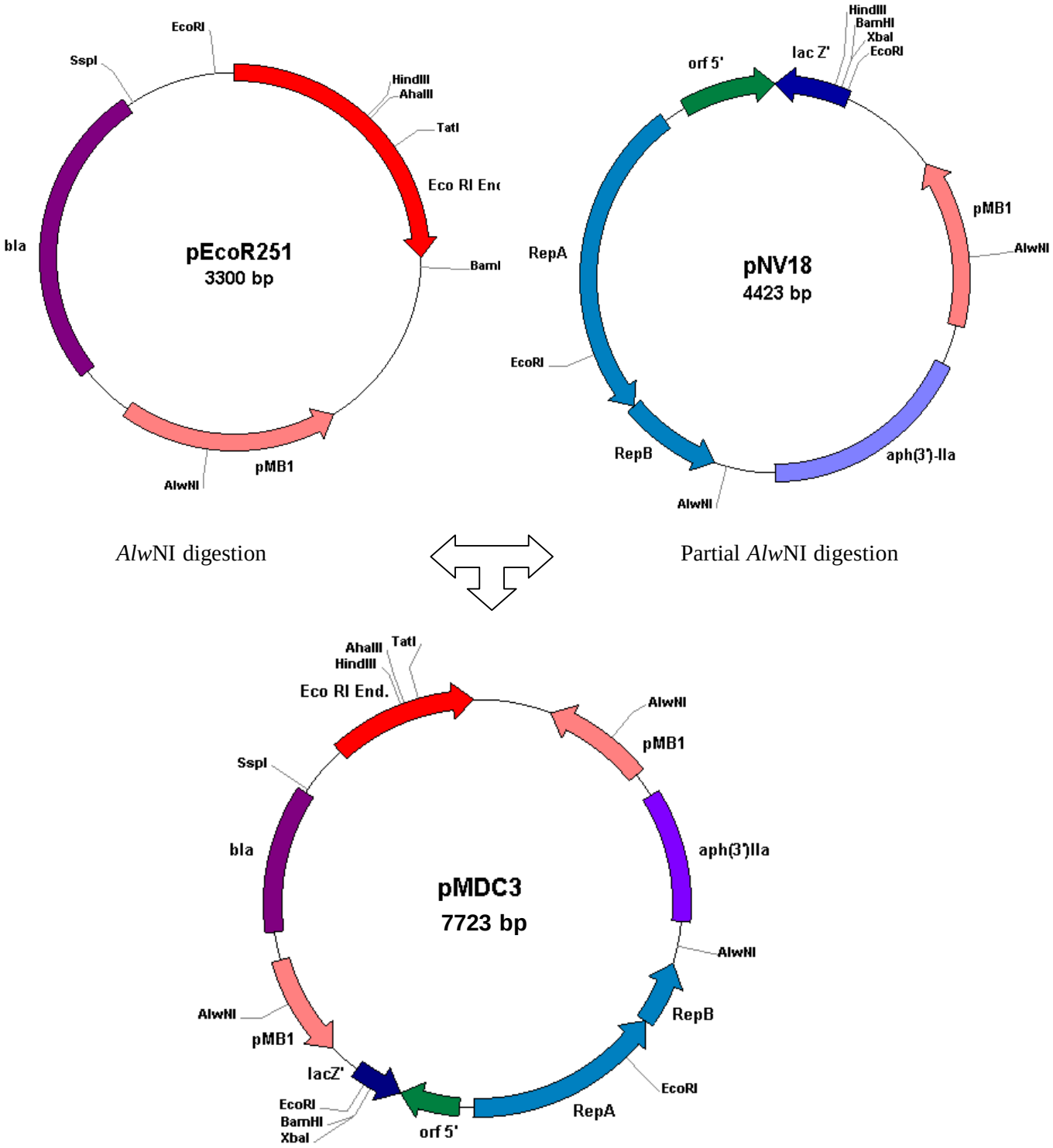


Fig. 3.4: Construction of vector pMDC3

pNV18 was subject to partial digestion with *Alw*NI and ligated to pEcoR251 restricted at the single *Alw*NI site to create pMDC3. Only one orientation was obtained. This vector was effectively transformed. An attempt was made to remove the additional pMB1 replicon and ampicillin resistance gene (*bla* determinant). pMDC3 was double digested with *Ssp*I, leading to a blunt end and *Xba*I which led to a sticky end. This was treated with Klenow to fill in the sticky end created by *Xba*I and religated. Oddly, no transformants resulted, although four deletion mutants were detected, all of which had part of the *RepA* gene missing.

3.4 Construction of vector pCCC2

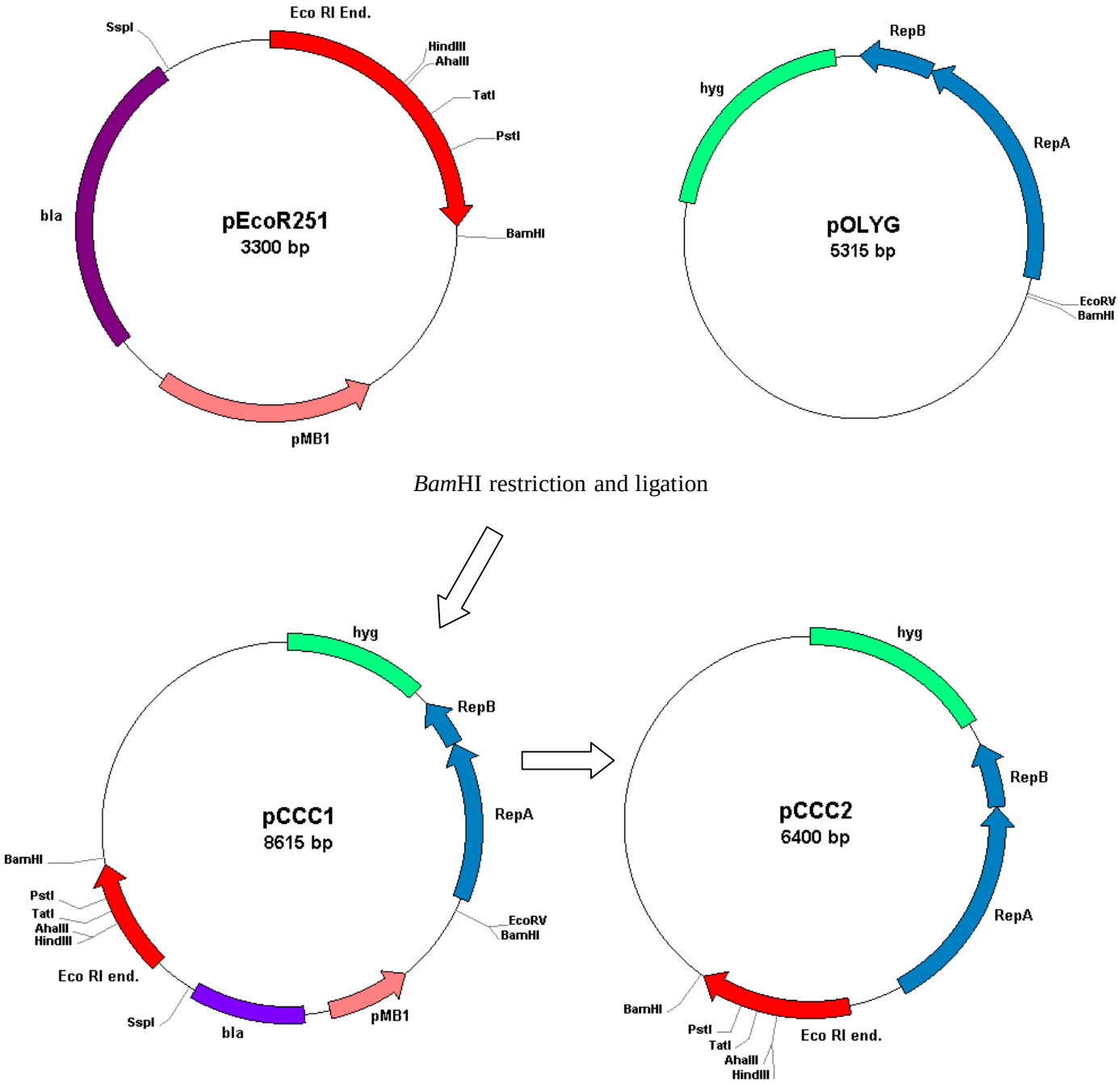


Fig. 3.5: Construction of vector pCCC2

pCCC1 was formed by the ligation of pOLYG to pEcoR251 at their respective *Bam*HI sites. Ligation of pOLYG to pEcoR251 resulted in an 8615 bp vector. To reduce the vector size, excess DNA (additional pMB1 replicon and *bla* determinant) was

removed by digesting the vector with *Ssp*I and *Eco*RV and religating the vector once again, leading to the formation of pCCC2.

Just four of the main strains were chosen to test the vector host range. Both vectors were disrupted by inserting DNA into the unique *Hind*III sites and electroporated into these strains [Table 3.5]. To test whether the suicide gene was active in the Gram positive hosts, intact vectors were also electroporated. Transformants were detected on plates in which the gene was disrupted and unexpectedly on plates in which the gene was not interrupted.

Table 3.4: Host range of vectors pMDC3 and pCCC2

Organism	**Strain**	**pMDC3**	**pCCC2**
Rhodococcus erythropolis	SQ1	+ [a]	+
Rhodococcus erythropolis	DSM 1069	+	+
Mycobacteria smegmatis	mc^2 155	+	+
Gordonia rubropertincta	ATCC 25593	+	+

[a] = vector DNA could not be re-extracted

Table 3.5: Time constants resulting from electroporation of these vectors

Organism	**Strain**	**pMDC3**	**pCCC2**	**No plasmid**
Rhodococcus erythropolis	SQ1	8.4	8.5	8.9
Rhodococcus erythropolis	DSM 1069	8.2	8.0	8.8
Mycobacterium smegmatis	mc^2 155	8.1	8.6	8.8
Gordonia rubropertincta	ATCC 25593	8.1	8.6	8.8

3.5 Vector structural stability

The structural stability of the vectors was evaluated by extracting the vectors from their Gram positive hosts, retransforming them into *E. coli* and performing digestions to establish if rearrangements or deletions had occurred. The size of bands expected was calculated from the restriction map of the expected vector. pCCC2 results coincided with the estimations made, revealing no significant rearrangement or deletions [Fig. 3.6]. Unexpectedly, pMDC3 could not be re-extracted from *R. erythropolis* SQ1. Thus no structural analysis could be done.

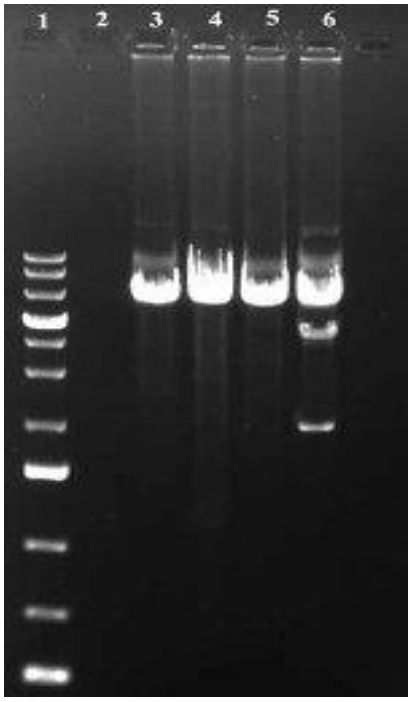


Fig. 3.6: Retransformation and re-extraction of pCCC2 from *E. coli*, digested with the following enzymes: Lanes **1**: DNA ladder molecular weight marker, **3**: *Hind*III, **4**: *Pst*I, **5**: *Bam*HI and **6**: *Bgl*II

3.6 Maintenance of suicide function

The re-extracted intact pCCC2 vector was transformed into an *E. coli* λ lysogen and non- lysogen strain to determine if the suicide gene was still functional. The presence of colonies in the lysogen strain and absence thereof in the non-lysogen strain confirmed the functionality of the endonuclease gene.

4. Discussion

The intention of this study was to develop positive selection vectors with the suicide function reliant on the *Eco*RI endonuclease gene. Additionally, broad host range vectors were required which could be easily electroporated. The latter traits were tested by electroporating into members of the species *Rhodococcus*, *Mycobacterium* and *Gordonia*.

The original developers of pNV18 and pNV19 intended these shuttle vectors for use in *Nocardia* spp., since this genus has limited applicable cloning vectors (Chiba *et al*., 2007). *Rhodococcus* and *Gordonia* species face the same limitation although the number of vectors developed for these genera is on the increase (Dabbs *et al*., 1990; Bahn *et al.,* 2005). In truth, *Mycobacterium* spp. have many useful vectors. The exploration of pathogenesis in this genus has spurred the development of numerous transformation systems. Hatfull and Jacobs (2000) listed 40 vectors developed for use in mycobacterial genetics, comprising cloning, expression and integrating plasmids.

As mentioned previously pAL5000 shares sequence similarity to replicons within unrelated bacteria. Thus, it can be presumed that this large number of vectors developed specifically for use in *Mycobacterium* spp. has the potential for applicability in other genera as well. With regard to *Gordonia* spp., recently constructed vectors based on pNC903 have been used to transform them. Vector pNC903 isolated from *R. rhodochrous* has an origin of replication similar to pAL5000 (Bahn *et al.,* 2005). Notably, it was transformable in 12 *Gordonia* spp. with efficiencies in the range of 10^2- 10^4 CFU/ /µg DNA (Arenskötter *et al.,* 2003). Similarly, the cryptic plasmid pFAJ2600 isolated from *R. erythropolis* N186/21 showed similarity to pAL5000 *RepA* and *RepB*. The vector based on this plasmid was transformed in *R. erythropolis, R. fascians, R. rhodochrous* and *R. ruber* (De Mot *et al.,* 1997).

Encouragingly both pNV vectors and pOLYG were transformable in all strains tested. Comparative studies were conducted on two other shuttle vectors, namely pCY104, a Nocardial vector and pK4 a *Rhodococcus* replicon vector. Yao and coworkers (1994) publication regarding pCY104 mentioned a transformation efficiency of 8 x 10^4

CFU/µg DNA in *N. asteroids*. In this study the vector yielded an equally high efficiency in *R. erythropolis* of ~ 4.9 x 10^5, though significantly poorer transformation in *Gordonia* and *Mycobacterium* spp. Both pK4 and pCY104 failed to transform *M. parafortuitum* and *Gordonia* spp. strains NB4 and NB13. It is possible that the replicons are either not recognized in these strains or electroporation parameters need to be adjusted to facilitate transformation. The low transformation efficiency of strains NB4 and NB13 with both pNV18 and pNV19 suggests the presence of a restriction modification system. In cases such as this the brief exposure of cells to a high temperature has proved to be effective in temporarily inactivating the restriction system (Engel, 1987).

The highest transformation of the pNV vectors and pOLYG was detected in *R. erythropolis* SQ1 at an efficiency of 4.0 x 10^5 and 2.8 x 10^6 transformants/ µg DNA respectively. This is similar to experiments reported by Chiba and colleagues (2007). These researchers reported an efficiency of pNV18 and pNV19 in *N. farcinica* IFM 10152 ranging between 2.4 x 10^5 to 1.3 x 10^6 CFU/ µg DNA. Unexpectedly, these mycobacterial replicon vectors were transformed at a lower efficiency in *M. smegmatis*, between 6.8 x 10^3 - 2.1 x 10^4 CFU/ µg DNA. This most likely is due to the transformation conditions. Common protocols of *M. smegmatis* transformation utilize different electroporation solutions and variable electroporation parameters (Pelicic *et al.*, 1997).

Studies have described that hygromycin carrying vectors have transformed mycobacterial strains which were non-transformable with kanamycin vectors. Stolt and Stoker (1996) carried out investigations on vectors pYUB12 and pUH4 which differ only with respect to their antibiotic resistance genes. pYUB12 carried a kanamycin selective marker and pUH4 a hygromycin selective marker. These authors claimed that the lower stability of pYUB12 could be attributed to the kanamycin gene placing a greater burden on the cell than the hygromycin gene. Similarly, pNV18 and pOLYG are alike with the exception of their antibiotic resistance markers and multiple cloning sites. The transformation of pOLYG yielded a higher efficiency than pNV18 in *M. smegmatis*. In this regard, there are many factors to consider in transformation.

pNV and pOLYG vector transformation into *Gordonia* spp. was low. Arenskötter *et al.* (2003) described pNC9503 and pNC9501, *E. coli – Rhodococcus* shuttle vectors which were electroporated into *G. polyisoprenivorans.* Initial transformation led to approximately 10^3 transformants/ µg DNA and 50% of these carried an identical 800 bp deletion. The transformation efficiency was improved to 4 x 10^5 CFU/ µg DNA and vector deletion prevented by applying heat shock. This was accomplished by incubation for 10 min. at 0^oC before and 6 min. at 46^oC after electroporation. This suggests, it might be possible to improve *G. rubropertincta* transformation efficiencies by inactivating the restriction system through heat shock (Arenskötter *et al.*, 2003).

Despite the efficiencies being low in *Mycobacterium* and *Gordonia* species these vectors are still sufficient for cloning purposes and can adequately be utilized for library construction. It should be noted that no optimization was attempted and thus these efficiencies can be improved upon.

In general vector DNA harvested from a Gram negative intended for use in a Gram positive leads to a reduced efficiency due to the presence of dam or dcm methylation. This can be improved upon by harvesting the DNA instead from a dam^-dcm^- strain or Gram positive related to the host strain. For example, Singer and Finnerty (1988) described pMVS301, which when harvested from a *Rhodococcus* strain led to a transformation efficiency of 1.9 x 10^5 CFU /µg DNA and lowered to 3.6 x 10^2 CFU/ µg DNA when harvested from *E. coli*. A similar situation was described by Yao and coworkers (1994) who noted a 10^2-10^3 drop in efficiency when harvesting DNA from *E. coli*.

From previously published articles the pNV vectors were transformed into several strains, namely *N. farcinica, N. asteroides, N. nova, N. cyriacigergica* and *R. equi* (Chiba *et al.*, 2007; Mangan *et al.*, 2005). From this study this host range has been extended to include *R. erythropolis* strains SQ1, 4277 and 1069, *Rhodococcus rhodochrous* RI8, *Rhodococcus opacus* Hl-PA1, *Mycobacterium smegmatis* mc^2 155, *Mycobacterium parafortuitum* 490, *Gordonia rubropertincta* 25593, *Gordonia australis* 554 and *Gordonia* spp. strains NB4 and NB13.

Apart from the endonuclease gene, two other counterselectable suicide genes exist, namely *sacB* and *rpsL*. *SacB* codes for levansucrase, which is responsible for the hydrolysis of sucrose and synthesis of levans. In the presence of sucrose its suicide function is initiated leading to an accumulation of levans and cell death (Hatfull and Jacobs., 2000). In essence this gene induces sucrose sensitivity. The gene *rpsL* works in a similar fashion. It confers dominant streptomycin sensitivity in streptomycin resistant strains (Hosted and Baltz, 1999). Mycobacterial vectors based on the use of these genes have been constructed. pPR insertional vectors carrying the *sacB* gene were used to generate a library which produced a high percentage of exchange mutants (Pelicic *et al.*, 1997). Likwise, pGOAL vectors containing *sacB* were used to create *M. tuberculosis* mutants to be screened for virulence (Parish and Stoker, 2000).

In pEcoR251 the *Eco*RI endonuclease gene is controlled by the P_R promoter. When this vector is transformed into a non-lysogen strain with an intact endonuclease gene, its expression leads to DNA digestion and resultantly the killing of the cell. To overcome this, DNA can be introduced into a unique site of the endonuclease gene, eliminating gene function and preventing cell death. In this regard only cells carrying DNA inserts will survive, revealing the practical use of selection vectors (Zabeau and Stanley, 1982; Dabbs *et al.*, 1990). Vector pDA71 is a well recognized *Rhodococcus* suicide vector based on the *Eco*RI gene. This vector and its predecessors have been used to clone genes involved in the degradation of azo dyes, rifampicin inactivation and pigment synthesis (Dabbs, 1998). From these studies it is clear that a positive selection feature is useful, offering many advantages.

pMDC and pCCC vectors based on the endonuclease gene were developed. Both constructs pMDC1 and pMDC2 were discovered to be non-viable. Host restriction was ruled out since this was not a problem with the original pNV vectors. A commonality between these two vectors was the use of the lacZ′ multiple cloning site region to insert the suicide vector. LacZ′ is the 3′ truncated region of the lacZ gene which holds the lacZ promoter operator element and codes for a portion of the peptide β-galactosidase. Presumably, in both pMDC1 and pMDC2 no transformants resulted due to overexpression of the suicide gene induced by the strong lacZ′ promoter. Despite the suicide gene occurring in the opposite orientation in pMDC1 it is believed

that it can still be expressed. In particular, the *Eco*RI endonuclease gene is in the opposite orientation in vector pDA37 and still functional (Gordhan, 1994).

Since the first two attempts to generate positive selection vectors had failed, another strategy was undertaken. In this case a restriction site outside the cloning region was used and both vectors were successfully ligated, generating pMDC3. Attempts to excise the excess DNA failed and just four deletion mutants were obtained. However, since part of the *Rep* region was missing preventing replication within a Gram positive, these clones were not characterized any further.

pMDC3 could not be re-extracted from strain SQ1. The reason for this is unknown, since this problem was not encountered with the original pNV18 vector. It is possible that the vector is unstable and thus this was caused by rapid loss of the vector or a less likely possibility would be integration into the genome. Whether this instability is limited to strain SQ1 or not still needs to be investigated. Colonies were present on plates in which the pMDC3 *Eco*RI endonuclease gene was disrupted and fewer colonies on plates in which the gene was intact. This implies that the suicide gene is not fully functional in this Gram positive host.

Conversely, no problems were encountered in the construction of pCCC2. The suicide gene was introduced and surplus DNA easily removed. In spite of the suicide gene being functional in *E. coli*, it was not effectively expressed in Gram positive bacteria. When transformed into *Rhodococcus* spp., *Mycobacterium* spp. and *Gordonia* spp., colonies carrying an intact endonuclease gene still grew. The construct was electroporated into SQ1, re-extracted and introduced into *E.coli*. Subsequent restriction analysis did not show any apparent signs of the vector having undergone either deletions or rearrangements. Furthermore, to confirm that the suicide function was not lost due to passage through the Gram positive strain it was retransformed into a lambda lysogen and non-lysogenic strain (*E. coli* MM294-4). The presence of colonies in the lysogen and absence thereof in the non-lysogen confirmed that the suicide function was still active.

Only preliminary experimental work was conducted on constructs pMDC3 and pCCC2. At the moment their applicability is limited. The unavailability of multiple

restriction sites coupled with the large size of pMDC3 makes it inconvenient. Additionally, its current instability makes it impractical to use. Both vectors are still unsuitable since the intact suicide gene is not entirely effective in Gram positives. More needs to be done to improve these vectors. PCR site directed mutagenesis could be used to introduce new useful cloning sites.

4.1 Concluding remarks

Although these vectors need to be improved upon they carry useful features in that they can be transformed using electroporation, a convenient and reproducible procedure when compared to PEG mediated protoplast transformation. Moreover, they possess a potentially wide host range. Taking other studies into account, the pNV vectors have been successfully transformed into 4 *Rhodococcus* spp., 2 *Mycobacterium* spp., 3 *Gordonia* spp., and 4 *Nocardia* spp. In addition pOLYG was transformed into 3 *Rhodococcus* spp., 2 *Mycobacteria* spp. and 3 *Gordonia* spp. The vector pCCC2 is structurally stable in Gram positive species and upon improvement holds potential as a useful cloning vector.

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Adaptation of conventional *Rhodococcus* spp. PEG-mediated transformation procedure for use with *S. lividans* and the generation of *S. lividans* mutants capable of regenerating in liquid broth

Chapter VII abstract

An *E. coli-Streptomyces* species positive selection shuttle vector was constructed and used to assess transformation efficiency in *S. lividans*. A modified *Streptomyces* species PEG-mediated transformation protocol was also developed which led to a three-fold lower efficiency than the conventional procedure commonly used. In addition a protocol was developed allowing for regeneration of Streptomycete clones in liquid broth.

1. Introduction

1.1 Actinomycetales

Actinomycetales (particularly Streptomycetes) remain renowned for the array of antimicrobials they synthesize, producing 85% of all known antibiotics. Moreover, antitumor, antiviral and antiparasitic elements have been identified in this genus (Walsh, 2003). Members of this order have always been widely used in industrial processes, however realization of their potential in bioremediation has drawn additional focus on this group of prokaryotes. Remarkable knowledge has been gained from the screening of the genomes of Streptomycetes.

1.2 Protoplast regeneration

The conventional *Streptomyces* spp. transformation protocol which is still used today was developed initially by Okanishi and workers in 1974, later modified by Bibb and colleagues in 1978 and finally optimized by Thompson and workers in 1982 (Kieser *et al.*, 2000). The procedure involves the basic steps which include the growth of the mycelia till late exponential phase, generation of protoplasts using lysozyme, the uptake of DNA in the presence of polyethylene glycol and finally its regeneration on specialized plates. Depending on the vectors and host strains used, this protocol normally results in approximately 10^6 transformants/ µg DNA (Bailey and Winstanley, 1986). This pioneering method has seen little change over the last 28 years. Moreover, it has been used as a platform for transformation into other bacterial species, particularly *Micromonospora* species (Kojic *et al.*, 1991).

1.3 Objectives

i) Development of a *Streptomyces*-*E. coli* shuttle vector

ii) Testing the transformation efficiency of *S. lividans* using an adapted *Rhodococcus* spp. procedure

iii) Development of a transformation technique allowing *S. lividans* to regenerate in liquid broth

2. Materials and methods

2.1 Development of *Streptomyces- E. coli* spp. positive selection shuttle vector

The *Streptomyces* spp. replicon vector pIJ702 was ligated to the *E. coli* replicon vector pEcoR251. pIJ702 was digested with *Bgl*II and pEcoR251 with *Bam*HI. These were ligated together, transformed into the *E. coli* lambda lysogen and screened for the presence of the joined vectors. The pEcoR251 vector was joined to pIJ702 in two orientations and subsequently named pLR591 and pLR592. Following verification of the ligated vectors, the suicide gene was disrupted through insertion of a genomic fragment and transformed into *S. lividans* TK23 for confirmation of replication of the vector as in sections 2.2-2.4.

2.2 Modified *Rhodococcus* spp. transformation protocol

2.2.1 *Preparation and storage of Streptomycete protoplasts*

10 ml of 0.5% LBSG media was added to a flask containing glass beads, inoculated with a dense mycelial suspension and grown for 36 - 40 h. The culture was aliquoted into 1 ml volumes into microfuge tubes and harvested (13 000 rpm; 30 sec.). The supernatant was discarded and the pellet washed in 1 ml B buffer. This was re-centrifuged (13 000 rpm; 30 sec.) and resuspended in B buffer containing 1 mg/ml lysozyme. This was then incubated at 37^{o}C for 30 min. with occasional inversion. One minute prior to the end of the incubation P buffer was prepared. After incubation the protoplast suspension was harvested by gently spinning in a bench top microfuge for 10 seconds, the supernatant discarded and the protoplasts washed in 1 ml B buffer. This was re-centrifuged (10 000 rpm; 30 sec.), the supernatant discarded and the pellet resuspended in 500 µl P buffer. The tubes were placed on ice in the 4^{o}C cold room for several hours. These were then stored in the -70^{o}C freezer and thawed when needed.

2.2.2 *PEG mediated transformation of Streptomyces spp. protoplasts*

0.5 g of PEG granules were sterilized under UV for 10 min. During this time, the protoplasts were rapidly thawed by placing them directly under cool water. 100 µl of the protoplast suspension was dispensed into Eppendorf tubes and the DNA added. A no DNA control was included. This was left to incubate at room temperature for 10 min. Five min. prior to the end of the incubation, P buffer was made, the PEG granules added to 1 ml of the buffer and dissolved via. vortexing. An equal volume of P-PEG buffer was added and the tube contents mixed by bubbling air through to mix the two phases. The contents of the tubes were spotted onto R2YE regeneration plates and spread using a glass pipette. The plates were incubated at 30^{o}C for 18 h and overlaid with thiostrepton (30µg/ml). This was then incubated at 30^{o}C for 3-4 days.

2.2.3 *Antibiotic overlay*

For each regeneration plate, 500 µl of 0.3 M sucrose was added to an Eppendorf tube and 132 µl of 5mg/ml thiostrepton added to this. This was suctioned to mix and spotted onto the agar surface. This was gently spread using a glass pipette and incubated.

2.3 Conventional *Streptomyces* spp. transformation procedure

Twenty-five ml of YEME was added to a baffled flask and inoculated with a dense spore suspension. This was incubated for 36-40 h at 30^{o}C. The culture was centrifuged (3000 rpm; 10 min.) and the supernatant discarded. This was resuspended in 15 ml of 10.3% sucrose and centrifuged (3000 rpm; 10 min.) and the supernatant discarded. The latter step was repeated and the mycelia resuspended in 4 ml lysozyme solution. This was incubated for 15 min. at 37^{o}C. Using a 5 ml pipette the culture was triturated three times and incubated for a further 15 min. 5 ml of P buffer was added and the suspension filtered through cotton wool. The protoplasts were harvested by centrifuging (3000 rpm; 7 min.), the supernatant decanted and 10 ml of P buffer added. This was stored at -70^{o}C for later use.

The protoplasts were thawed and 100 µl aliquoted into Eppendorf tubes. 10 µl of DNA was added and mixed by tapping. 200 µl T buffer was added and mixed by blowing bubbles through the solution. This was spread onto R2YE plates and incubated at 30^{o}C for 18 h. Each plate was overlaid as in section 2.2.3.

2.4 Generation of *Streptomyces* spp. protoplasts capable of liquid broth regeneration

2.4.1 *Liquid broth regeneration*

Protoplasts were prepared as in section 2.2.1 using mutated cells as prepared in section 2.4.3. Transformation was conducted as in section 2.2.2 with the following modifications: Following the addition of the P-PEG, the contents of the tube were added directly to 15-20 ml of liquid RM broth or liquid R2YE broth in a Petri dish and gently swirled to evenly distribute the cells. This was incubated at 30^{o}C (without agitation) for 18 h. To each liquid regeneration plate 50 µg/ml final concentration of thiostrepton was added and placed on a tilting shaker for 7 days to allow for full regeneration. The cells were washed, diluted appropriately and spread directly onto selective media.

2.4.2 *Selection of liquid regeneration mutants*

Streptomyces spp. protoplasts were prepared as in section 2.2.1 and the transformation conducted as in section 2.2.2 with a slight modification; no thiostrepton was added to the liquid broth. The cells were allowed to regenerate in non-selective broth for 7 days. These few spontaneous mutant cells capable of regenerating in liquid broth were plated onto non-selective R2YE media. Cells were streaked in order to isolate single colonies. These colonies were then used for transformation as in section 2.2.1 and 2.2.2.

2.4.3 *Curing of liquid regeneration mutants*

The cells capable of surviving on thiostrepton overlaid R2YE plates due to the uptake of the recombinant vector were then cured by the following means. These cells were

grown and protoplasts were created as in section 2.2.1 and plated onto non-selective R2YE regeneration media. This led to the generation of a lawn of cells. Thus, cells were restreaked onto non-selective regeneration media in order to isolated single colonies. These colonies were patched onto LA non-selective plates and correspondingly onto LA thiostrepton (30 µg/ml) plates. Colonies not capable of growth on the thiostrepton plates yet capable of growth on the non-selective media plates were considered cured liquid broth regenerative mutants.

2.4.5 *Addition of ions for regeneration*

Several parameters were modified and the effect on transformation efficiency analyzed. The addition of 2 times the concentration of Ca^{2+} ions, included the addition of 0.58 % $CaCl_2$ into the RM liquid broth. The addition of 2 times the concentration of Mg^{2+} ions, included the addition of 2.5 % $MgCl_2$ into the RM liquid broth. The addition of cesium ions included the addition of 5 µl of 10% CsCl into protoplasts into which the DNA had just been added.

3. Results and discussion

The procedure followed for the construction of vector pLR591was done as by Hill and colleagues (1989). These researchers isolated the joined vectors in one orientation as shown in Fig. 3.1 (left). I was able to isolate the pEcoR251 vector in the opposite orientation as well and named this pLR592 [Fig. 3.1 (right)]. I verified results obtained by Hill and coworkers (1989) by establishing that the suicide gene was functional in *E. coli* and that it had the ability to replicate in *S. lividans*. Unique restriction sites include *Bgl*II and *Hind*III with the former site giving an additional six isocaudomer possibilities. Within *E. coli* the copy number of pLR591 was estimated to be between 50 to 100 copies per cell and in *S. lividans* was calculated as 20 to 100 copies per cell (Hill *et al.*, 1989). Additionally, the vector was found to be structurally stable (Hill *et al.*, 1989). Since pLR591 had been extensively tested by Hill *et al.* (1989) I chose to use this vector for further work.

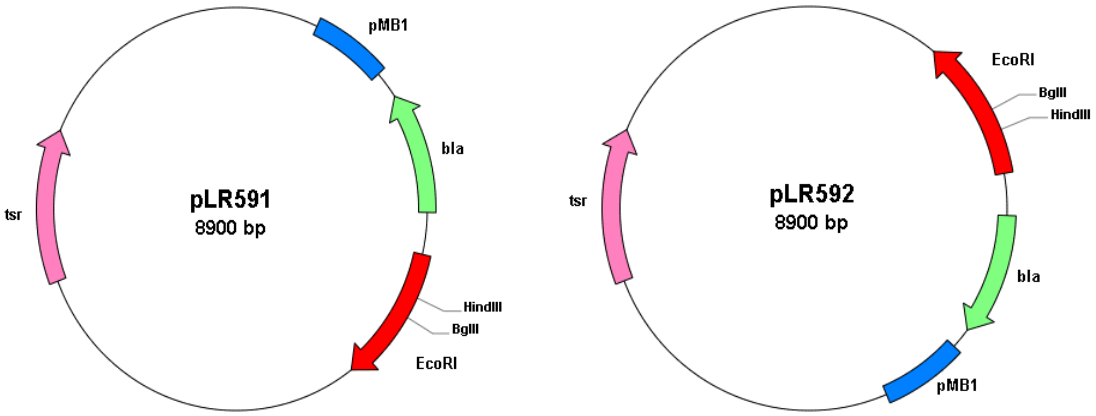

Fig. 3.1: *Streptomyces*- *E. coli* shuttle vectors (left) pLR591 and (right) pLR592. Drawn with Plasm software.

The procedure adopted for use in the transformation of *S. lividans* in this study is a traditional *Rhodococcus* spp. transformation protocol. Through the slight modification of key parameters I was able to effectively transform the streptomycete host. These parameters were taken from the conventional *Streptomyces* spp. method designed by Thompson and coworkers (1982). The changes made include the following: (i) the use

of late exponential phase cells, (ii) the incubation of the protoplasts in 1mg/ml lysozyme for 30 min., (iii) plating of the cells onto R2YE plates and (iv) for selection, an antibiotic overlay at 18 h. To be candid there are very few advantages to the use of this procedure over that of the conventional procedure. The protoplast preparation is quicker, since these are not triturated through a pipette or filtered through cotton wool. Only one buffer is used (B buffer and from this the P buffer is made), opposed to the use of lysozyme solution, P buffer and T buffer with the traditional method. Also, B buffer can be stored indefinitely.

The *Rhodococcus* spp. procedure makes use of RM regeneration media which does not contain trace elements or amino acid supplements as does R2YE. Transformation onto these plates reduced efficiency by 2.5 times compared to R2YE media [Fig. 3.2]. The efficiency on the R2YE media was three fold lower than the conventional method designed by Thompson and colleagues (Kieser *et al.*, 2000). Interestingly, Hill and workers (1989) reported $9x10^3$ - $1x10^4$ transformation efficiency when transforming *S. lividans* with pLR591 propagated in an *S. lividans* host. They reported that propagation of the DNA in *E. coli* K514 led to between 2-7 transformants/µg DNA, a considerable 10^4 fold decrease. The values represented in this study are due to transformation from DNA extracted from a methylation proficient *E. coli* strain. Hence, it is possible that DNA extracted from a methylation deficient host could appreciably increase the transformation efficiency.

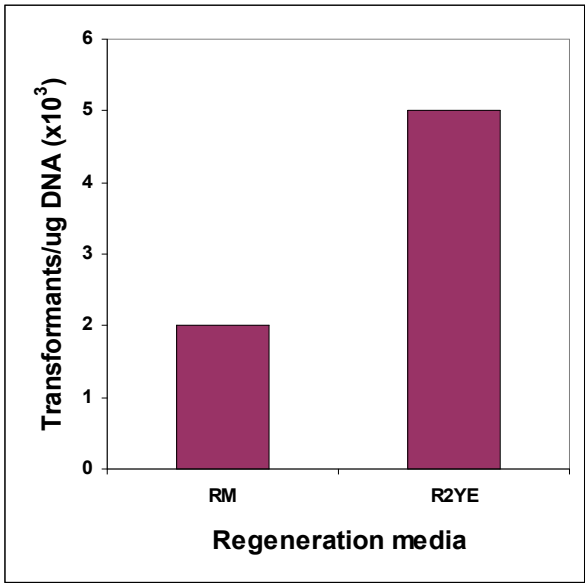

Fig. 3.2: Comparison of transformation efficiency on regeneration media

The screening of *Streptomyces* spp. is a tedious process that involves the individual patching of clones onto selective media. Due to their growth below the agar surface they cannot like other bacteria be screened simply through pooling of the library clones, appropriate dilution and spreading onto selective media; which allows thousands of colonies to be conveniently screened within a short period of time. The additional problem with Streptomycetes is that they possess one of the largest bacterial genomes meaning that screening for a phenotype of interest often involves the patching of several thousand clones (Rose *et al.*, 2005). Hence, it is a long process. Thus, a procedure to amerliorate the screening process through liquid regeneration was sought. Multiple attempts to regenerate protoplasts in liquid broth were unsuccessful. This was believed to be due to the complex life cycle of the host strain. Clearly the ability of protoplasts to revert back to their mycelial state was problematic. One unusual feature noted in the liquid media was the presence of a few biofilm-like cells, yet when transferred onto thiostrepton plates they were unable to grow, showing that they had in fact not taken up the vector. It was hypothesized that these were mutant cells capable of liquid regeneration. Since the common *S. lividans* protoplasts could not regenerate efficiently the decision was made to use the mutated cells for transformation. Unfortunately, this meant that the procedure could not be made applicable to regular *S. lividans* cells and must make use of a mutant. Attempts however to transform DNA into the mutant and regenerate it in liquid media failed. Our paralleled attempt to transform the mutant and regenerate it on solid R2YE media however was successful. I then cured the aforementioned strain using a method desribed by Okanishi *et al.* (1982). The curing process was highly effective, with 60 % of the cells tested losing the vector. I avoided the use of ethidum bromide and mitomycin C since this could induce unwanted mutations which could be problematic at a later stage. Once the strain was cured, my attempt to introduce the recombinant vector pLR591 and regenerate *S. lividans* in liquid broth was successful. I then attempted to optimize the procedure by slight variations of parameters.

The first factor was to compare agitation versus incubation under a stationary condition. It was noticed that agitation favored regeneration while cultures which remained stationary saw a 14 times reduction in regeneration [Fig. 3.3]. I attributed this to Streptomycetes being obligate aerobes and the static condition negatively influencing its development.

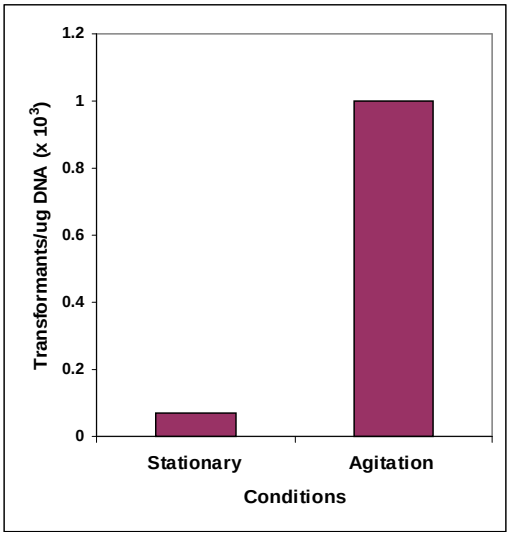

Fig. 3.3: Comparison of agitation vs. stationary incubation on transformation efficiency

Okanishi and coworkers (1974) wrote an innovative paper describing aspects that influence the reversion of *Streptomyces* protoplasts to their filamentous state through regeneration on specialized agar media. They identified several components that effected regeneration, namely $MgCl_2$, $CaCl_2$, phosphate, casamino acid and sucrose concentrations in addition to nitrogen and buffer type. In particular, their experimentation detailed the effect of Ca^{2+} and Mg^{2+} ions. They documented that the absence of either of these components caused protoplast leakage to occur (Okanishi *et al.*, 1974). Prior research suggested the role of Mg^{2+} is to prevent the release of lipid from the plasma membrane thus stabilizing the protoplasts while calcium ions effectively prevent lysis of the protoplasts in a hypertonic solution (Okanishi *et al*, 1974). I monitored the effect of these components by individually increasing the concentration two times. An increase in Ca^{2+} with respect to Mg^{2+} and inversely an increase in Mg^{2+} with respect to Ca^{2+} reduced the regeneration efficiency [Fig. 3.4]. Okanishi *et al.* (1974) reported that a two and a half times increase in Mg^{2+} led to 37 % regeneration in *S. griseus* and 50 % regeneration in *S. venezuelae*. A two and a half times increase in Ca^{2+} resulted in a 41 % and 44 % regeneration in *S. griseus* and *S. venezuelae* respectively. Ultimately, this showed that a balance of the two components is required. Madon and Hutter (1991) tested the use of alkaline cations to improve transformation. Potassium, rubidium and cesium were found to be the most effective cations used in the transformation of *A. mediterranei*. Similarly, I tested

the effect of cesium and found that it increased the transformation efficiency by five times [Fig. 3.4].

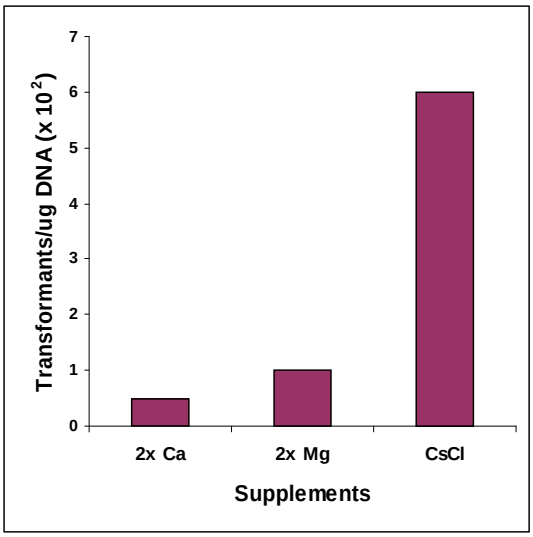

Fig. 3.4: Comparison of the supplementation of regeneration broth with 2X calcium chloride and 2X magnesium chloride. The effect of the addition of cesium chloride (CsCl) to the DNA

I detected a definite reduction in transformation using RM solid agar media and decided to check whether the same pattern would be seen in liquid RM broth. The transformation efficiency in RM versus R2YE broth revealed a 25 % decline in the use of RM media [Fig. 3.5].

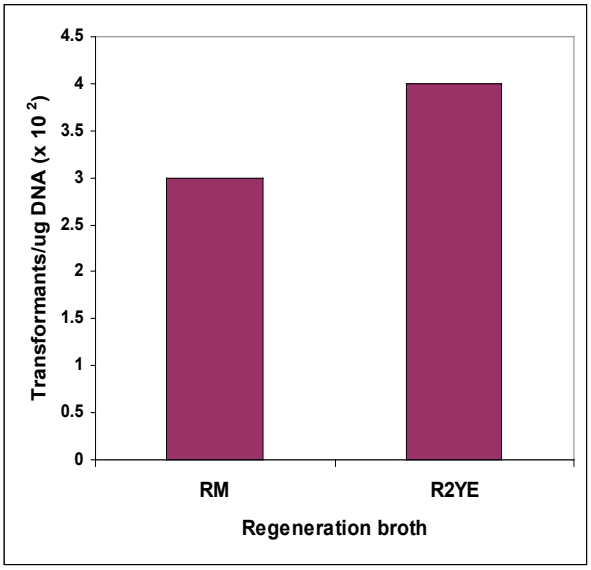

Fig. 3.5: Comparison of RM vs. R2YE regeneration broth on transformation efficiency

In summary the optimal conditions include gentle agitation following the addition of the selective antibiotic, the addition of CsCl to the DNA, the use of 1.25% $MgCl_2$ and 0.29% $CaCl_2$ and the use of R2YE regeneration broth.

There were a few drawbacks to the liquid broth regeneration that are still being worked out. It was found that the liquid broth was more easily contaminated by fungal growth than the solid agar media used. This was easily solved by adding the antifungal agent nystatin (50 µg/ml). I believe that making the strain resistant to rifampicin would be beneficial in preventing bacterial contamination. The highest transformation efficiency was recorded as $6x10^2$ transformants/ µg DNA, which is disappointingly low. Correspondingly, this is not high enough for this method to be used in the screening of streptomycete libraries. However, the adjustment of parameters as seen with the addition of cesium shows that the efficiency can be further improved and probably requires the amendment of key parameters.

3.1 Concluding remarks

I was able to show that regeneration in liquid broth is possible. The generation of the mutants to be used in the procedure can be done easily and quickly. The adoption of this method might also prove to work effectively with other Streptomycete hosts and possibly other bacterial species. It should be noted that this is a technique still in the preliminary stages of experimentation and with appropriate adjustments could potentially lead to quicker screening of *Streptomyces* spp.

3.2 Final Conclusion

This project looked briefly into the well studied area of antibiotic production and the lesser acknowledged fields (involving actinomycetes) of dye decolorization and phytase production. A central theme extended through the dye and rubber based projects which is the expression of genes in different hosts and the analysis of the outcome. As mentioned before the differences in decolorization by microbes can be attributed to the complex dye structures as well as culture conditions and genetic variations. Taken together, this reflects the difficultly in establishing a 'biological system' for dye biodegradation. Addressing the first issue regarding dye structures; over 100 000 dyes exist, falling into over 15 different dye classes, thus finding a single microbial species capable of degrading such a wide array of structures is impossible. Additionally, it is rare for a single species to be capable of degrading more than one of the dye classes proficiently. The use of a consortium could prove more practical, however one has to consider that this would need to include several bacterial species that would require an ability to work harmoniously and tolerate a harsh environment. With many bacterial species the problem of out-competing other important strains becomes evident, clearly another situation with many variables and as such difficult to control.

In this study the isolates managed well in the breakdown of triphenylmethane dyes, however struggled to degrade the sulfonated dyes. Looking more closely at the literature with regard to the degradation of sulfonated azo dyes – it is can be stated that cofactor dependent reductases and oxidases are more appropriate. Thus, I propose that the cloning and expression of a cofactor reductase and oxidase (similar to non-stringent substrate specific fungal oxidases) would greatly enhance the mineralization of this dye class. As such, I believe a combination of reductases, relevant synthases, hydrolases and oxidative enzymes would prove most useful in the treatment of triphenylmethane, non-sulfonated and sulfonated azo dye classes.

Ultimately, these projects set out to establish the effect of heterologous expression of certain genes. Results were variable, these genes might not be expressed effectively in recombinant hosts, as observed with the *lcp* gene, be extremely restrictive in activity as with the *DsbD* homologue or could adopt novel functionalities as with the crystal

violet mineralizing genes. The expression of three genes displaying different mechanisms of activity allowed eight of sixteen dyes to be broken down. This was a small-scale study but can be used to resonate a strategy which can be adapted in order to deal with a spectrum of dye structures.

3.3 What these studies add to the field

The identification of isolates not previously implicated with these activities:

- This is the first study to associate *Amycolatopsis* spp. with azo and triphenylmethane dye biodegradation as well as phytase production
- *M. fulvum* is only the fourth Gram negative rubber degrader to be identified
- *Tsukamurella* spp. was linked to cholesterol break down

Genes

- All genes identified in the triphenylmethane and azo projects have never before been associated with dye biodegradation

Techniques

- The regeneration of *Streptomyces* spp. in liquid broth
- *Streptomyces* spp. regeneration through the adaptation of a *Rhodococcus* spp. PEG-mediated transformation procedure
- The use of Taussky-Shoor as a means to identify phytase producers on solid media

Concepts

- Heterologous expression of genes and monitoring of the outcome
- Synergism of genes contributing to phenotypic expression

4. References

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6. Appendices

6.1 Appendix A: Supplementary data

Table 6.1: Inhibition of indicator strains by tested isolates

Strains	***S. aureus* 64-1**	***B. subtilis* IA3**	***R. erythropolis* SQ1**
BA1	-	-	-
Gam	-	-	-
Reu	-	-	-
Berlin	+	+	+
Hak	-	-	-
Cal	-	-	-
Pasa	+	+	+
Bot1	+	+	+
FHome	-	-	-
WitsP	+	+	+
SY3	+	+	+
SY5	+	+	+
SY6	+	+	+
HY	-	-	-
WITS	-	-	-
Bedd	-	+	-
H2	-	-	-
H3	+	+	+
BotY	-	+	-
Bot2	-	-	-
Est	-	-	-
Chiba	-	-	-
Yeo	-	-	-
Hunt	-	-	-
S. lividans TK23	-	-	-
YeoE	-	-	-
HZ	-	-	-
HZWS	+	+	+
HZBS	-	-	+
25593	-	-	-
A554	-	-	-
NB4	-	-	-
HLPA1	-	-	-
RI8	-	-	-
4277	-	-	-
P. aeruginosa	-	-	-
S. aureus	-	-	-
S. marcescens	-	-	-
M. luteus	-	-	-
M. flavus	-	-	-
Agrobacterium spp.	-	-	-
B. subtilis	-	-	-
SQ1	-	-	-

Table 6.2: Dyes tested which isolates were capable of decolorizing in the presence of selected carbon sources

	Dyes (10 µg/ml) and carbon supplementation (0.5%)														
	Eriochrome					Orange II					Congo red				
	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20
Strains															
BA1	-	-	+	-	-	-	-	-	-	+	-	+	+	+	+
Gam	-	-	+	-	-	-	-	-	-	-	-	+	+	-	+
Reu	-	-	+	-		-	-	-	-	+	+	+	+	-	+
Berlin	-	-	+	-	-	-	-	-	-	+	+	+	+	-	+
Hak	-	-	+	-	-	-	-	-	-	+	+	+	+	-	+
Cal	-	-	+	-	-	-	-	-	-	+	+	+	+	+	+
Pasa	-	-	+	-	-	-	-	-	-	+	-	+	+	-	+
Bot1	-	-	+	-	-	-	-	-	-	-	-	+	+	-	+
FHome	-	-	+	-	+	-	-	-	+	+	+	+	+	-	+
WitsP	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
SY3	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
SY5	-	-	+	-	+	-	-	-	+	+	-	+	+	-	+
SY6	-	-	-	+	+	-	-	-	+	+	-	-	-	-	+
HY	-	-	+	-	-	-	-	-	-	+	+	+	+	-	+
WITS	-	-	+	-	-	-	-	-	+	-	-	+	+	-	+
Bedd	-	-	+	-	-	-	-	-	+	+	-	+	+	-	+
H2	-	-	+	-	-	-	-	-	-	+	-	-	+	-	+
H3	-	-		-	-	-	-	-	+	+	-	-	-	-	+
BotY	-	-	+	-	-	-	-	-	-	+	-	+	+	+	-
Bot2	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+

	Dyes (10 µg/ml) and carbon supplementation (0.5%)														
	Eriochrome					Orange II					Congo red				
	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20
Strains															
Est	-	-	-	-	-	-	-	-	-	+	-		-	-	+
Chiba	-	-	+	-	-	-	-	-	+	+	-	+	+	-	+
Yeo	-	-		-	-	-	-	-	-	+	-	+	-	+	+
Hunt	-	-	+	-	-	-	-	-	+	+	-	+	-		+
TK23	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
YeoE	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-
HZ	-	-	+	-	-	-	-	+	-	+	-	+	+	+	+
HZBS	-	-	-	-	-	-	-	-	-		-	-	-	+	+
HZWS	-	-	-	-	+	-	-	-	+	+	-	-	-	-	+
25593	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A554	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NB4	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-
HLPA1	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-
RI8	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-
4277	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	+	-	+	-	-	-	+	+	-	+	+	-	+
S. aureus	-	-	-	-	-	-	-	-	+	+	-	-	-		-
S. marcescens	-	-	+	-	-	-	-	+	+	-	-	-	+	+	+
M. luteus	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
M. flavus	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
pE 1913	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IA3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SQ1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	Dyes (10 µg/ml) and carbon supplementation (0.5%)														
	Crystal violet					Amido black					Fast green				
	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20
Strains															
BA1	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
Gam	-	-	-	-	-	-	-	-	+		-	-	-	-	-
Reu	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
Berlin	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Hak	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
Cal	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Pasa	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
Bot1	-	-	-	-	-	-	-	+	+		-	-	-	-	-
FHome	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
WitsP	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
SY3	-	-	-	-	-	-	-	-		+	-	-	-	-	-
SY5	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
SY6	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-
HY	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
WITS	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
Bedd	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
H2	-	-	-	-	-	-	-	+	+	+		-	-	-	-
H3	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
BotY	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Bot2	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-

	Dyes (10 µg/ml) and carbon supplementation (0.5%)														
	Crystal violet					Amido black					Fast green				
	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20
Strains															
Est	-	-	-	-	-	-	-		+	+	-	-	-	-	-
Chiba	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
Yeo	-	-	-	-	-	-	-	+		+	-	-	-	-	-
Hunt	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
TK23	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-
YeoE	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
HZ	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-
HZBS	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
HZWS	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
25593	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-
A554	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
NB4	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-
HLPA1	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
RI8	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-
4277	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-
P. aeruginosa	-	+	+	+	+	-	-	-	+	+	-	-	-	-	-
S. aureus	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
S. marcescens	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-
M. luteus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M. flavus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pE 1913	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IA3	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
SQ1	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-

	Dyes (10 µg/ml) and carbon supplementation (0.5%)														
	Scarlet					Ponceau					Janus green				
	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20
Strains															
BA1	-	-	-	+	+	+	-	-	-	-	+	-	-	-	-
Gam	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Reu	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-
Berlin	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Hak	-	+	-	-	+	+	-	-	-	-	+	-	-	-	-
Cal	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
Pasa	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
Bot1	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-
FHome	-	-	-	+	+	-	-	-	-	-	+	+	+	-	-
WitsP	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
SY3	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
SY5	-	-	-	+	+	-	-	-	-	-	+	-	-	+	-
SY6	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-
HY	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-
WITS	-	-	-	+	+	-	-	-	-	-	+	+	-	+	-
Bedd	-	-	-	+	+	-	-	-	-	-	+	+	-	+	-
H2	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
H3	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
BotY	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bot2	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-

	Dyes (10 µg/ml) and carbon supplementation (0.5%)														
	Brown					Brilliant green					Tartrazine				
	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20
Strains															
Est	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Chiba	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-
Yeo	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-
Hunt	-	-	-	-	-		-		-	-	-	-	-	-	-
TK23	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-
YeoE	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-
HZ	-	-	+	-	-	-	-		-	+	-	-	-	-	-
HZBS	-	-	-	-	-	-	-		+	-	-	-	-	-	-
HZWS	-	-	-	-	-	-	-		-	-	-	-	-	-	-
25593	-	-	+	-	-	+	-	+	+	+	-	-	-	-	-
A554	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
NB4	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-
HLPA1	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-
RI8	-	-	-	-	+	+	-	+	+	+	-	-	-	-	-
4277	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-
P. aeruginosa	-		-	-	-	-	-	+	-	-	-	-	-	-	-
S. aureus	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
S. marcescens	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-
M. luteus	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-
M. flavus	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-
pE 1913	-	-	-	-	-	-	-		-	-	-	-	-	-	-
IA3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SQ1	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-

	Dyes (10 µg/ml) and carbon supplementation (0.5%)														
	Brown					Brilliant green					Tartrazine				
	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20
Strains															
BA1	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Gam	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-
Reu	-	+		-	-	-	-	-	-	-	-	-	-	-	-
Berlin	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-
Hak	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Cal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pasa	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
Bot1	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
FHome	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-
WitsP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SY3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SY5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SY6	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
HY	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
WITS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bedd	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BotY	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bot2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	Dyes (10 µg/ml) and carbon supplementation (0.5%)														
	Scarlet					Ponceau					Janus green				
	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20	Dye	Succinate	Glucose	Tween 80	Tween 20
Est	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Chiba	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Yeo	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Hunt	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
TK23	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-
YeoE	-	-	-	-	+	-	-		-	-	-	-	-	-	-
HZ	-	+	+	-	+	-	-	+	-	-	-	-	-	-	-
HZBS	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
HZWS	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
25593	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
A554	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
NB4	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
HLPA1	-	-	-	-		-	-	-	-	-	-	-	-	-	-
RI8	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
4277	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-
S. aureus	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
S. marcescens	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-
M. luteus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M. flavus	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
pE 1913	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IA3	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
SQ1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

6.2 Appendix B: Suppliers of chemicals, software and equipment

6.2.1 *Suppliers of chemicals used:*

Product	**Supplier**	**Location of supplier**
ExamTex powdered rubber gloves	Ansell	Malaysia
Glucose	Associated chemical enterprises	
Certified PCR agarose	BioRad	Hercules, California
Cesium chloride Ethidium bromide Restriction enzymes Sodium dodecyl sulfate	Boehringer Mannheim	Germany
Polyethylene glycol 6000 (PEG 6000)	Fluka	-
Fuchsin	Gurr	-
Molecular weight markers Restriction enzymes T4 DNA ligase All PCR reagents	MBI Fermentas	Hanover, USA
Ammonium chloride Chloroform Ethanol Isopropanol Magnesium chloride Methanol Potassium acetate Sodium chloride Tris (hydroxymethylaminomethan) Tween 80	Merck	Modderfontein, South Africa
Brain heart infusion Peptone P Technical agar Tryptone Yeast extract	Oxoid	Basingstoke, UK
Boric acid powder Calcium chloride hexahydrate Phenol Sodium hydroxide pellets Amaranth Amido black Biebrich scarlet Brilliant green Congo red Crystal violet Eriochrome black T Fast green Janus green Ponceau S	Saarchem	Krugersdorp, South Africa
Cinnamyl alcohol	Sigma	Aston Manor, South

Ferulic acid Glycine Kanamycin Naladixic acid NTG Orange II Parahydroxy benzoic acid Rifampicin Starch Tartrazine Vanillic acid Veratric acid Sodium phytate Calcium phytate		Africa
Ampicillin Hygromycin Klenow Lysozyme Proteinase K Ribonuclease	Roche	Randburg, South Africa

6.2.2 *Suppliers of kits used:*

Commercial kit	**Company**	**Location of company**
GFX™ micro plasmid prep kit	Amersham Biosciences	UK
MicroSeq® 500 primers	Applied Biosystems	Warrington, UK
pGEM-T Easy	Promega	Madison, USA
Qiagen QIAquick gel extraction kit	Qiagen	West Sussex, UK

6.2.3 *List of manufacturers of equipment and software used:*

Equipment	**Manufacturer**	**Location of manufacturer**
J2-21 centrifuge L7-55 ultracentrifuge	Beckman	USA
Biorad Gel doc system	Biorad	Japan
Video graphic printer	Sony	Japan
Sequencing machine	Spectromedix LCC sequencer	-
LabWorks 4.5 image acquisition and analysis software	UVP	Cambridge, UK
JSM-840 scanning electron microscope	-	-
Spectronic 601 spectrophotometer	Milton Roy company	West Germany

6.2.4 *Website addresses of software used:*

i) Plasm – http://www.bio-log.biz/index.php?page=plasm

ii) PubMed Blast – http://www.ncbi.nlm.nih.gov/blast/Blast.cgi

iii) FramePlot - http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl

6.3 Appendix C: Solutions and Media

6.3.1 *Antibiotic Stock Solutions:*

Antibiotic	**Concentration (mg/ml)**	**Solvent**
Ampicillin	50	70% ethanol
Chloramphenicol	4	100% ethanol / methanol
Hygromycin	50	PBS
Kanamycin	50	Sterile water
Rifampicin	10	Methanol
Streptomycin	50	Water (filter sterilized)
Thiostrepton	50	DMSO

6.3.2 *Media:*

Luria Bertani agar (LA)

Tryptone	1 g
Yeast extract	0.5 g
Sodium chloride	0.5 g
Technical agar	1.5 g
Distilled water	100 ml

½ Luria Bertani agar (LA)

Tryptone	1 g
Yeast extract	0.5 g
Sodium chloride	0.5 g
Technical agar	0.75 g
Distilled water	100 ml

Luria Bertani broth (LB)

Tryptone	1 g
Yeast extract	0.5 g
Sodium chloride	0.5 g
Distilled water	100 ml

LBSG

Sucrose	10 g
Glycine	1-3 g
Tryptone	1 g
Yeast extract	0.5 g
Sodium chloride	0.5 g
Distilled water	100 ml

Brain heart infusion

Brain heart infusion	11.1 g
Technical agar	4.5 g
Distilled water	300 ml

YPD media

Yeast extract	1 g
Peptone	2 g
Dextrose	2 g
Agar	1.5 g
Distilled water	100 ml

YEME (yeast extract-malt extract medium)

Yeast extract	3 g
Peptone	5 g Malt
extract	3 g
Glucose	10 g
Sucrose	340 g
Distilled water	1000 ml

Phytase screening media

Dextrose	2 g
Tryptone	1 g
NaCl	0.5 g
KCl	0.01 g
Agar	2 g
Distilled water	100 ml

Phosphate supplemented plates

Tryptone	1 g
Yeast extract	0.5 g
Sodium chloride	0.5 g
Technical agar	1.5 g
Stock solution III	
x 10	10
Distilled water	90 ml

Latex agar

10X stock III	
solution	30 ml
Ammonium chloride	0.3 g
Distilled water	120 ml

Technical agar	4.5 g
Distilled water	150 ml

Both solutions prepared in separate beakers and combined once autoclaved. Afterwards 0.3 ml of liquid latex is added.

10X stock III solution (liquid minimal media)

K_2HPO_4. $3H_2O$	91.7 g (or K_2HPO_4 69.95 g)
KH_2PO_4	26.8 g
$MgSO_4$	1.0 g
Distilled water	up to 1000 ml

Once autoclaved add 0.1 g NH_4Cl to every 100 ml of solution before use. Solution either stored at room temperature with 10 ml chloroform or at -20^oC.

Minimal media agar

10X stock III solution	30 ml
Ammonium chloride	0.3 g
Distilled water	120 ml
Technical agar	4.5 g
Distilled water	150 ml

Both solutions prepared in separate beakers and combined once autoclaved.

0.1 M potassium phosphate buffer

0.2 M KH_2PO_4

27.2 g KH_2PO_4
Made up to 1000 ml with sterile distilled water

0.2 M K_2HPO_4

34.8 g K_2HPO_4
Made up to 1000 ml with sterile distilled water

Refer to table 5.4.1 to see the volumes of 0.2 M KH_2PO_4 and 0.2 M K_2HPO_4 which need to be combined to obtain the desired pH.

Regeneration media

Tryptone	3 g
Yeast extract	1.5 g
Sodium chloride	0.9 g
Sucrose	30.9 g
Glucose	1 g
$MgCl_2$	1 g
Distilled water	250 ml

Microwaved till sucrose is fully dissolved, after which the following was added:

Technical agar	5.5 g

After autoclaving the solution was cooled to 60^oC and the following was added:

TES	10 ml
$CaCl_2$	6 ml

KH_2PO_4	3 ml
Rifampicin (10mg/ml)	0.9 ml

Plates with a constant volume of 22 ml were poured on a horizontal surface, allowed to solidify and dried for two days at 37^{o}C.

Sloppy Agar

Tryptone	1 g
Yeast extract	0.5 g
Sodium chloride	0.5 g
Technical agar	0.75 g
Distilled water	100 ml

Starch agar (3%)

Technical agar	2 g
Peptone	0.5 g
Beef extract	0.3 g
NaCl	0.5 g
Distilled water	99 ml
pH to 7.2	

Add starch (3%) after media has been autoclaved and cooled.

6.3.3 *Miscellaneous Solutions:*

Ribonuclease

Ribonuclease	10 mg
Distilled water	1 ml

Solution heated at 95^{o}C for 20 min.

25% Tween

Tween 80	2.5 ml
Distilled water	7.5 ml

Solution placed in 100^{o}C waterbath till well dissolved and filter sterilized.

TE buffer

0.5M EDTA	2 ml
1M Tris-HCl	1 ml
Distilled water	97 ml

1M Tris-HCl (pH 8.0)

Tris base	12.11 g
Distilled water	50 ml
pH to 8.0 with HCl	
Add remaining water to give total volume of 100 ml	

0.5M EDTA

EDTA	18.61 g
Distilled water	50 ml

pH with potassium hydroxide or sodium hydroxide to 8.0
Add remaining water to give total volume of 100 ml

1M NaCl

NaCl	0.58 g
Distilled water	10 ml

6.3.4 *Transformation solutions:*

i) *E. coli $CaCl_2$ mediated transformation*

Glucose (20%)

Glucose	2g
Distilled water	10 ml

Transformation buffer

1M Tris HCl (pH 8.0)	1 ml
Calcium chloride (hexahydrate)	2.2 g
Distilled water	99 ml

ii) *PEG mediated transformation*

TES buffer (pH 7.2)

TES	5.73 g
Distilled water	100 ml

pH to 7.2

Basal buffer (B buffer)

Sucrose	10.3 g
K_2SO_4	25 mg
$MgCl_2.\ 6H_2O$	202 mg
0.25M TES (pH 7.2)	10 ml
Distilled water	87.5 ml

Protoplast buffer (P buffer)

B buffer	5 ml
KH_2PO_4	50 µl
$CaCl_2$	125 µl

P-PEG buffer

PEG	0.5 g
P buffer	1 ml

PEG was sterilized for 5 min. by UV and vortexed vigorously for 10 min. to dissolve.

1M $CaCl_2$

CaCl2	21.9 g
Distilled water	100 ml

0.5% KH_2PO_4

KH_2PO_4	0.5 g
Distilled water	100 ml

iii) *Solutions for Streptomyces transformation*

Trace element solution

$ZnCl_2$	40 mg
$FeCl_3.6H_2O$	200 mg
$CuCl_2.2H_2O$	10 mg
$MnCl_2.4H_2O$	10 mg
$Na_2B_4O_7.10H_2O$	10 mg
$(NH_4)6Mo_7O_{24}$.$4H_2O$	10 mg

T buffer

Sucrose (10.3%)	25 ml
Distilled water	75 ml
Trace element solution	0.2 ml
K_2SO_4 (2.5%)	1 ml

To 9.3 ml of the above solution add:

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

For use add 3 parts by volume of the above solution to 1 part by weight of PEG, previously sterilized by autoclaving

P buffer

Sucrose	103 g
K_2SO_4	0.25 g
$MgCl_2.6H_2O$	2.02 g
Trace element solution	2 ml
Distilled water to	1000 ml

L buffer

Sucrose (10.3%)	100 ml
TES buffer (5.73% pH 7.2)	10 ml
K_2SO_4 (2.5%)	1 ml
Trace element Solution	0.2 ml
KH_2PO_4 (0.5%)	1 ml
$MgCl_2.6H_2O$	

(2.5M)	0.1 ml
$CaCl_2$ (0.25M)	1 ml

Just before use dissolve lysozyme in a sample of the solution to 1mg/ml and filter sterilize

R2 Medium

Sucrose	103 g
K_2SO_4	0.25 g
$MgCl_2.6H_2O$	10.12 g
Glucose	10 g
Difco casaminoacids	0.1 g
Distilled water	800 ml

Place 2.2 g Difco Bacto agar in each 250 ml flask and pour in 80 ml of the solution. Autoclave mixture.

At time of use, remelt medium and add the following to each flask:

KH_2PO_4 (0.5%)	1 ml
$CaCl_2.2H_2O$ (3.86%)	8 ml
L-proline (20%)	1.5 ml
TES buffer (5.73%)	10 ml
Trace element solution	0.2 ml
NaOH (1N)	0.5 ml

6.3.5 *Miniprep Solutions:*

i) *E. coli miniprep solutions:*

Solution I

Glucose	0.9 g
1M Tris-HCl (pH 8.0)	2.5 ml
0.5M EDTA	2 ml
Distilled water	95.5

Solution II

NaOH	0.8 g
SDS	1 g
Distilled water	100 ml

Solution is not autoclaved. Mixture is placed in 40^oC water bath until fully dissolved.

Solution III

Potassium acetate	29.4 g
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

ii) *Miniprep solutions for gram positives*

TE saturated phenol

Phenol	10 g
TE buffer	10 ml

Solution stored in bottle covered in foil.

TE-SDS (10% SDS)

SDS	1 g
TE buffer (pH 8.0)	10 ml

Mixture placed in 40^{o}C water bath to dissolve.

6.3.6 *Media and solutions for agarose gel preparation:*

Agarose gel

Agarose	0.4 g (0.4 % gel) or 0.8 g (0.8% gel) or 2 g (2% gel)
0.5X TBE	100 ml

Solution dissolved in the microwave or by autoclaving.

5X TBE

Tris base	54 g
Boric acid	27.5 g
0.5M EDTA (pH 8.0)	20 ml

Made up to 1000 ml with distilled water

0.5M TBE

5X TBE	100 ml
Distilled water	900 ml

Ethidium bromide

Ethidium bromide powder	10mg/ml
Distilled water	

Solution stored in bottle covered with foil. Mixture then vortexed briefly and left in 40^{o}C water bath till fully dissolved.

Tracking dye

Bromophenol blue	25 mg
Xylene cyanol	25 mg
Glycerol	5 ml
0.25M EDTA	5 µl

6.3.7 *Mutagenesis solutions:*

i) Ultra-violet mutagenesis

8-Methoxypsoralen
1mg of 8-methoxypsoralen was added to 1ml of absolute ethanol

ii) NTG mutagenesis

NTG

NTG powder	1 mg
0.02M Tris-HCl (pH8.5)	1 ml

Heat briefly (5 sec intervals in the microwave) till fully dissolved.

0.02M Tris-HCl pH 8.5

Tris base	0.21 g
Distilled water	100 ml
pH to 8.5 with HCl	

Phosphate buffer
10mM K_2HPO_4 titrated with KH_2PO_4 to pH 7.0

6.3.8 *Carbon sources:*

Carbon sources were added to minimal media plates made with noble agar.

Glucose

Glucose	0.1 g
Distilled water	1 ml

Solution was heated at 30^{o}C till dissolved and filter sterilized.

Sucrose

Sucrose	0.1 g
Distilled water	1 ml

Solution was heated at 30^{o}C till dissolved and filter sterilized.

Lignin components:

Vanillic acid

Vanillic acid	0.1 g
Methanol	1 ml

Ferulic acid

Ferulic acid	0.1 g
Methanol	1 ml

Veratric acid

Veratric acid	0.1 g
Methanol	1 ml

Syringic acid

Syringic acid	0.1g
Methanol	1 ml

Solutions made separately and dissolved by placing in 40^{o}C water bath. All components were combined to give a final concentration of 0.02 g/ 100 ml.

Nylon components:

Adipic acid

Adipic acid	0.1 g
Distilled water	1 ml

Aminocaproic acid

Aminocaproic acid	0.1 g
Distilled water	1 ml

Caprolactam

Caprolactam	0.01 g
Distilled water	3 ml

Solutions made separately and dissolved by autoclaving. All components were combined to give a final concentration of 0.02 g/ 100 ml.

Dyes

Amaranth
Amido black
Biebrich scarlet
Brilliant green
Congo red
Crystal violet
Eriochrome black T
Fast green
Janus green
Orange II
Ponceau S
Tartrazine
Malachite green
Basic Fuchsin
Indigo
Bismarck brown Y

20mg/ml solutions were made by dissolving the powder in sterile distilled water. Solutions were stored at -20^{o}C.

6.3.9 *SEM solutions*

Gluteraldehyde (3%)

Gluteraldehyde	0.3 ml
Distilled water	9.7 ml

6.3.10 *Schiff's test*

Schiff's reagent

Basic fuchsin	9 g
1N HCl	90 ml
Potassium metabisulfite	9 g
Activated charcoal powder	0.5g

After adding fuchsin to 600 ml boiling water the mixture is shaken, allowed to cool to 50^{o}C and filtered. HCl and Potassium metabisulfite is then added, mixed and left overnight in a foil covered bottle, after which the solution is filtered. Charcoal is then added the following day.

Sulfite solution

$Na_2S_2O_5$	5 g
HCl (37-38%)	5 ml
Distilled water	95 ml

6.4 Appendix D: Calculations

S. tendae average insert size = 4739
Pseudonocardia spp. average insert size = 2445
S. griseus average insert size =
S. flavogriseus average insert size =
with probability (p) is taken as 95% (0.95)
and genome size (b) as 8 Mbp

N (*S. tendae*) = ln (1-p) / ln (1- a/b)
= ln (0.05) / ln (0.9994)
= 4991 clones

N (*Pseudonocardia* spp.) = ln (1-p) / ln (1- a/b)
= ln (0.05) / ln (0.9997)
= 9984 clones

N (*S. griseus*) = ln (1-p) / ln (1- a/b)
= ln (0.05) / ln (0.9998)
= 14977 clones

N (*S. flavogriseus*) = ln (1-p) / ln (1- a/b)
= ln (0.05) / ln (0.9996)
= 7765 clones

Rf = distance travelled by the compound/ distance travelled by solvent front

Table 5.4.1: pH table for 0.1 M potassium phosphate buffer

pH	**0.2 M KH_2PO_4 (ml)**	**0.2 M K_2HPO_4 (ml)**
6	17.54	2.46
6.5	13.7	6.3
7	7.8	12.2
7.5	3.2	16.8
8	1.06	18.94

6.5 Appendix E: DNA ladders

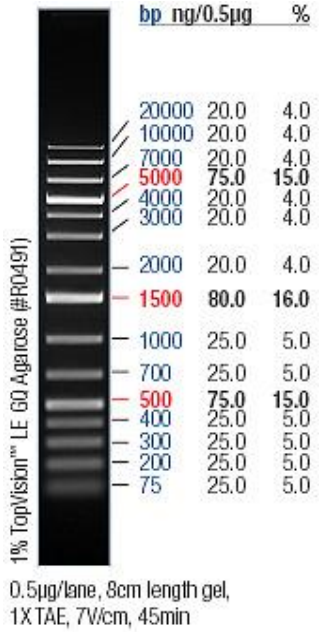

Fig. 6.5.1: GeneRuler^{TM} 1kb DNA ladder plus

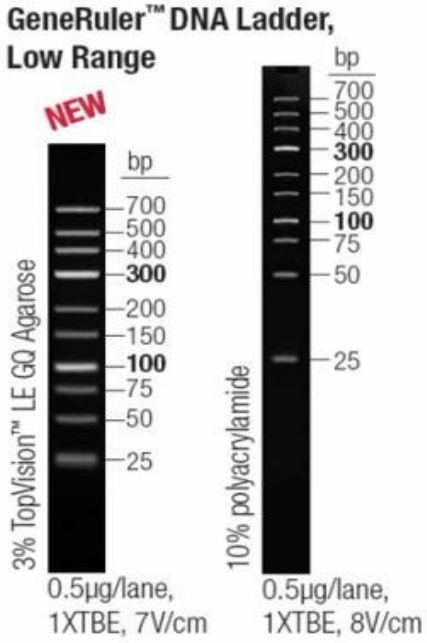

Fig. 6.5.2: GeneRuler^{TM} DNA ladder low range

6.6 Appendix F: Vectors

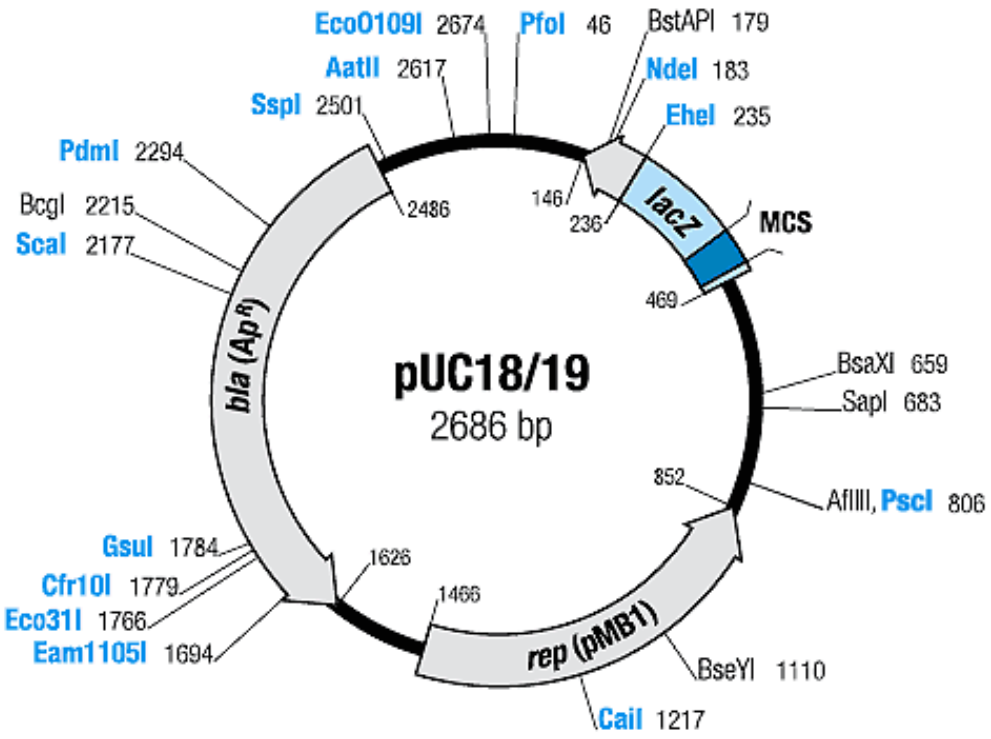

Fig. 6.6.1: Restriction map of pUC18/19

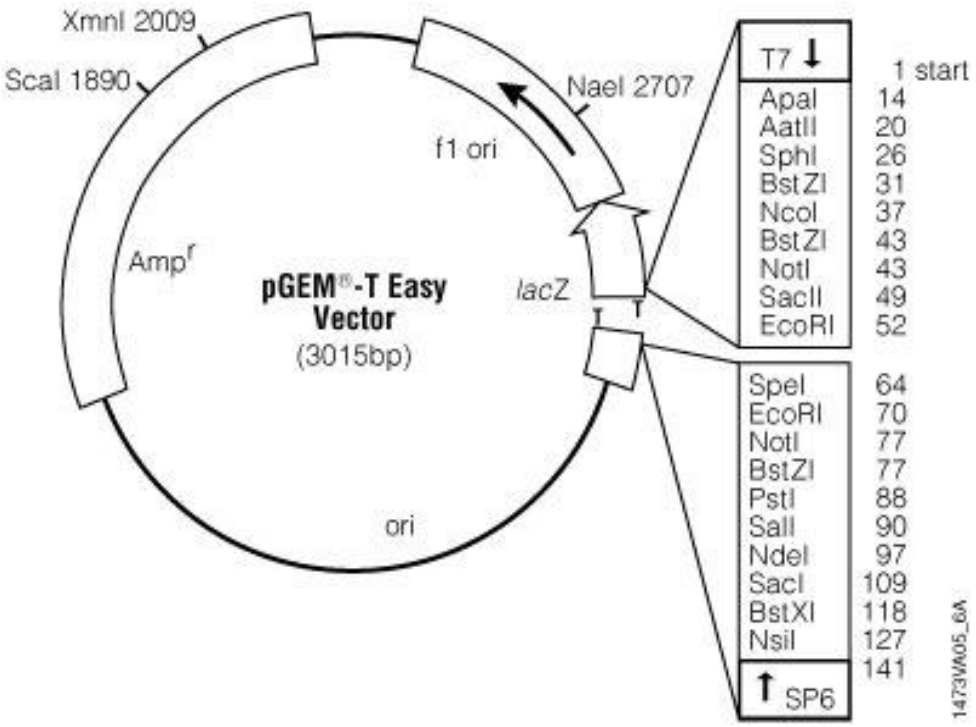

Fig. 6.6.2: Restriction map of pGEM-T Easy

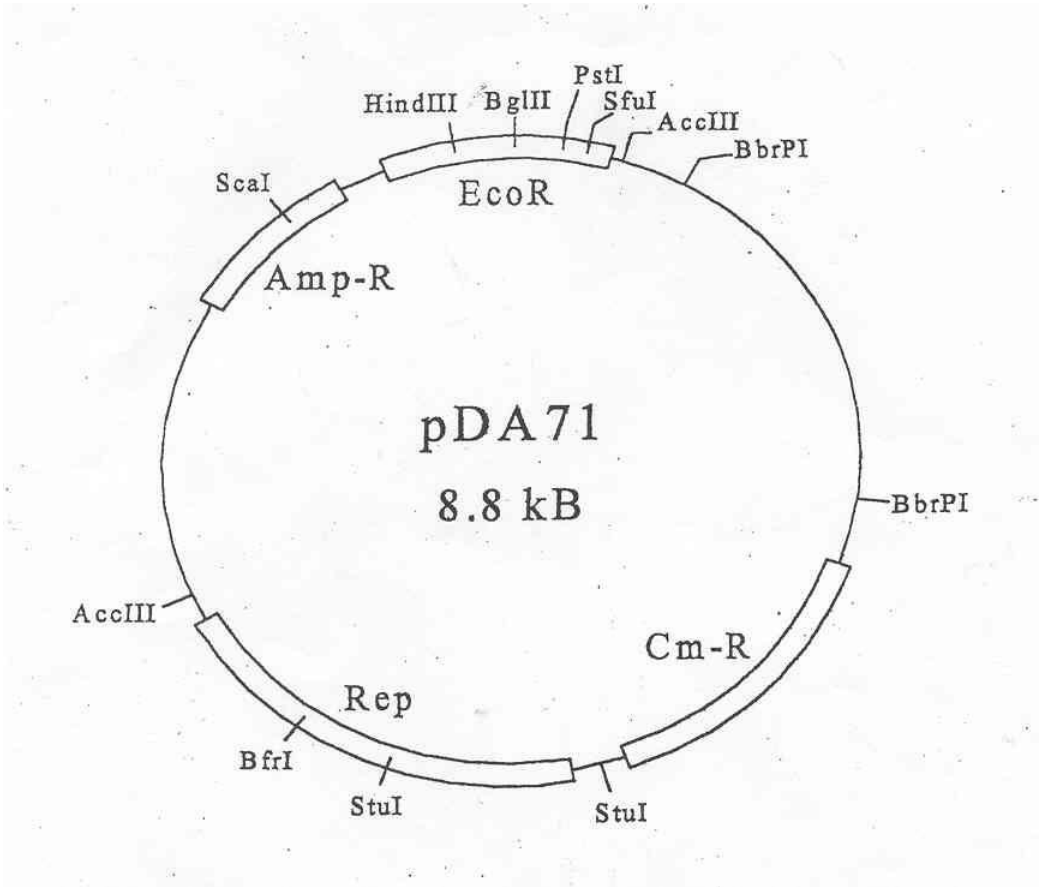

Fig. 6.6.3: Restriction map of pDA71