

https://dspace.org/

Molecular biological characterisation of the novel Rifampicin inactivation mechanism in Nocardioform bacteria

Andersen, Susan Jean

1996

http://hdl.handle.net/10539/22180

Andersen, Susan Jean (1996) Molecular biological characterisation of the novel Rifampicin inactivation mechanism in Nocardioform bacteria, University of Witwatersrand, Johannesburg, <http://wiredspace.wits.ac.za/handle/10539/22180>

Downloaded from a DSpace repository. https://dspace.org/

3.2.1.4 pDA30 and pEcoR251 as cloning vectors 65
3.2.1.5 Survival of *E.coli* plasmid DNA in nocardioforms 66
3.2.2 Construction of pDA37 and pDA38 . . . 67
3.3 pDA37 IN THE CLONING OF AN *iri* GENE . . . 74
3.3.1 Construction of the genomic library of ATCC4277 DNA 74
3.3.2 Isolation of DNA carrying *iri*$^+$ gene . 76
3.3.3 Rifampicin inactivation by the cloned gene 80
3.4 SUBCLONING OF THE *iri* GENE FROM *R.erythropolis* 86
3.4.1 Constructing a restriction map . . . 86
3.4.2 Small *BglII* fragment 87
3.4.3 *HindIII* fragment 87
3.4.4 Elimination of a further 1,75kb . . . 89
3.4.5 Large *BamHI-BglII* fragment 90
3.4.6 *Asp718* fragment 90
3.4.7 *NotI* fragment 92
3.4.8 Exonuclease *Bal31* deletions 93
3.4.9 Subcloning attempts with *EcoRI*, *NarI*, *PvuII* and *SmaI* 95
3.4.10 Insertion of the *BamHI-BglII* fragment into pDA71 98
3.4.11 Mapping of more restriction sites within the *BamHI-BglII* fragment . . . 100
3.4.12 Removal of *AgeI*, *BsiWI* and *ClaI* fragments and the insertion of an *SfuI* fragment 102
3.4.13 Elimination of two *BclI* sites 103
3.4.14 Summary of subcloning experiments . . 104
3.5 FURTHER WORK WITH *iri*$^+$ CLONED DNA 106
3.5.1 Complementation of other rifampicin hypersensitive mutants by cloned DNA 106
3.5.2. The effect of *iri*$^+$ DNA on sensitivity to other antibiotics 110

2.16.5 Immunological detection 46
2.17 DNA SEQUENCING 46
2.17.1 Preparation of DNA for sequencing . . 46
2.17.1.1 Double stranded DNA 46
2.17.1.2 Single stranded phagemid DNA . . 47
2.17.1.3 Single stranded DNA from M13 clones 47
2.17.2 Sequencing reactions 48
2.17.2.1 Using a pUC sequencing kit (Boehringer-Mannheim) 48
2.17.2.2 Using a Sequenase Version 2.0 kit (United States Biolabs) 48
2.17.3 Electophoresis of labelled DNA . . . 49
2.18 THIN-LAYER CHROMATOGRAPHY OF RIFAMPICIN INACTIVATED PRODUCTS 49
2.19 MAXICELL PROTEIN EXPRESSION SYSTEM 50

3.0 RESULTS 51
3.1 ANSAMYCIN INACTIVATION BY NOCARDIOFORM BACTERIA 52
3.1.1 Sensitivity of strains to ansamycins 52
3.1.2 Inactivation of rifampicin by *R.erythropolis* 52
3.1.3 Inactivation of other ansamycins . . 55
3.1.4 Inactivation by ansamycin producing organisms 57
3.1.5 Non-inactivation of streptolydigin . 58
3.1.6 Protein isolates from nocardioform supernatants 58
3.1.7 Non-inactivating mutants 60
3.2 SHUTTLE VECTOR CONSTRUCTION 60
3.2.1 Preliminary Experiments 60
3.2.1.1 Optimisation of Nocardioform Transformation 60
3.2.1.2 Origin of pDA30 62
3.2.1.3 Origin of pEcoR251 63

2.8.1 Small scale preparation of DNA from nocardioforms 34
2.8.2 Small scale preparation of DNA from *E.coli* 34
2.9 TRANSFORMATIONS 35
2.9.1 Protoplast transformation of nocardioforms 35
2.9.2 Electroporation of nocardioforms . . 36
2.9.3 Competent cell transformation of *E.coli* 36
2.9.3.1 With plasmid 36
2.9.3.2 With phage 37
2.10 CLONING BY COMPLEMENTATION 37
2.11 CONSTRUCTION OF A RESTRICTION MAP 38
2.12 RECOVERY OF DNA FROM AGAROSE GELS 39
2.12.1 From low melting agarose 39
2.12.2 Freeze-squeeze method 39
2.12.3 Electroelution 40
2.13 SUBCLONING 40
2.13.1 By insertion of fragments into the BglII site of pDA37 40
2.13.2 Elimination of specific restriction fragments 40
2.13.3 Exonuclease digestion 41
2.13.4 Insertion of restriction fragments into other vectors 41
2.13.5 Analysis of coding function 42
2.14 POLYACRYLAMIDE GEL ELECTROPHORESIS 42
2.15 PREPARATION OF SAMPLES FOR PAGE 43
2.15.1 From supernatant 43
2.15.2 From *E.coli* 43
2.15.3 From nocardioforms 43
2.16 SOUTHERN HYBRIDISATION 44
2.16.1 Blotting 44
2.16.2 Probe labelling 45
2.16.3 Hybridisation 45
2.16.4 Washing 46

2.0 METHODS 21
2.1 BACTERIAL STRAINS 22
2.1.1 Nocardioform bacteria 22
2.1.2 *Escherichia coli* stains 23
2.1.3 *Bacillus subtilis* strain 24
2.1.4 Replica plating 24
2.2 PLASMIDS 25
2.2.1 Nocardioform plasmids 25
2.2.2 *E.coli* plasmids 25
2.2.3 Shuttle plasmids 26
2.3 PHAGES 26
2.4 ANTIBIOTIC INACTIVATION ASSAYS 27
2.4.1 Plate assay 27
2.4.2 Quantitative assay 28
2.4.3 Rate determination 28
2.4.4 *In vitro* assays 28
2.5 SELECTION OF SPONTANEOUS MUTANTS 29
2.6 BULK ISOLATION OF DNA 29
2.6.1 Large scale preparation of DNA from nocardioforms 29
2.6.2 Large scale preparation of plasmid from *E.coli* 30
2.6.3 Chromosomal DNA extraction from *E.coli* 31
2.7 DNA MANIPULATIONS 31
2.7.1 DNA precipitation 31
2.7.2 Digestions 31
2.7.3 Phenol-chloroform extractions 32
2.7.4 Dephosphorylation 32
2.7.5 Blunt ending of DNA 33
2.7.6 Ligation of DNA 33
2.7.7 Agarose gel electrophoresis 33
2.7.7.1 Fragment size determination and quantification 33
2.7.7.2 Photography of agarose gels . . 34
2.8 PLASMID SCREENS 34

TABLE OF CONTENTS

ABSTRACT i

DECLARATION ii

DEDICATION iii

ACKNOWLEDGEMENTS iv

PUBLICATIONS FROM THIS THESIS v

TABLE OF CONTENTS vi

LIST OF TABLES xiv

LIST OF FIGURES xvii

1.0 INTRODUCTION 1

1.1 NOCARDIOFORM BACTERIA 2

1.1.1 Habitat 2

1.1.2 Taxonomy 2

1.1.3 Uses 4

1.1.4 Pathogenic nocardioforms 5

1.2 RIFAMPICIN 8

1.2.1 Ansamycins 8

1.2.1.1 Rifamycins 8

1.2.1.2 Streptovaricins 10

1.2.1.3 Other ansamycins 11

1.2.2 Chemical aspects of rifampicin . . . 12

1.2.3 Antimicrobial action of rifampicin . 13

1.2.4 Rifampicin in chemotherapy 14

1.3 ANTIBIOTIC RESISTANCE 15

1.4 INACTIVATION OF RIFAMPICIN 17

1.5 AIMS OF THIS WORK 19

PUBLICATIONS FROM THIS THESIS

Dabbs ER, Gowan B, Andersen SJ (1990) Nocardioform arsenic resistance plasmids and construction of *Rhodococcus* cloning vectors. *Plasmid* 23:242-247

Andersen SJ and Dabbs ER (1991) Cloning of nocardioform DNA confering the ability to inactivate rifampicin. *FEMS Microbiol.Lett.* 79:247-250

Dabbs ER, Gowan B, Quan S, Andersen SJ (1995) Development of improved *Rhodococcus* plasmid vectors and their use in cloning genes of potential commercial and medical importance. *Biotechnology (Moscow)* 9:129-135

Dabbs ER, Yazawa K, Mikami Y, Miyaji M, Andersen SJ, Morisaki N, Iwasaki S, Shida O, Tagaki H, Kadowaki K (1995) Rifampicin inactivation by *Bacillus* species. *J. Antibiot.* 48:815-819

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to all the people who have encouraged me in producing this work; to Dr Eric Dabbs for giving me a solid foundation in microbial genetics and the expertise that is required to work in this field, for giving me the opportunity to work on my own and develop the skills required to continue after the Ph.D.; to the staff and postgraduates of the Department of Genetics for their availability and willingness that made things easier; to Selwyn Quan for his untiring willingness to help in all situations, with thinking through results, with battling together to get the sequencing up and running, all computer difficulties and the production of the figures that appear in this thesis; to Dr Bhavna Gordhan for her friendship, her encouragement and her availability, not only in the "Forty Days" but throughout the work; to Elvera D'Andrea whose help made my life easier; to Professor Valerie Mizrahi and the staff of the Molecular Biology Unit of the SAIMR for pointing me in the right direction of sequencing single stranded DNA; to Wendy Aykroyd and Judy Masterson for helping me with typing from time to time; to Graham Aykroyd for taking colour photographs; to my family, my parents - Dick and Margaret Andersen, my sister - Wendy Aykroyd, my boyfriend - Adrian van Niekerk, thank you for putting up with me, for believing in me and for praying for me; to all who have prayed with me and for me and stood with me, you know who you are, thank you. I wouldn't have got this far without your love and the love, faithfulness, strength and joy of our Lord Jesus.

I acknowledge and appreciate the financial contributions made by the University of the Witwatersrand in their Senior Bursary Program, the Foundation for Research and Development and the part-time employment offered by the SAIMR.

DEDICATION

This is dedicated to my family.

DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination at any other university.

_____

27 day of March, 19 94.

ABSTRACT

Rifampicin is one of the major antibiotics used in the treatment of *Mycobacterium tuberculosis*. This organism causes tuberculosis. Other related nocardioform bacteria which include the *Rhodococci* are opportunistic pathogens in AIDS patients. These organisms cause tuberculosis-like disease and are currently treated with rifampicin and other drugs. The presence of a low level rifampicin resistance mechanism was identified in seven rhodococcal strains and five other related and unrelated bacteria. This work describes the cloning of the gene responsible for rifampicin inactivation from two of the rhodococcal strains, *Rhodococcus erythropolis* and *Rhodococcus equi*. A *Rhodococcus-E.coli* shuttle vector was constructed and used to build a genomic library of *Rhodococcus erythropolis* ATCC12674. The gene was cloned by complementation of a non-inactivating mutant. The gene from *Rhodococcus equi* was cloned in the same way. The subcloned DNA from *Rhodococcus equi* was sequenced and shown to contain an open reading frame (ORF) of 1437bp with an ATG start codon. Regulatory sequences similar to those found in *Streptomyces* were identified upstream of the start codon. The ORF encoded a 479 amino acid sequence that is homologous to flavoprotein monooxygenases involved in antibiotic biosynthesis and monooxygenases involved in the degradation of aromatic compounds. The enzyme was expressed in *E.coli* and was shown to need FAD and utilise NADPH as a cofactor. The presense of this gene may have implications for the correct diagnosis and treatment of *Rhodococcus equi* infections with particular relevance to the third world.

Ask and it will be given to you; seek and you will find; knock and the door will be opened to you. For everyone who asks receives; he who seeks finds; and to him who knocks, the door will be opened. (Matthew 7:7-8)

MOLECULAR BIOLOGICAL CHARACTERISATION OF THE NOVEL RIFAMPICIN INACTIVATION MECHANISM IN NOCARDIOFORM BACTERIA

Susan Jean Andersen

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg 1996

people die every day as a result of tuberculosis. There are five first-line drugs currently used in treatment: isoniazid, rifampicin, streptomycin, pyrazinamide and ethambutol. Of these isoniazid and rifampicin are the most effective. Random mutations in a population of dividing cells occasionally produce a strain that is resistant to a particular drug. For this reason patients are treated with a combination of drugs to prevent drug resistant strains arising.

Leprosy, a disease frequently mentioned in biblical narratives, is the result of *M.leprae* infection. The onset of leprosy is insidious. It is the cooler issues of the body that are affected: skin, superficial nerves, nose, pharynx, larynx, eyes and testicles. Neurological disturbances can be seen from nerve infiltration and thickening with resultant anaesthesia and related symptoms. *M.leprae* has not been cultivated *in vitro* however inoculated armadillos develop extensive lepromatous leprosy. Armadillos spontaneously infected with leprosy have been found (quoted in Jawetz *et al*, 1982). The 2 drugs used effectively against infection are dapsone and rifampicin.

Several other mycobacterial strains are opportunistic pathogens often found in immunosuppressed patients, especially HIV patients. These include *M.avium-intracellulare* complex, *M.scrofulaceum, M.marinum* and *M.ulcerans*.

Nocardia species, specifically *N.asteriodes* and *N.brasiliensis*, are the main causes of nocardiosis. This is an opportunistic pulmonary disease of humans that may spread to the rest of the body. These organisms may produce mycetoma. Usually infection produces pneumonia but in some cases it is sub-clinical. If the bacteria spread through the blood to the brain abscesses are likely to form. Nocardiosis is more common in immunocompromised individuals (Jawetz *et al*, 1982).

Rhodococcus strains can be pathogenic to humans, animals or plants. *R.fascians* causes witches-broom or leafy galls in

need two hydroxyl groups on the ring to perform this function. The monooxygenases recognise acyclic compounds that contain at least one hydroxyl group and then hydroxylates another carbon either *ortho* or *para* to the first one. Nearly all bacterial pathways for the degradation of aromatic compounds transform initial substrates into intermediates that carry two or more hydroxyl groups on the aromatic ring. If two of the hydroxyl groups on a substrate are in the *ortho* position relative to each other, the ring cleavage by the dioxygenases occurs either between the hydroxyl groups (intradiol cleavage) or at a bond proximal to one of the two hydroxyl groups (extradiol cleavage). Most systems that have been described involve cleavage of *ortho* hydoxylated compounds, however a few have been described for *para* hydroxylated compounds. In these cases ring cleavage occurs between the proximal hydroxyl and the next substituent (Harayama *et al*, 1992).

1.1.4 Pathogenic nocardioforms

Several species of nocardioform bacteria are pathogenic to humans or animals or plants. The mycobacteria and the diseases they cause are most well known. They are pathogenic in many organisms including humans, guinea pigs, fowls and cattle.

Tuberculosis, as mentioned earlier, is caused by *Mycobacterium tuberculosis*. Other bacteria that cause tuberculosis-like disease include *M.bovis* and *M.kansasii* (Jawetz *et al*, 1982). These organisms are spread most commonly in droplets through coughing and sneezing and to a lesser extent through contact with contaminated objects. Those most at risk are the poor, the homeless, those with suppressed immunity and those who care for them (Benetar, 1991). Tuberculosis kills more people than any other infectious disease with about 3 million deaths each year. Another one billion people are believed to be infected (Strebel and Seager, 1991). These people have a 10% chance of developing the clinical disease in a life time, however if they are HIV positive the risk increases by 8% per annum. In South Africa 36

R. erythropolis. The type strain ATCC4277 does not clump significantly. Over 60 traits have been used in the development of a linkage map (Adams and Brownell, 1976).

Serological methods show antigens common with *Corynebacteria, Mycobacteria* and *Nocardia* and that ribosomes account for many of the cross reactions. All members grow well on standard media, they also grow well on simple media using amino acids and nitrate as nitrogen sources and a host of sugars and organic acids as carbon sources.

1.1.3 Uses

The Rhodococci are extremely versatile in their metabolic capabilities. Of pharmacological interest is the ability of these organisms to convert cholesterol into substances which are the precursors of steroid hormones or oral contraceptives (Ferreira *et al*, 1984). These organisms also utilise some unusual compounds such as acyclic hydrocarbons, nitroaromatic compounds and polycyclic hydrocarbons. In a study of soil and marine environments in Kuwait these bacteria were most abundant and more efficient than other bacteria known to be active oil degraders (Sorkhoh *et al*, 1990). They are also known for their ability to degrade lignin-related compounds and humic acid, as well as surfactants (MacDonald *et al*, 1981), pesticides (Ferguson and Kate, 1981) and acrylamide (Arai *et al*, 1981). The degradation of phenol (Houder *et al*, 1981) and pyrene (Walter *et al*, 1991) has also been described.

The ability to degrade cyclic compounds is dependent on enzymes that are able to cleave ring structures. There are several ways in which this might happen. The ring may be directly cleaved or it may be modified first, providing a substrate for the enzyme that is involved in cleavage. The enzymes involved are usually oxygenases, either monooxygenases or dioxygenases which incorporate one or two molecules of oxygen into the substrate. Dioxygenases are the enzymes that cleave the aromatic ring. They

mycelia, which in some cases is short lived and breaks up into bacillary or coccoid elements, that may be flagellated. These bacteria can be divided into two types - the oxidative forms which are soil dwellers and the fermentative types that occur in the natural cavities of man or animals. The cell walls contain meso-diaminopimelic acid, tuberculostearic acid and mycolic acids of variable carbon-chain length.

The genus *Rhodococcus* was resurrected and continues to provide a niche for actinomycetes previously assigned to such genera as *Arthrobacter, Brevibacterium, Corynebacterium, Mycobacterium,* and *Nocardia* (Goodfellow and Cross, 1984). The genera which are currently classified as nocardioform actinomycetes include *Mycobacteria, Corynebacteria, Nocardia, Gordona, Tsukamurella* and *Rhodococcus* (Goodfellow, 1986).

One of the strains used in this study, *Rhodococcus erythropolis* has previously been designated *Jansenia canicruria* which was once included in *Mycobacterium rhodochrous, Nocardia calcarea, Nocardia erythropolis* and *Nocardia globula* (Goodfellow and Minnikin, 1977). *Rhodococcus equi* was originally assigned to the genus *Corynebacterium*, it spent a brief time in the genus *Mycobacterium* under the name *M.equi* before being reassigned to the *Rhodococcus*.

The taxon *Rhodococcus* currently accommodates 14 species according to the 1986 edition of Bergey's manual of Systematic Bacteriology. The *Rhodococcus* species are Gram positive, partially acid fast, aerobic, chemo-organotrophic, catalase positive and have a high GC ratio of 63 - 72%. They are non motile and some strains produce feeble aerial hyphae. They have no distinctive morphological features other than the ability to form hyphae and fragment into rods and cocci. Considerable heterogeneity is evident.

Genetic studies have been hampered by slow growth and the tendency of the organism to clump, thus work has centred around

1.1 NOCARDIOFORM BACTERIA

1.1.1 Habitat

Nocardioform bacteria are predominantly soil dwelling microorganisms, found across the spectrum of soil types. They have been associated with the roots of dune plants, in virgin and ploughed land as well as in forest soil and peat bog (Cross et al, 1976). Some species possess mechanisms that enable them to degrade humic acid and hydrocarbons which suggests that they are able to degrade certain plant materials including leaf surface waxes.

Although most nocardioforms are non-pathogenic, the soil is thought to be a reservoir of some pathogenic forms. These result in cutaneous infections on the feet, actinomycetoma pedis, or on the back from carrying soil contaminated sacks. These bacteria have also been isolated from fresh water but in low numbers. They are present in cow dung, pasture grass and in activated sludge and there is some evidence for marine nocardioforms. Other species have a symbiotic relationship with blood sucking arthropods. Studies suggest that these insects do not reach sexual maturity in the absence of the bacteria. These bacteria have been isolated from their alimentary tracts (Cross et al, 1976).

1.1.2 Taxonomy

Nocardioform bacteria have a checkered taxonomic history. They are classified as actinomycetes. However the same strain, depending on the observer, may be classified in the genus *Corynebacterium, Arthobacter, Mycobacterium* or *Nocardia*. This was the perception of Gordon in 1966. Since then more in depth studies have been carried out resulting in the refining of the taxonomy.

The actinomycetes are filamentous bacteria. They generally form

1.0 INTRODUCTION

Figure 3.21 Deletions of the 8,3kb fragment and there concomitant phenotypic expression 104
Figure 3.22 Deletions of the 5kb *BamHI-BglII* fragment and there concomitant phenotypic expression 105
Figure 3.23 Hybridisation of *R.erythropolis* DNA and *E.coli* DNA with the 8,3kb fragment 114
Figure 3.24 Hybridisation of *iri*$^+$ DNA to *R.erythropolis*, *R.australis*, *M.smegmatis* and *E.coli* DNA . 115
Figure 3.25 Transformants from C1 library complementing the Ri8-1 mutation 118
Figure 3.26 *R.equi* complementing DNA digested with *BglII* 120
Figure 3.27 Restriction map of 5kb complementing DNA fragment from *R.equi* 122
Figure 3.28 Inactivation of rifampicin by two cloned *iri* genes from *Rhodococcus* species 124
Figure 3.29 Plasmid c screened for deletions of *AgeI*, *Asp718*, *ClaI* and *NotI* fragments 125
Figure 3.30 Summary of the deletions made of 5kb *BamHI-BglII* fragment from *R.equi* 131
Figure 3.31 Diagrammatic representation of sequencing strategy 139
Figure 3.32 Open reading frame in *BclI-ClaI* 140
Figure 3.33 The sequence of the *iri* gene and flanking regions 142
Figure 3.34 Absorption spectra of rifamycin compounds 157
Figure 3.35 TLC analysis of rifamycin compounds . . . 159
Figure 3.36 Southern blot analysis for homology with *iri* and the rifamycin producer organism . . . 162
Figure 4.1 Comparison of deduced amino acid sequences of the aminoterminal of the *iri* gene . . . 181
Figure 4.2 Comparison of deduced amino acids from the flavin binding region of the *iri* gene . . 182
Figure 4.3 Structures of KRM 1648 and KRM 1657 . . . 189

LIST OF FIGURES

Figure 1.1	Structures of rifamycins	10
Figure 1.2	Structures of streptovaricins	11
Figure 3.1	Inactivation of rifampicin by two rifampicin sensitive *R.erythropolis* strains	53
Figure 3.2	Inactivation of rifampicin by rifampicin resistant strains	54
Figure 3.3	Restriction map of pDA30	63
Figure 3.4	Restriction map of pEcoR251	64
Figure 3.5	Agarose gel of *BamHI* and *BglII* digests of putative shuttle vectors,showing different orientations	69
Figure 3.6	Diagrammatic representation of both orientations of the shuttle vector	70
Figure 3.7	Restriction map of pDA37	73
Figure 3.8	*BglII* digested DNA from plasmid screens of 16x transformants	75
Figure 3.9	An agarose gel of transformants #1-#6 . .	79
Figure 3.10	Inactivation of rifampicin	83
Figure 3.11	Inactivation of rifampicin by *R.erythropolis* strains resistant to rifampicin	84
Figure 3.12	Chloroform extraction of rifampicin and rifampicin degradation products in the presence and absence of the *iri* gene . . .	85
Figure 3.13	Preliminary restriction map of #6	86
Figure 3.14	*HindIII* fragments from different complementing clones	88
Figure 3.15	*Asp718* deleted plasmid	91
Figure 3.16	*NotI* deletions of the fragment	92
Figure 3.17	A plasmid screen of Ri8-1 containing RV-Sca	96
Figure 3.18	Diagrammatic representation of pDA71 . . .	97
Figure 3.19	Both orientations of the *BamHI-BglII* fragment ligated into pDA71	99
Figure 3.20	Detailed restriction map of 5kb *BamHI-BglII* fragment	102

Table 3.17	Comparison of rifampicin inactivation between culture containing recombinant plasmids and those cured of these plasmids	81
Table 3.18	Rates of inactivation by *R.erythropolis* .	85
Table 3.19	Restriction enzyme analysis of the fragment containing the rifampicin inactivating gene from ATCC4277	100
Table 3.20	Inactivation of rifampicin by hypersensitive mutants containing the *iri*$^+$ clone	107
Table 3.21	Rifampicin inactivation by DNA complementing the Ri14 mutation	109
Table 3.22	Increase in rifampicin resistance conferred to Ri mutants by cloned DNA	110
Table 3.23	Resistance conferred by *iri*$^+$ DNA to ansamycins	111
Table 3.24	Sensitivity to streptolydigin	112
Table 3.25	Inactivation of erythromycin by *R.erythropolis*	113
Table 3.26	Comparative numbers of restriction sites in 5kb inserts from *R.erythropolis* and *R.equi*	122
Table 3.27	Complementation of the rifampicin hypersensitivity mutation in Ri8-1 by deletion and insertion plasmids of *R.equi* DNA	127
Table 3.28	Inactivation and decolorisation of rifampicin mediated by deletion and insertion plasmids of *R.equi* DNA	127
Table 3.29	BLASTN results of similarity to *iri* gene .	143
Table 3.30	BLASTP results of similarity to Iri polypeptide	143
Table 3.31	Expression of *iri* genes in *E.coli*	150
Table 3.32	Sensitivity of CSR603 to successive bursts of 50J/m^2	152
Table 3.33	Cofactor requirements of Iri	153
Table 3.34	Decolorisation of rifamycin compounds by Iri	156

LIST OF TABLES

Table 1.1	Ansamycins and their producer organisms .	12
Table 2.1	Nocardioform bacteria	22
Table 2.2	Rifampicin hypersensitive mutants	23
Table 2.3	*E.coli* strains	24
Table 2.4	*B.subtilis* strain	24
Table 2.5	Nocardioform plasmids	25
Table 2.6	*E.coli* plasmids	25
Table 2.7	Shuttle plasmids	26
Table 2.8	Bacteriophages	26
Table 3.1	Sensitivity of various strains to ansamycin drugs	52
Table 3.2	Inactivation and decolorisation of 20μg/ml rifampicin by various strains of bacteria	55
Table 3.3	Inactivation of rifamycin SV and streptovaricin by various strains of bacteria	56
Table 3.4	Zone inhibition assay to identify the antibiotic released by DSM40512	58
Table 3.5	Optimisation of glycine concentration for transformation	61
Table 3.6	Compatible cohesive ends	65
Table 3.7	pDA30 as a cloning vector	66
Table 3.8	Transformation of CW22 with pDA30 and pDA26	67
Table 3.9	Transformation of λMM294 with pEcoR251 ligated DNA	68
Table 3.10	ShV1, ShV3 and ShV 4 as cloning vectors .	71
Table 3.11	ShV15 as a cloning vector	72
Table 3.12	Efficiency of pDA37 on transformation . .	73
Table 3.13	Optimisation of DNA ratios for ligation .	74
Table 3.14	Sensitivity of mutants to rifampicin . . .	77
Table 3.15	Resistance to rifampicin conferred by #1-#6	80
Table 3.16	Inactivation of rifampicin by complementing transformants	80

3.9.4 Construction of further plasmids for overexpression 147
3.9.5 Enhanced expression of *iri* in *E.coli* 149
3.9.6 Protein expression using maxicells . 150
3.9.7 Cofactors required by Iri for function 153
3.9.8 Intracellular location of enzyme . . 153
3.9.9 Inhibition studies with methimazole . 154
3.9.10 Effect of Iri on rifamycin derivatives 155
3.9.11 Effect of Iri on various aromatic compounds 160
3.9.12 *A.mediterranei* and *iri*$^+$ DNA 161

4.0 DISCUSSION 163
4.1 *Rhodococcus equi* 164
4.2 RIFAMPICIN RESISTANCE 167
4.3 RIFAMPICIN INACTIVATION 169
4.4 CLONING VECTORS 172
4.5 CLONING *iri* GENES 175
4.6 SEQUENCING 177
4.7 MONOOXYGENASES 179
4.8 POLYKETIDE HYDROXYLASES 183
4.9 RIFAMPICIN AND OTHER RIFAMYCINS 188
4.10 CONCLUSION 191

5.0 APPENDICES 192
APPENDIX A 193
APPENDIX B 201
APPENDIX C 204

6.0 REFERENCES 206

3.5.3 Homology with DNA from other strains 113
3.6 CLONING *iri*$^{+}$ DNA FROM OTHER NOCARDIOFORMS 116
3.6.1 From *R.australis* A554 116
3.5.2 From *A.oxydans* C1 117
3.6.3 From *R.erythropolis* DSM1069 119
3.6.4 From *R.equi* ATCC14887 119
3.6.4.1 Constructing a restriction map . 121
3.6.4.2 Inactivation of ansamycins by the *R.equi* clone 123
3.7 SUBCLONING OF *R.equi* FRAGMENT 124
3.7.1 Elimination of *AgeI*, *Asp718*, *ClaI* and *NotI* fragments 124
3.7.2 Insertion of the *ClaI* fragment into pDA71 126
3.7.3 Complementation, inactivation and decolorisation studies on newly constructed p. .s 126
3.7.4 Elimination of the two *BclI* sites . . 128
3.7.5 Attempted deletion of *BstYI* fragment 129
3.7.6 Insertion of *SmaI-ClaI* fragment . . . 129
3.8 SEQUENCING A RIFAMPICIN INACTIVATION GENE 131
3.8.1 Production of subclones in pUC vectors 132
3.8.2 Sequencing of pUC subclones 132
3.8.3 Sequencing single-stranded templates 134
3.8.4 Sequencing of *Sau3A* partial digests . 137
3.8.5 Subclones made with *SacI* and *ClaI* . . 138
3.8.6 Computer aided analysis of sequenced DNA 140
3.9 PROTEIN EXPRESSION AND CHARACTERISATION OF *iri* GENE 145
3.9.1 Checking existing plasmids for overexpression 145
3.9.2 Shifting reading frames in *BclI-ClaI* 146
3.9.3 Checking for overexpression in *Rhodococcus* 147

of the active drug to an inactive derivative. The last of these would be characterised by the reduction in rifampicin concentration in the media containing *R.erythropolis* cultures prior to resumption of growth.

The results of the experiments suggested that this nocardioform species had a means of inactivating rifampicin. Further experiments were performed to exclude the possibility that the antibiotic was simply being absorbed out of the medium by the cells. Changes in the absorption spectrum of the drug during the course of the putative inactivation were monitored.

This approach was facilitated by the isolation of a spontaneous mutant of *R.erythropolis* resistant to high levels of rifampicin, ATCC12674R1. Changes in the absorption spectrum were much easier to monitor when more rifampicin was present in the medium, than when the wild-type strain was employed. The mutant inactivated the antibiotic faster than the parental strain probably because the inhibitory effects of rifampicin were absent in the mutant, allowing a faster rate of synthesis of the putative inactivating enzyme. Results of experiments done on absorption spectra demonstrated that the reduction in bacteriostatic activity was not principly the result of the antibiotic being absorbed out of the medium by the cells of the nocardioform.

A lag was invariably observed before a culture challenged with rifampicin started to inactivate the compound, suggesting that this activity was inducible rather than constitutive. The possibility was investigated by adding chloramphenicol, an inhibitor of protein synthesis, to *R.erythropolis* strain 12674R1 cultures at the same time as rifampicin.

It was found that a culture of nocardioform in which protein synthesis was blocked did not act on the rifampicin molecule in the manner previously observed. This also strongly suggested that the activity responsible was inducible rather than constitutive.

protein synthesis by the drug

* alteration of membrane proteins leading to decreased penetration of the antibiotic or an efflux mechanism that results in decreased accumulation in the cell
* an increased production of antibiotic sensitive protein
* an increased production of a metabolite that antagonises the antibiotic
* the introduction by a plasmid of a gene coding for a bypass to a blocked metabolic step
* the diversion to an alternate pathway circumventing blockage by the antibiotic

1.4 INACTIVATION OF RIFAMPICIN

In work by Koch and Gross (1979) evidence was presented for the inactivation of rifampicin by *E.coli*. They attributed the ability of susceptible bacteria to survive in the presence of the drug to an inducible detoxification mechanism. Later Dabbs (1987) identified a rifampicin inactivation mechanism in some nocardioform species.

Most of his work was done on *Rhodococcus erythropolis* strain ATCC12674. This work showed that cultures of this organism challenged with rifampicin stopped growing. However, after a lag phase of 6-8 hours, growth resumed. The same level of rifampicin resistance was observed in single colonies isolated from cultures of this organism, irrespective of whether they had been exposed to this antibiotic or not. This, together with the shortness of the lag phase that occurred before growth resumed after the antibiotic challenge, suggested that the selection of rifampicin resistant mutants was unlikely.

Dabbs (1987) deduced that this organism possessed some sort of inducible resistance mechanism. Several mechanisms were possible including reduced cell permeability to the drug, modification of DNA-dependant RNA polymerase to avoid inhibition and conversion

bacillus, if we do not adopt better ways of arresting the trend towards resistance than those in current use". (Lowbury and Ayliffe, 1974)

We are halfway through those forty years and the publicity of the South African National Tuberculosis Association (SANTA) now carries the slogan "Help us cure Tuberculosis while its still curable".

Resistance to antibiotics seems to have been around for as long as the antibiotics themselves. In the absence of antibiotics a mutation to resistance is generally disadvantageous to the cell. In some cases essential proteins are altered so as not to be affected by the drug, resulting in some cases in a loss of efficiency. A case of resistance to antiseptics was reported as far back as 1887 when *Bacillus subtilis* became "acclimatised" to mercuric chloride and boric acid (Kossiakoff MG, 1887). Resistance to arsenicals in trypanosomes was detected in 1907 (Ehrlich P, 1959) and parafuchsin resistance in these same organisms in 1909. The use of sulfonamides in chemotherapy saw resistant pneumococci by 1940. In 1941 penicillin proved ineffective in the treatment of some patients and by 1944 streptomycin resistant meningococci and gonococci had appeared (Koch, 1981).

The studies done on the mechanisms of resistance show that these are based on genetic changes that induce or alter specific proteins that can be found on plasmids, transposons or in the chromosome. Koch (1981) classifies the biochemical bases of antibiotic resistance into nine categories :

* enzymatic detoxification
* genetic alteration of an enzyme so that is no longer binds to the antibiotic and it therefore not inhibited by it
* genetic alteration of a protein component of protein synthetic machinery so that is fails to bind the antibiotic
* alteration of rRNA by methylation preventing inhibition of

the drug of choice in the treatment of tuberculosis (Maggi et al, 1966). It is not usually administered alone but in conjunction with one or two other antitubercular drugs. The drug has been used against *M.leprae*, *Staphylococcus aureus*, *Bacteroides* and *Neisseria gonorrhoea*. It has also been successfully used in cases of chronic granulomatous disease, mycobacterial cervical adenitis, acne, bronchitis, brucellosis, gastroenteritis, meningitis, *Legionella* pneumonia. It is also an anti-trachoma agent, but has no effect on fungi. High doses given to mice show no carcinogenic effects (Glaz, 1974).

The incidence of side-effects is usually influenced by the administration level. The toxic effects that have been reported are many and varied (Wade A, 1979). Gastro intestinal side-effects include nausea, vomiting, diarrhoea, anorexia and abnormalities in liver function. Fatalities have been reported after administration to patients with pre-existing liver disorders. Altered kidney function and renal failure have been reported due to hypersensitivity. Other side effects include skin rashes, urticaria, eosinophilia, leucopenia, thrombocytopenia, purpura, haemoptysis as well as confusion, drowsiness, weakness, ataxia, dizziness, peripheral neuropathy, blurred vision, transient hearing loss and menstrual irregularities.

1.3 ANTIBIOTIC RESISTANCE

The discovery of antibiotics and their subsequent application to the chemotherapy of bacterial disease revolutionised clinical medicine. However, the rise in drug resistant strains of many pathogenic bacteria has heralded warnings such as this one :

"We have good reason to be anxious about the future. It seems likely that in forty years time a doctor may recall the lifespan of antibiotic therapy as a short episode in the long art of medicine; that, at least, might become the outcome for certain bacteria, such as *Staphylococcus aureus* and the tubercle

Resistance to rifampicin is conferred by the mutational alteration of the β-subunit of RNA polymerase. The mutation rate from sensitivity to resistance is of the order of 10^{-7}. This resistance is due to a chromosomal DNA mutation. Rifampicin is one of the few chemotherapeutic agents for which no transferable resistance has been observed.

Studies on the growth inhibitory effects of rifampicin have lead Romero *et al* (1971) to the conclusion that resistant strains may not always contain resistant RNA polymerases. They reckon that overall resistance is due to resistant RNA polymerase and permeability of the cell.

Rifampicin has also been found to deplete levels of cellular glutathione in *M.smegmatis*. Glutathione functions in the detoxification of various xenobiotics (Kumar *et al*, 1991).

1.2.4 Rifampicin in chemotherapy

Rifampicin is well absorbed after oral administration. It is widely distributed through the body with 75-88% being bound to plasma proteins. It can cross into the cerebrospinal fluid, enter bone, cross the placenta and is found in milk. *In vivo* it is deacetylated to form desacetylrifampicin which has antibacterial activity. The antibiotic is excreted in bile and so it is used to treat biliary infections.

Rifampicin induces drug metabolising enzymes in the liver. It is also responsible for the increased metabolism of oral contraceptives, hydrocortizone, hexobarbitone, tolbutamide and its own metabolism. This drug may also reduce the activity of anticoagulants, corticosteroids, oral hypoglycaemic drugs, anticonvulsants and oral contraceptives.

Rifampicin is active against both extracellular and intracellular organisms. It is effective against Gram positive bacteria especially against the mycobacteria. For many years it has been

It has a chemical formula C43 H58 N4 O12 and a molecular weight of 823,0. It is also 3- (4-methylpiperazin-1-yliminomethyl) rifamycin SV.

This compound is an odourless red-brown crystalline powder that is slightly soluble in water and acetone but is soluble in chloroform and methanol. In a 1% suspension it has a pH of 5-6,5. At pH 7,4 it has broad poorly defined maxima at 237, 254, 334 and 475nm.

1.2.3 Antimicrobial action of rifampicin

Rifampicin is an inhibitor of DNA-dependant-RNA polymerase. It binds to the enzyme inducing a conformational change in the enzyme (Wehrli and Staehelin, 1970). The antibiotic has little effect on the formation of the first phosphate diester bond but it does inhibit the synthesis of the next bond by interfering with the binding of the incoming purine nucleoside triphosphate to the active site of the enzyme. However, once the synthesis of the RNA chain progresses beyond the second or third phosphate diester bonds, further chain elongation is insensitive to the action of rifampicin. The drug does not prevent the binding of RNA polymerase to DNA (Sippel and Hartmann, 1970).

It is not known what bonds hold the rifamycin-enzyme complex together. Although the binding is tight it is reversible so no covalent linkage is involved. Known data seems to suggest that the oxygens at C1, C8, C21 and C23 may be involved in the formation of hydrogen bonds with specific enzyme functional groups. Modifications made at C3 and C4, which are on the opposite side of the aromatic ring, give active compounds (Horn and De Ranter, 1084). It is the structure of the substituents that alters the antibacterial activity and the transport through the bacterial wall and membrane. As the rifamycins enter the cells by passive diffusion their physiochemical properties, especially their lipophilicity, are expected to be major factors in their abilities to cross the cell barrier.

Table 1.1 Ansamycins and their producer organisms

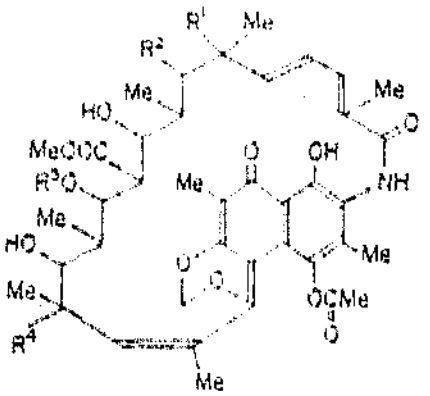
Producer	Product	Activity
Napthalenic chromaphore		
Micromonospora halophitica	Halomycin A,B,C	Antibacterial
Streptomyces tolypophorus	Tolypomycin Y	Antibacterial
Streptomyces collinus	Napthomycin	Antibacterial, Antifungal
Streptomyces X12384	Napthomycin	Antibacterial, Antifungal
Streptomyces E784	Actamycin	Antibacterial, Antifungal
Streptomyces achromogenes	Rubradirins	Antibacterial

Producer	Product	Activity
Benzenic chromophore		
Streptomyces hygroscopicus	Geldanamycin	Antiprotozoal Antitumour
	Herbimycins A,B	Herbicidal
Nocardia C15003	Ansamitocins	Antitumour
Nocardia C14919	Macbecins I,II	Antitumour

1.2.2 Chemical aspects of rifampicin

Rifampicin is the name accepted by the World Health Organisation (WHO), however rifampin is the name recognised in the United States of America. This semi-synthetic antibiotic is one of the 500 derivatives prepared by Ciba of Basel and Lepetit of Milan in collaboration with one another (Kradofler, 1968).

prevalent and streptovaricin C is less so. These compounds bind to prokaryotic RNA polymerase and have also been shown to inhibit the reverse transcriptase of oncogenic viruses. Pox virus replication is inhibited by the streptovaricins which are also active against a mouse leukaemia virus. Streptovaricin is both tuberculostatic and tuberculocidal. Studies have shown satisfactory treatment in animals but not in humans (Kersten and Kersten, 1974).



	R^1	R^2	R^3	R^4
Streptovaricin A	OH	OH	$COCH_3$	OH
B	H	OH	$COCH_3$	OH
C	H	OH	H	OH
D	H	OH	H	H
E	H	=O	H	OH
G	OH	OH	H	OH
J	H	$OCOCH_3$	H	OH

Figure 1.2 Structures of streptovaricins

1.2.1.3 Other ansamycins

Below is a table of other known ansamycins, their producer organisms and their activities (Lancini, 1983).

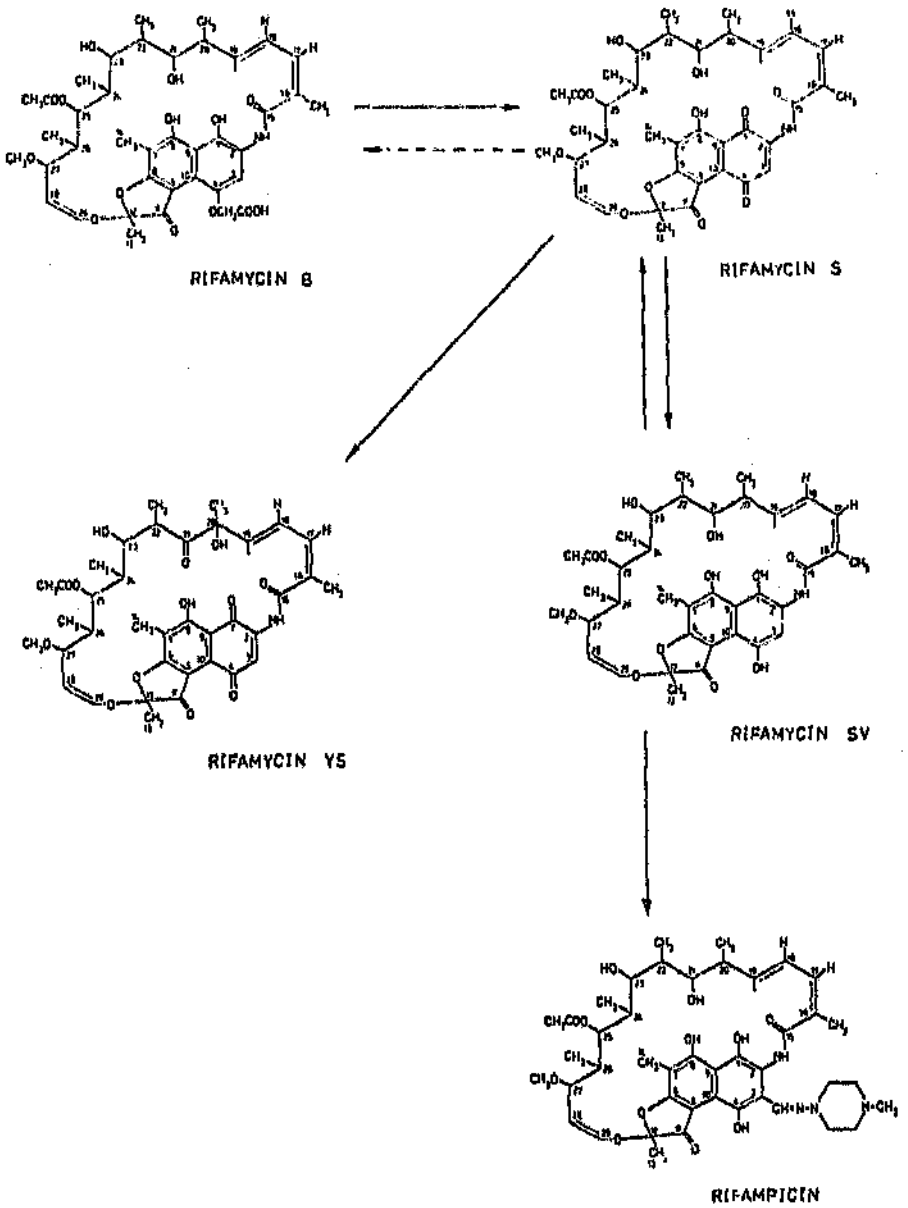


Figure 1.1 Structures of rifamycins

1.2.1.2 Streptovaricins

This is a complex of seven components produced by *Streptomyces spectabilis*. This microorganism was originally isolated from soil samples collected in Dallas. Streptovaricins A and B are most

collected in France. This organism was later classified as *Nocardia mediterranei* and more recently as *Amycolatopsis mediterranei*. The first rifamycin to be isolated was rifamycin B. Later it was shown that through mild oxidation and hydrolysis of this compound rifamycin S was produced. In turn the mild reduction of rifamycin S resulted in the production of rifamycin SV.

The rifamycins are structurally made up of a naphthohydroquinone nucleus fused to a five member ring and a 17 membered ansa chain connected to C2 and C12.

Rifamycin SV was introduced into clinical practice in 1961. It is strongly active against acid-fast bacilli including *M.leprae*. The drug is not active against resting bacteria but exerts a bactericidal effect on multiplying bacteria. It is active against most Gram positive bacteria including *S.aureus* but is less strongly active against *Brucella* and *Pasteurella* species and Gram negative organisms. The antibiotic inhibits RNA polymerase by forming stable complexes with it. However the RNA polymerase of eukaryotes is insensitive to the antibiotic. It is poorly absorbed through the gastro-intestinal tract.

Rifamycin B and rifamycin S were used to prepare a number of semi-synthetic rifamycins. Of all the semi-synthetic derivatives produced rifampicin is the most widely used.

1.2 RIFAMPICIN

1.2.1 Ansamycins

Rifampicin belongs to a class of antibiotics known as the ansamycins. This name was proposed by Prelog and Oppolzer (1973). Ansa means handle in Latin. These antibiotics have a characteristic chemical structure of an aromatic moiety spanned by an aliphatic bridge (or handle). This bridge joins the aromatic moiety at 2 non-adjacent positions. Of all the ansamycins discovered so far only two different aromatic moieties have been identified. Some have a naphthalene moiety and the others have a benzene origin.

These ansamycins are natural compounds produced by bacteria. By 1983 twenty ansamycin producers had been independently isolated (Lancini, 1983).

The structural differences in these antibiotics clearly correspond to the differences in their biological activity. The naphthalene ansamycins include the rifamycins, streptovaricins, actamycin, napthamycin tolipomycins and halomycins (Perlman D, 1977). These are antibacterial agents that act, for the most part, by inhibiting RNA polymerase. The benzene ansamycins include geldamycin and herbmycins, these are active on eukaryotic cells where they interfere with the cell replication mechanism.

The rifampicins are the only ansamycins that are industrially produced. Most of the studies that have been carried out have been done on this group and to a lesser degree on the streptovaricins and geldamycin.

1.2.1.1 Rifamycins

There are many rifamycins. The first producer organism to be identified was then classified as *Streptomyces mediterranei* (Margalith and Beretta, 1960). It was isolated from soil samples

dicotyledonous plants and the malformation of bulbs in monocotyledonous plants (Miller *et al*, 1980). Very few cases of human disease caused by rhodococcal strains have been reported. *R.rubropertinctus* was isolated from a patient with a tuberculosis-like disease (Hart *et al*, 1988). In a study conducted in Germany from 1979-1991 only one case of *R.equi* infection and 7 cases of *R.erythropolis* infection were treated (Schaal and Lee, 1992).

In total 30 cases of *R.equi* had been documented in 1991 (Harvey and Sunstrum, 1991). This organism is a well known animal pathogen first described by Magnusson in 1923 (cited by Emmons *et al*, 1991) as the cause of pneumonia in foals. Foals infected in the first 2 months of life usually develop lung abscesses and die (Lipsky *et al*, 1982). Horses over 3 months usually survive the infection (Smith, 1966). *R.equi* is also reported to cause urogenital infections, ulcerative enteritis and mediastinal lymph adenitis (Wilson, 1955), cervical adenitis in pigs (Smith, 1966) pneumonia, urogenital infections and lymph adenitis in cattle and fatal septicemic infections in sheep (Dennis and Bamford, 1966). These organisms are only moderately pathogenic in laboratory animals (Lipsky *et al*, 1982). The bacteria are found in the soil of animal runs where they survive for about a year.

The first human infection with *R.equi* (Golub *et al*) was reported in 1967 and the first case in an HIV patient (Sane and Durack) in 1986. Infection occurs mainly through the respiratory route with pneumonia as the commonest manifestation. As it often forms abscesses treatment is by antibiotics and sometimes by surgical resection. Of the reported cases, only one patient was not immunocompromised and there was an overall mortality rate of 20%. Rifampicin demonstrates excellent activity against isolates *in vitro* but very few drugs have been studied *in vivo* (Harvey and Sunstrum, 1991).

Following a 30 minute incubation at 37°C the lysate was centrifuged in a 50Ti rotor at 40 000 rpm for 30 minutes. DNA was decanted off and 4,4g caesium chloride was added and dissolved. After centrifugation at 15 000 rpm for 15 minutes, the DNA solution was poured out from under the scum. Ethidium bromide was added and the refractive index adjusted to 1,391-2 before it was loaded into Quickseal tubes and centrifuged for 16 hours at 40 000 rpm in a VTi65.2 vertical rotor. After extracting the DNA band, it was decolorised using a TE saturated butanol solution. The DNA was diluted with 1,6 volumes of TE and precipitated. The precipitate was dried and resuspended in TE or 3mM Tris, pH 8,0 and stored frozen.

2.6.2 Large scale preparation of plasmid from *E.coli*

Bacteria were grown in 100ml of LB containing 100µg/ml ampicillin to maintain the plasmid. Pelleted cells were resuspended in 3,25ml of Solution 1 and incubated at room temperature for 5 minutes then 6,5ml of Solution 2 was added and the contents mixed well. After 15 minutes at room temperature 5ml of Solution 3 was added, vigorously shaken and placed in an ice-water slurry for 10 minutes. The preparation was centrifuged for 15 minutes at 16 000 rpm and the decanted supernatant warmed to room temperature. Isopropanol (0,6 volumes) was mixed in and nucleic acid pelleted by centrifugation for 10 minutes at 15 000 rpm at room temperature or above. This DNA was washed with 5ml of ethanol and dried in a vacuum desiccator for 15 minutes. To the nucleic acid resuspended in 4,2ml TE, 4,4g of caesium chloride and 0,4ml of ethidium bromide were added, the refractive index adjusted to 1,389-1,390 and the preparation loaded into a Quickseal tube which was centrifuged for 16 hours at 40 000 rpm in a VTi65.2 rotor. The plasmid band was illuminated under long wave length ultra-violet light and extracted using a wide bore needle. The ethidium bromide was removed with TE-saturated butanol and the DNA was diluted with 1,6 volumes of TE and precipitated with 2,5 volumes ethanol. The precipitate was vacuum dried then resuspended in either TE or 3mM Tris, pH 8.0. The DNA solutions

1µl each of required co-factors at varying concentrations. These reactions were allowed to proceed from 2 hours to overnight. The active drug was assayed using the plate based method.

2.5 SELECTION OF SPONTANEOUS MUTANTS

Bacteria were grown to stationary phase, 100µl of culture was spread on an LA plate containing a high concentration of the drug. These were incubated for 4 to 5 days. Colonies that grew up were streaked onto the appropriate antibiotic containing plates.

Some of the nocardioform strains did not naturally maintain some of the smaller plasmids. Spontaneous mutants able to maintain these plasmids were obtained by transforming these strains with the plasmid DNA. The number of transformants was dramatically reduced. The transformants that then grew up were cured by growing in the presence of 20µg/ml ethidium bromide. These cells were washed and diluted 10^{-6} before 100µl was spread on an LA plate. The colonies were patched out on LA and a plate containing the selective pressure for the presence of the plasmid. Those colonies that grew only on the broth plate were subjected to plasmid screen analysis for the presence of the plasmid.

2.6 BULK ISOLATION OF DNA

2.6.1 Large scale preparation of DNA from nocardioforms

Organisms were grown for 2-3 days in TYG broth. Harvesting of the cells was achieved by centrifugation at 6000 rpm for 10 minutes. These were resuspended in 5ml 10mM Tris, 10% sucrose with 5mg/ml lysozyme and incubated at 37°C for 30 minutes. The cells were pelleted at 8000 rpm for 5 minutes and resuspended in 4ml TE with 1mg proteinase K dissolved in it. To this was added TE containing 10% SDS or as much as was needed to achieve partial clearing.

2.4.2 Quantitative assay

At the time of assay 1ml of the culture was harvested. The supernatant was removed after centrifugation, boiled and stored at -20°C until an assay using aliquots from each time interval could be undertaken. Of the thawed supernatants 100µl was added to a test-tube containing 4,9ml LB, a 1:100 dilution of the assay organism and the appropriate concentration of the antibiotic to which it was resistant. A set of standards was set up in the same way containing known concentrations of antibiotic in place of the supernatant. The turbidity of the cultures was measured by determining the OD at 540nm over the next few days. The OD was determined using a Spectronic 601 spectrophotometer. A standard curve was constructed of the OD of the assay organism versus the concentration in the antibiotic standards and from this the concentration in the supernatants of the experimental strains was determined.

2.4.3 Rate determination

Samples of the supernatant of inactivating cultures were taken every 20 - 30 minutes. The concentration of rifampicin in each sample was determined. These values were plotted on a semi-log graph against time. The rate of inactivation could be determined by measuring the slope of the graph.

2.4.4 *In vitro* assays

Cell extract was prepared by harvesting 25ml of *E.coli* culture containing appropriate plasmids. The cells were washed twice in assay buffer. They were resuspended in 2,5ml of assay buffer and sonicated for 3x 30 seconds on ice. The cell extract was then used directly in *in vitro* assays or stored in 20% glycerol at -20°C. Assays were done in a final volume of 200µl. Included in this mix was 10µl cell extract, 2-20µl rifampicin in methanol and

2.4 ANTIBIOTIC INACTIVATION ASSAYS

ALL RECIPES FOR SOLUTIONS CAN BE FOUND IN APPENDIX A

The ability of different strains and plasmid containing mutants to inactivate rifampicin and related antibiotics was assayed using two techniques, one a plate assay and the other a liquid assay. A preculture of each strain was grown to stationary phase in TY broth. An aliquot of 100µl of this preculture was inoculated into 5ml of fresh TY broth. These cultures were allowed to grow into exponential phase when the optical density was determined at 540nm. The cultures were equilibrated to the same optical density and then challenged with the antibiotic. For hypersensitive strains 2,0µg/ml was added and for resistant strains 20µg/ml was added. The time when the antibiotic was added was taken as time zero and assays carried out at various times after that. Various assay organisms were used, each containing an antibiotic resistance and each sensitive to rifampicin or the related antibiotics. These organisms include a spectinomycin, streptomycin resistant mutant of *Arthrobacter oxydans*, C1SS; a spectinomycin resistant *Bacillus subtilis*, 1A3-1.

2.4.1 Plate assay

A 100µl aliquot of the assay organism which had been grown in LB was spread on an LA plate containing a third of the normal concentration of agar to allow for easier diffusion of the antibiotic and an appropriate concentration of the antibiotic to which the assay organism was resistant. Wells were cut into the agar with the blunt end of a pasteur pipette. These wells were each filled with 60µl of the culture being tested. Each sample was boiled to denature any protein that could continue inactivating the drug after sampling. The plates were incubated at 4°C to allow for diffusion of the antibiotic. Subsequently they were placed at 37°C until the zones of inhibition became apparent.

2.2.3 Shuttle plasmids

These were stored frozen in *E.coli* lambda lysogens. This was in 33% glycerol at -70°C. Purified shuttle vectors were stored in TE or 3mM Tris, pH 8,0 at -20°C.

Table 2.7 Shuttle plasmids

Plasmid	Size	Characteristics	Origin
pDA26	14,5kb	As^R, Amp^R	E.Dabbs
pDA29	14,5kb	As^R, Amp^R	E.Dabbs
pDA37	14,5kb	As^R, Amp^R, *EcoR1*	This work
pDA38	14,4kb	As^R, Amp^R, *EcoR1*	This work
pDA71	8,8kb	Cm^R, Amp^R, *EcoR1*	Dabbs and Quan

All solutions not specified in the text are listed in the appendix.

2.3 PHAGES

The phages used were all derivatives of M13. They were stored as phage supernatants at 4°C. They were propagated by inoculating phage supernatant into LB with a 1:100 dilution of an overnight culture of JM101. These were grown with vigorous aeration for 5 hours. The supernatants were then harvested.

Table 2.8 Bacteriophages

Phage	Origin
M13K07	M.Kitakawa
M13mp18	V.Mizrahi
M13mp19	V.Mizrahi

2.2 PLASMIDS

2.2.1 Nocardioform plasmids

These were stored at -70°C in the appropriate nocardioform strains. Once the DNA had been purified from the strain it was resuspended in TE or 3mM Tris, pH8,0 or in water and kept frozen at -20°C.

Table 2.5 Nocardioform plasmids

Plasmid	Size	Characteristics	Origin
pDA21	15,0kb	As^R	E.Dabbs
pDA30	11,2kb	As^R	E.Dabbs
B9	9,5kb	As^R	E.Dabbs
Bc1-2	8,2kb	Cm^R	E.Dabbs

2.2.2 *E.coli* plasmids

These were maintained frozen in their host strains at -70°C. Once they had been purified from the strain they were stored frozen at -20°C in TE or 3mM Tris, pH8,0, or water.

Table 2.6 *E.coli* plasmids

Plasmid	Size	Characteristics	Origin
pACYC184	4,3kb	Cm^R, Amp^R	E.Dabbs
pBR322	4,4kb	Tc^R, Amp^R	Boehringer-Mannheim
pEcoR251	3,3kb	Amp^R, *EcoR1*	E.Dabbs
pUC118	3,2kb	Amp^R, M13, *lacZ*	M.Kitakawa
pUC19	2,7kb	Amp^R, *lacZ*	E.Dabbs
pGEM+3(f)	2,9kb	Amp^R, *lacZ*	H.Venter

Table 2.3 *E.coli* strains

Strain	Relevant characteristics	Origin
MM294	*endA1, thi-1, hsdR17,*	B.Bachmann
λMM294	λlysogen of MM294	E.Dabbs
MM294-1	MM294, Rif^R	E.Dabbs
GM161-1	*dam-4, supE44, hsdS1,* Rif^R	B.Bachmann
GM2929-1	*dcm-6, dam13::Tn9, recF143 hsdR2, supE44,* Rif^R	B.Bachmann
JM101	*supE44, endA1, Δ(lac-proAB)*	
JM109	*recA1, supE44, endA1, hsdR17, Δ(lac-proAB)*	M.Kitakawa
CSR603	*recA1, uvrA6*	M.Kitakawa

2.1.3 *Bacillus subtilis* strain

These were stored in 33% glycerol at -70°C and on old dried plates. They were grown overnight at 37°C with vigorous aeration in LB or on LA plates. Fresh cultures were always used.

Table 2.4 *B.subtilis* strain

Strain	Characteristics	Origin
1A3-1	*cysA14, purA26, trpC2,* Spc^R	Bacillus Genetic Stock Center

2.1.4 Replica plating

In order to monitor the growth response of specific isolates on several plates which each contained different additives, single bacterial colonies were replica plated.

CW22 was produced by curing ATCC12674 of its large plasmid. Subsequently mutants were made of this strain that were hypersensitive to rifampicin. These are tabled below.

Table 2.2 Rifampicin hypersensitive mutants

Mutant	Characteristics	Origin
Ri1	As^R, Cd^R, Cm^R, rif^{HS}	E.Dabbs
Ri2	rif^{HS}	E.Dabbs
Ri3	rif^{HS}	E.Dabbs
Ri4	rif^{HS}	E.Dabbs
Ri6	rif^{HS}	E.Dabbs
Ri7	*iri*$^-$	E.Dabbs
Ri8	*iri*$^-$	E.Dabbs
Ri8-1	*iri*$^-$, Sm^R	This work
Ri9	rif^{HS}, Sm^R	S.Andersen
Ri10	rif^{HS}, Sm^R	S.Andersen
Ri11	rif^{HS}, Sm^R	S.Andersen
Ri12	rif^{HS}, Sm^R	S.Andersen
Ri13	rif^{HS}, Sm^R	S.Andersen
Ri14	*iri*$^-$, Sm^R	S.Andersen

where *iri*$^-$ lacks the ability to inactivate rifampicin and is hypersensitive to it.

Rifampicin resistant mutants were made of all nocardioform strains.

2.1.2 *Escherichia coli* stains

All *E.coli* strains were stored in 33% glycerol at -70°C and grown on LA or in LB. Strains were usually grown at 37°C overnight. However lambda lysogens of the strains were grown at no higher than 33°C to prevent the induction of the lytic cycle.

2.1 BACTERIAL STRAINS

2.1.1 Nocardioform bacteria

The strains used in this study were stored at -70°C in 33% glycerol. They were recovered by streaking onto tryptone-yeast agar (TYA) plates or Luria-Bertani agar (LA) plates. These plates were incubated at 28°C for between one and three days. When the strains were grown in liquid culture single colonies were inoculated into TY or LB broth. The cultures were aerated on a rotary drum at room temperature. If bulk cultures were grown the flasks were placed in a rotary water bath at 28°C. In some cases 0,5% Tween 80 was added to decrease clumping.

Table 2.1 Nocardioform bacteria

Strains	Characteristics	Origin
Amycolatopsis mediterranei DSM46095	Rifamycin Producer	German Culture Collection
Arthrobacter oxydans C1		E.Dabbs
Mycobacterium smegmatis DSM43756		German Culture Collection
Streptomyces		
lividans TK 54		E.Dabbs
spectabilis DSM40512	Streptovaricin Producer	German Culture Collection
Rhodococcus		
australis		
A448		N.Ferreira
A554		N.Ferreira
equi ATCC14887		N.Ferreira
erythropolis		
ATCC4277		N.Ferreira
ATCC12674	As^R, Cd^R, Cm^R	N.Ferreira
CW22	As^S, Cd^S, Cm^S	E.Dabbs
DSM1069		German Culture Collection
fascians DSM20669		German Culture Collection
rubropertinctus ATCC25594		N.Ferreira

2.0 METHODS

to inactivate rifampicin. By selecting for the complementation of the mutation the gene could be isolated.

However, cloning vectors for these strains at the beginning of this work had not been well developed or characterised so this was also done.

Several other *Rhodococcus* species were tested to see if their response to rifampicin resembled that of strain ATCC12674. Similar results were obtained, although rates of inactivation were invariably slower than those found with strain ATCC12674. *M.smegmatis* was also tested, a nocardioform which is not a member of the genus *Rhodococcus*. This strain behaved similarly with respect to rifampicin except the time scale was several times greater, which is in accordance with the slower growth rate of this organism.

Thus this work showed that *R.erythropolis* strain ATCC12674 possessed the ability to overcome the inhibitory effects of the antibiotic rifampicin by inactivating the drug. Some other nocardioforms appeared to possess a similar capacity, although to a lesser extent that strain ATCC12674. The approximate 50-fold increase in resistance resulting from the presence of this activity is comparable to that conferred by rifampicin resistance mutations in organisms such as *E.coli* and *B.subtilis* (Wehrli and Staehelin, 1971; Halling *et al*, 1977). The gene responsible for this was designated *iri* for inactivation of rifampicin.

1.5 AIMS OF THIS WORK

This work follows on directly from the work of Dabbs (1987) cited above. The purpose has been to investigate this novel rifampicin inactivation mechanism in the hope that these findings would aid in our understanding of rifampicin resistance in nocardioform bacteria. This may then enhance the knowledge of rifampicin resistance in the mycobacteria which in turn could lead to the modification of rifampicin or the use of appropriate DNA for detection of drug resistance genes.

The approach used in this work was to use mutants of *R.erythropolis* strain ATCC12674 that were unable to inactivate rifampicin as recipients. These recipients would be transformed with a genomic DNA library constructed from a strain that is able

2.13.5 Analysis of coding function

At the various stages in the subcloning procedure the *iri*⁻ mutant that had been transformed with each subclone of the original fragment in a vector, was patched onto a LA plate containing 40mM arsenate or 40µg/ml Cm and onto another similar plate that also contained 0,3µg/ml rifampicin. The complementation of the rifampicin hypersensitivity mutation was taken as an indication that the coding region had still been maintained in this latest construct. An absence of complementation indicated that a portion of the coding region was no longer present. On occasion a rifampicin inactivation assay was performed to verify the presence or absence of inactivation activity.

2.14 POLYACRYLAMIDE GEL ELECTROPHORESIS

Separating gels containing 10% acrylamide and stacking gels containing 6% acrylamide were prepared from a stock solution of 30% acrylamide and 0,8% bis-acrylamide which had been filtered. The gels were polymerised by the addition of TEMED and ammonium persulphate. Ten centimetre separating gels of 1,5mm thickness were poured with a stacking gel of 2,5cm. An equal volume of sample loading dye to sample was added. The proteins were dissociated by boiling the sample in the sample loading buffer for 5 minutes. Electrophoresis was carried out at a constant voltage of 120V until the bromophenol blue marker reached the bottom of the gel (in about 6 hours). The gels were stained overnight with a 0,1% coomasie brilliant blue solution R250 made up in a 5:4:1 solution of methanol: distilled water: acetic acid. The gels were destained by repeated washing on a 5:1:4 solution of menthol: acetic acid: distilled water.

ligation. The ligated DNA was transformed into MM294-1 and transformants screened. The DNA isolated by plasmid screen was digested with either *EcoRI* or *Asp718* to determined whether the fragment had been deleted. These digestions were run on agarose gels and visualised under UV light.

2.13.3 Exonuclease digestion

Exonuclease *Bal31* acts on linear DNA. Plasmids were linearised by digestion with either *BglII* or *HindIII* using the B buffer supplied by Boehringer-Mannheim. Digestions were done in a volume of 20μl. After digestion the following components were added to make up the buffer for *Bal31* exonuclease: 18μl B buffer, 100μl 1M NaCl, 60 μl dH_2O, 2μl 1M $CaCl_2$. 0,5units of the exonuclease was added and the digestion proceed for 2 hours at room temperature. The *Bal31* was then removed by phenol-chloroform extraction. The salts of the buffer were removed through ethanol precipitation. The DNA was resuspended in ligation buffer, ligated overnight and transformed into the nocardioform strain the next day.

2.13.4 Insertion of restriction fragments into other vectors

pDA37 was not the only vector used in the subcloning approach. Nocardioform plasmids that had unique restriction sites in non-essential regions of the plasmid were also used. These plasmids were prepared using the bulk preparation protocol. These plasmids were digested in the appropriate buffers. They went through a routine phenol-chloroform extraction, precipitation and resuspension in ligation buffer. Restriction fragments were prepared by isolation from low gelling agarose gels. These fragments were restricted with the same enzymes as the vectors or with enzymes that produced compatible cohesive ends. These were ligated into the vectors and transformed into the nocardioform.

1,5ml Eppendorf. This was washed with phenol and chloroform several times to remove unwanted agarose. The DNA was precipitated, dried and resuspended for further use.

2.12.3 Electroelution

Purified DNA was restricted and separated on an agarose gel. The fragment of interest was excised from the gel in the same manner as previously described. This gel fragment was placed in dialysis tubing in TBE buffer. This was returned to the electrophoresis chamber where it was subjected to a further 100V for 15 minutes. The current was then reversed for 30 seconds to dissociate the DNA from the dialysis tubing. The TBE containing the DNA was then transferred from the dialysis tubing to a sterile Eppendorf where it was washed with phenol and chloroform. The DNA was precipitated, dried and resuspended in TE for further use.

2.13 SUBCLONING

2.13.1 By insertion of fragments into the BglII site of pDA37

BglII digested DNA produces compatible sticky ends for ligation with *BamHI, BclI, BstYI* and *Sau3A*. Thus DNA digested by any of these enzymes could be ligated into the *BglII* site of pDA37. This ligated DNA was transformed into *E.coli*. The insertions were visualised after plasmid screen analysis.

2.13.2 Elimination of specific restriction fragments

A cloned *BglII* fragment was isolated on low gelling agarose. This purified fragment which was resuspended in the appropriate restriction enzyme buffer was digested with either *EcoRI* or *Asp718*. After 2 hours of digestion, the enzyme was removed using phenol and chloroform. The precipitated DNA was resuspended in ligation buffer and ligated overnight. pDA37 was treated in the same manner. A ratio of 4:1 insert to pDA37 was used for the

two or three other enzymes.

2.12 RECOVERY OF DNA FROM AGAROSE GELS

2.12.1 From low melting agarose

Low gelling agarose was made up in TBE and later TAE at a concentration of 0,8%. Digested DNA was run on the gels until the relevant band was clearly separated from the other bands. Gels were run at 4°C at 100-150V. The bands of interest were cut out of the gel using a scalpel blade whilst viewing in UV light. The pieces of agarose were placed in Eppendorf tubes and melted at 60°C for 15-30 minutes. These were then extracted 4 times with phenol and once with chloroform. The first phenol spin was done at room temperature and all the subsequent centrifugation steps were done at 4°C. The DNA was then ethanol precipitated and resuspended in an appropriate buffer.

2.12.2 Freeze-squeeze method

This is a modification of the method described by Tautz and Renz in 1983. DNA that had been purified on a CsCl gradient and subsequently digested was separated on an agarose gel. The appropriate fragment was cut out of the gel using a scalpel blade whilst viewing under UV light. The fragment was equilibrated in the dark with 10 volumes of 0,3M sodium acetate (pH 7,0) containing 0,1mM EDTA. This was done for 30-45 minutes with occasional shaking.

Once it had been equilibrated the gel slice was transferred to a 0,6ml Eppendorf tube which had been punctured in the bottom and plugged with glass wool. The tube had also been washed by placing it in a 1,5ml Eppendorf tube together with 150µl of sterile water and microfuging it. These tubes containing the gel slices were frozen at -20°C for 1 hour. They were then centrifuged immediately at 4°C for 5 minutes. The DNA could be found in the

was transformed using the nocardioform method into Ri8-1. A representative number of transformants (for a greater than 95% probability of obtaining the gene of interest) were obtained and these colonies were washed off the plates with 1-2ml of LB containing 40mM arsenate. As a control Ri8-1 colonies containing pDA30 were washed off in a similar manner. These transformants were shaken at room temperature for 2 hours to disperse the clumping. They were diluted serially from 10^{-1} to 10^{-6} and 100µl was spread on LA plates containing 40mM arsenate and 0,3µg/ml rifampicin. Cultures were equilibrated to similar optical densities. Only the colonies growing up would contain the *iri*$^{+}$ gene as 0,3µg/ml rifampicin is inhibitory to Ri8-1 alone. The colonies that grew up were screened for the presence of a plasmid. A control of a strain carrying the vector alone was done.

2.11 CONSTRUCTION OF A RESTRICTION MAP

The DNA to be mapped was digested using various enzymes. Enzymes that cut the DNA only once or twice and in some cases three times were used to construct the map. Single digests using only one enzyme were compared with double digests of the DNA. If possible all combinations of enzymes were used in double digestions. Each time a double digest was done, single digests of each of the enzymes used were performed simultaneously. All digestions were run on agarose gels. Gels with a concentration of 0,4% were used to more accurately size larger fragments (>2kb) when compared with λII markers. Gels of 1,2% were used to size the smaller fragments (<2kb) relative to λIII markers (for sizes of λ fragments see appendix).

The sizes of the fragments were obtained by reading the sizes off a standard curve drawn from the distance moved of the λ markers. A comparison of the fragments that were maintained in double digests with those that were not, led to the elucidation of the restriction map. Most sites were placed on the map relative to

dispensed in 0,2ml aliquots into prechilled Eppendorf tubes then kept for 2 hours on ice. The DNA in ligation buffer or TE was added to the cells and incubated at 0°C for a further 30 minutes with occasional agitation. After heatshocking at 42°C for one minute, 0,5ml of LB was added. These were incubated at 37°C for one hour without shaking. The cells were then spread gently on LA containing 200µg/ml ampicillin. These plates were placed at 37°C overnight whereafter the number of colonies were counted and further manipulations carried out.

When the recipient strain contained the temperature sensitive lambda lysogen all the steps previously carried out at 37°C were performed at 33°C.

2.9.3.2 With phage

The preparation of cells was similar to the cells prepared for plasmid. However the preculture of JM101 had to be grown in minimal media to maintain the F' factor. The first wash of the cells was with 0,1M $MgCl_2$ and then with $CaCl_2$ as before. For each transformation 0,2ml of competent cells were used. M13 clones in [illegible] were added, mixed and left at 4°C for 0,5-2 hours. The cells [illegible] heat-shocked at 42°C for 2 minutes just prior to plating. To the heat-shocked cells 0,2ml of a 3-4 hour growth of JM101 (grown from a 1:100 dilution in LB) was added together with 3ml salt agar that had been kept liquid at 46°C. This was gently mixed and poured onto a prewarmed LA plate. These were left to set on a flat surface for 15 minutes before being placed at 37°C overnight. For colour selection X-gal and IPTG were added to the agar before plating.

2.10 CLONING BY COMPLEMENTATION

The *R.erythropolis* ATCC12674 mutant Ri8-1 which contained the *iri*⁻ mutation served as a host. The library of the wild type *iri*⁺ *R.erythropolis* ATCC4277 which had been built up in *E.coli*

counted and further manipulations carried out.

2.9.2 Electroporation of nocardioforms

Cultures of 100ml were grown to an OD_{540} of 0,5. These cells were centrifuged at 6000 rpm for 10 minutes. The supernatant was discarded and the cells resuspended in 50ml of sterile water. These were centrifuged again, as before, to eliminate any salts. They were resuspended in 1ml of 30% PEG 6 000. Aliquots of 100µl were frozen at -70°C. Samples were thawed on ice before electroporation. Only 2µl of DNA was added to each sample, mixed and incubated on ice for a further 5 minutes. These were then electroporated under the following conditions:

0,2 cm cuvettes; 2,5kV; 25µF; 400Ω

One millilitre of LB was added immediately after electroporation, the sample was transferred into an Eppendorf tube and left to recover at 26°C for an hour. 100µl was then plated on an LA plate containing 200µg/ml streptomycin and 20µg/ml kanamycin. After five hours the plates were underlayed with chloramphenicol at a final concentration of 40µg/ml. Colonies were visible in 2-3 days.

2.9.3 Competent cell transformation of *E.coli*

2.9.3.1 With plasmid

A volume of 0,5ml of an overnight preculture of MM294-1 was inoculated into 50ml LB. The cells were grown with vigorous shaking at 37°C to a density of approximately 5×10^7 cells/ml or an OD_{540} of 0,2, then chilled on ice for 10 minutes and centrifuged at 6000 rpm for 10 minutes at 4°C in a prechilled rotor (Beckman JA-20). The cells were resuspended in half the original culture volume in an ice-cold solution of Tris-$CaCl_2$, placed in an ice water bath for 15 minutes and then centrifuged again. The supernatant was discarded and the cells resuspended in one fifteenth of the original volume in Tris-$CaCl_2$ which was

microfuge and the cells resuspended in 80µl of Solution 1. This remained at room temperature for 5 minutes before 160µl of Solution 2 was added and mixed. This stood at room temperature for 5 minutes whereafter 120µl of Solution 3 was vigorously mixed in. After 5 minutes on ice this mixture was centrifuged for 1 minute and the supernatant removed to a fresh Eppendorf tube to which an equal volume of isopropanol was added. This was left to stand at room temperature for 5 minutes. DNA was precipitated by microfuging on the bench top for 5 minutes. The DNA was washed with cold ethanol. It was dried and resuspended in 20µl of 3mM Tris, pH 8,0 containing 1mg/ml ribonuclease. After resuspension the volume was increased to 100µl and 15µl was loaded on an agarose gel. The rest was stored frozen for further examination with restriction endonucleases. These digestions were carried out overnight.

2.9 TRANSFORMATIONS

2.9.1 Protoplast transformation of nocardioforms

Cultures were grown in TYGS broth to a post-exponential pre-stationary phase when they were divided into 1 ml aliquots and either used immediately or stored frozen for future use. The cells were pelleted and resuspended in P buffer containing 5mg/ml lysozyme. This was incubated at 37°C for 30 minutes. The cells were then washed in 0,5ml P buffer and gently resuspended in 0,5ml P buffer. These were dispensed in 80µl aliquots to which 1-10µl of plasmid DNA in TE was added. A 50% weight per volume solution of polyethylene glycol 1000 in P buffer was made; 80µl of this was mixed in with the cells and the DNA and left for 4-5 minutes before being gently spread on protoplast regeneration plates. These plates were incubated at 28°C for 10 hours after which 0,5ml of a sodium arsenate-sodium arsenite solution was introduced beneath the agar in the plate. The final concentration in each plate was 60mM arsenate and 10mM arsenite. The plates were incubated for a further 5-10 days at 28°C. The colonies were

the gels. λII markers were run on 0,4% gels and λIII markers were run on 1,2% gels. A standard curve of molecular weight versus distance from the wells was drawn which was used to determine the size of fragments. The concentration of DNA was determined by visual comparison with a known concentration and size of one of the λ fragments (See Appendix for formula).

2.7.7.2 Photography of agarose gels

Gels were viewed and photographed on a UV transilluminator using a Polaroid camera and Polaroid 667 black and white film.

2.8 PLASMID SCREENS

2.8.1 Small scale preparation of DNA from nocardioforms

Strains were grown in TYG broth containing 40mM arsenate. Aliquots of 1ml were harvested and resuspended in the same volume of Tris-sucrose to which 5mg of lysozyme had been added. The cells were incubated for 1 hour at 37°C with agitation. They were then pelleted and resuspended in 280µl TE to which 40µl of 10% SDS in the same buffer was added and gently mixed. After 15 minutes at room temperature, 40µl of 4,5M sodium acetate pH 5,6 was added and gently mixed. Samples were placed on ice for 30 minutes then microfuged for 20 minutes at 4°C. The supernatant was extracted once with phenol and once with chloroform then the nucleic acid was ethanol precipitated. After air drying it was resuspended in 20µl TE containing 1mg/ml ribonuclease. Mixed with loading buffer, samples were analyzed by electrophoresis in agarose gels buffered with TBE.

2.8.2 Small scale preparation of DNA from *E.coli*

Single colonies were inoculated into 5ml of LB containing 100µg/ml ampicillin and incubated over night at 37°C with aeration. A millilitre of overnight culture was pelleted in a

minutes at 37°C. This was then extracted once with phenol and chloroform to remove the enzyme. The phosphatased DNA was then precipitated and resuspended in ligation buffer.

2.7.5 Blunt ending of DNA

DNA was blunt ..ded using the Klenow fragment of DNA polymerase I. This enzyme functions in most buffers and so could be used after restriction endonuclease digestion of the DNA or prior to ligation. A mixture of the four deoxynucleotide-triphosphates was added and one unit of Klenow enzyme. This was incubated at room temperature or at 30°C for 30 minutes. Extraction of the enzyme with phenol and chloroform could then follow.

2.7.6 Ligation of DNA

DNA which had been previously digested and had had the enzyme removed was precipitated and resuspended in 1x ligation buffer provided by Boehringer-Mannheim. Ligations were performed in a final volume of 20µl containing all the DNA to be ligated. In most cases 0,3µl of T4 DNA ligase was sufficient to ligate the DNA in 17 hours when it was placed at 14°C. This DNA was then used in transformations.

2.7.7 Agarose gel electrophoresis

Agarose was prepared in 0,5xTBE buffer at concentrations of 0,4; 0,8 and 1,2%. Gels were prepared using 25ml of agarose to which 1,5µl of 10mg/ml ethidium bromide was added. These were set, level, at 4°C. Samples had 2µl of sample loading dye added before they were loaded into the wells of the gel. The running buffer was 0,5xTBE containing 0,6 µg/ml ethidium bromide. Gels were run at between 80V and 120V at room temperature.

2.7.7.1 Fragment size determination and quantification

Digested DNA was run together with molecular weight markers on

enzyme was removed. The DNA was treated with TE-saturated phenol and then with chloroform:isoamyl alcohol (24:1) before being precipitated, dried and resuspended in an appropriate buffer.

With Exonuclease *Bal31*

The plasmid DNA was first linearised using endonucleases *BglI*. or *HindIII* in 20μl medium buffer (Boehringer-Mannheim). To this was added 18μl B buffer (Boehringer-Mannheim), 100μl 1M NaCl, 60μl water, 2μl 1M $CaCl_2$ and 0,5 units *Bal31*. This was incubated at room temperature (25°C) for 15 minutes to 2h. The enzyme was removed through treatment with phenol and chloroform before being precipitated, dried and resuspended in ligation buffer (Boehringer-Mannheim).

2.7.3 Phenol-chloroform extractions

To remove enzymes and other contaminating proteins from DNA solutions, including agarose, they were subjected to extraction with phenol and chloroform. The phenol used had previously been saturated with TE. Between 80μl and 200μl of phenol was added to the solution and inverted several times. This was microfuged for 5 minutes when the upper layer was aspirated. On the occasions when more than one phenol wash was necessary a few microlitres of 1M Tris pH 8,0 was added to increase the pH, to prevent DNA being lost at the interface. Afterwards a volume of chloroform equal to that of the phenol was added. This was well mixed before being microfuged for 30 seconds. The upper layer was aspirated once again.

2.7.4 Dephosphorylation

DNA was linearised by digestion with an appropriate enzyme. This was phenol and chloroform extracted to remove the enzyme afterwards. The DNA was precipitated and resuspended in 50μl of CIP buffer obtained from Boehringer-Mannheim. A microlitre of calf intestinal phosphatase was added and incubated for 30

were stored frozen at -20°C.

2.6.3 Chromosomal DNA extraction from *E.coli*

The procedure followed was similar to that for total DNA extraction from nocardioform bacteria, except that CsCl was dissolved before the 50Ti centrifugation step thus eliminating the clearing centrifugation step.

2.7 DNA MANIPULATIONS

2.7.1 DNA precipitation

In order to precipitate the DNA, NaCl was added to the DNA solution at a final concentration of 100mM. Two and a half volumes of cold ethanol were added and then centrifuged for 20 minutes in a microfuge at 4°C. The supernatant was then discarded and the tubes blotted on paper towel. These were dried under vacuum in 15 minutes. The DNA was then resuspended in either TE, 3mM Tris pH 8,0 or restriction enzyme buffer.

2.7.2 Digestions

With Endonucleases

DNA which had been stored at -20°C in TE was precipitated, dried and resuspended in the appropriate restriction enzyme buffer provided by the suppliers of the enzymes. Chromosomal DNA was digested for between 3h and 16h at the specified temperature, mostly 37°C, and plasmid DNA was restricted for between 1h and 3h. Double and triple digestions were performed simultaneously where possible in a common buffer. Where the procedure had been done for the purpose of determining fragment sizes or the existence of enzyme sites these digestions were loaded onto agarose gels. Where the process had been carried out for subsequent ligation or treatment with alkaline phosphatase the

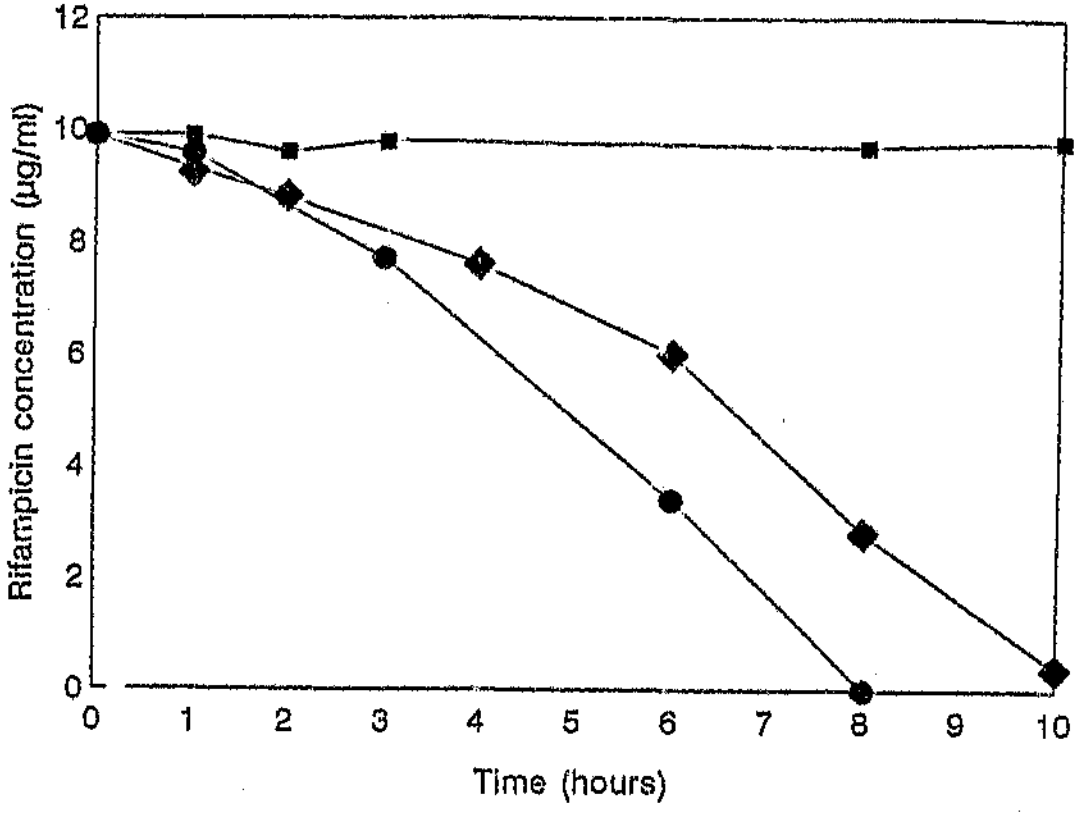


Figure 3.2 Inactivation of rifampicin by rifampicin resistant strains of ATCC12674 (●) and ATCC4277 (◆), where (■) is a media control.

3.1.3 Inactivation of rifampicin by other strains

As higher concentrations of rifampicin could be more accurately measured using zone diffusion assays, all the strains used were made resistant to 100µg/ml rifampicin. These were spontaneous resistant mutants.

Log phase cultures of the rifampicin resistant mutants were

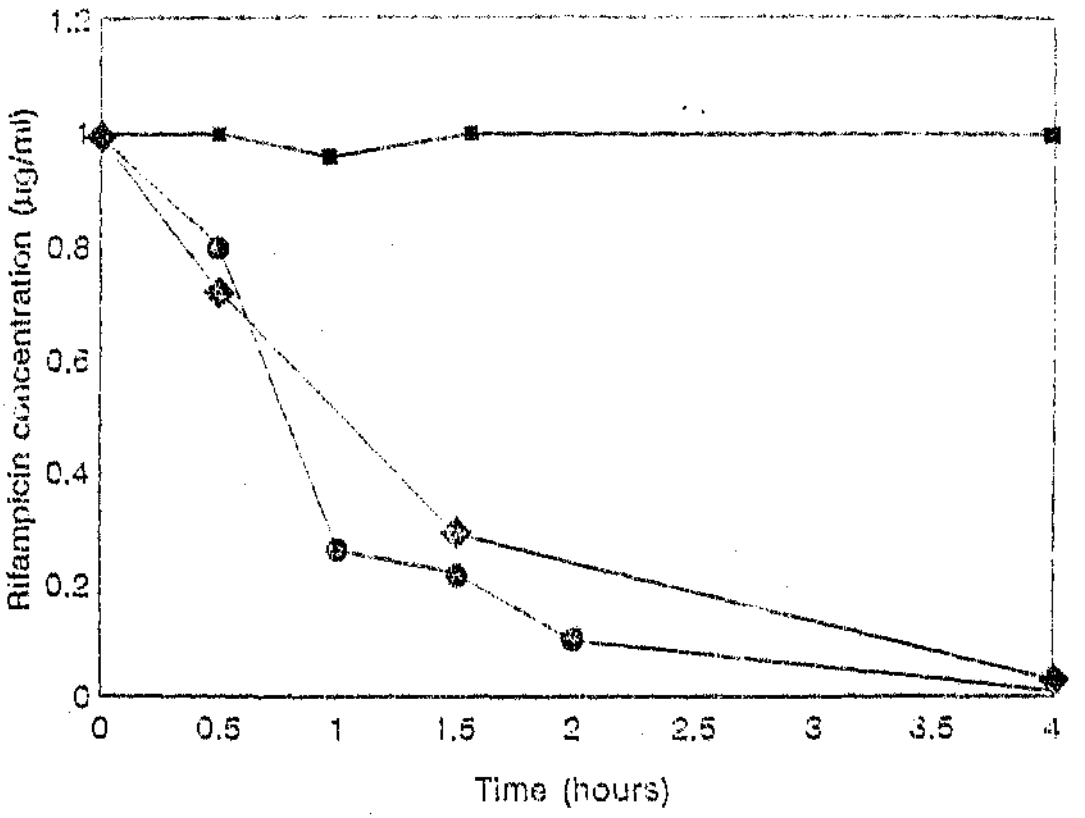


Figure 3.1 Inactivation of rifampicin by two rifampicin sensitive *R.erythropolis* strains ATCC12674 (●) and ATCC4277 (◆), where (■) is a media control.

3.1 ANSAMYCIN INACTIVATION BY NOCARDIOFORM BACTERIA

3.1.1 Sensitivity of strains to ansamycins

I investigated the ability of strains of nocardioform bacteria available in our laboratory to inactivate rifampicin, rifamycin SV and streptovaricin.

To establish the sensitivity range of these organisms to these antibiotics, some of these strains were replica plated onto LA plates containing different concentrations of the drugs.

Table 3.1 Sensitivity of various strains to ansamycin drugs. Growth of bacteria is indicated with +.

Strain	LA	Rifampicin 0,1	1	10	Rifamycin SV 0,1	1	10	Streptovaricin 0,1	1	10 µg/ml
ATCC12674	+++	+++			+++			+++	+++	
CW22	+++	+++			+++			+++	+++	
ATCC4277	+++	+++			+++			+++	+++	
ATCC14887	+++	+++			+++			+++	+++	
C1S	+++	+++			+++			+++	+++	

3.1.2 Inactivation of rifampicin by *R.erythropolis*

Previous work, for my M.Sc. thesis (1989), revealed that the *R.erythropolis* strains grew well on plates containing a maximum concentration of 0,3µg/ml of rifampicin. It was also shown previously that through spontaneous mutation to rifampicin resistance of greater than 100µg/ml, the inactivation remained unaffected. This is the result of the resistance mutation occurring in the *rpoB* gene and not in the gene responsible for inactivation. This can be seen in the following graphs, these experiments were repeated for this work.

3.0 RESULTS

container and the pH adjusted to 2-3 with 2N HCl. To this 1ml of ethyl acetate was added and the resulting mixture was vortexed and centrifuged. 0,5ml of this extract was transferred to a fresh tube and dried under vacuum. The pellet was dissolved in 20µl acetone. 10µl was spotted onto TLC plates (Merck 5715.001 Precoated silica gel $60F_{254}$). The liquid phase for running was chloroform:methanol 4:1 or methanol:chloroform 9:1.

2.19 MAXICELL PROTEIN EXPRESSION SYSTEM

E.coli, strain CSR603, containing appropriate plasmids were grown overnight in K medium at 37°C. An inoculum of 0,1ml of the preculture was introduced into 10ml of K medium containing ampicillin and grown at 37°C until an OD_{540} of 0,3 was reached. The culture was then transferred to a petridish and exposed to UV irradiation in a UV Stratolinker 1800, Stratagene. The cells were then transferred into flasks and aerated at 37°C. After an hour 0,1ml of a 10mg/ml cycloserine solution in phosphate buffer was added to the irradiated cells and the incubation continued over night at 28°C. The *iri* gene was expressed to a significant level at 28°C and in the presence of B1. All the media in this experiment contained B1. The cells were washed twice with Hershey's salts before being resuspended in 1,5ml of Hershey's medium. The cells were starved in this medium for 90 minutes before 3µl of ^{35}S-methionine was added at 15µCi/ml. Incubation continued for 90 minutes at 28°C. Cells were harvested and resuspended in 60µl of water to which 30µl of 3x loading buffer was added. Each sample was boiled for 4 minutes prior to loading on an SDS PAGE gel. The rest of the sample was stored at -20°C. The gel was stained and destained before being dried and autoradiographed.

incubated at room temperature for 2-5 minutes. 3,5µl of the labelling reaction was added to each termination tube and continued to incubate at 37°C for 5 minutes. The reaction was stopped by adding 4µl of stop solution. Samples were heated to 75°C for 2 minutes immediately before 3µl was loaded.

In order to read sequence close to the primer 1µl of Mn buffer was added at the labelling step. The presence of Mn^{2+} ions reduces the average length of DNA synthesised at the termination step intensifying bands corresponding to sequences close to the primer. These samples were loaded onto gels that were run until the bromophenol blue was 80% of the way down the gel.

2.17.3 Electophoresis of labelled DNA

Gels were run using the Hoeffer Pokerface II gel system. They were run at constant power of 100 Watts against a TBE buffer. 6% acrylamide gels were poured using the flat gel method of Chang and Wu (1992) that utilises capillary action to draw the acrylamide solution between the plates. This method did not need taping of the plates and there was no leaking of gels. Short runs (until the bromophenol blue came off) and longer runs (until the xylene cylanol or more came off) were performed. The gels were transferred onto blotting paper and dried under vacuum for 40 minutes. They were then placed in an X-Ray cassette with an X-Ray film. The films were developed between 1 and 7 days later. The procedure involved 5 minutes in developer, 1 minute in water and a further 5 minutes in fixer. The films were then washed and air dried. The sequences were read manually over a light box. The data was analyzed using several different software packages.

2.18 THIN-LAYER CHROMATOGRAPHY OF RIFAMPICIN INACTIVATED PRODUCTS

The supernatant of a culture challenged with 30µg/ml rifampicin was obtained by centrifugation. 2ml were decanted into a fresh

resuspended in 10µl water. Of this 1µl was analyzed on an agarose gel and the remainder used for 2-3 sequencing reactions.

2.17.2 Sequencing reactions

2.17.2.1 Using a pUC sequencing kit (Boehringer-Mannheim)

Double stranded DNA was denatured using NaOH and EDTA for 5-10 minutes. This was neutralised and precipitated by adding 3M NaAc and ethanol. To the dried DNA, 15µl water, 1µl of sequencing primer (2,5pmol), 1,5µl of 10x reaction buffer and 2µl 16µCi [^{35}S]dATP-α-S was added and incubated at 37°C for 15 minutes. Meanwhile 2µl of each of the corresponding dNTP/ddNTP mixtures were pipetted into tubes (A,C,G,T). To the DNA primer mix 2 units of Klenow enzyme was added and mixed. 3µl of this was pipetted into each of the four (A,C,G,T) tubes. These were placed immediately into a water bath at 37°C for 20 minutes. The reaction was chased by adding 1,5µl of a dideoxynucleotide mixture to each tube and incubating at the same temperature for a further 15 minutes. The reaction was terminated by adding 4µl of formamide buffer. Immediately before loading the samples on the sequencing gel they were heated for 3 minutes at 95°C and quick cooled on ice. 2µl was loaded into each slot.

2.17.2.2 Using a Sequenase Version 2.0 kit (United States Biolabs)

If double stranded DNA was used it was denatured as above. For annealing of primers to template DNA 1µl of primer was added to 7µl of DNA with 2µl of reaction buffer. This was heated at 65°C for 2 minutes and then allowed to cool for 15-30 minutes until the temperature was below 35°C. This was then placed on ice. While cooling 2,5µl of termination mixture was added to appropriate tubes (A,C,G,T). Both dGTP and dITP reactions were carried out on this GC rich DNA. To the ice cold annealed mixture 1µl 0,1M DTT, 2µl diluted labelling mix, 0,5µl [^{35}S]dATP and 2µl of diluted Sequenase polymerase was added. This was mixed and

2.17.1.2 Single stranded phagemid DNA

Overnight cultures of pUC118 clones in JM109 were grown in LB containing 100µg/ml ampicillin. 30µl of culture was added to 30µl of M13K07 lysate (titre > 10^{10} pfu/ml) and incubated at 37°C for 20 minutes. Subsequently 3ml of 2xYT broth was added and this was incubated at the same temperature with good aeration for 14-18 hours. After the first hour of incubation 100µg/ml ampicillin and 70µg/ml kanamycin were added. Cells were collected by centrifuging the culture at 4°C. The supernatant was transferred to a sterile Eppendorf and 200µl of a PEG-NaCl solution was added. This was mixed thoroughly by vortexing and left at room temperature for 15 minutes. Phage particles were collected by a 5 minute centrifugation at 4°C. The supernatant was discarded. Care was taken to remove all traces of PEG. The pellet was resuspended in 100µl TE by vigorous vortexing. This was then extracted with phenol and chloroform and precipitated with 10% 3M sodium acetate (vol/vol) and ethanol. DNA was washed with 70% ethanol and dried under vacuum. The DNA was resuspended in 20µl of TE.

2.17.1.3 Single stranded DNA from M13 clones

M13 clones in JM101 were grown for 5 hours in 2xYT medium. Phage containing supernatant from 1,5ml of culture was obtained by centrifugation. This was poured into a tube containing 200µl of the PEG NaCl solution which was mixed well and placed on ice for 10 minutes. To precipitate the phage, samples were centrifuged for 10 minutes and the PEG solution carefully removed. The pellet was resuspended in 100µl TE and placed on ice for 10 minutes. This was centrifuged again for 5 minutes to precipitate insoluble bacterial degradation products. The supernatant was drawn off and transferred to a fresh tube containing 10µl of 3M NaAc and 250µl phenol/chloroform (1:1). This was vortexed thoroughly and centrifuged. The top phase was carefully removed and re-extracted with phenol/chloroform and again with chloroform. The DNA was precipitated with ethanol and dried under vacuum. DNA was

2.16.4 Washing

Membranes were washed twice for 5 minutes at room temperature in solution 1 and twice for 15 minutes at 68°C in solution 2.

2.16.5 Immunological detection

The membranes were washed for 1 minute in buffer 1. The antibody conjugate was diluted to 150mU/ml in buffer 1 and the membrane was incubated in 20ml of the diluted antibody-conjugate solution for 30 minutes. The membrane was washed twice with buffer 1 for 15 minutes to remove any unbound antibody-conjugate. The membrane was equilibrated for 2 minutes in 20ml of buffer 3 and then incubated in the dark with 10ml of the colour solution in a plastic container. Bands were visible from between 10 minutes and 20 hours after the addition of the colour solution. Once the bands had developed the membrane was placed in TE for 5 minutes and then air dried. The membrane could be photographed or photocopied.

2.17 DNA SEQUENCING

2.17.1 Preparation of DNA for sequencing

2.17.1.1 Double stranded DNA

DNA was prepared using the method of alkaline lysis. However because of the need for very clean DNA, samples were extracted with phenol between 1 and 4 times and once with chloroform. On some occasions DNA was precipitated with PEG. DNA that had been precipitated with ethanol was resuspended in 27μl of water. To this 4μl of a 4M NaCl and 9μl of a 30% PEG solution was added. The DNA was placed on ice for 20 minutes and the centrifuged at 4°C for the same length of time. The PEG solution was aspirated and the pellet was washed with 70% ethanol before it was dried in a vacuum desiccator.

membrane was then placed in sterile distilled water. The gel was removed from the neutralising solution and placed on the support in an inverted position. The wet nitrocellulose filter was placed on the gel. Upon this were placed 2 pieces of 3mm Whatman paper that had been soaked in 2xSSC. A stack of paper towels, cut slightly smaller than the gel were placed on top of the filter paper. A 300g weight was balanced on a glass plate on top of the stack of towels. DNA transfer proceeded overnight. In the morning the upper layers were removed, the positions of the wells marked on the membrane with a ball point pen and the filter soaked in 6xSSC for 5 minutes to remove residual agarose. The membrane was allowed to dry on paper towel for 30 minutes at room temperature before it was baked between 2 pieces of Whatman 3mm paper at 80°C for 2 hours under vacuum to fix the DNA to the filter.

2.16.2 Probe labelling

Linearised DNA was purified with a phenol-chloroform extraction and an ethanol precipitation. The DNA was resuspended in 15ml sterile water. Single stranded DNA was obtained by boiling the sample for 10 minutes in a water bath and then chilling quickly in an ice-water slurry. 2µl of dNTP labelling mixture was added to the freshly denatured DNA. A microlitre of Klenow enzyme was added. The reaction mixture was incubated at 37°C overnight. In the morning 3µl of 0,2M EDTA pH 8,0 was added to stop the reaction. The DNA was then precipitated with 2,5µl 4M LiCl and 75µl prechilled ethanol, dried under vacuum and resuspended in 50µl TE.

2.16.3 Hybridisation

Membranes were prehybridised for between 2 and 4 hours at 68°C in 20 ml of hybridisation solution. This solution was agitated from time to time. The prehybridisation solution was removed and replaced with 5ml of fresh hybridisation solution containing the single stranded, labelled DNA. This was incubated at 68°C overnight.

in Tris-sucrose with lysozyme. This was incubated for 1 hour at 37°C. The cells were washed twice to remove the lysozyme. They were resuspended in 60µl of water to which 30µl of 3x loading dye was added. Each sample was mixed vigorously to achieve lysis of the cells. The samples were boiled for 5-10 minutes before they were loaded onto the gel.

2.16 SOUTHERN HYBRIDISATION

In this procedure a non-radioactive DNA labelling and detection kit was used. This was supplied by Boehringer-Mannheim. The probe was labelled by random primed incorporation of digoxigenin-labelled deoxyuridine triphosphate. Immunological methods were used to detect this labelled sequence. An antibody conjugate of antidigoxigen alkaline phosphatase was used. Visual detection of the probe was obtained by an enzyme catalysed colour reaction with 5-bromo-4-chloro-3-indalyl phosphate (x-phosphate) and nitroblue tetrazolium salt (NBT).

2.16.1 Blotting

DNA was blotted onto nitrocellulose membranes in the described manner by Sambrook *et al* (1989). After electrophoresis, the DNA in the gel was denatured by soaking the gel for 45 minutes in 100ml of denaturing solution on a rotary platform. The gel was rinsed briefly in sterile water before being placed in 100ml of neutralising solution for 30 minutes. The gel was soaked for a further 15 minutes in fresh neutralising solution with constant agitation. The transfer apparatus was prepared while the gel was soaking. This comprised of a piece of perspex, wider and longer than the gel, supported on a plastic dish. The dish was filled with transfer buffer (10xSSC). A wick of Whatman 3mm paper was placed over the perspex and suspended in the transfer buffer. At each stage in the proceedings as different things were placed on the perspex they were smoothed out using a pipette. A piece of nitrocellulose filter was cut out to the size of the gel. This

2.15 PREPARATION OF SAMPLES FOR PAGE

2.15.1 From supernatant

The parental strain ATCC12674 was grown in TY medium. An aliquot was taken from the preculture and inoculated into 30ml of TY medium. When the culture was in exponential phase the antibiotic was added at a concentration of 1μg/ml. At time intervals of 24 hours, 48 hours and 72 hours, 10ml aliquots were pelleted at 15,000 rpm for 15 minutes in a Beckman JA-20 centrifuge. The protein in the supernatants was precipitated by adding on average 6,4g of ammonium sulphate or as much as would saturate the supernatant. This sample was then centrifuged at 20 000 rpm for 20 minutes and the supernatants discarded. The proteins were resuspended in 750μl of sterile distilled water and dialysed against distilled water overnight to remove the ammonium sulphate. The sample was decanted and frozen at -80°C before being lyophilised overnight on a Virtis lyophiliser. Each sample was resuspended in 60μl of sterile distilled water before an aliquot was taken for loading onto a polyacrylamide gel.

2.15.2 From *E.coli*

Extracts of proteins from *E.coli* were prepared from 1,5ml of stationary phase culture. The cells were harvested by centrifugation. Each sample was resuspended in 60μl of water to which 30μl of 3x loading dye was added. The samples were mixed and boiled for 5-10 minutes before 20μl of each was loaded on the gel.

2.15.3 From nocardioforms

In order to weaken the cell walls to facilitate lysis the cultures were grown in TYG with an appropriate antibiotic if necessary. When the culture was in late log phase 20μg/ml ampicillin was added to further weaken the walls. At stationary phase the cells were harvested by centrifugation and resuspended

ATCC14887 was digested with either *BclI* or *BglII* or *BamHI* and ligated to pDA30. These constructs were transformed into CW22.

Table 3.7 pDA30 as a cloning vector

Plasmid	Number of Transformants
None	2
pDA30 + *BglII* 14887	4
pDA30 + *BclI* 14887	8
pDA30 + *BamHI* 14887	150

Plasmid screens of transformants confirmed that DNA had been inserted into pDA30.

CW22 chromosomal DNA digested with *BglII* was ligated into the *BglII* site of pEcoR251. Numerous transformants containing inserts were obtained.

3.2.1.5 Survival of *E.coli* plasmid DNA in nocardioforms

In earlier work (Andersen, M.Sc dissertation, 1989) mutants were made of CW22. These mutants were hypersensitive to rifampicin and some of them were unable to inactivate the antibiotic. Since they might possess a restriction system it was necessary to determine the fate of pEcoR251 in CW22. pDA26, which is pEcoR251 with a nocardioform plasmid ligated into the *BglII* site, had been constructed by Dabbs (unpublished). CW22 was transformed with this chimaera.

ATCC14887 was digested with either *BclI* or *BglII* or *BamHI* and ligated to pDA30. These constructs were transformed into CW22.

Table 3.7 pDA30 as a cloning vector

Plasmid	Number of Transformants
None	2
pDA30 + *BglII* 14887	4
pDA30 + *BclI* 14887	8
pDA30 + *BamHI* 14887	150

Plasmid screens of transformants confirmed that DNA had been inserted into pDA30.

CW22 chromosomal DNA digested with *BglII* was ligated into the *BglII* site of pEcoR251. Numerous transformants containing inserts were obtained.

3.2.1.5 Survival of *E.coli* plasmid DNA in nocardioforms

In earlier work (Andersen, M.Sc dissertation, 1989) mutants were made of CW22. These mutants were hypersensitive to rifampicin and some of them were unable to inactivate the antibiotic. Since they might possess a restriction system it was necessary to determine the fate of pEcoR251 in CW22. pDA26, which is pEcoR251 with a nocardioform plasmid ligated into the *BglII* site, had been constructed by Dabbs (unpublished). CW22 was transformed with this chimaera.

structural integrity of the endonuclease was destroyed. They showed that it was useful as a positive selection cloning vehicle which could be employed for regulated over production of hybrid proteins. pEcoR251 was made by combining the above two approaches. It is a plasmid of 3346 bp that contains the *EcoRI* endonuclease gene fused to a portion of the *cro* gene of λ under the control of the λ promoter. The plasmid also contains an *E.coli* origin of replication and a gene for ampicillin resistance.

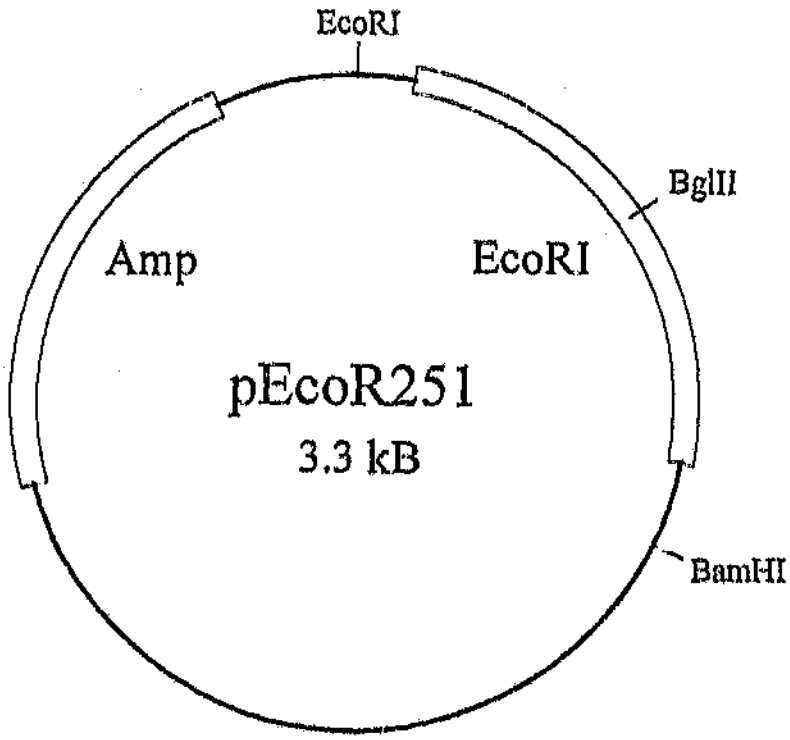


Figure 3.4 Restriction map of pEcoR251

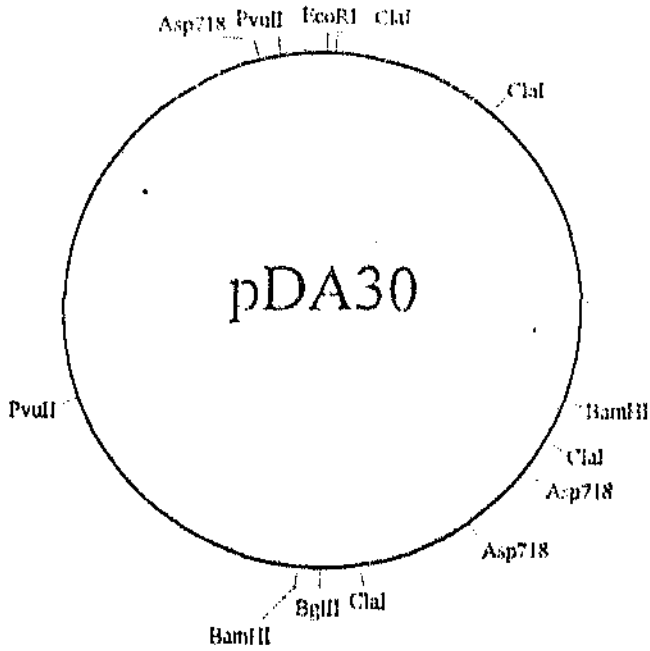


Figure 3.3 Restriction map of pDA30

3.2.1.3 Origin of pEcoR251

pEcoR251 is a positive selection or suicide vector. It produces a gene product that is lethal for certain bacterial hosts. However insertion of a foreign segment of DNA into the gene inactivates it. The production of this vector was based on work done by Zabeau and Stanley (1982) and work done by Cheng and Modrich (1983).

Zabeau and Stanley produced a vector which enhanced expression of a *cro*β-galactosidase fusion proteins under the control of the P_R promoter of bacteriophage λ. This plasmid was made by joining DNA segments carrying the P_R promoter and the start of the *cro* gene of λ to the *lac Z* gene fragment carried by another plasmid. Recombininant plasmids which directed the synthesis of high levels of β-galactosidase were conditionally lethal and could only be propagated when the P_R promoter was repressed.

Cheng and Modrich produced a plasmid which contained the *EcoRI* endonuclease gene down stream from the P_L promoter of λ. It did not yield transformants after introduction into *E.coli* unless the

nocardioform species *R.erythropolis* strain ATCC12674 and the other plasmid, pEcoR251, from *E.coli*. Neither of these plasmids are naturally occurring. They are both the result of considerable genetic manipulation.

3.2.1.2 Origin of pDA30

A large plasmid of about 120kb was isolated by Dabbs and Sole (1987) from ATCC12674. This was done using a primarily genetic approach. An unstable genetic element was identified which conferred resistance to cadmium chloride, chloramphenicol, sodium arsenate and sodium arsenite. No plasmids were visible on agarose gels and this was thought to be the result of integration of the plasmid with the chromosome. To disrupt the integration of the elements, plasmid DNA was packaged using the generalised transducing nocardiophage Q4 (Dabbs, 1987). These were transduced into a derivative of ATCC12674, CW22, which had been spontaneously cured of resistance to those heavy metals and chloramphenicol. Transductants were selected for increased resistance and these showed plasmid bands on agarose gel electrophoresis. These plasmids were chimeras of the unstable genetic element and part of the Q4 phage genome.

A plasmid was transformed into a related *Rhodococcus* strain ATCC14887 (*R.equi* restricts ATCC12674 DNA and is not lysed by Q4 phage). This produced a lot of smaller plasmids. The smallest being pDA21. When this plasmid was transformed into CW22 it showed resistance to sodium arsenate and sodium arsenite and it retained the Q4 phage immunity. A restriction map was made of pDA21 and it was shown to be 15,3kb in size. pDA21 was further reduced in size by digesting it with a number of restriction enzymes and then ligating them. It was found that an *EcoRI* fragment and a couple of *BamHI* fragments were not essential. The resulting plasmid was pDA30 and was about 11,2kb in size (Dabbs *et al*, 1990).

These conditions included optimal growth phase, the absence of agitation at the lysozyme step, the volume of protoplasts, the concentration of the selective agents - arsenate and arsenite - and the time of addition of the selective agents.

The glycine concentration of the broth also needed to be optimised. Glycine weakens the cell wall by interfering with the glycine - glycine bonds in the cell wall. A preliminary tolerance experiment, where CW22 was grown with glycine concentrations varying between 0 and 8%, showed that the optimum concentration lies between 2% and 6%. Cells were prepared in 3%,4% and 5% glycine for transformation. A 1:100 inoculum of a stationary phase culture was introduced and samples were taken at regular intervals over a 78h period. The samples were frozen. After all the samples had been taken, they were thawed and transformed with 0,1µg of pDA30. After 6 days the following results were obtained.

Table 3.5 Optimisation of glycine concentration for transformation

	Number of Transformants		
Time (h)	3% gly	4% gly	5% gly
18	0	0	0
30	0	0	0
42	24	4	10
54	112	183	272
66	173	214	500
78		215	254

The greatest number of transformants were obtained from cells grown for 66h in 5% glycine OD_{540} of 3,05. A bulk culture of CW22 in TY + 5% glycine was grown to an OD_{540} of 3,00. One millilitre aliquots were frozen for future use.

The approach that was followed to construct the *Rhodococcus* - *E.coli* shuttle vector involved the ligation of two plasmids of different origins. One plasmid, pDA30, was derived from a

samples exposed to rifampicin. Sodium tauracholate samples showed an extra low molecular weight band while ampicillin samples showed an extra high molecular weight band.

Additional experiments were done including using the protease inhibitor PMSF. These failed to make the situation any clearer. As the original experiment by Dabbs (1987) required the culture to be in stationary phase for at least 40 hours, the cells were probably being lysed by this time. This presented the possibility that the enzyme was actually an intracellular enzyme.

3.1.7 Non-inactivating mutants

To clone the gene responsible for inactivation by complementation a non-inactivating mutant was needed. In work done prior to this thesis, 14 mutants were produced by E.Dabbs and myself. These were hypersensitive to rifampicin and three were clear non-inactivating mutants: Ri7, Ri8 and Ri14. These mutants were of strain CW22 a derivative of *R.erythropolis* ATCC12674.

In this work the other *R.erythropolis* strains ATCC4277 and DSM1069 which had been mutagenised were screened for hypersensitive mutants. Of 224 ATCC4277 colonies screened no mutants were obtained. Three potential hypersensitive mutants were obtained from DSM1069. On replica plating only one of these proved to be hypersensitive. However inactivation assays of this mutant, Ri15, showed that it still inactivated rifampicin.

3.2 SHUTTLE VECTOR CONSTRUCTION

3.2.1 Preliminary Experiments

3.2.1.1 Optimisation of Nocardioform Transformation

Most of the conditions for transformation of CW22 had been optimised previously (Andersen SJ, M.Sc. Dissertation, 1989).

Attempts were made to identify this protein. Protein was isolated from the supernatants of rifampicin challenged and non-challenged cultures and separated on polyacrylamide gels. It had been reported by Dabbs (1987) that inactivation of the drug was detectable only with supernatants from cultures maintained in stationary phase for at least 40 hours. For this reason supernatants were prepared from cultures at varying times in order to detect when this protein was released.

Samples of 10ml were taken after 2 days and every 12 hours for a further 2 days. After preparing the samples half of each was loaded on a polyacrylamide gel. After staining and destaining very faint bands could be seen. However more sample was needed if any differences were to be determined.

Larger samples of 20ml were taken and processed. No bands were seen in the samples from supernatants of cultures not exposed to the antibiotic. Many bands were seen in the samples exposed to rifampicin. In fact it appeared as though the samples had been lysed.

Cultures were exposed to other agents that lyse cells. These included 10µg/ml ampicillin, 10µg/ml fusidic acid and 0,02% sodium tauracholate. Supernatants of cultures exposed to these agents during log phase were harvested 8 hours later. After centrifugation no pellets were visible, however water was added to resuspend low concentrations of protein. These samples were dialysed but not lyophilised before electrophoresis. The gel was blank except for the lane from the ampicillin challenged culture which showed definite bands. Repetition of this experiment indicated that the samples needed to be concentrated further.

Lyophilisation of the samples increased the protein yield with bands appearing in most lanes. The lanes that were blank were those not exposed to any additional agent. Although it was difficult to determine, no extra bands could be identified in the

Table 3.4 Zone inhibition assay to identify the antibiotic released by DSM40512

Assay organism:	Spc^R	Rif^R	Stv^R
50µg/ml stv in LB:	large zone	no zone	no zone
DSM40512 supernatant:	small zone	small zone	small zone

This shows that the substance being produced is not streptovaricin for if it was there would be no zone on the plate assayed with the Stv^R assay organism. Checking the DSM catalogue it became apparent that DSM40512 produces two products other than streptovaricin: actinospectacin and prodigiocin were therefore probably responsible for producing the small zones of inhibition. There also appeared to be a cross resistance between rifampicin and streptovaricin in *B.subtilis*.

3.1.5 Non-inactivation of streptolydigin

Another antibiotic that also inhibits RNA polymerase is streptolydigin. Attempts were made to see if the drug was inactivated. Both *A.oxydans* and *B.subtilis* did not give zones of inhibition at similar concentrations to the ansamycins. Zones appeared at 500µg/ml on *B.subtilis* lawns. This concentration inhibited the growth of most strains tested and none of them gave any indication of inactivation. Those tested included *R.erythropolis*, *R.australis*, *R.fascians*, *R.rubrapertinctus*, *S.spectabilis*, *S.lividans* and *M.smegmatis*.

3.1.6 Protein isolations from nocardioform supernatants

Previous work done in this laboratory by Dabbs (1987) showed that the supernatants of cultures challenged with sub-inhibitory concentrations of rifampicin were able to inactivate the drug. This seemed to indicate that a protein was being released into the supernatant that had inactivating ability.

and *R.fascians*. These were then tested for streptovaricin inactivation and better definition of zones were obtained. These revealed that both A554 and *R.fascians* totally inactivated streptovaricin.

3.1.4 Inactivation by ansamycin producing organisms

The antibiotic producing organisms, *Amycolatopsis mediterranei* (rifamycins) and *Streptomyces spectabilis* (streptovaricins), were tested for the presence of inactivation mechanisms.

A.mediterranei was tested for its level of resistance to rifampicin. As reported by Hardisson *et al*, (1984) this strain was found to be resistant to at least 100µg/ml rifampicin. The strain was grown under the same conditions as the other nocardioforms. Once the strain had entered log phase it was challenged with 20µg/ml rifampicin. Assay of the supernatant 20 hours later showed that there had been no inactivation of the antibiotic.

S.spectabilis DSM40512 was grown to stationary phase and seeded on a broth plate containing 100µg/ml streptovaricin. A lawn of bacteria grew up showing that the organism was resistant to the antibiotic it produced.

Inactivation assays showed that most of the antibiotic was inactivated. However a small zone of inhibition on the *B.subtilis* lawn was consistently observed. A culture was grown and assayed without the addition of the antibiotic and the small zone was still present. This seemed to indicate that an antibiotic was being produced. To determine whether the product was streptovaricin, assay organisms resistant to rifampicin (Rif) and one resistant to streptovaricin (Stv) were made. The assay was repeated using the regular spectinomycin (Spc) resistant organism and both the newly made mutants.

rifamycin SV and streptovaricin. Thus the rifampicin resistant mutants produced for the earlier work could also be used to assay for inactivation with these drugs.

Rifamycin SV was assayed under the same conditions as rifampicin. These conditions were not optimal for streptovaricin. No zones of inhibition could be seen using 10μg/ml streptovaricin. Optimisation showed that 50μg/ml streptovaricin and either a 10^{-2} dilution of C1S or *B.subtilis* were more definitive.

Table 3.3 Inactivation of rifamycin SV and streptovaricin by various strains of bacteria

Strain	10μg/ml RSV	50μg/ml STV
R.erythropolis		
ATCC12674	YES	NO
ATCC4277	YES	NO
DSM1069	YES	YES
R.equi	YES	NO
R.rubropertinctus	NO	NO
R.australis		
A448	YES	NO
A554	YES	YES
R.fascians	YES	YES
*A.oxydans*C1S	YES	NO
B.subtilis	NO	YES

M.smegmatis was also tested. This is a much slower growing strain. The cell free control containing LB and the antibiotic was hydrolysed before any detectable differences were observed.

Surprisingly the *B.subtilis* strain which was able to inactivate rifampicin was not able to inactivate rifamycin SV, but was able to inactivate streptovaricin. This was repeated more than three times using the plate assay and twice using a quantitative assay in liquid culture. In each case the result was confirmed, *B.subtilis* 1A3-1 did not inactivate rifamycin SV.

As the zones of inhibition were not as clear for these ansamycins, streptovaricin resistant mutants were made of A554

challenged with 20μg/ml of the antibiotic then monitored for one or two days. In this time inactivation assays were performed and colour changes observed. Each of these was repeated at least twice.

Table 3.2 Inactivation and decolorisation of 20μg/ml rifampicin by various strains of bacteria

Strain	Inactivated	Decolorised
R.erythropolis		
ATCC12674	YES	YES
ATCC4277	YES	YES
DSM1069	YES	YES
R.equi	YES	YES
R.rubropertinctus	NO	NO
R.australis		
A448	YES	NO
A554	YES	NO
R.fascians	YES	NOT TESTED
A.oxydans C1S	YES	YES
S.lividans	YES	NO
M.smegmatis	YES	YES
B.subtilis	YES	NO
E.coli	YES	YES

Of all the strains tested only *R.rubropertinctus* did not inactivate rifampicin under the same conditions as the others. This assay consistently obtained the same result. Most of the strains also decolorised the antibiotic, notable exceptions being the *R.australis* strains A448 and A554, *S.lividans* and *B.subtilis*.

3.1.3 Inactivation of other ansamycins

We assayed these strains for their ability to inactivate other ansamycin antibiotics, rifamycin SV (RSV) and streptovaricin (STV). Both are naturally occurring antibiotics with rifampicin being a semi-synthetic derivative of the rifamycins and streptovaricin being a different class of ansamycin.

Replica plating revealed that resistance to rifampicin at the *rpoB* locus also increased the level of resistance to both

there is a 100% efficiency of ligation of inserts. In this case where there has been a 72% efficiency, N = 7922. So a library of a minimum of 8000 transformants was constructed.

During the construction of the library plasmid screens of transformants were regularly done to determine the number of inserts and the size of the inserts. The origin of the inserted DNA was also verified by blotting *BglII* digested DNA from MM294-1 and ATCC4277 and probing with the DNA inserted into pDA37 from several of the transformants.

Probes were prepared by digesting the plasmids obtained from the transformants with *BglII*. This DNA was separated on a 0,8% low melting agarose gel. The inserts were purified and labelled with non-radioactive digoxygenin. The kit used was the "DNA labelling and detection kit" from Boehringer-Mannheim. These fragments of DNA were used to probe nitrocellulose membranes onto which digested MM294-1 and ATCC4277 DNA had been blotted. Hybridisation was detected using the immunological visualisation procedure. All the probes bound to the ATCC4277 DNA and none to the MM294-1 DNA confirming the origin of the inserted DNA.

A library of 20 500 transformants was constructed with an average insert size of 3,3kb. Of the transformants 79% contained detectable inserts, that is inserts greater than 200bps. Thus the probability of any gene being present in this library is greater than 97,5%.

Transformants were washed off the plates using LB containing 50µg/ml ampicillin and 30µg/ml rifampicin. Aliquots were stored in glycerol at -70°C and an aliquot was inoculated into 200ml of the above media in order to do a bulk extraction of the library.

3.3.2 Isolation of DNA carrying *iri*⁺ gene

As the method of cloning was by complementation, a mutant unable to inactivate rifampicin would be the recipient strain. In

1 2 3 4 5 6 7 8

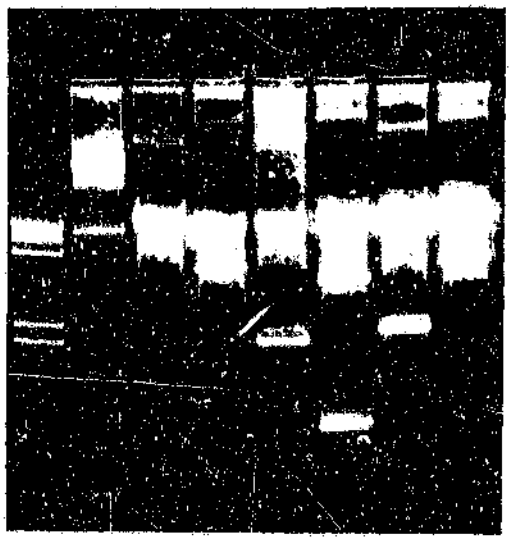


Figure 3.8 *BglII* digested DNA from plasmid screens of 16x transformants, lane 1: molecular weight marker

Inserts were detected in 6 out of 13 transformants from the 8x ligation and in 13 out of 13 transformants from the 16x ligation. All subsequent ligations were performed with a ratio of 1:2 vector to insert. The average insert size of the latter was determined to be 3,02kb. This figure is needed to determine the approximate number of transformants needed for a library (Appendix).

The genome size was taken to be 7,42 x 10^6 bps (genome size for *M.smegmatis* has been estimated at 3 x 10^9Da). Thus N = 9669 where

1 2 3 4 5 6 7 8

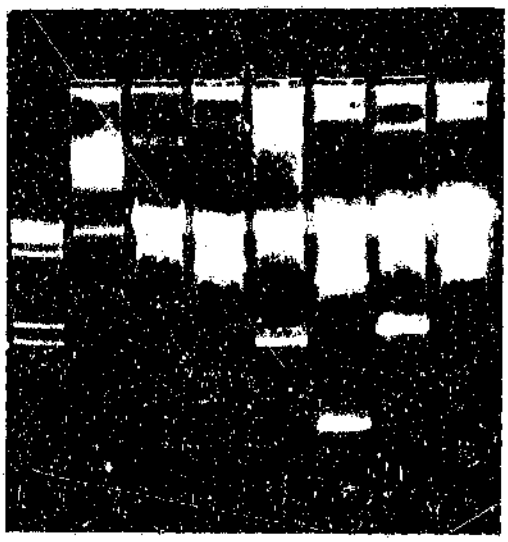


Figure 3.8 SgIIT digested DNA from plasmid screens of 16x transformants, lane 1: molecular weight marker

Inserts were detected in 5 out of 13 transformants from the 2x ligation and in 13 out of 16 transformants from the 16x ligation. All subsequent kigations were performed with a ratio of 1:2 vector to insert. The average insert size of the latter was determined to be 3,93kb. This figure is needed to determine the approximate number of transformants needed for a library (Appendix).

The genome size was taken to be 3,47 x 10^7 bps (genome size for *M.smegmatis* has been estimated at 3 x 10^9Da). Thus N = 8668 where

3.3 pDA37 IN THE CLONING OF AN *iri* GENE

In principle, the source of DNA used to construct the library which was to be transformed into the mutant Ri8 could be its parental strain. However the presence of a *rec* system in nocardioforms would lead to recombination between plasmid-borne DNA and homologous sequences in the chromosome. To reduce such recombination, a library was constructed from another nocardioform strain ATCC4277. This strain was also found to inactivate rifampicin with the concomitant changes in the absorption spectrum of the antibiotic. DNA from this strain was subjected to partial digestion with *BglII* and ligated with pDA37 DNA digested with the same enzyme. The vector was not treated with alkaline phosphatase to prevent recircularisation because of the positive selection function of the plasmid.

3.3.1 Construction of the genomic library of ATCC4277 DNA

In order to establish optimum conditions for ligation, varying amounts of chromosomal DNA were ligated into a uniform amount of pDA37 and the number of transformants determined.

Table 3.13 Optimisation of DNA ratios for ligation

DNA	Number of Transformants
None	1
pDA37	0
pBR322	10^4
pDA37 cut and ligated	2
pDA37 + 2 x chromosomal	23
pDA37 + 4 x chromosomal	14
pDA37 + 8 x chromosomal	26
pDA37 + 16x chromosomal	20

Plasmid screens of colonies from both the 2x and 16x chromosomal plates were performed to evaluate the sizes of the inserts in each case.

Table 3.13 Efficiency of pDA37 on transformation

	pDA37	1:4 pDA37 + inserts
µl/transformation	1	100
Number of transformants	3	1015
Concentration of DNA	9,1ng/µl	117,4ng/µl
Transformants/µgDNA	330	87

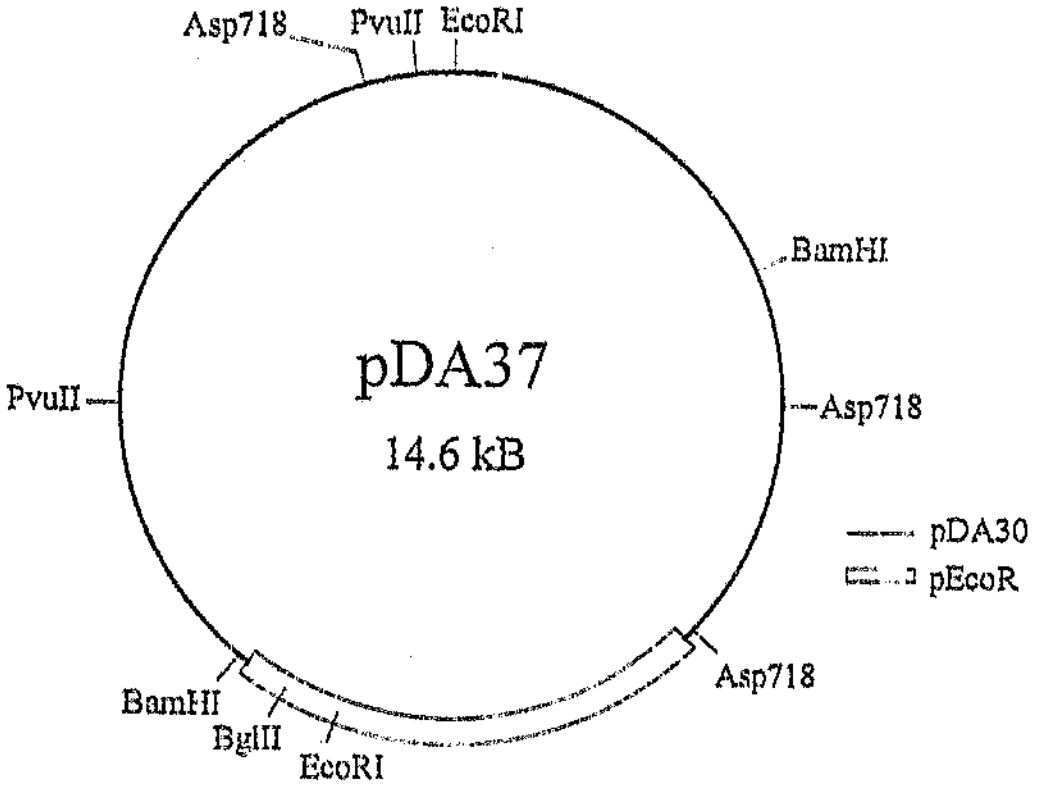


Figure 3.7 Restriction map of pDA37

Table 3.11 ShV15 as a cloning vector

Plasmid	Number of transformants
None	2
pDA30	1164
ShV15	105
ShV15 + 14887	450

Plasmid screens of transformants confirmed that there were inserts in ShV1 and ShV24.

On the basis of these results it was decided to use ShV24 as a cloning vector. This orientation was given the designation pDA37 and the other orientation pDA38.

A transformation with DNA isolated from both nocardioform and *E.coli* backgrounds was done using both MM294 and λMM294 as recipients. This was to determine if the *EcoRI* endonuclease gene was still functional. Many colonies were obtained in the lambda containing strain while none were obtained in MM294. The number of transformants were relatively low, so significant comparison, resulting from the strain from which the DNA was extracted, could not be made. In order to determine the efficiency of transformation in the nocardioforms, the concentration of the DNA was determined and compared with the number of transformants obtained, this was determined relative to a known concentration of DNA on an agarose gel.

migrated faster in agarose gels. If these could be used as effective shuttle vectors it would be advantageous as they are smaller plasmids. Each isolate was prepared in bulk and purified on a CsCl gradient. These were transformed into MM294 and the λ lysogen of the strain. The transformation results showed that the *EcoRI* endonuclease gene was still functioning. In order to determine if these vectors could be used to build libraries a representative of each orientation, ShV1 and ShV4, as well as the smaller ShV3 and ShV15 were digested with *BglII*. Chromosomal DNA from ATCC14887 digested with *BglII* was ligated into these. They were subsequently transformed into MM294. Plasmid screens of the transformants showed that each vector contained some inserts and that ShV1 contained more than the others. DNA from this pool of clones was transformed into ATCC4277. After ten days, the following results were observed.

Table 3.10 ShV1, ShV3 and ShV 4 as cloning vectors

DNA	Number of transformants
None	2
pDA30	700+
ShV1	3
ShV1 + 14887	123
ShV3	2
ShV3 + 14887	175
ShV4	3
ShV4 + 14887	1015

Similar concentrations of DNA had ben used, indicating that there was some advantage in using ShV4, this could be due to orientation.

A transformation of ShV15 + chromosomal DNA and ShV15 alone, where the concentration of ShV15 was increased ten fold, produced the following results after ten days.

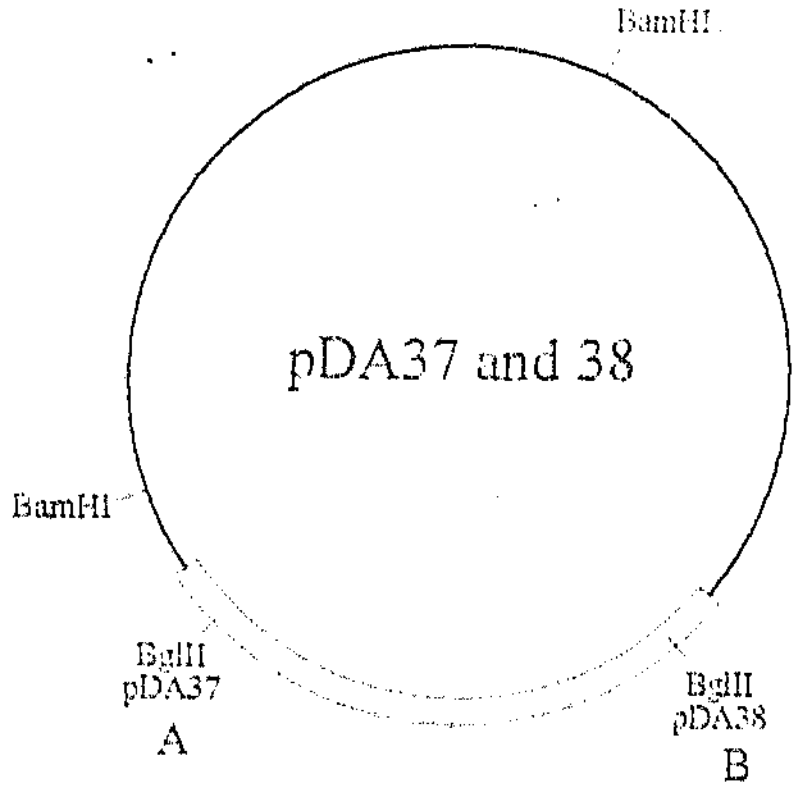


Figure 3.6 Diagrammatic representation of both orientations of the shuttle vector

A unique *BglII* site could be found at either position A or position B depending on the orientation of the constituent plasmids. The potential shuttle vectors have been identified with their *BglII* sites at the following positions:

Position A : ShV 4,5,34 called pDA37
Position B : ShV 1,11 called pDA38

ShV3 and ShV15 were deletions of original constructs since they

1 2 3 4 5 6 7

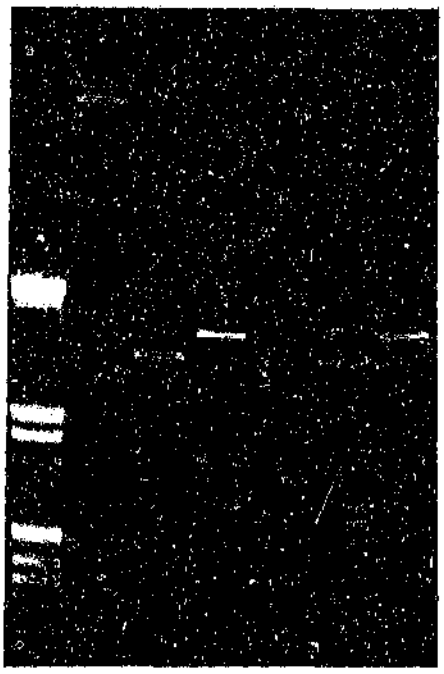


Figure 3.5 Agarose gel of *BamHI* and *BglII* digests of putative shuttle vectors, showing different orientations.
Lane 1: λIII molecular weight marker
2: ShV1 uncut
3: ShV1 digested with *BamHI* and *BglII*
4: ShV1 digested with *PvuII*
5: ShV24 uncut
6: ShV24 digested with *BamHI* and *BglII*
7: ShV24 digested with *PvuII*

be useful for positive selection vector construction.

After the original plasmids were digested with the appropriate enzymes an aliquot of each sample was run on a gel to verify complete digestion. The pEcoR251 was phosphatased before being ligated to pDA30. An equal volume of uncut pEcoR251 was passed through the alkaline phosphatase treatment to determine the amount of DNA lost through this process. Several different ligation mixtures were prepared. Half of each was transformed into λMM294. Results are shown below.

Table 3.9 Transformation of λMM294 with pEcoR251 ligated DNA

Ligation Mixture	Number of Transformants
No DNA	0
pEcoR251 uncut	~1244
pEcoR251 uncut, phosphatased	~484
pEcoR251 cut	27
pEcoR251 cut, ligated	~750
pEcoR251 cut, phosphatased	2
pEcoR251 cut, phosphatased, ligated	7
pEcoR251 cut, phosphatased, ligated to pDA30	7

The 7 colonies that potentially carried shuttle vectors were screened to determine their plasmid content. There was one putative shuttle vector (ShV4). When the other half of the above ligation mixtures were transformed into λMM294 68 colonies grew that potentially contained shuttle vectors. Twenty-four transformants were screened for plasmids, of these 6 contained putative shuttle vectors: ShV's 1, 3, 5, 11, 15 and 24. The DNA was digested with *BamHI* and *BglII* to determine the orientations of the plasmids and both were found.

Table 3.8 Transformation of CW22 with pDA30 and pDA26

Plasmid	Number of Transformants
No plasmid	0
0,1µg pDA30	564
0,1µg pDA26	550

There did not appear to be a significant decrease in the number of transformants. So it was decided that the mutants already made could be used without the introduction of any further mutations.

Some of these transformants were replica plated onto LA plates containing different concentrations of ampicillin. pDA30 grew well at 10µg/ml while pDA26 thrived at 15µg/ml. There was no increase in resistance to arsenate or arsenite in *E.coli*.

λMM294 grew on 2µg/ml ampicillin but not on 5µg/ml. However λMM294 containing pEcoR251 grew well on 1000µg/ml ampicillin.

3.2.2 Construction of pDA37 and pDA38

pDA30 contained a unique *BglII* site in a non-essential region of the plasmid. pEcoR251 had a unique *BamHI* site that did not interrupt any of the critical regions of this plasmid. These plasmids were digested with these enzymes which produce compatible cohesive ends, phosphatased and ligated. They were subsequently transformed into λMM294 and transformants screened for inserts. Initially doublets and triplets of pEcoR251 as well as deletion mutants were obtained. The 14 deleted plasmids were screened with various enzymes to identify the missing region. A smaller functional plasmid would facilitate construction of a smaller shuttle vector. All the plasmids had lost both their *BamHI* and *BglII* sites. This indicated that the deletions had occurred within the endonuclease gene. These plasmids would not

Table 3.8 Transformation of CW22 with pDA30 and pDA26

Plasmid	Number of Transformants
No plasmid	0
0,1µg pDA30	564
0,1µg pDA26	550

There did not appear to be a significant decrease in the number of transformants. So it was decided that the mutants already made could be used without the introduction of any further mutations.

Some of these transformants were replica plated onto LA plates containing different concentrations of ampicillin. pDA30 grew well at 10µg/ml while pDA26 thrived at 15µg/ml. There was no increase in resistance to arsenate or arsenite in *E.coli*.

λMM294 grew on 2µg/ml ampicillin but not on 5µg/ml. However λMM294 containing pEcoR251 grew well on 1000µg/ml ampicillin.

3.2.2 Construction of pDA37 and pDA38

pDA30 contained a unique *BglII* site in a non-essential region of the plasmid. pEcoR251 had a unique *BamHI* site that did not interrupt any of the critical regions of this plasmid. These plasmids were digested with these enzymes which produce compatible cohesive ends, phosphatased and ligated. They were subsequently transformed into λMM294 and transformants screened for inserts. Initially doublets and triplets of pEcoR251 as well as deletion mutants were obtained. The 14 deleted plasmids were screened with various enzymes to identify the missing region. A smaller functional plasmid would facilitate construction of a smaller shuttle vector. All the plasmids had lost both their *BamHI* and *BglII* sites. This indicated that the deletions had occurred within the endonuclease gene. These plasmids would not

The above figure demonstrates clearly the colour change that was identified by Dabbs (1987), and confirms that this colour change is coded for by the same plasmid that is responsible for rifampicin inactivation.

3.4 SUBCLONING OF THE *iri* GENE FROM *R.erythropolis*

3.4.1 Constructing a restriction map

The clone used in developing the restriction map was #6. Fragment sizes from the gels comparing #3 and #6 were determined. Because these digestions were of the insert still in the vector it was difficult to identify the fragments in the insert. Thus *BglII* digested plasmid was separated on a low melting agarose gel and both the inserted fragments were purified. Single and double digests using the following enzymes were done: *EcoRI*, *HindIII*, *PvuII*, *BamHI* and *Asp718*. These were separated on both 0,4% and 1,2% gels in order to size the fragments more accurately. It was noted if certain bands were maintained or cut by the second enzyme. The sizes were determined and a preliminary map constructed. The insert size was 8,5kb. As there were several spaces in the map more enzymes were tried in order to fill the gaps. Attempts were made using enzymes that had only one or two sites in the fragment. Of the following enzymes *EcoRV* and *StuI* cut once, *NotI* cut twice and *DraI* and *ScaI* did not cut. These sites were mapped.

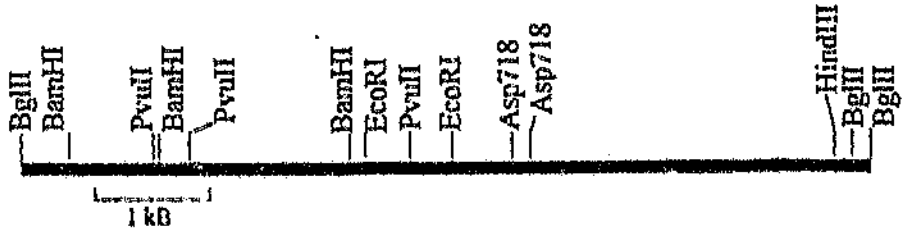


Figure 3.13 Preliminary restriction map of #6

Table 3.18 Rates of inactivation by *R.erythropolis*.

Strain/Plasmid	Rate of Inactivation
ATCC4277	47ng/min
ATCC12674	55ng/min
#6 in Ri8-1	135ng/min

A distinct colour change was noted with the inactivation of the antibiotic. Rifampicin is soluble in chloroform and chloroform is not missable with water but separates from it. Bulk cultures of Ri8-1 containing either pDA30 or #6 were grown and challenged with 50μg/ml of rifampicin. After 20h the antibiotic was extracted by adding 5ml of chloroform and shaking well. The chloroform was then aspirated from under the culture medium.

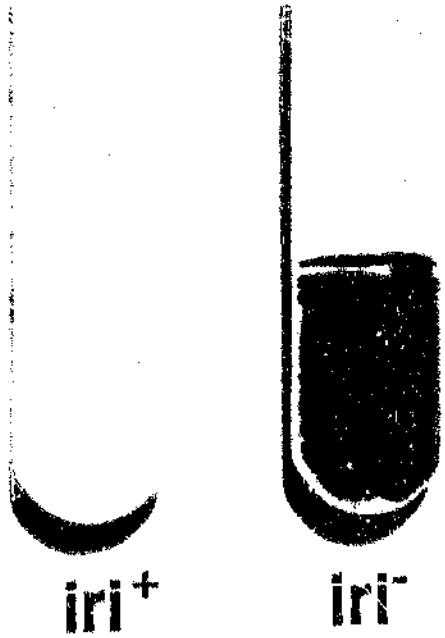


Figure 3.12 Chloroform extraction of rifampicin and rifampicin degradation products in the presence and absence of the *iri* gene

density and challenged with 10μg/ml of the antibiotic. Samples were taken every half hour for the first four hours and then every two hours up until 10 hours. The inactivation curves are shown in the figure below.

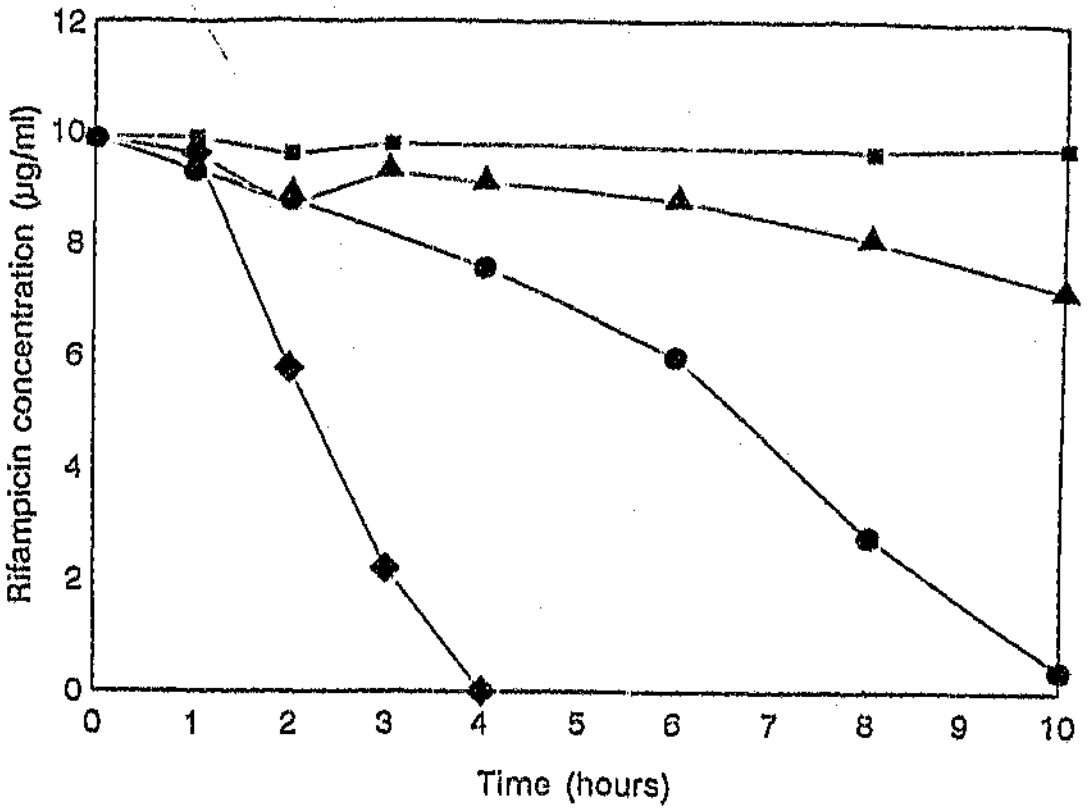


Figure 3.11 Inactivation of rifampicin by *R.erythropolis* strains resistant to rifampicin. (◢) - #6, (●) - CW22, (▲) - ATCC4277, (■) - cell free control.

Values for the concentrations of rifampicin present in a specific culture at a given time were plotted on semi-log graph paper. By measuring the gradients of these lines the rates of inactivation could be determined.

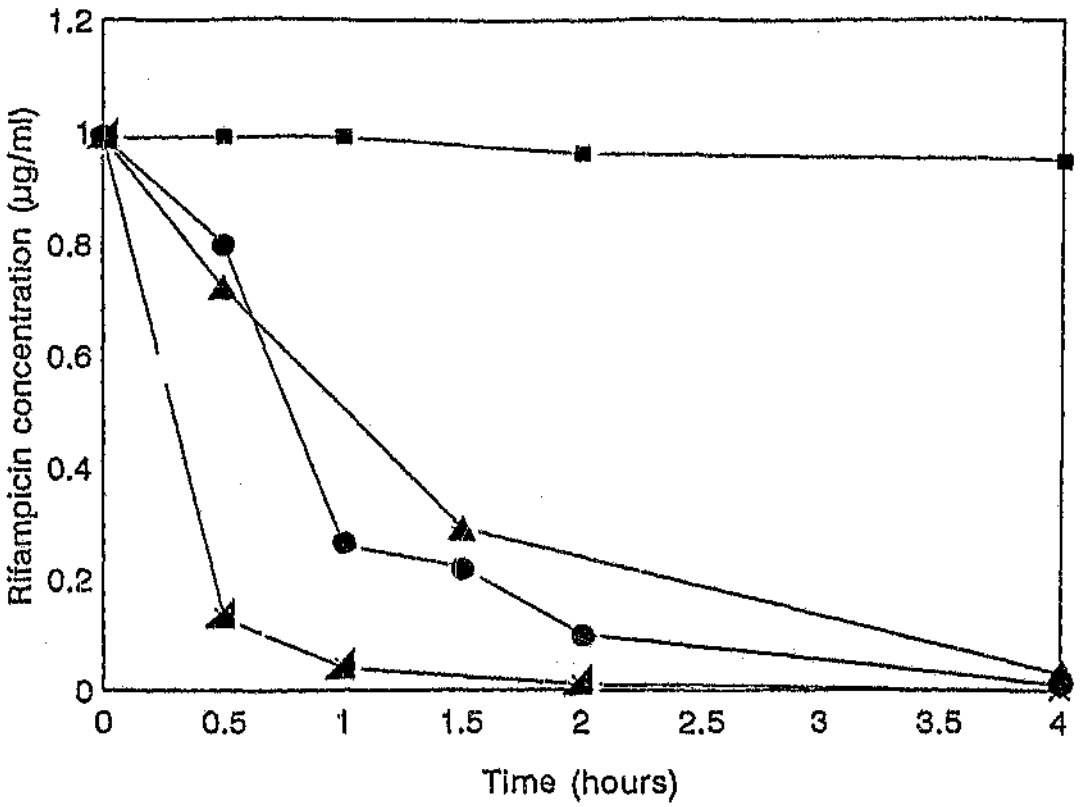


Figure 3.10 Inactivation of rifampicin by *R.erythropolis*. (◆) - #6, (●) - ATCC4277, (▲) - Ri8-1, (■) - cell free control.

The plasmid bearing strain carrying the *iri* gene was inactivating the rifampicin more quickly than the wild type strains. This could be ascribed to a copy number effect with multiple copies of the plasmid in each cell.

Similar experiments were done using higher concentrations of rifampicin and rifampicin resistant mutants of the strains. Cultures in log phase were equilibrated with respect to optical

work had used *B.subtilis* 1A3-1 (spectinomycin resistant). This organism gave larger zones of inhibition at low antibiotic concentrations, hence it was used as an assay organism for liquid culture. This was not successful. In retrospect, it could be seen that this organism was sensitive to 10ng/ml rifampicin and the concentrations of rifampicin to be measured were an order of magnitude or two greater. This organism had also been useful because of the spectinomycin resistance marker. The streptomycin resistance marker of *A.oxydans* C1S was no longer useful as Ri8-1 now contained a similar resistance. For this reason a spectinomycin resistance mutation was introduced into *A.oxydans* C1S by selecting for a mutant spontaneously resistant to 200µg/ml spectinomycin. This mutant was designated *A.oxydans* C1SS and it was used in all subsequent quantitative assays.

Cultures of #6, ATCC4277 - the organism from which the DNA was cloned, ATCC12674 - t parental strain of Ri8-1 and Ri8-1 were grown to log phase. These were equilibrated to similar cell density before being challenged with 1µg/ml rifampicin. Samples were taken at regular intervals. Initially a useful time scale, that included inactivation by the strain with the slowest rate, was ascertained. In other words when the slowest inactivating culture had completely inactivated the antibiotic. After repeating this experiment four times taking samples every hour or every two hours for 16 hours it was clearly shown that Ri8-1 did not inactivate rifampicin in this time period. It was also clear that most of the inactivation took place in the first two hours and tailed off from 4h to 8h. The experiment was repeated taking samples every half hour for the first two hours and every two hours after that. The results of this are recorded in the figure below.

To ensure that the rifampicin inactivation was due to the presence of the plasmids, cultures of each were cured of the plasmid by growth in the presence of 20μg/ml of ethidium bromide. These cultures were then assayed for inactivation of the antibiotic in parallel with those still containing the plasmids. These results are shown below.

Table 3.17 Comparison of rifampicin inactivation between culture containing recombinant plasmids and those cured of these plasmids

Strain/Plasmid	0h	4h	8h
No cell control	*	*	*
#1	*		
#3	*		
#4	*		
#5	*		
#6	*		
Cured:#1	*	*	*
#2	*	*	*
#3	*	*	*
#4	*	*	*
#5	*	*	*

where * indicates the presence of rifampicin in the culture medium.

These results confirmed that the ability to inactivate rifampicin was encoded by genes on the recombinant plasmids. The plasmid in transformant #6 was chosen for more detailed investigation into the process of inactivation. To do this an assay was used that would quantitate the concentration of rifampicin in the culture at a given time. This assay worked on the same principle as the plate assay method, however it was done in liquid culture so that spectrophotometric readings could be taken of the growth of the assay organism in order to determine the drug concentration.

Previously (S.Andersen, M.Sc Dissertation 1989) a method had been developed using *A.oxydans* C1S (streptomycin resistant) as an assay organism. This strain has been used for both plate and liquid assays. The plate assays carried out in this part of the

Table 3.15 Resistance to rifampicin conferred by #1-#6

Strain	LA	Rifampicin in µg/ml 0,1	0,2	0,3	0,4	0,5	0,6
ATCC12674	+++	+++	+++	+++			
pDA30 in Ri8	+++						
pDA37 in Ri8	+++						
#1	+++	+++	+++	+++	+++	+++	
#2	+++						
#3	+++	+++	+++	+++	+++	+++	
#4	+++	+++	+++	+++	+++	+++	
#5	+++	+++	+++	+++	+++	+++	
#6	+++	+++	+++	+++	+++	+++	

where +++ indicates growth; all plates contained 40mM arsenate for plasmid maintenance.

Assays revealed that strains carrying these plasmids inactivated rifampicin.

3.3.3 Rifampicin inactivation by the cloned gene

Rifampicin inactivation assays were carried out on several of the transformants. Samples of the supernatants were assayed every 2,5 hours for 7,5 hours. The cultures were challenged with 1µg/ml rifampicin. The following results were obtained.

Table 3.16 Inactivation of rifampicin by complementing transformants

Strain/Plasmid	0h	2,5h	5h	7,5h
No cell control	*	*	*	*
pDA30	*	*	*	*
ATCC12674	*	*	*	
#1	*			
#2	*	*	*	*
#3	*			
#4	*			
#5	*			

where * indicates the presence of rifampicin.

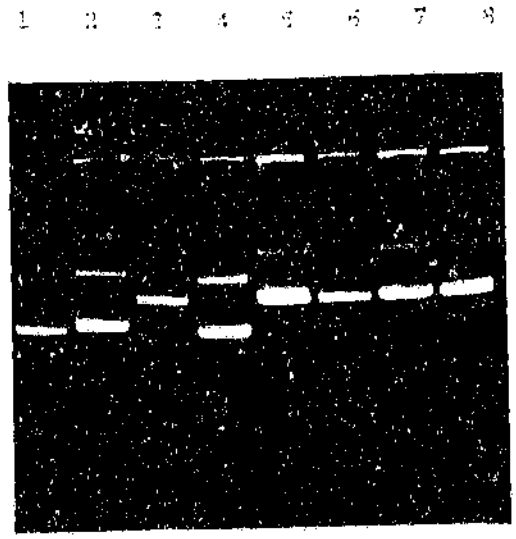


Figure 3.9 An agarose gel of transformants #1-#6, pDA37 in lanes 1 and 2, tranformants #1-#6 in lanes 3-8

The plasmids were all extracted and purified on CsCl gradients for further analysis. After digestion with *BglII* and separation on an agarose gel it became evident that each contained the same sized inserts except for one which did not have an insert. Each had two *BglII* fragments inserted into pDA37, one of about 9kb and the other about 0,2kb.

Two of these plasmids were chosen for further analysis #3 and #6. Identical digestions were done of each one: Single and double digests with *BglII*, *HindIII*, *EcoRI* and *PvuII*. There was no significant difference between these plasmids.

When several of these clones were tested for increased resistance to rifampicin it was found that the level of resistance had increased 6 fold over R16-1. It was also more than twice the level of resistance in the wild type ATCC14674. This is not surprising as the gene is now located on a plasmid, it is probably in multiple copies in the cell.

transform the genomic library. The transformation efficiency was 53 100 transformants/µg of pDA30. As heterologous DNA reduces the transformation efficiency on average 100 fold, 531 transformants/µg genomic library DNA could be expected.

To check for reversion in Ri8-1 aliquots of cultures of the strain and the same containing pDA30 were spread on broth plates supplemented with 0,3 or 0,6 or 1,0µg/ml rifampicin. The plates spread with the plasmid containing strain were also supplemented with 40mM arsenate. After three days at 26°C no colonies had appeared on the plates thus showing a clear mutant.

About 34 000 transformants resistant to arsenate and arsenite were obtained. These were washed into broth supplemented with 60mM arsenate. From this suspension 0,1ml (containing 8×10^5 colony forming units) was spread on a plate supplemented with 40mM arsenate and 0,3µg/ml rifampicin. As a control, a similar amount of cells of strain Ri8-1 transformed with pDA30 was spread on an identical plate.

After 72h of incubation at 26°C, there were 38 colonies on the plate spread with cells transformed with the library but no colonies were observed on the plate spread with cells transformed with pDA30.

Cultures were grown from six of these colonies and screened for plasmids. In each case, the plasmid was of a similar size and migrated substantially slower in agarose gels than the vector itself. Half of the DNA prepared from the plasmid screens was transformed back into *E.coli*. This was to ensure that we still had the plasmid in case it reintegrated into the host chromosome. DNA prepared from *E.coli* was also easier to manipulate.

transform the genomic library. The transformation efficiency was 53 100 transformants/μg of pDA30. As heterologous DNA reduces the transformation efficiency on average 100 fold, 531 transformants/μg genomic library DNA could be expected.

To check for reversion in Ri8-1 aliquots of cultures of the strain and the same containing pDA30 were spread on broth plates supplemented with 0,3 or 0,6 or 1,0μg/ml rifampicin. The plates spread with the plasmid containing strain were also supplemented with 40mM arsenate. After three days at 26°C no colonies had appeared on the plates thus showing a clear mutant.

About 24 000 transformants resistant to arsenate and arsenite were obtained. These were washed into broth supplemented with 60mM arsenate. From this suspension 0,1ml (containing 8×10^5 colony forming units) was spread on a plate supplemented with 40mM arsenate and 0,3μg/ml rifampicin. As a control, a similar amount of cells of strain Ri8-1 transformed with pDA30 was spread on an identical plate.

After 72h of incubation at 26°C, there were 38 colonies on the plate spread with cells transformed with the library but no colonies were observed on the plate spread with cells transformed with pDA30.

Cultures were grown from six of these colonies and screened for plasmids. In each case, the plasmid was of a similar size and migrated substantially slower in agarose gels than the vector itself. Half of the DNA prepared from the plasmid screens was transformed back into *E.coli*. This was to ensure that we still had the plasmid in case it reintegrated into the host chromosome. DNA prepared from *E.coli* was also easier to manipulate.

previous work by Dabbs and Andersen (M.Sc. Dissertation, 1989) 14 mutants of CW22 hypersensitive to rifampicin were isolated after screening 4000 colonies. On checking their inactivation profiles three classes of mutants were identified. Those that inactivated the antibiotic at the same rate as the wild type, those that inactivated slower than the wild type and three of them Ri7, Ri8 and Ri14 that did not inactivate rifampicin at all.

Representatives from each class were streaked out from glycerol stocks and their levels of hypersensitivity to rifampicin were checked by replica plating. The wild type strain ATCC12674 was also plated. They were all spotted onto a broth plate and broth plates containing different concentration of the antibiotic.

Table 3.14 Sensitivity of strains to rifampicin

Strain	Rifampicin in µg/ml				
	LA	0,1	0,2	0,3	0,4
ATCC12674	+++	+++	+++	+++	
Ri1	+++				
Ri6	+++	+++	+++		
Ri7	+++				
Ri8	+++				
Ri10	+++				

where +++ indicates growth

Ri8 was chosen as the recipient strain. As it did not have any other antibiotic resistant marker Ri8 was made resistant to 200µg/ml streptomycin by selecting for spontaneous mutant on a streptomycin containing plate. This mutant was designated Ri8-1. It was still hypersensitive to rifampicin and did not inactivate it.

Bulk cultures of Ri8-1 were grown up in TYGS containing 3% or 5% glycine. Twenty-four 1ml samples were taken every 24 hours. An aliquot from each sampling was transformed with 10ng of pDA30 to find the most transformable batch. These would be used to

there is a 100% efficiency of ligation of inserts. In this case where there has been a 72% efficiency, N = 7922. So a library of a minimum of 8000 transformants was constructed.

During the construction of the library plasmid screens of transformants were regularly done to determine the number of inserts and the size of the inserts. The origin of the inserted DNA was also verified by blotting *BglII* digested DNA from MM294-1 and ATCC4277 and probing with the DNA inserted into pDA37 from several of the transformants.

Probes were prepared by digesting the plasmids obtained from the transformants with *BglII*. This DNA was separated on a 0,8% low melting agarose gel. The inserts were purified and labelled with non-radioactive digoxygenin. The kit used was the "DNA labelling and detection kit" from Boehringer-Mannheim. These fragments of DNA were used to probe nitrocellulose membranes onto which digested MM294-1 and ATCC4277 DNA had been blotted. Hybridisation was detected using the immunological visualisation procedure. All the probes bound to the ATCC4277 DNA and none to the MM294-1 DNA confirming the origin of the inserted DNA.

A library of 20 500 transformants was constructed with an average insert size of 3,3kb. Of the transformants 79% contained detectable inserts, that is inserts greater than 200bps. Thus the probability of any gene being present in this library is greater than 97,5%.

Transformants were washed off the plates using LB containing 50µg/ml ampicillin and 30µg/ml rifampicin. Aliquots were stored in glycerol at -70°C and an aliquot was inoculated into 200ml of the above media in order to do a bulk extraction of the library.

3.3.2 Isolation of DNA carrying *iri*$^+$ gene

As the method of cloning was by complementation, a mutant unable to inactivate rifampicin would be the recipient strain. In

3.4.10 Insertion of the *BamHI-BglII* fragment into pDA71

The advantages of inserting the 5kb fragment into pDA71 were twofold. Firstly, as there has been a reduction in size of the plasmid there are more unique restriction sites as restriction sites have been lost from the vector. Secondly, chloramphenicol selection in nocardioform transformations facilitates the growth of transformants in half the time of arsenic selections.

The 8,3kb original fragment was separated from pDA37 by *BglII* digestion and electrophoresis on a low melting agarose gel. This fragment was digested with *BamHI* and ligated to *BglII* digested pDA71. Recombinants could therefore contain any of four fragments. The appropriate one would be identified by the presence of the *Asp718* fragment.

When no transformants were obtained and all appropriate controls showed that the transformation was working, it appeared as if the problem lay with the ligation. Several times the procedure was repeated with new phenol, different numbers of phenol extractions, increasing the ligase concentration and increasing the ligation time to 48 hours. The insert DNA was also digested with *DraI* in addition to *BglII* and *BamHI*. *DraI* cuts three times in the 8,3kb fragment but not once in the 5kb fragment. This would theoretically cut down on the number of unwanted inserts.

The literature seemed to indicate that a combination of TBE buffer, ethidium bromide and short wave length UV light reduced ligation efficiency 1000 times (Sambrook *et al*, 1989) So the transition was made to TAE buffer, ethidium bromide and long wave length UV light. The first attempt under these conditions yielded 13 transformants. Only two had sizable insertions which on digestion were shown to be dimers. However subsequent transformations of DNA purified and ligated in the way described above yielded many transformants. Of these 40 were screened in order to get the fragment inserted in both orientations. Transformants were first screened for the presence of the *Asp718*

These plasmid containing strains were subsequently cured of these plasmids by growing them in the presence of 20μg/ml ethidium bromide. The stationary phase culture was diluted 10^{-6} and spread on an LA plate. Of the 44 colonies that grew up only one grew when patched onto the selective plate. The absence of the plasmids was confirmed by screening.

Numerous attempts were made to insert the above mentioned fragments into pDA64. The inserts were purified on low melting agarose and ligated to phosphatased pDA64. None of these were successful. Several changes were made to both the agarose purification and phosphatasing procedures, but without success.

pDA64 was cut down further by Quan and Dabbs, 1992, and ligated to pEcoR251 to produce an 8,8kb positive selection shuttle vector that was maintained with chloramphenicol in the nocardioform. This plasmid was designated pDA71.

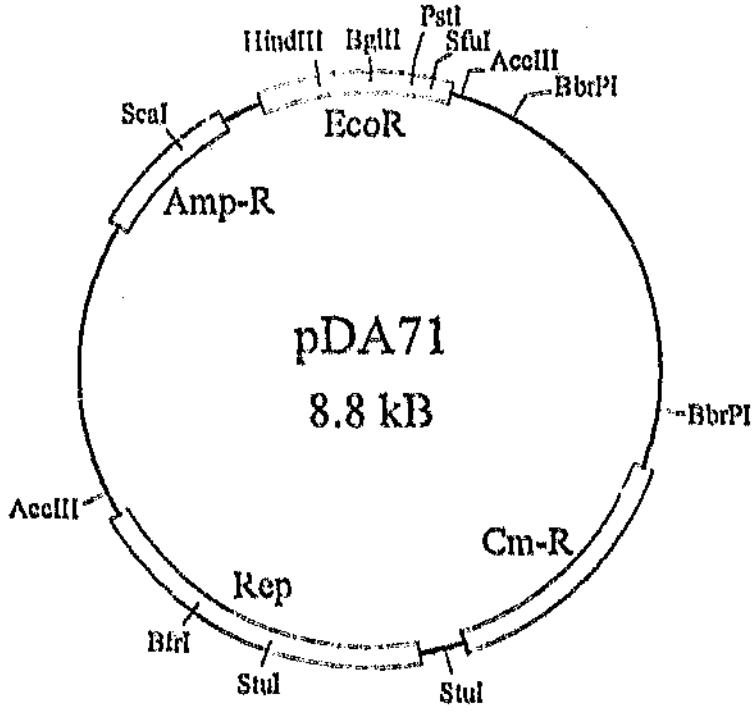


Figure 3.18 Diagrammatic representation of pDA71

was used. pDA64 is a plasmid of the same lineage as pDA30's predecessor, pDA21. It had a chloramphenicol resistance gene inserted and the arsenic genes deleted. This vector also contains a unique *ClaI* site, a *BsiWI* site, an *Asp718* site and a blunt ending *XmnI* site in a non essential region. It would therefore be possible to insert the *PvuII-NarI* fragment into the *ClaI-XmnI* region and the *SfuI-NarI* fragment into the *ClaI* site as all produce compatible sticky ends. It should also be possible to introduce the *SfuI-SmaI* fragment as *SmaI* produces a blunt end. A *PvuII-SmaI* fragment which has blunt ends at both ends would be more difficult to ligate in.

However before any of these procedures could be carried out Ri8-1 had to made highly transformable to chloramphenicol resistance plasmids. Ri8-1 was transformed with a large plasmid known as RV-Sca which is not easily maintained in this strain.

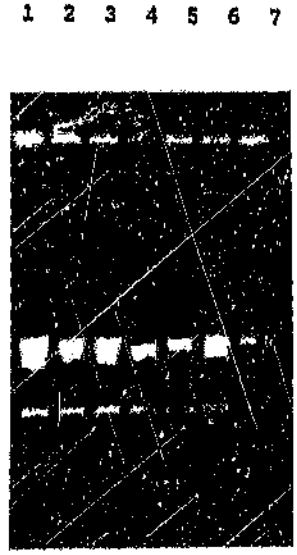


Figure 3.17 A plasmid screen of Ri8-1 containing RV-Sca

inserting it into several vectors other than pDA37.

The first attempt was with the *EcoRI* fragment that extended from the site in the fragment through the *Asp718* sites to the *EcoRI* in the pEcoR251 portion of pDA37. This fragment was to be ligated to the *EcoRI* site of pDA30. The vector containing the 5kb *BamHI-BglII* fragment was digested with *EcoRI* and separated on a regular agarose gel. The appropriate fragment was cut out of the gel and electroeluted in dialysis tubing. pDA30 was cut and phosphatased. Ligation mixtures were transformed into Ri8-1. Many tiny colonies grew that showed no growth on patching. However one patched colony grew up but was unable to grow in the presence of 0,3µg/ml rifampicin.

No plasmid was seen initially on a screen of the uncut DNA. When the DNA was digested with *EcoRI* an insert of approximately 7kb was observed. Since this was larger than expected, the experiment was repeated obtaining the fragment by the freeze-squeeze method This was not successful.

The second fragment to be used was the *PvuII-NarI* fragment. The strategy was to clone this into the *EcoRV* and *ClaI* sites of pACYC184. *NarI* and *ClaI* produce compatible cohesive ends and *PvuII* and *EcoRV* produce blunt ends. Insertion would be determined by sensitivity to tetracycline. This recombinant would be digested with *BamHI* and ligated to the *BglII* of pDA30. This recombinant would be transformed into the nocardioform and tested for inactivating ability.

This was done twice using 4 times as much insert as vector that had been freshly isolated. Each time plasmid screens of transformants showed no inserted DNA although the vector had been phosphatased.

To eliminate the two step procedure of cloning first into an *E.coli* vector and then ligating it a nocardioform plasmid, pDA64

reactions were carried out using *BglII* digested DNA and *HindIII* digested DNA in tandem.

These plasmids were treated with *Bal31*, ligated and transformed into Ri8-1. Only 7 transformants grew, one from a *BglII* digested plasmid and 6 from *HindIII* digested plasmids. After plating onto LA plates containing 40mM arsenate with and without 0,3µg/ml rifampicin, growth showed that 5 of the 7 still complemented the mutation whereas two did not. Further analysis of these plasmids showed that the two deleted plasmids had lost both *EcoRI* and both *Asp718* sites.

Another transformation of Ri8-1 with *Bal31* treated plasmids yielded 4 colonies from *HindIII* digested plasmids and more than 80 colonies from *BglII* digested plasmids. Of these 21 were analyzed further by extracting the plasmids using the miniprep method and then digesting with various enzymes including *Asp718*, *EcoRI*, *NarI*, *NotI*, *PvuII*, *SfuI* and *SmaI*.

To summarise the results of these digestions: Plasmids linearised with *HindIII* and then subjected to *Bal31* were unable to inactivate rifampicin when they had lost the *Asp718* fragment and the *SmaI* site. Sites found before the *Asp718* sites were checked and found to be missing. Those that were able to inactivate the antibiotic had lost one *EcoRI* site but not the *PvuII* site.

Plasmids linearised with *BglII* and then treated with *Bal31* were unable to inactivate rifampicin when they had lost the *Asp718* sites and those preceding them. Those that were able to inactivate the drug had lost the one *NotI* site and [illegible] *NarI* sites.

As the plasmids obtained from this process had either been significantly digested or hardly digested they confirmed the significance of the region containing the *Asp718* fragment but they did not succeed in establishing the boundaries for the DNA involved in rifampicin inactivation.

An attempt was made to insert the *NotI* fragment into pDA21. pDA21 was a 15kb predecessor of pDA30 that contained a unique *NotI* site in a non-essential region. The *NotI* fr t was separated on a regular agarose gel and subsequently electroeluted. After ligation the mixture was transformed into Ri8-1. The colonies that grew were very small and howed no growth on patching. Another attempt was made to insert the fragment. It was purified using a freeze-squeeze method. This was inserted into pDA21 which had a chloramphenicol resistance introduced into it. It proved unsuccessful. This experiment was not repeated as one *NotI* site is found within the *Asp718* fragment which appeared to be in the gene. Therefore it would not yield additional information.

3.4.8 Exonuclease *Bal31* deletions

Exonuclease digestions of the plasmid were done to locate the region of the plasmid responsible for the inactivation of rifampicin. Because of the nature of *Bal31* unique restriction sites needed to be present on either side of the fragment. The plasmid was then linearised at that position and subjected to endonuclease digestion. The 5kb fragment in pDA37 has a unique *BglII* site which could be used. Unfortunately there was no unique site on the other - of the fragment. Digestion of this plasmid with *HindIII* showed two sites 5kb apart, one in the vector and one in the insert. However with *Bal31* digestion, both ends of the DNA is digested. This means that the plasmid linearised with *BglII* and digested with *Bal31* could lose the *HindIII* site that is found adjacent to the *BglII* site in the insert. If this was recircularised there would be only one *HindIII* left on the plasmid, on the other end of the insert. Thus plasmids would be available for *Bal31* digestions from both sides.

Plasmid screens of transformants in *E.coli* showed that on *HindIII* digestion 50% had lost one *HindIII* site.

One of these plasmids which had lost a *HindIII* site was extracted in bulk and purified on a CsCl gradient. Subsequently *Bal31*

cloned DNA fragment.

3.4.7 *NotI* fragment

As there were no *NotI* sites in pDA37 and 2 in the fragment no phosphatasing was necessary. The vector containing the 5kb insert was digested with *NotI* and then ligated. Of the transformants that were screened 64% had lost the *NotI* fragment.

1 2 3 4 5 6 7 8 9 10 11 12

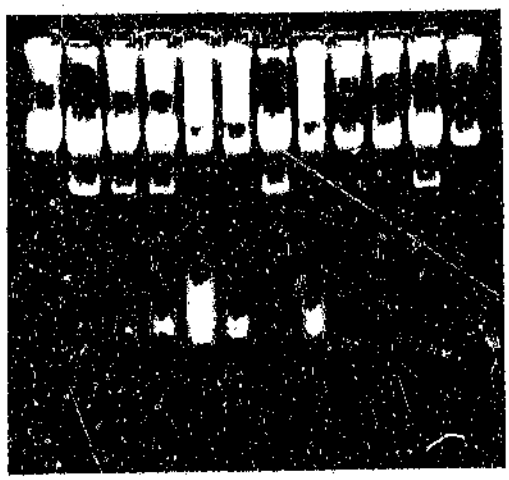


Figure 3.16 *NotI* deletions of the fragment
lanes 1, 5, 6, 8, 10, 12

One isolate of the deleted plasmids was transformed into R18-1. The mutation was not complemented and the plasmid was unable to induce inactivation or decolorisation of rifampicin.

1 2 3 4 5 6 7 8 9



Figure 3.15 *Asp718* deleted plasmid - lane 2

This plasmid was transformed into R18-1. It did not complement the mutation neither did it inactivate nor decolorise rifampicin. This gave the first indication of the position of the gene on the

Minipreps from this organism yields a substantially greater amount of DNA than equivalent preparations from nocardioforms. This DNA was then digested with various enzymes to determine the extent of DNA that had been eliminated, these enzymes included *BamHI*, *EcoRI*, *HindIII* and *PvuII*.

They all still contained the *HindIII* site, but had lost sites from the other end of the fragment. Further digestions showed that the *Asp718*, *EcoRI* and *NotI* sites were maintained. The smallest had lost both *PvuII* sites. Rifampicin inactivation assays of these subclones showed that all inactivated the drug.

From this information it appeared that the largest *BamHI-BglII* fragment contained the gene.

3.4.5 Large *BamHI-BglII* fragment

The large *BglII* fragment in pDA37, #6, was digested with *BamHI* and *BglII*. These fragments were separated on a low melting agarose gel and the 5kb fragment extracted. This DNA was ligated into the *BglII* site of pDA37 and transformed into MM294-1. An *Asp718* digestion showed the characteristic presence of the 300bp fragment. This construct facilitated inactivation and decolorisation of rifampicin.

3.4.6 *Asp718* fragment

Not all the *BamHI-BglII* fragment isolated for the previous experiment was used. The remaining fragment was phosphatased, phenoled, chloroformed, precipitated and resuspended in B buffer. It was then digested with *Asp718*. This was later resuspended in ligation buffer and ligated to *BglII* digested pDA37. Only 7 transformants were obtained, however one of these had lost the small *Asp718* fragment.

plasmid complemented the Ri8 mutation. Rifampicin was still inactivated and decolorised so this fragment was not involved.

3.4.4 Elimination of a further 1,75kb

As *BamHI* and *BclI* produce compatible cohesive ends to *BglII*, These enzymes were used to digest the 8,3kb fragment that had been purified from low melting agarose. The intention was to ligate these fragments into the *BglII* site of pDA37 and to select for complementation of the Ri8 mutation. Several attempts with each enzyme did not yield the desired result. The low ligation efficiency was shown later so be affected by the use of TBE buffer.

Another attempt was made by partially digesting the large fragment with *Sau3A*. This four base pair cutter also produces cohesive ends compatible with *BglII*. These digests were separated on low melting agarose. The DNA was cut out in three sections according to size: greater than 4,5kb, between 2kb and 4,5kb and smaller than 2kb. These fractions were purified and ligated into pDA37. A large number of colonies grew on the control transformed with cut and self ligated pDA37. This occurred when digested plasmid was prepared in large quantities and just thawed when needed. By preparing DNA afresh each time the problem was overcome. Fresh phenol was also prepared for the removal of the agarose from newly digested fragment.

Eventually 71 transformants were obtained. These were then pooled and used to inoculate a bulk culture. DNA was purified on a CsCl gradient and later dialysed against TE. This pool of inserts was eventually transformed into Ri8-1. Seventy colonies grew up on the regeneration plates. These were patched onto the LA plates containing 40mM arsenate with and without 0,3µg/ml rifampicin. Of these 20 grew up on the antibiotic containing plate. Plasmid screens showed that all contained large inserts. The 8 smallest were examined further. These were transformed into *E.coli*. Minipreps from this organism yields a substantially greater

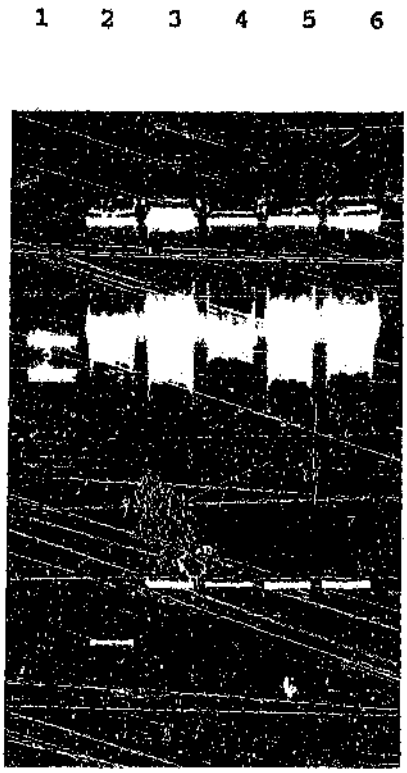


Figure 3.14 *HindIII* fragments from different complementing clones

Of the 10 tested 7 had 750bp fragments and 3 had 500bp fragments. No plasmids were found inserted in the opposite direction. Purified DNA of #6 was digested with *HindIII*. After phenol and chloroform were used to remove the enzyme, the DNA was precipitated to remove the salts and was resuspended in ligation buffer. This digested DNA was ligated over night and transformed into *E.coli* MM294-1. Transformants were screened and some were found that no longer contained the small *HindIII* fragment. This

In order to sequence the *iri* gene it would be helpful if the region on this fragment could be narrowed down and surplus DNA cut away. The approach most often used was deletion mapping.

In each case of deletion or insertion the following checks were made: 1. Did the new plasmid complement the Ri8 mutation?
2. Did the new plasmid cause inactivation of rifampicin?
3. Did the new plasmid cause decolorisation of the drug?

3.4.2 Small *BglII* fragment

The large *BglII* fragment was inserted into pDA37 and transformed into *E.coli*. Plasmid was purified by CsCl gradient centrifugation and transformed into Ri8-1. This fragment complemented the mutation - thus the small fragment was not necessary. The small fragment was nevertheless purified, ligated and transformed. On its own it did not complement the mutation. The large fragment still inactivated and decolorised rifampicin.

3.4.3 *HindIII* fragment

Digestions of 10 of the clones originally isolated showed that all contained a small *HindIII* fragment. There was however a difference in the sizes of these fragments. Some were about 750bp in size and others about 500bp. However this had no effect on the ability to inactivate rifampicin, indicating that the DNA contained in the *HindIII* fragment was not part of the gene or its regulatory elements.

Table 3.22 Increase in rifampicin resistance conferred to Ri mutants by cloned DNA

Strain	LA	Rifampicin in µg/ml 0 1	0,3	0,5	1,0	1,5	2,0
Bcl2 in Ri7	+++						
#16 in Ri7	+++	+++	+++	+++	+++	+++	
pDA71 in Ri8	+++						
#16 in Ri8	+++	+++	+++	+++	+++	+++	
Bcl2 in Ri10	+++						
#16 in Ri10	+++	+++	+++	+++			
Bcl2 in Ri11	+++						
#16 in Ri11	+++	+++	+++	+++	+++	+++	
Bcl2 in Ri12	+++	+++					
#16 in Ri12	+++	+++	+++	+++	+++	+++	
Bcl2 in Ri13	+++	+++					
#16 in Ri13	+++	+++	+++	+++	+++	+++	
Bcl2 in Ri14	+++						
#16 in Ri14	+++	+++	+++	+++	+++	+++	
Bcl2 in CW22	+++	+++	+++				
#16 in CW22	+++	+++	+++	+++	+++	+++	

where +++ indicates growth; each plate contained 40µg/ml chloramphenicol for plasmid maintenance.

In each case the presence of the cloned DNA increased the level of resistance to rifampicin at least 10 fold.

3.5.2. The effect of *iri*$^+$ DNA on sensitivity to other antibiotics

Previous studies in our lab showed that an increase in rifampicin resistance in *Rhodococcus* produced a concomitant increase in resistance to fusidic acid and erythromycin. This study, (H.Golob, M.Sc dissertation, 1989) showed that the Ri mutants used here still inactivated fusidic acid. It was decided that the original strain and the Ri8-1 mutant, both containing either *iri*$^+$ or *iri*$^-$ plasmids be screened for increased resistance to other antibiotics.

First of all the ansamycins were tested and the following results obtained.

Table 3.21 Rifampicin inactivation by DNA complementing the Ri14 mutation

Strain	Inactivation
Ri14 + pDA30	NO
Ri14 + #6(used previously)	NO
Ri14 + 8,3kb + 4kb	YES
Ri14 + 8,3kb + 4kb	YES
Ri14 + 8,3	YES
Ri14 + 8,3kb + 4kb	YES

These results seemed to indicate that the 8,3kb fragment did cause rifampicin inactivation. It was decided to check the plasmid contained in the original Ri14 transformant, the one used in the earlier experiments. Since no plasmid was isolated from Ri14 it must have been a spontaneous arsenate resistant mutant.

Thus the presence of the 8,3kb fragment conferred the ability to inactivate rifampicin. All the mutants inactivated rifampicin faster if they contained the plasmid of cloned DNA. This however could be due to a copy number effect. Several of these mutants were screened for complementation of the hypersensitivity and for increased resistance to rifampicin. For these replica plate experiments, mutants were transformed with pDA71 containing the 5kb *BamHI-BglII* fragment, #16. The control was a nocardioform plasmid containing a chloramphenicol resistance, Bcl2. The results are contained in the table below.

using higher concentrations of the drug. The challenge was made using 10µg/ml rifampicin. All the rest besides Ri14, showed faster inactivation in the presence of the cloned DNA than in its absence.

This seemed to indicate that the mutation in the Ri14 was not complemented by the genes on #6. Thus it should be possible to pull out a complementing clone if the ATCC4277 *BglII* library was transformed into Ri14. This was done and 34 000 transformants were obtained. They were washed off, pelleted and resuspended in LB containing 40mM arsenate. This was serially diluted to 10^{-5} and spread on LA plates containing 40mM arsenate and 0,3µg/ml rifampicin. An equivalent dilution of pDA30 in Ri14 was spread on similar plates as a control. Of the colonies that grew up 51 were patched onto LA plates containing 40mM arsenate with and without 0,3µg/ml rifampicin. Some colonies of Ri14 transformed with pDA37 were also patched. All 51 grew on the rifampicin containing plate but none of the pDA37 did. This indicated that the hypersensitive mutation had been complemented.

DNA was prepared from several colonies and then transformed directly into *E.coli*. DNA was then extracted from these bacteria and subjected to restriction enzyme analysis. In each case a control of the previously cloned DNA was digested and run alongside these digestions. Of 10 transformants examined, all contained an 8,3kb *BglII* fragment and 3 contained an additional 4kb *BglII* fragment. The digestion patterns of the plasmids containing the 8,3kb insert were identical to the previously cloned DNA after restriction with *EcoRI* and *NotI*.

It appeared as if the same fragment had been cloned again. In order to see if this extra 4kb insert was important for rifampicin inactivation, assays were done using the pair or Ri14 plasmid containing strains used earlier and four of the newly isolated plasmids. Of the four, three contained the extra 4kb fragment and one only the 8,3kb fragment. The results are presented below.

Table 3.20 Inactivation of rifampicin by hypersensitive mutants containing the *iri*$^{+}$ clone

Mutant	Plasmid	0h	2h	6h
Ri2	pDA37	*	*	
Ri2	#6	*	*	*
Ri3	pDA37	*	*	
Ri3	#6	*	*	
Ri4	pDA37	*	*	
Ri4	#6	*	*	*
Ri6	pDA37	*	*	
Ri6	#6	*		
Ri7	pDA37	*	*	*
Ri7	#6	*		
Ri9	pDA37	*		
Ri9	#6	*		
Ri10	pDA37	*	*	
Ri10	#6	*	*	
Ri11	pDA37	*	*	
Ri11	#6	*		
Ri12	pDA37	*		
Ri12	#6	*		
Ri13	pDA37	*		
Ri13	#6	*	*	
Ri14	pDA37	*	*	*
Ri14	#6	*	*	*
Cell free control		*	*	*

* indicates the presence of the drug due to a zone of inhibition on a lawn of *B.subtilis*.

Ri6, Ri7, and Ri11 showed faster inactivation in the presence of *iri*+ DNA. Ri3, Ri9, Ri10 and Ri12 showed inactivation at similar rates. To overcome this the experiment would be repeated with samples being taken more frequently. Ri14 did not inactivate during the time period sampled. Ri2, Ri4 and Ri13 showed results in the reverse of what was expected.

On the repeat of the experiment Ri12 and Ri13 inactivated faster than the control. For the others this was not clear. For this reason rifampicin resistant mutants were made of plasmid containing strains so inactivation assays could be carried out

From these figures it can be seen that approximately 5,8kb has been excluded from the fragment as being non essential for the inactivation of rifampicin and that the region responsible has been narrowed down to 2,5kb.

3.5 FURTHER WORK WITH *iri*$^+$ CLONED DNA

3.5.1 Complementation of other rifampicin hypersensitive mutants by cloned DNA

Earlier it was mentioned that several mutants had been made that were hypersensitive to rifampicin. In order to see if these mutations were complemented by the cloned DNA, plasmids were transformed into them. In addition the vector was also transformed into them to be used as a control. The following mutants were used: Ri2, Ri3, Ri4, Ri6, Ri7, Ri9, Ri10, Ri11, Ri12, Ri13 and Ri14. Ri1 had been made from ATCC12674 which is arsenic resistant and so could not be used to select for plasmid containing colonies. Ri5 was very slow growing and Ri8 had been used in the cloning of the gene. All of these mutants were hypersensitive although some inactivated rifampicin. Some inactivated at the same rate as wild type others inactivated more slowly (Andersen SJ, M.Sc.Dissertation, 1989).

Subsequently rifampicin inactivation assays were done on each pair of mutants. Samples were taken at regular intervals to see if the clone containing mutants inactivated faster than the vector alone containing mutants. Cultures were equilibrated to the same optical density before they were challenged with 2μg/ml rifampicin. The results are represented below.

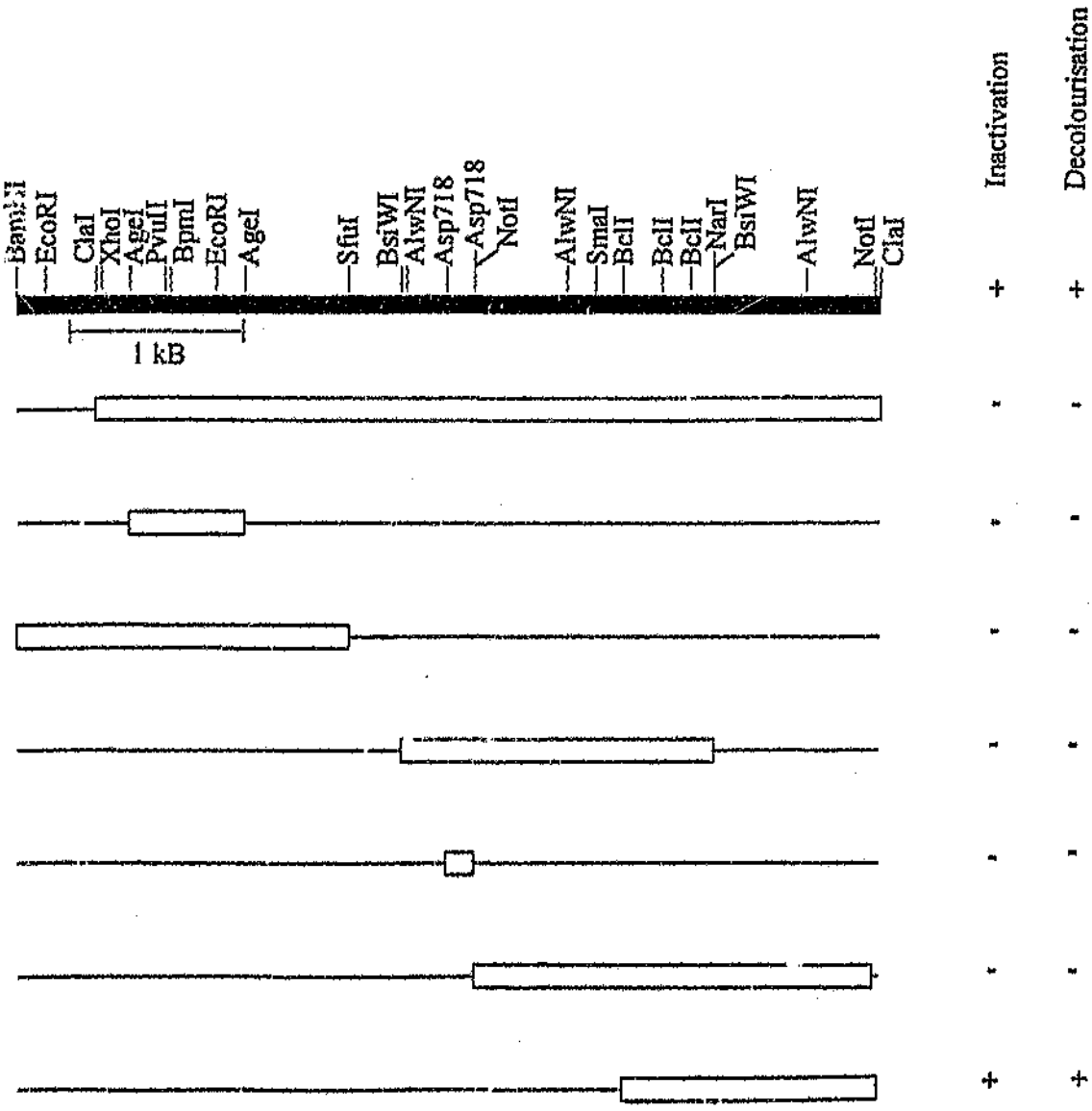


Figure 3.22 Deletions of the 5kb *BamHI-BglII* fragment and there concomitant phenotypic expression. Blocks indicate deletions.

3.4.14 Summary of subcloning experiments

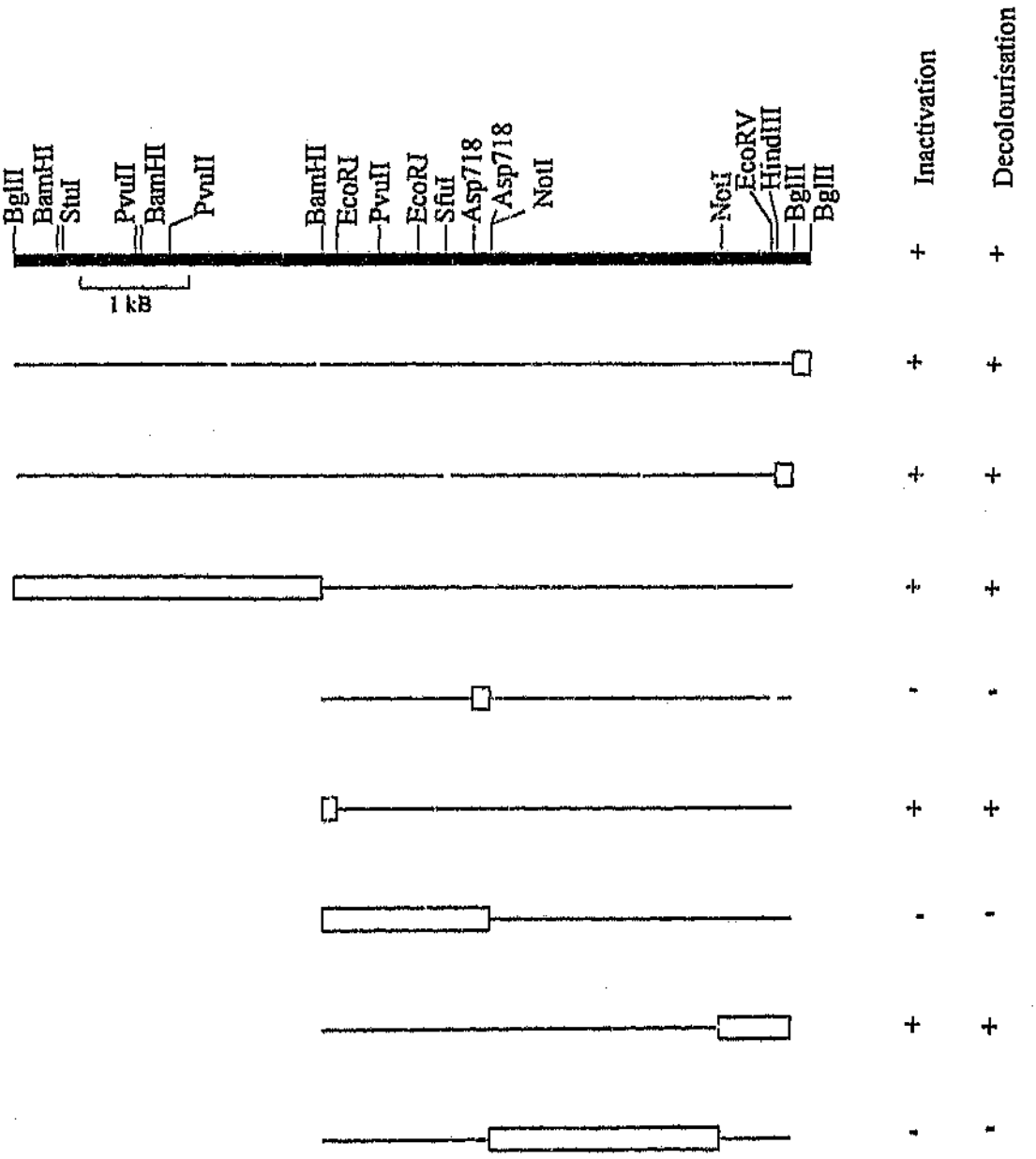


Figure 3.21 Deletions of the 8,3kb fragment and there concomitant phenotypic expression. Blocks indicate deleted regions.

using Klenow enzyme, to pDA37, the *BglII* site having been similarly treated, failed. Since the *AgeI* deletion revealed that at least one *AgeI* site was involved in the gene the information obtainable from the BsiWI ligation would not increase the knowledge already obtained.

The *SfuI* fragment was inserted into the *SfuI* site of pDA71. This plasmid in Ri8-1 and Ri8-1R confirmed that the *SfuI* site between the *AgeI* and *Asp718* sites is within the gene responsible for rifampicin inactivation. There was no complementation, inactivation or decolorisation.

3.4.13 Elimination of two *BclI* sites

Thus it appeared that most of the DNA on the *AgeI* side of *Asp718* fragment was necessary for function whereas the distal end had not been clearly defined between the *Asp718* and the *NarI* sites. Three *BclI* sites were located in this region. Mapping of the *BclI* sites shows that it should be possible to insert the larger *BclI* fragment into pDA71.

Firstly #16 was digested with *HindIII*, this produced a 5kb fragment which was purified on low melting agarose. This fragment was then digested with *BclI*. pDA71 was digested with both *BglII* and *HindIII*. The digested fragment was then ligated into pDA71 and transformed into *E.coli*. Transformants were screened for plasmids containing the characteristic *Asp718* fragment. These were then screened to ensure that the other *BclI* sites had been eliminated. One of these was transformed into Ri8-1 and Ri8-1R. This construct complemented the mutation, inactivated and decolorised rifampicin.

lost or were still maintained.

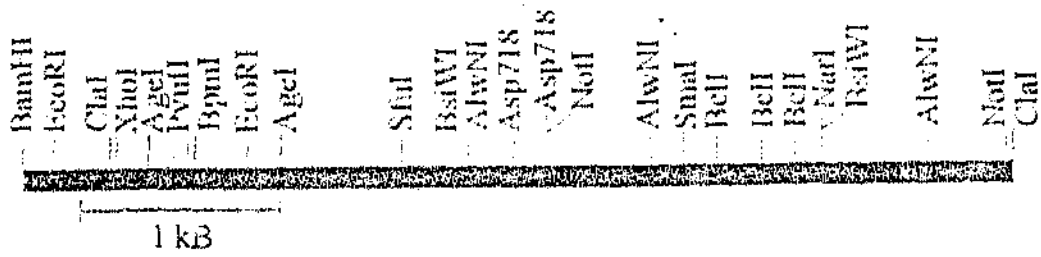


Figure 3.20 Detailed restriction map of 5kb *BamHI-ClaI* fragment

3.4.12 Removal of *AgeI*, *BsiWI* and *ClaI* fragments and the insertion of an *SfuI* fragment

As can be seen from the previous restriction map there are two *AgeI* sites, two *BsiWI* sites and two *ClaI* sites. None of these enzymes cut pDA71. It was therefore relatively simple to make deletion mutants using these enzymes. Plasmid DNA was digested with the above enzymes, then the enzyme was removed by phenol and chloroform extractions. The DNA was ligated and transformed into *E.coli*. Transformants were screened and the DNA digested with the original enzyme to determine the presence or absence of the fragment. In each case several transformants were found to have lost the fragment and so the deletion mutant was formed.

These were the transformed into Ri8-1 and Ri8-1R, the rifampicin resistant strain. None of them complemented the mutation, none inactivated or decolorised rifampicin. In each case part or all of the gene had been deleted.

Attempts to ligate the *BsiWI* fragment, that had been blunt ended

DdeI	many	*	*
DraI	0	*	
EagI	many	*	*
EcoRI	2		
EcoRV	1		
EcoR4711I	0	*	
FspI	0	*	
HaeIII	many	*	*
HindII	many	*	*
HindIII	1		
HpaI	0	*	
MluI	0	*	*
MscI	2	*	
NaeI	many		
NarI	2		
NcoI	1	*	*
NdeI	0	*	*
NheI	0	*	*
NotI	2		
NruI	3	*	*
NsiI	0	*	*
PflMI	0	*	*
PvuII	1		
PstI	1	*	*
RsrII	0	*	
SalI	many	*	*
Sau3A	many		
ScaI	0	*	
SfiI	0		
SfuI	2		
SmaI	1		
SnaBI	0	*	
SnoI	many	*	*
SpeI	0	*	
SphI	0	*	*
SspI	0	*	
StuI	0		
StyI	many	*	*
SwaI	0	*	*
TaqI	many	*	*
XbaI	0	*	*
XcmI	0	*	*
XhoI	1	*	*

Most of the enzymes that cut once, twice or three times were mapped onto the fragment. Initially digestions were done using the vector containing the fragment. However for finer mapping the fragment was purified on low melting agarose and then subjected to further restriction. Single and double digests aided in the formation of the map. Later enzyme sites were confirmed by digesting deletion and insertion mutants to see if sites had been

3.4.11 Mapping of more restriction sites within the *BamHI-BglII* fragment

As the amount of DNA in this plasmid has been significantly reduced from 23,1kb to 13,8kb, the number of restriction sites would also be significantly reduced. If unique sites or pairs of sites could be determined further analysis of the fragment would be facilitated. In order to determine whether the sites were found in the fragment or in pDA71, both pDA71 and #39 were digested with the same enzyme and run in parallel on a gel so the number of fragments could be compared. At various stages of this work different enzymes were tested the results are recorded in the table below.

Table 3.19 Restriction enzyme analysis of the fragment containing the rifampicin inactivating gene from ATCC4277. * indicates strain background.

Enzyme	Number of sites	*dam*⁻	*dcm*⁻
AatII	many	*	*
AccI	many	*	*
AgeI	2	*	*
AlwnI	3	*	*
AluI	many	*	*
ApaI	0	*	*
AscI	0	*	
AspI	many	*	*
Asp718	2	*	*
AvaI	many	*	*
AvaII	many	*	*
AvrII	0	*	
BamHI	1		
BanI	many	*	*
BbrPI	2		
BclI	3	*	
BglI	many	*	*
BglII	1		
BpmI	1	*	*
BsaBI	0	*	*
BsiHKI	many	*	*
BsiWI	2	*	
Bst1107I	0	*	*
BstEI	1	*	*
Bsu36I	0	*	
ClaI	2	*	

fragment. These samples were then digested with *BglII* to ensure that there was only one site and the 8,3kb fragment had not been inserted. These samples were then digested to make sure the that the *EcoRI* fragment was present and to see if both orientations were present. It was however the *HindIII* digestion that gave conclusive evidence of both orientations. The orientation that had a 5kb *HindIII* fragment was sample #16 and the other containing a 500bp *HindIII* fragment was sample #39.

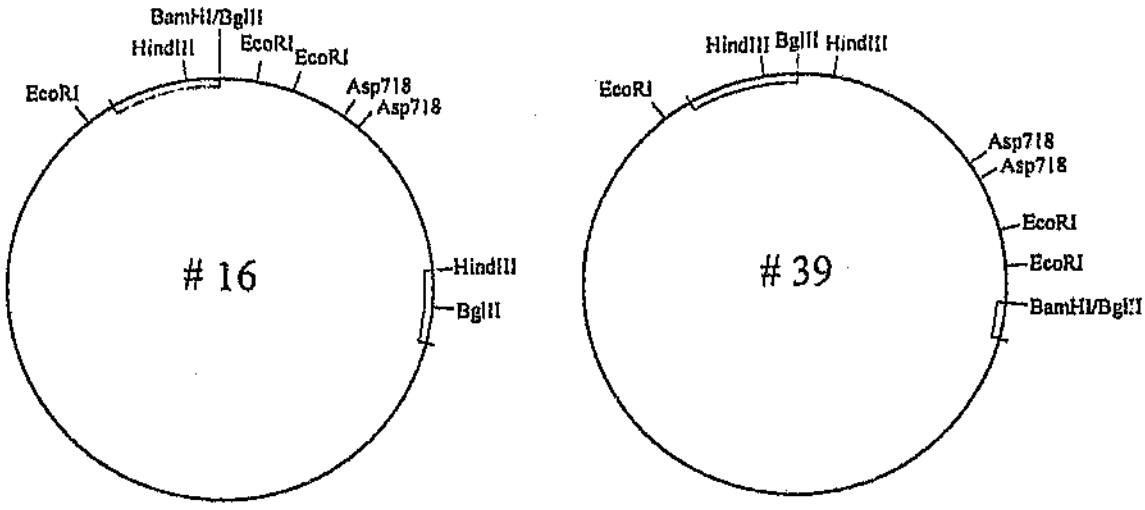


Figure 3.19 Both orientations of the *BamHI-BglII* fragment ligated into pDA7'

Table 3.26 Comparative numbers of restriction sites in 5kb inserts from *R.erythropolis* and *R.equi*

Enzyme	Number of sites in 5kb fragment from *R.erythropolis*	*R.equi*
AgeI	2	2
AlwnI	3	3
Asp718	2	2
BclI	3	3
BpmI	1	1
BsiWI	2	2
BstYI	2	2
ClaI	2	2
EcoRV	0	0
MscI	2	2
NarI	2	2
NcoI	1	1
NotI	2	2
PvuII	1	0
PstI	0	0
SfuI	2	2
SmaI	1	1
XhoI	1	1

Interestingly there is no *PvuII* site in the *R.equi* insert. A restriction map of the 5kb fragment was made by doing double digestion with various of the above enzymes and then confirming the places by digesting the fragment alone, which had been purified from low melting agarose. Owing to the high degree of similarity between the two fragments it would not be unexpected if the enzyme sites were in similar places.

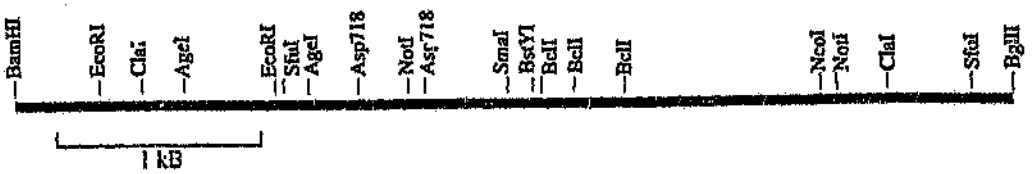


Figure 3.27 Restriction map of 5kb complementing DNA fragment from *R.equi*

All the complementing plasmids had an 8kb insert and one contained another larger *BglII* fragment. These were also digested with *Asp718* and *EcoRI* to see if the 8kb plasmid was the same in each plasmid. Identical banding patterns were found for each 8kb insert. A small *Asp718* fragment similar to the characteristic *Asp718* fragment found in *R.erythropolis* gene was identified as well as a similar sized *EcoRI* fragment. This prompted comparative digestions of the *R.erythropolis* and *R.equi* clones with different enzymes. These inserts were very similar. The characteristic *Asp718* fragment is the same size in both as is the *EcoRI* fragment. Both have a small *HindIII* fragment but it is smaller in the *R.equi* clone. The *R.equi* clone has a different banding pattern with *PvuII* but has a similar banding pattern with *SfuI*.

To ensure that these are really different pieces of DNA they were digested with enzymes that cut frequently. Different banding patterns were obtained with digestions by *AvaI*, *SalI*, *SmaI* and *StyI*.

3.6.4.1 Constructing a restriction map

As these fragments were so similar knowledge gained from mapping the previous clone would be used to map this fragment and localise the gene on it. A double digestion of both fragments with *BamHI* and *BglII* showed that the *R.equi* clone also contained a 5kb *BamHI-BglII* fragment. This fragment was purified on low melting agarose and ligated into pDA71 that had been cleaved with *BglII*. Of seven transformants (a-g) screened only one contained the 5kb insert - c. This plasmid was extracted in bulk from GM2929, a *dam*$^-$ and *dcm*$^-$ strain, and purified on a CsCl gradient. This DNA was then digested with enzymes that cut only once, twice or three times in #39 - the equivalent insert from *R.erythropolis* in pDA71. The following results were obtained.

complemented the mutation in Ri8-1. Plasmids were extracted from cultures inoculated from the patched transformants. The DNA was transformed into *E.coli* and then extracted for further analysis. Digestions were done with *BglII* as this enzyme should cut the insert out of the vector. Seven of the transformants were analyzed in this manner.

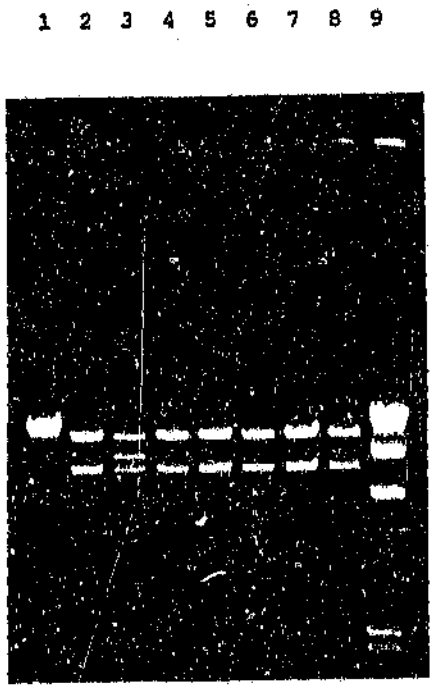


Figure 3.26 *R.equi* complementing DNA digested with *BglII*.
Lane 1: pDA37
Lanes 2-8: Complementing plasmids
Lane 9: DNA molecular weight markers

42 hours. This could mean that the inserted DNA was part of another gene involved in rifampicin resistance, either *rpoB* or some component of the cell wall.

3.6.3 From *R.erythropolis* DSM1069

This *BamHI* library was constructed by S.Moola for her M.Sc. dissertation, 1992. This library was extracted from *E.coli* and transformed into Ri8-1. 16 000 transformants were obtained. These were washed off, pelleted and resuspended. Serial dilutions were spread on selective plates. One set of selective plates contained 40mM arsenate and 0,3µg/ml rifampicin. Another set contained 40mM arsenate and 2,5µg/ml streptovaricin. This second set of plates were used in an attempt to select for a streptovaricin inactivating gene as well. Initially one transformant was observed. Later more nocardioform colonies grew on the selective plates, however they had also begun to grow on the pDA30 control plates. Some of these were isolated and grown up for plasmid screens. DNA was extracted and transformed into *E.coli*. As this library had been constructed from *BamHI* digested DNA ligated into the *BglII* site of pDA37, these plasmids could not be digested with *BglII* to determine the presence of inserts. Digestions were done with *Asp718* and *PvuII*. Neither enzyme revealed any extra bands or an increase in size of existing bands. No inserts were found.

3.6.4 From *R.equi* ATCC14887

This *BglII* library had been constructed by B.Gowan for her Ph.D thesis, 1994. The DNA was also extracted from *E.coli* and transformed into Ri8-1. 30 000 transformants were washed off the plates, diluted and spread on selection plates together with the pDA30 revertant control. After three days numerous colonies had grown up amongst the contaminants. These were patched onto 40mM arsenate with and without 0,3µg/ml rifampicin. All the colonies grew on both plates. Their plasmids contained DNA that

1 2 3 4 5 6 7 8 9

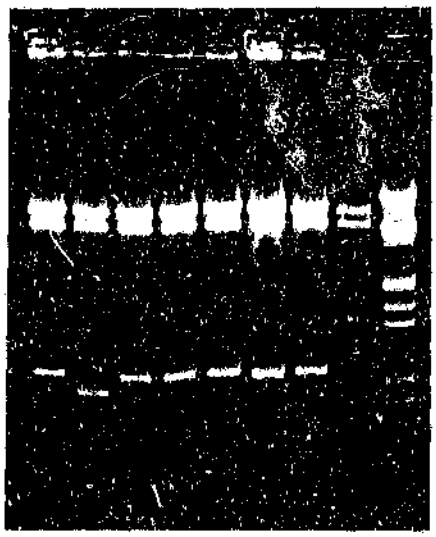


Figure 3.25 Transformants from C1 library complementing the Ri8-1 mutation. Lanes 1-7: the seven transformants, lane 8: pDA37, all digested with *HindIII* and *BamHI*, lane 9: DNA MW markers.

This gel shows that some DNA has been inserted into the *BglII* site. The insert is probably only 160bp in size. Some of the original culture in Ri8-1 was plated on a 40mM arsenate plate containing 50μg/ml rifampicin to produce resistant mutants. Inactivation assays of these cells showed no inactivation after

time as the revertants it was quite possible that the plasmid was pDA37 alone.

3.5.2 From *A.oxydans* C1

This *BglII* library had been constructed by S.Brown for her M.Sc Dissertation (1990). This library was extracted from *E.coli* and transformed into Ri8-1. Initially two transformations yielded 8 700 and 7 700 colonies respectively. These were spread on selection plates but nothing grew up. Later another transformation yielded a further 1 200 transformants. These were spread on selective plates and after 3 days many colonies had grown up and none on the control plates. There were 685 colonies on the plate where the culture had been diluted 10^{-1}, 80 colonies on the 10^{-2} plate and 5 colonies on the 10^{-3} plate. Seven these colonies together with #6, as a control, were grown up and had their plasmids extracted. These were immediately transformed into *E.coli*. Many colonies grew up. These were scraped off the plates and a plasmid screen was done of them. The uncut plasmid ran together with pDA37. A *BglII* digestion showed that there was no detectable insert. As these all appeared to be similar a few were chosen for further analysis. They showed similar banding patterns to pDA37 with *Asp718*, *BglII*, *BamHI*, *PvuII* and *SfuI*, although there was some indication that a band may be slightly larger. However digestion with *EcoRI* gave an extra band.

As there was a *HindIII* site close to the *BglII* insertion site in the vector as well as a *BamHI* site on the other side - this is the pDA30 portion of the vector - a double digestion of these transformants was done.

Hybridising sequences were identified in *R.australis* but not in *M.smegmatis*. Even when washing conditions were made less stringent and considerably more DNA was loaded on the gel no clear band of hybridisation could be seen.

So it was concluded that the DNA encoding the rifampicin inactivating ability from ATCC4277 has homology with other rhodococcal strains but not with *M.smegmatis* or *E.coli* under the experimental conditions used.

3.6 CLONING *iri*$^+$ DNA FROM OTHER NOCARDIOFORMS

As most of the nocardioforms inactivated rifampicin, and a genomic libraries of others had been built by other workers in our laboratory, these were used to select for other inactivating genes.

3.6.1 From *R.australis* A554

This organism inactivates rifampicin but does not decolorise it. A *BglII* library was constructed by A. Goldman (M.Sc.Dissertation,1991). This library was extracted from *E.coli* and transformed into Ri8-1. The first transformation yielded 10 050 transformants. These were washed off and spread on selective plates. One colony grew up but it looked like a streptomycete. The library in Ri8-1 was spread again. Once again no complementing clones were obtained. Another transformation of this library into Ri8-1 yielded 4 200 transformants. After spreading these on selective plates only one colony grew up. It grew up at the same time as revertants on the pDA30 control. A plasmid screen did not show the presence of a plasmid. To be certain DNA was extracted again and transformed into *E.coli*. Transformants grew up. These were screened for plasmid. Digestion of the plasmid DNA with *BglII* showed one clear band the same size as pDA37. No insert was detected. Either there was no insert or the insert was very small. As this originally grew up at the same

It is clear that there is a considerable degree of homology between the *R.erythropolis* strains ATCC12674 and ATCC4277. It is also apparent that DNA is found on different sized *BglII* fragments. No hybridisation was detected with the *E.coli* strain.

Next DNA was extracted from *R.australis* A448, the non-decolorising inactivator, and *M.smegmatis*. This was digested and once again gels were run to determine the relative concentrations of DNA. The probe that was used was a 3kb *PvuII-NarI* fragment. This included the critical region containing the *Asp718* fragment. It was purified from low melting agarose and labelled as before. DNA was blotted, hybridised and visualised.

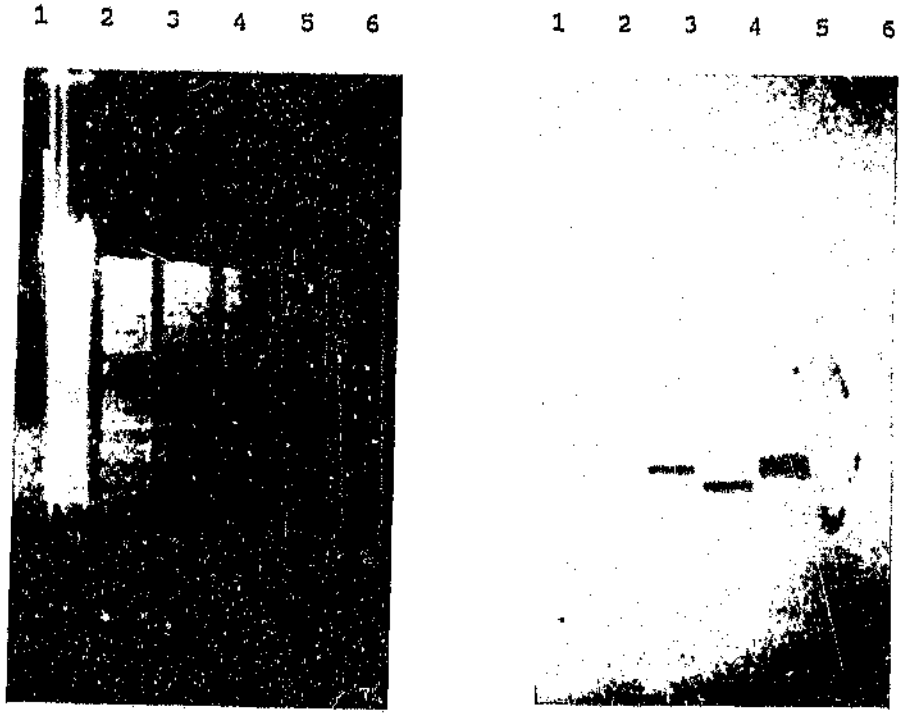
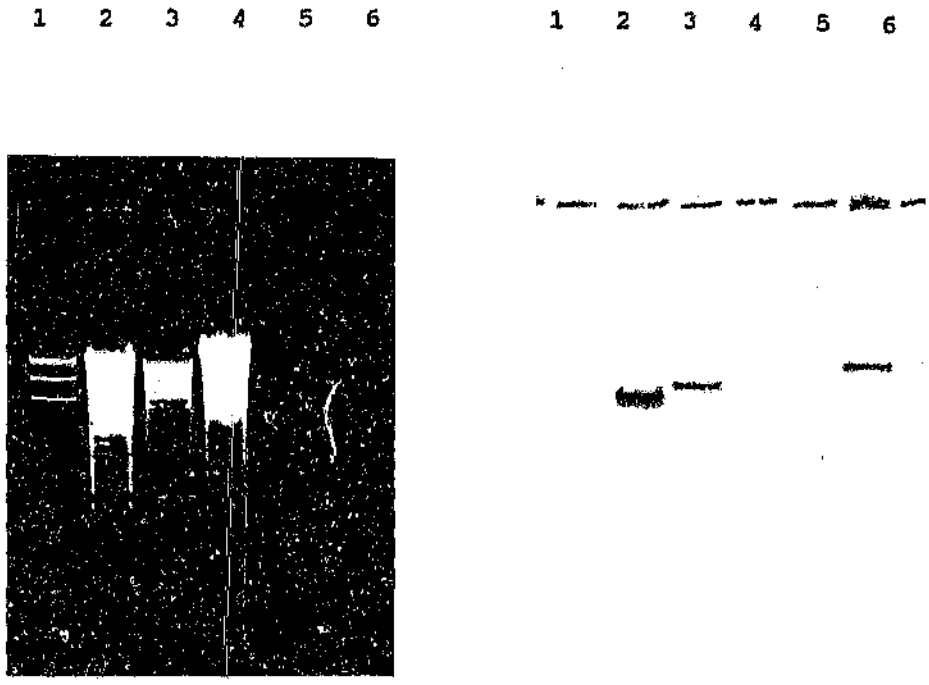


Figure 3.24 Hybridisation of *iri*$^+$ DNA to *R.erythropolis*, *R.australis*, *M.smegmatis* and *E.coli* DNA.
a) 0,8% agarose gel that was blotted
b) Nitrocellulose membrane
Lane 1: λMM294; lane 2: ATCC12674; lane 3: ATCC4277; lane 4: A448; lanes 5 and 6: *M.smegmatis*



(a) (b)

Figure 3.23 Hybridisation of *R.erythropolis* DNA and *E.coli* DNA with the 8,3kb fragment.
a) 0,8% agarose gel that was blotted
b) Nitrocellulose filter
lane 1:DNA molecular weight marker; lane 2: ATCC12674; lane 3:ATCC4277; lane 4:λMM294; lane 5:pDA37; lane 6:#6

challenged with 5µg/ml erythromycin. Samples taken once daily for two days showed on assay that the erythromycin had been inactivated.

A preliminary investigation showed that erythromycin was almost completely inactivated in 3 hours by late log phase cultures, and that the mechanism was independent of the cloned DNA.

Table 3.25 Inactivation of erythromycin by *R.erythropolis*. Relative zone sizes are indicated and blanks show no visible zones.

Strain	3h	5h	7h
No cells control	Large	Large	Large
CW22 + pDA30			
Ri8-1 + pDA30	Small		
Ri8-1 + #6			
ATCC12674	Small		

Another series of experiments carried out using the cloned DNA involved probing other DNA for homologous sequences.

3.5.3 Homology with DNA from other strains

In the preliminary experiments the entire 8,3kb fragment was purified from low melting agarose. This was labelled with digoxygenin using the "Non-radioactive DNA labelling and detection kit" from Boehringer-Mannheim. Chromosomal DNA was prepared from ATCC12674, ATCC4277 and λMM294. The chromosomal DNA was digested with *BglII* over night. Aliquots of the DNA were separated on a 0,8% agarose gel to ascertain their relative concentrations. A gel was loaded with similar concentrations of chromosomal DNA, pDA37 and #6. This was blotted overnight onto nitrocellulose. The nitrocellulose filter was hybridised with the 8,3kb fragment.

Table 3.24 Sensitivity to streptolydigin

Strain	LA	Streptolydigin in µg/ml 2,5	5,0	7,5	10,0	12,5	15,0	17,5
pDA30 in CW22	+++	+++	+++	+++	+++	+++	+++	
#6 in CW22	+++	+++	+++	+++	+++	+++	+++	
pDA30 in Ri8	+++	+++	+++	+++	+++	+++		
#6 in Ri8	+++	+++	+++	+++	+++	+++	+++	

where +++ indicates growth; 40mM arsenate - plasmid maintenance.

Other antibiotics were tested over a range of concentrations and no difference in resistance was found. The following drugs were tested and the strains found to be resistant to the following concentrations: DDS(dapsone) 100 µg/ml
Erythromycin 0,2µg/ml
Fusidic acid 0,7µg/ml
Isoniazid > 300 µg/ml
Kasugamycin > 500 µg/ml
Nalidixic acid 40 µg/ml

When testing with kanamycin as increase in resistance was noted in both plasmid containing strains of Ri8-1. Both Ri8-1 and CW22 carry streptomycin resistance mutations. These were introduced separately and so could be due to different mutations. As streptomycin and kanamycin are both aminoglycoside antibiotics it is possible that the resistance introduced into Ri8-1 increases resistance to kanamycin.

It was concluded that the cloned DNA was not responsible for the concomitant increases in resistance to fusidic acid or erythromycin. As inactivation of fusidic acid had been previously shown an investigation into the fate of erythromycin was undertaken.

A preliminary investigation showed that 5µg/ml of the antibiotic produced a well defined zone of inhibition on the lawn of *B.subtilis*. Nocardioform strains were grown into log phase and

Table 3.23 Resistance conferred by *iri*$^+$ DNA to ansamycins. It can be seen that the presence of the *iri*$^+$ DNA increases resistance to the ansamycins - rifampicin, rifamycin SV and streptovaricin.

a) Rifampicin

Strain	LA	Rifampicin in μg/ml 0,1	0,3	0,5	1,0	1,5	2,0
pDA30 in CW22	+++	+++	+++				
#6 in CW22	+++	+++	+++	+++	+++	+++	
pDA30 in Ri8	+++						
#6 in Ri8	+++	+++	+++	+++	+++	+++	

b) Rifamycin SV

Strain	LA	Rifamycin SV in μg/ml 1	2		4	5
pDA30 in CW22	+++	+++	+++			
#6 in CW22	+++	+++	+++	+++	+++	
pDA30 in Ri8	+++	+++				
#6 in Ri8	+++	+++	+++	+++	+++	

c) Streptovaricin

Strain	LA	Streptovaricin in μg/ml 1	2	3	4	5	6
pDA30 in CW22	+++	+++	+++	+++	+++	+++	
#6 in CW22	+++	+++	+++	+++	+++	+++	
pDA30 in Ri8	+++	+++	+++	+++			
#6 in Ri8	+++	+++	+++	+++	+++	+++	

where +++ indicates growth; each plate contained 40mM arsenate for plasmid maintenance.

Testing with another inhibitor of RNA polymerase, streptolydigin, the following results were obtained.

progressively worse so other conditions were altered. The termination reactions were carried out at higher temperatures 42°C and 50°C. Times for elongation were shortened from 5 minutes to 4 and then 3 minutes. In spite of this banding across all four lanes was consistently being achieved.

A method for sequencing "dirty" DNA was discovered and tried (Disdell *et al*, 1992). This was a modification of the Sequenase protocol. The annealing reaction was at 100°C for 2-3 minutes and then snap cooled. The standard labelling "cocktail" was added and mixed. This labelling reaction was immediately split and aliquoted into the appropriate termination mix. These were incubated at 37°C before the stop solution was added. This method was not that successful.

Further reading and communications seemed to indicate that single-stranded sequencing was far more efficient than double-stranded sequencing.

3.8.3 Sequencing single-stranded templates

As some of the subclones were in the phagemid pUC118, single-stranded DNA was prepared from these. High yields of DNA were obtained but the X-rays were blank.

Although it would require further subcloning I decided to reclone everything into M13mp18 and M13mp19. This was not without its difficulties as two of the four subclones proved hard to subclone. The sequences obtained were a dramatic improvement on all previous attempts. From this point on consistently decent sequences were obtained reading 250-360 bps with only one or two stops across all four lanes. On the rare occasion no stops were obtained.

The reactions were always loaded on the gel in alphabetical order ACGT. As this DNA has a high GC content these reactions were run side by side. Parallel dGTP and dITP reactions were run to

DNA was prepared using the standard method of alkaline lysis. Initially the X-ray films were totally blank. Various strategies were adopted for obtaining sequencing ladders. These included different conditions for annealing the primers. The kit suggested 15 minutes at 37°C. This was changed to 65°C for 2 minutes with a gradual decrease in temperature to below 35°C as suggested in the Sequenase kit. This produced a faint blur on the autoradiograms. Next fresh solutions of denaturation buffer were used. In addition time periods of 5, 10, 15 and 30 minutes were tried for denaturation as well as stepping up the temperature to 37°C. The possibility existed that the primers were not binding so reactions were carried out using the forward primer as well as ones using the reverse primer. There was not much change in the state of the of the autoradiograms. No difference was noted if the urea was leached out of the gels before drying or not. Also extra phenol extractions and chloroform extractions were carried out to make sure that the DNA was clean. Klenow enzyme from different sources was also used.

Since this kit was no longer available from Boerhinger-Mannheim the Sequenase Version 2.0 kit was obtained from United States Biolabs. Using this kit tracks could be seen on the X-ray film. However as these tracks were unclear it was decided that other makes of film should be used. Two other makes of film that were available in the department were exposed to see if better resolution was obtained. This was not the case. The film was then exposed for longer time, up to 5 days. This increased the banding marginally. Next comparisons were made using primers from both kits as these primers bind at different places. Faint banding could be seen with both of these. However banding across all four lanes occurred quite regularly. DNA was purified using more phenol extractions and PEG precipitations. Since banding was still not of acceptable quality DNA was purified on a CsCl gradient. This yielded the best results to date with about 100 bp being read at a stretch. All the subclones were purified in this manner and parallel dGTP and dITP reactions were run on the sequencing gels. However the sequencing results became

decision was made on which one would be sequenced. As *R.equi* is emerging as an opportunistic pathogen in AIDS patients and these people are being treated with rifampicin, the gene from this organism was the one chosen for sequencing.

A pUC sequencing kit (Boehringer-Mannheim) had been obtained previously. This was a kit for sequencing double stranded DNA. For this reason all the subclones that were made were ligated into pUC vectors. The two vectors that were available in our laboratory were the phagemid pUC118 and the plasmid pUC19.

3.8.1 Production of subclones in pUC vectors

Analysis of the ± 2kb *BclI-ClaI* fragment showed four potential sites for subcloning into the multiple cloning site of the pUC series of vectors. These sites would produce fragments of approximately the following sizes:
AgeI-Asp718 fragment of 520bp inserted into *AvaI* and *Asp718*,
Asp718-Asp718 fragment of 320bp inserted into the *Asp718* site,
Asp718-EcoRI fragment of 365bp inserted into the same sites and
EcoRI-BclI fragment of 750bp inserted into the *BamHI-EcoRI* sites of both pUC118 and pUC19 vectors.

The vectors were prepared by usual methods with only the *Asp718* digest being phosphatased to prevent religation. None of the other digestions would cause the formation of compatible cohesive ends. Constructs were transformed into JM109. This would facilitate single stranded DNA isolation should that be needed at some stage.

3.8.2 Sequencing of pUC subclones

^{35}SdATP was used for labelling the DNA. It has a longer half-life so would be more cost effective, the bands on autoradiograms were reported to be sharper than with ^{32}P and the lower energy of the β-particles provided greater safety.

of the pBR322 inserted plasmid revealed that the whole *ClaI* fragment had been inserted.

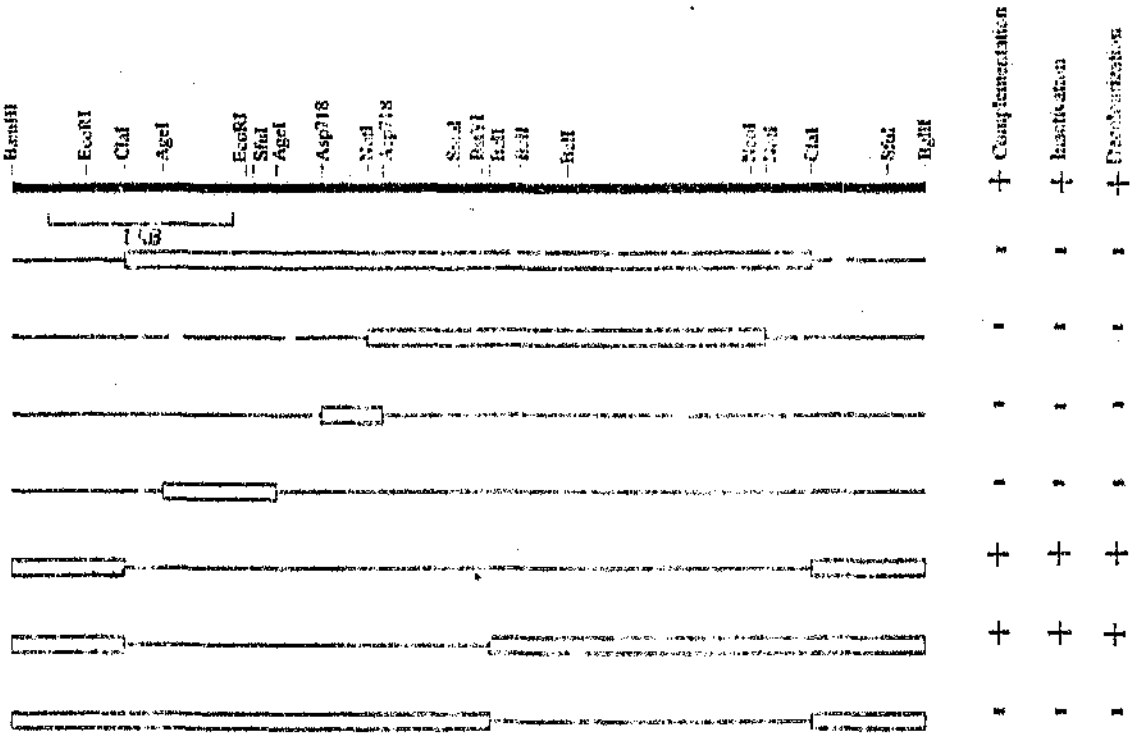


Figure 3.30 Summary of the deletions made of 5kb *BamHI-BglII* fragment from *R.equi*. Blocks indicate deletions

3.8 SEQUENCING A RIFAMPICIN INACTIVATION GENE

As rifampicin inactivating genes had been cloned from two different *Rhodococcus* species - *R.erythropolis* and *R.equi* a

enzyme. The pDA71 was treated with alkaline phosphatase to prevent self ligation. Subsequently both DNA segments were ligated and transformed into *E.coli*. Transformants were screened for inserts. Sixteen of forty transformants screened contained inserts larger than pDA71 but smaller than pDA71 containing the *ClaI* fragment. Initially these were digested with *EcoRI*. These all exhibited three sites. That is what would be expected if the DNA containing the critical region had been inserted. However none of these contained the *Asp718* fragment.

This process was repeated, the only difference was that pDA71 was digested with *SfuI* before being blunt ended. Only six transformants were obtained and none of these had plasmids containing the *Asp718* fragment.

This precipitated the second strategy: pBR322 was transformed into a *dam*⁻, *dcm*⁻ *E.coli* strain and subsequently purified from it. This was necessary as it was later cut with methylation sensitive *ClaI*. It was also cut with *EcoRV* a blunt end producing enzyme. This was ligated to the *ClaI* fragment that had been cut with *SmaI*. This may have been more successful as only one blunt end ligation was involved. These were transformed into *E.coli*. The transformants were patched onto LA plates containing 35µg/ml chloramphenicol with and without 35µg/ml tetracycline. When DNA is inserted into the *EcoRV-ClaI* sites of pBR322 it inactivates the tetracycline resistance gene. All the transformants were only able to grow on the chloramphenicol plate. Ten of these were screened for plasmids containing the *Asp718* fragment. They all had it. Blunt end cloning has a low efficiency and affected the success of these experiments.

Simultaneously pDA30 had been digested with *BglII* and phosphatased. One of the *Asp718* containing plasmids was digested at its unique *BamHI* site and ligated to pDA30. This ligation mixture was transformed into Ri8-1 by electroporation. The transformation directly into the nocardioform would select for pDA30 containing an insert. Unfortunately further investigation

decolorise rifampicin. This further reduced the area of interest to 2kb.

3.7.5 Attempted deletion of *BstYI* fragment

An attempt was made in order to determine the significance of the *BstYI* site that is internal to the *BclI* site. *BstYI* produces cohesive ends compatible with *BglII*. Thus an approach similar to that of *BclI* was undertaken. After the *ClaI* fragment had been purified on low melting agarose it was digested with *BstYI*. This was ligated into the *BglII* and *SfuI* sites of pDA71. The ligation mixture was split, with 2µl being electroporated into Ri8-1 and 18µl being transformed into a *dam*⁻, *dcm*⁻ strain of *E.coli*. *BstYI* is sensitive to methylation. In *E.coli*, 7 transformants were obtained as against 181 transformants from Ri8-1. The *E.coli* transformants were screened. Three of the seven contained *Asp718* fragments, these however also maintained their *BstYI* fragment. As these had not yielded further information the Ri8-1 colonies were analyzed. These were first patched onto chloramphenicol plates with and without 0,3µg/ml rifampicin. Of the 56 transformants patched 36 complemented the mutation. Sixteen of these were screened, half complementing the mutation the others not. All the complementing plasmids contained the entire *ClaI* fragment. Most of the non-complementing transformants contained the vector alone. One of these however showed an *Asp718* fragment and an extra *BstYI* site over and above the two present in the *ClaI* fragment. Further digestions did not unravel this rearrangement. So the *SmaI* site was targeted.

3.7.6 Insertion of *SmaI-ClaI* fragment

Several strategies were adopted to clone this fragment from *R.equi*.

The first: the *ClaI* fragment was purified on low melting agarose and digested with *SmaI*. At the same time pDA71 was digested with *BglII*. Both pieces of DNA were made blunt ended with Klenow

In order to eliminate non-essential DNA the following experiments were done.

3.7.4 Elimination of the two *BclI* sites

For the purpose of establishing the relevance of the *BclI* site closest to the critical region the following process was undertaken.

The *ClaI* fragment was purified from low melting agarose. This was digested with *BclI* and ligated into the *BglII* and *SfuI* sites of pDA71. Only eight transformants were obtained. These were screened first of all for the absence of the *BclI* tiny fragments. Of the eight, five had lost the tiny fragments. As two different *BclI-ClaI* fragments were generated by this digestion it was necessary to identify which fragments had been ligated in. Digestion with *Asp718* would identify the plasmids that contained the critical region. One of these contained the *Asp718* fragment. However when the plasmid was digested with *ClaI*, *NotI* and *SfuI* and run on a gel in parallel with plasmid c digestions, similar banding patterns were observed that seemed to indicate an undeleted fragment.

As the reasons for this were unclear the whole process was repeated. Over 100 transformants were obtained. Of these several were screened for the presence of plasmids larger than pDA71 but smaller than the vector containing the *ClaI* fragment. Several contained smaller inserts that also had *Asp718* fragments.

The vector containing the *BclI-ClaI* fragment was transformed into Ri8-1 by electroporation. Transformants were patched onto LA plates containing 20µg/ml chloramphenicol with and without 0,3µg/ml rifampicin. The plasmids complemented the hypersensitivity mutation. A culture was grown and spread on a 50µg/ml rifampicin plate containing chloramphenicol selective pressure in order to obtain rifampicin resistant mutants of Ri8-1 containing the plasmid. These were shown to inactivate and

Table 3.27 Complementation of the rifampicin hypersensitivity mutation in Ri8-1 by deletion and insertion plasmids of *R.equi* DNA

Plasmid	Complementation of rif^{HS} mutation
Plasmid c	YES
ΔAge	NO
ΔAsp	NO
ΔCla	NO
ΔNot	NO
+Cla	YES

Ri8-1R transformants were grown in LB and 20μg/ml chloramphenicol. When the cultures were in exponential phase they were challenged with 20μg/ml rifampicin. Supernatants were assayed for rifampicin inactivation using the plate method, in order to determine whether inactivation had occurred. The decolorisation or lack thereof was also recorded for each culture.

Table 3.28 Inactivation and decolorisation of rifampicin mediated by deletion and insertion plasmids of *R.equi* DNA

Plasmid	Inactivation	Decolorisation
Plasmid c	YES	YES
ΔAge	NO	NO
ΔAsp	NO	NO
ΔCla	NO	NO
ΔNot	NO	NO
+Cla	YES	YES

This set of experiments have resulted in the homing in on the portion of the fragment involved in the inactivation of rifampicin. This function was to be found on the 3,5kb *ClaI* fragment. A stretch of about 560bp that was part of the gene was identified. This is between the innermost *AgeI* site and the *Asp718* site furthest from it.

restriction map.

These deleted plasmids were transformed into Ri8-1 and its rifampicin resistant derivative. Transformants were screened for the presence of the plasmid by doing a miniprep of DNA. They all contained the plasmids.

3.7.2 Insertion of the *ClaI* fragment into pDA71

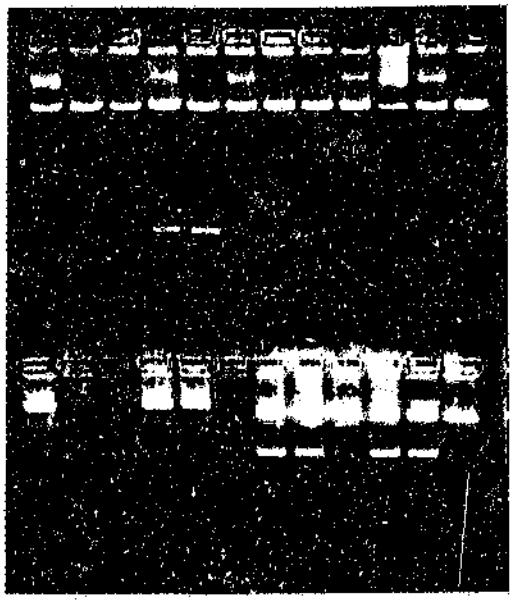
Another plasmid was also prepared, an insertion plasmid. The *ClaI* fragment was purified from low melting agarose and inserted into the *SfuI* site of pDA71. These enzymes produce compatible cohesive ends. Eight transformants were obtained. These were screened for plasmids.

The plasmids were digested with *EcoRI*. These were separated out on an agarose gel. The insertion would have to have one *EcoRI* site less than plasmid c and have fragment sizes adding up to the equivalent of pDA71 and the *ClaI* fragment combined (12,3kb). Of the eight plasmids screened two contained *ClaI* inserted fragments. The inserted plasmids were transformed into Ri8-1 and the rifampicin resistant derivative of it.

3.7.3 Complementation, inactivation and decolorisation studies on newly constructed plasmids

The Ri8-1 transformants of both inserted and deleted plasmids were patched onto 20µg/ml chloramphenicol plates with and without 0,3µg/ml rifampicin, together with transformants containing the intact plasmid c. This was to test for the complementation of the hypersensitivity mutation.

1 2 3 4 5 6 7 8 9 10 11 12



13 14 15 16 17 18 19 20 21 22 23 24

Figure 3.29 Plasmid c screened for deletions of *AgeI*, *Asp718*, *ClaI* and *NotI* fragments.
Lanes 1-6: Potential ΔAge cut with *AgeI*
7-12: Potential ΔAsp cut with *Asp718*
13-18: Potential ΔCla cut with *Asp718*
19-24: Potential ΔNot cut with *NotI*

The potential ΔCla plasmids were digested with *Asp718* as the strain into which these ligation mixtures were transformed was *dam*$^+$. *ClaI* is methylation sensitive and would not cut DNA isolated from this strain. Thus an enzyme was chosen which had sites within the *ClaI* fragment so its presence or absence could be established in this way. All the four enzymes used produced deleted plasmids.

These plasmids were used to confirm the sites on the restriction map. The loss of an *EcoRI* in ΔCla confirmed that one *EcoRI* site could be found on either side of a *ClaI* site. Digestion of ΔAge with *EcoRI* and *SfuI* confirmed the presence of these sites within the *AgeI* fragment. The loss of a *NotI* site in ΔAsp and the loss of an *Asp718* site in ΔNot substantiated their positions on the

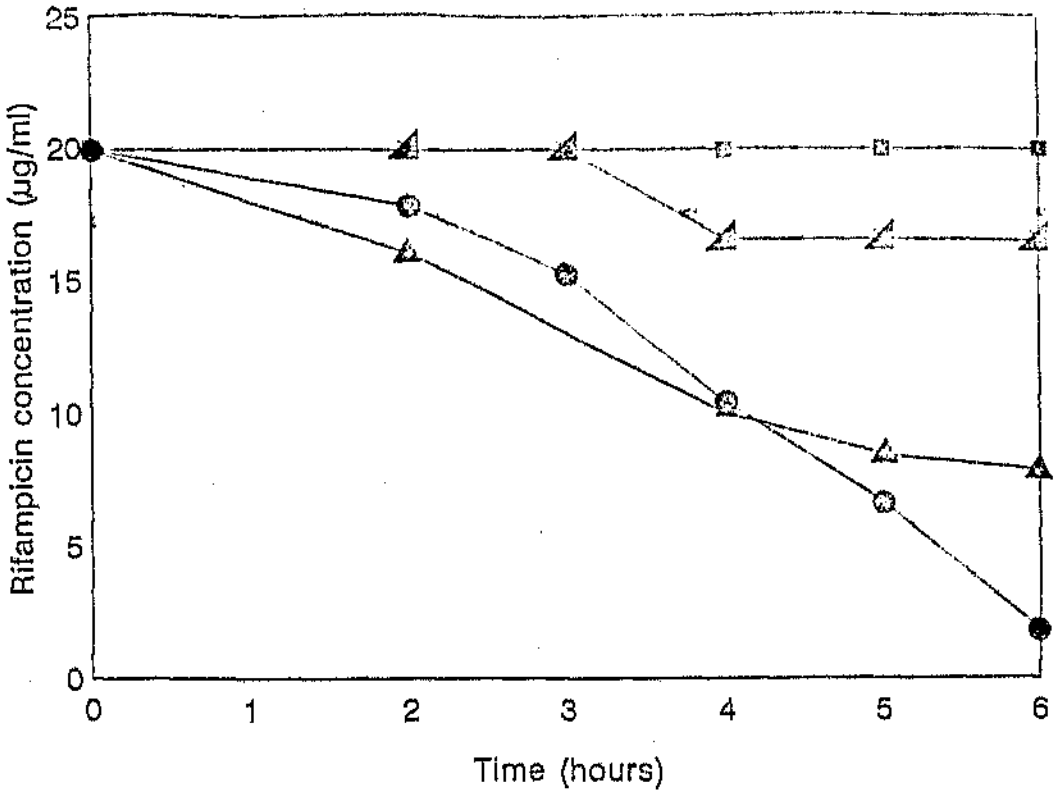


Figure 3.28 Inactivation of rifampicin by two cloned *iri* genes from *Rhodococcus* species. (▲)-from *R.erythropolis*, (●)-from *R.equi*, (◢)-Ri8-1 containing pDA71, (■)-cell free control.

3.7 SUBCLONING OF *R.equi* FRAGMENT

3.7.1 Elimination of *AgeI*, *Asp718*, *ClaI* and *NotI* fragments

As *AgeI*, *Asp718*, *ClaI* and *NotI* each cut the fragment twice and as there are no sites for these enzymes in the vector, they could be used to make deletions of the insert.

The purified plasmid c was aliquoted and d ed with each of the four enzymes. The enzyme was removed .. xtraction with phenol and chloroform. The DNA was ligated and transformed into *E.coli*. As many colonies as were obtained several were screened.

3.6.4.2 Inactivation of ansamycins by the *R.equi* clone

The 5kb *BamHI-BglII* fragment in pDA71 or plasmid c was transformed into the rifampicin resistant derivative of Ri8-1. This facilitated more accurate determination of antibiotic inactivation. A culture was grown to stationary phase and then subcultured into four tubes. These were grown into log phase before being challenged with the following antibiotics: 10µg/ml rifampicin, 10µg/ml rifamycin SV, 50µg/ml streptovaricin and 50µg/ml streptolydigin. A cell free control was prepared for each antibiotic. After 20 hours samples were taken and inactivation determined using the plate assay. Plasmid c caused the inactivation of rifampicin and rifamycin SV. This is consistent with previous data obtained for *R.equi*. Streptovaricin was not inactivated. This is also consistent with previous data. Streptolydigin did not cause zones to be formed on the assay plates, not even on the cell free control. This confirms that *B.subtilis* is not an effective assay organism for this drug.

Quantitative rifampicin assays were done comparing inactivation by the *R.erythropolis* and *R.equi* clones. The plasmids used in this experiment were both pDA71 carrying the 5kb *BamHI-BglII* fragment from each clone. They were also inserted in the same orientation. Samples of cultures challenged with 20µg/ml rifampicin were taken over a 6 hour period. During this time the antibiotic was decolorised. There was not a significant difference in the rates of inactivation as can be seen in the graphs below. The cultures had been equilibrated to similar optical densities before being challenged.

in any one specific construct. It was possible that the introduction of an inducing agent may alter this situation. A new range of concentrations was used including a concentration below 10μg/ml. These were 5, 10 and 20μg/ml rifampicin. All the plates were spread with IPTG before the strains were patched out on them. All grew at 5μg/ml rifampicin. Several of the transformants grew at 10μg/ml, namely, ΔAsp, *BclI-ClaI* in pDA71, *ClaI-ClaI* in pUC118 and c in pDA71. There was one that grew at 20μg/ml and that was *BclI-ClaI* in pDA71. It did not grow very well but there was some growth. This was unusual as IPTG should not induce the lambda promoter.

3.9.2 Shifting reading frames in *BclI-ClaI*

A possibility existed that the gene was not in frame with the *E.coli* fusion gene. This would prevent expression of the gene. To alter the reading frame the following steps were taken. *BclI-ClaI* and c both in pDA71 were linearised at the unique *HindIII* site that is found in the vector. Each plasmid was made blunt ended with Klenow enzyme and then ligated. This induced a frame shift. Klenow enzyme works in a variety of buffers including most that are commonly used for DNA manipulation. Twice the Klenow enzyme was introduced into ligation buffer 30 minutes before ligation. The reactions were carried out at 30°C and 37°C. These failed to produce and transformants. Instead of introducing the Klenow enzyme into ligation buffer it was added to the restriction digest mixture when the restriction should have reached completion. These transformants were patched onto rifampicin containing plates. There was no increase in resistance to the drug. In order to check if there had in fact been a frame shift as proposed, DNA was extracted from these transformants. This DNA was digested with *HindIII* and *Asp718*. The double digestion was also carried out on the original plasmids that had not been subjected to frame shift attempts. It was necessary to do a double digestion in order to show loss of the site rather than no cutting if only *HindIII* had been used. These digests showed that some had lost the site while others had not. There

3.9 PROTEIN EXPRESSION AND CHARACTERISATION OF *iri* GENE

3.9.1 Checking existing plasmids for overexpression

Previously an *iri* gene, which had been cloned from *M.smegmatis*, was found to be expressed in *E.coli* (Quan, unpublished). This gene increased the level of resistance of *E.coli* to rifampicin. The gene product was also seen as a distinct band on a PAGE gel. With this in mind the following experiments were undertaken. At this stage the gene was in the process of being sequenced and the orientation of the gene on the fragment was unknown as well as the positions of the start and stop codons.

Various fragments of the original cloned DNA were ligated into different plasmids that contained different *E.coli* promoters. The 5kb *BamHI-BglII* and the 2kb *BclI-ClaI* in pDA71 were under the possible control of the P_L promoter of lambda. The *ClaI-ClaI* fragment was inserted into pUC118 which has a P_{lac} promoter. The *BclI-ClaI* fragment was also cloned into pGEM3(f)+ also having a P_{lac} promoter. These plasmids all had different segments inserted into different sites (the multiple cloning sites of pUC118 and pGEM3(f)+ are different).

Control plasmids in this series of experiments included pGEM3(Z)f+ and the deletion mutant of o ΔAsp. These plasmids were all transformed into a rifampicin sensitive strain of *E.coli*, JM101. The transformants were then patched onto LA plates containing 100μg/ml ampicillin and various concentrations of rifampicin.

In the first series plates containing 10, 20 and 30μg/ml of rifampicin were used. All the transformants grew at 10μg/ml but not on either of the others. The range was narrowed down to 10, 12, 14, 16 and 18μg/ml of the drug. All the transformants grew on the control plate but not on any of the plates containing rifampicin. Although there was no growth at the lowest concentration, there was also no notable increase in resistance

All the BLAST results at both the nucleotide and amino acid levels showed homology to hydroxylases which are also referred to as monooxygenases. These enzymes are flavin-dependant. The highest scoring similarities were to hydroxylases involved in polyketide biosynthetic pathways followed closely by those involved in the degradation of aromatic compounds.

The earliest sequence comparison studies attempted to produce end to end alignments of complete sequences, also known as global alignments. However, it has been recognised that most genes and proteins are modular and mosaic structures often consisting of several distinct domains and/or repeated copies of domains, the order of which may be rearranged by several genetic mechanisms. Thus attention has shifted to algorithms for local alignment, and virtually all database search strategies today seek local alignments. Once the BLAST programs have found some aligned segments, the segments are considered in their totality for their statistical significance. The BLAST search algorithm seeks a type of local alignment called a high-scoring segment pair (HSP). An HSP is a pair of sequence segments, one drawn from the query sequence and the other from the database sequence, that may be aligned without gaps to achieve a high score. In order to be reported by BLAST, the score must meet or exceed a score threshold. The Smallest Sum Probability scores would be 0.00 for identical sequences, very similar sequences have probabilities that tend towards zero.

The local alignments identified, with the queried *iri* gene and its translated product, two major regions of homology and three minor regions.

Homology searches were conducted using the BLAST series of programs. The results of the BLASTN nucleotide search are recorded below.

Table 3.29 BLASTN results of similarity to *iri* gene

Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)	N
db|D38214|STMCTS	Streptomyces aureofaciens cts4, cts6...	770	9.3e-58	2
emb|X87093|SFURDABCF	S.fradiae urdE,F,A,B,C,D genes	672	4.6e-55	3
gb|U20[illegible]82|SVU20382	Streptomyces venezuelae C-16 hydroxy...	566	3.2e-38	2
gb|M80[illegible]|STMTCREP	Streptomyces glaucescens tcm operon.	447	1.0e-26	2
gb|M98557[illegible]|FLVBPENTA	Flavobacterium sp. pentachlorophenol...	405	1.0e-22	1
gb|U10405|SPU10405	Streptomyces purpurascens ATCC 25489...	295	4.6e-16	2
gb|L04488|TCRPHD	Trichosporon cutaneum phenol hydroxy...	295	1.5e-13	1
gb|M35097|PJPTFDCD	Plasmid pJP4 (from Alcaligenes eutro...	289	4.9e-13	1
gb|U32188|PPU32188	Pseudomonas putida plasmid pEST4011 ...	213	1.1e-06	1
emb|X68438|PFPOBA	P.fluorescens pobA gene for p-hydrox...	199	1.5e-05	1
gb|M23173|PSEPOBA	P.aeruginosa p-hydroxybenzoate hydro...	199	1.5e-05	1

The results of the BLASTP amino acid alignments are recorded below.

Table 3.30 BLASTP results of similarity to Iri polypeptide

Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)	N
gp|X87093|SFURDABCF_1	putative oxygenase [Streptomyces f...	383	9.2e-113	
gp|D38214|STMCTS_1	cts8 [Streptomyces aureofaciens]	378	2.8e-107	
gp|U20382|SVU20382_2	C-16 hydroxylase [Streptomyces vio...	285	6.7e-54	5
sp|P42535|PCPB_FLASP	PENTACHLOROPHENOL 4-MONOOXYGENASE ...	261	1.1e-44	5
pir|A40640|A40640	pentachlorophenol-4-monooxygenase ...	261	1.1e-44	5
gp|M80674|STMTCREP_4	hydroxylase [Streptomyces glaucesc...	221	1.9e-37	4
sp|P39880|TCMG_STRGA	TETRACENOMYCIN POLYKETIDE SYNTHESI...	221	2.2e-37	4
sp|Q05355|HYDL_STRHA	PUTATIVE POLYKETIDE HYDROXYLASE (E...	223	2.5e-36	5
pir|B49918|B49918	schC protein - Streptomyces halstedii	214	5.0e-35	5
gp|U10405|SPU10405_5	RdmE [Streptomyces purpurascens]	195	2.9e-33	4
sp|P15245|PH2M_TRICU	PHENOL 2-MONOOXYGENASE (EC 1.14.13...	173	1.8e-31	6
gp|L04488|TCRPHD_1	phenol hydroxylase [Trichosporon c...	173	1.8e-31	6
sp|P31020|PHEA_PSESP	PHENOL 2-MONOOXYGENASE (EC 1.14.13...	172	3.0e-31	5
gp|M57500|E12PHEAB_3	phenol monooxygenase [Plasmid pEST...	172	3.1e-31	5
sp|P27138|TFDB_ALCEU	2,4-DICHLOROPHENOL 6-MONOOXYGENASE...	167	1.9e-30	5
gp|U18082|SPU18082_2	aklavinone C-11 hydroxylase [Strep...	161	6.3e-29	4
gp|U32188|PPU32188_2	2,4-dichlorophenol hydroxylase [Ps...	166	1.6e-28	5
gp|U09844|SPU09844_1	aklavinone C-11 hydroxylase [Strep...	144	1.7e-26	4
sp|Q01551|TBUD_PSEPI	PHENOL 2-MONOOXYGENASE (EC 1.14.13...	142	3.4e-14	2
pir|A45730|A45730	phenol 2-monooxygenase (EC 1.14.13...	142	3.4e-14	2
pir|S37051|S37051	4-hydroxybenzoate 3-monooxygenase ...	95	4.7e-11	3

42bp down from the stop codon and the other 128bp away. The Frame Program seems to suggest that there may be another open reading frame starting downstream of the stop codon. No obvious Shine-Delgarno sequences were identified upstream of potential ATG or GTG start codons.

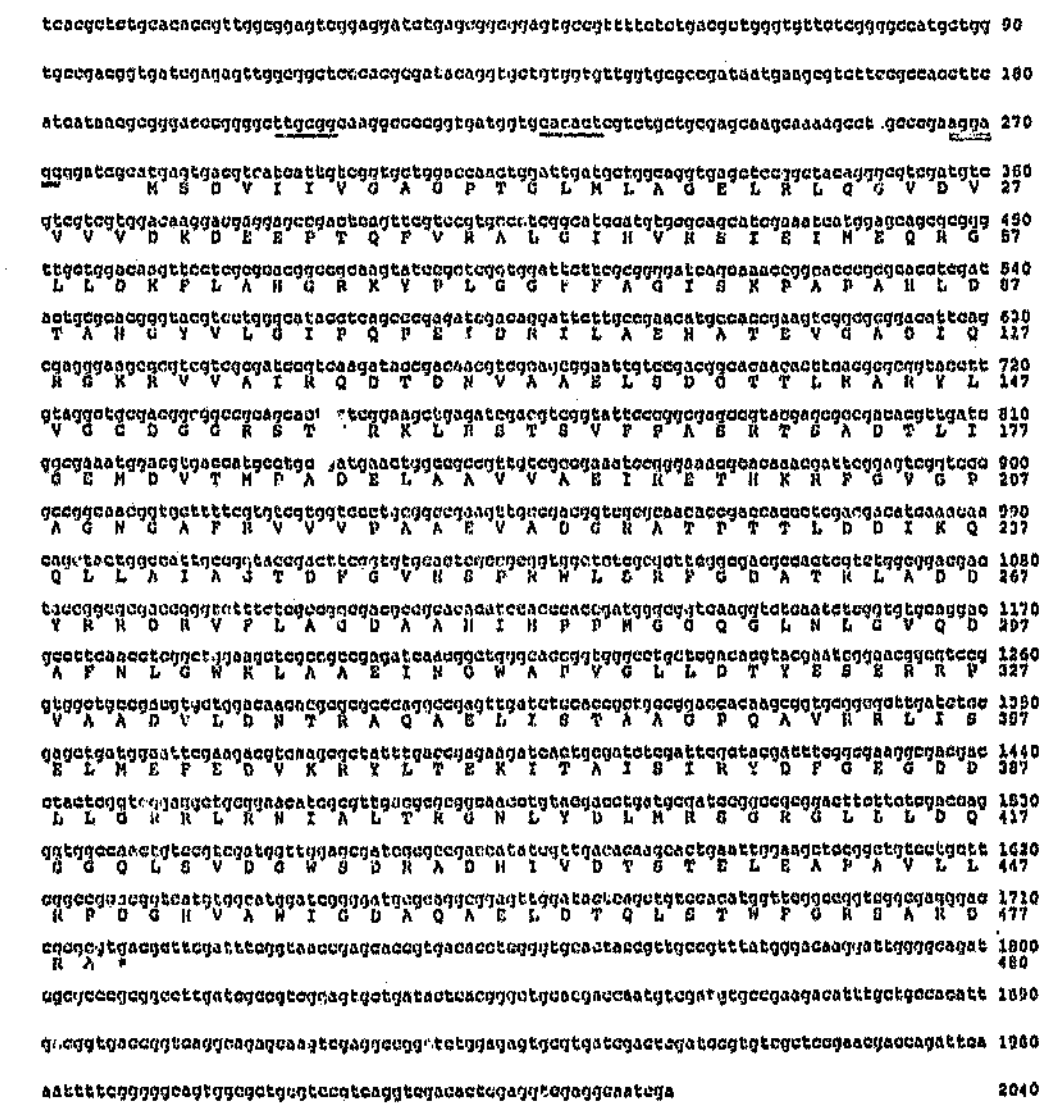


Figure 3.33 The sequence of the *iri* gene and flanking regions. The Shine-Delgarno sequence is underlined twice and the -35 and -10 sequences once.

In a study analyzing DNA upstream of start codons in *Streptomyces* a conserved Shine-Delgarno sequence was identified. This sequence, (a/g)GGAGG, was identified from 5-12 nucleotides upstream of the initiation codon (Strohl WR, 1992). A similar sequence, AGGAGG, was found to extend from -13 to -8 (where the A of the start codon is recorded as 1 and the nucleotide preceding it is designated -1). This was in keeping with the *Streptomyces* model. Analysis of the sequence upstream of other ATG or GTG codons on either side of the putative start codon did not have any similarity to the Shine-Delgarno motif. This study showed that only 29 of 139 promoters that were studied belonged to a group which was similar to those recognised by eubacterial RNA polymerases containing σ^{70}-like subunits. The other 110 putative promoter regions contained a wide diversity of sequences. Potential promoter sequences were also identified: -35 sequence TTGCGG and the -10 sequence CACACT which were -35 and -10 relative to each other. This would make the C at 242 a potential transcription start site. It has also been reported that the DNA within the promoter regions of several streptomycetes has significant potential for forming secondary structures like hair-pin loops (Sinclair R and Bibb MJ, 1989; Murakami T *et al*, 1989; Pulido *et al*, 1988; Rebollo *et al*, 1989). The upstream region of this gene was analyzed for hair-pin loops. A possible hair-pin loop was identified that encompassed the -35 region that had been identified as a possible promoter site:

```
          g   c   gat
  /gcaag ccc ggt
g ------ --- ---
  \cgttc ggg cca
          g   c   ggg
```

No comprehensive study had been made of mycobacterial or rhodococcal promoters at the time of writing.

Analysis of the sequence downstream of the stop codon was undertaken. Searches for possible stem-loop structures that may act as terminators revealed two possibilities. The one beginning

3.8.6 Computer aided analysis of sequenced DNA

The sequence had been entered onto the computer using the Esee Version 3.0 package (Eric Cabot, 1987 and 1994) and initial analysis done using the Frame program which was designed for streptomyces. Since there is an evolutionary relationship between the strepomycetes and rhodococci showing similar GC content this package seemed appropriate to use for analysis.

The DNA sequence was 2042bp in length with a GC content of 63%. The Frame program offered 5 codon usage tables over a range of 60-75% GC content. The appropriate codon usage table for 60-66% was used. This was used by the computer to translate the sequence into the six different reading frames and look for open reading frames. The codon usage analysis identified the open reading frame as running from the *BclI* site towards the *ClaI* site. The start codon was identified as an ATG at 280bp and the stop codon TGA at 1716bp. The open reading frame was 1438bp encoding a polypeptide of 479 amino acids.

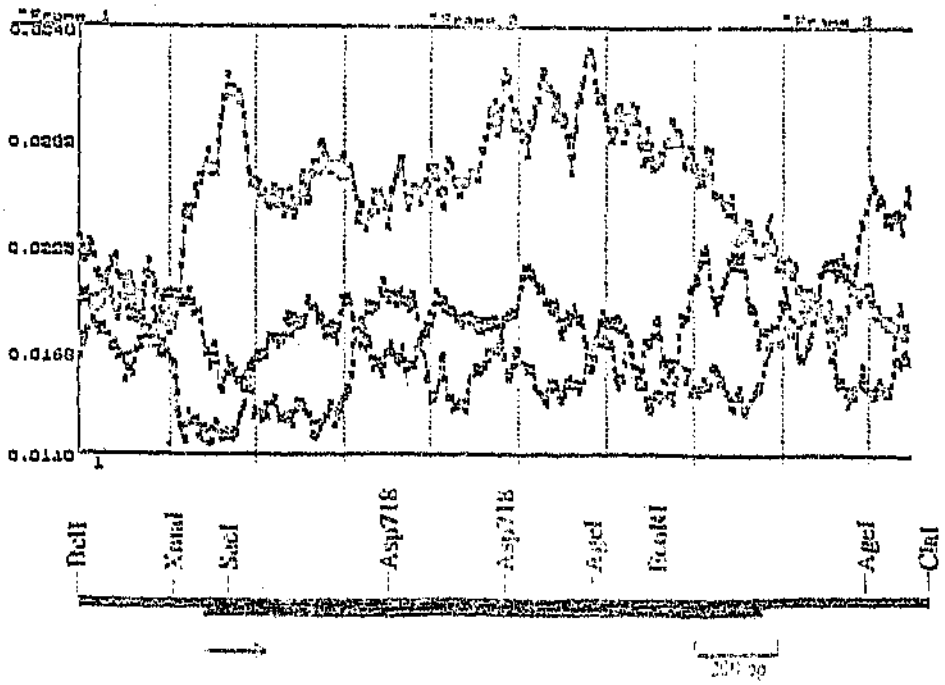


Figure 3.32 Open reading frame in *BclI-ClaI*

These constructs run in parallel with dITP reactions eliminated all but one of the compressions and stops that were producing frameshifts in this region. By sequencing both strands I had hoped to eliminate that one stop. In order to eliminate it DMSO was added to annealing and labelling reactions for both dGTP and dITP based samples. This succeeded in unravelling the secondary structure that had prevented elucidation of this sequence in the past.

The *BclI-ClaI* fragment had now been sequenced as far as the *AgeI* site. There still remained ± 150bp between *AgeI* and *ClaI*.
The *ClaI-EcoRI* fragment containing the *AgeI* site was cloned into M13mp18 and sequenced from the *ClaI* site. This provided the last section of the DNA as well as the overlap with the *AgeI* junction. The sequence showed the presence of the stop codon confirming that it was not just a shift in reading frame. Each subclone that was used in the construction of the entire sequence was sequenced between 2 and 8 times in order to eliminate error.

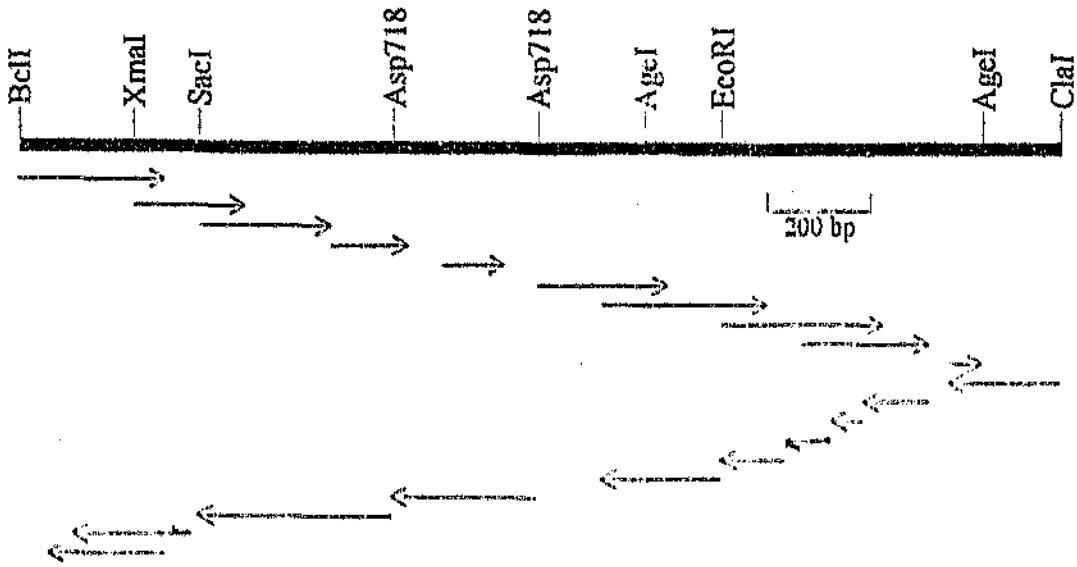


Figure 3.31 Diagrammatic representation of sequencing strategy

Microbiology, Ohio State University, 1993) based on their codon usage, it showed that the gene was jumping out of reading frame in several places in the large *BclI-Asp718* fragment. Also there was a change in reading frame near the *AgeI* site.

3.8.5 Subclones made with *SacI* and *ClaI*

Within the *BclI-Asp718* fragment sequence analysis revealed the presence of a *SacI* site centrally placed on the fragment. *SacI* is present in the multiple cloning site of M13. Earlier analysis of the sequence for extra cloning sites had shown no *SacI* sites. The digestion was performed again and showed no sites. An isoschisomer , *Eql136* was available. This enzyme produces a blunt end. Digestion with this enzyme confirmed the sequence data that a *SacI* site was present. However cloning attempts with the blunt ending isoschisomer was not successful. New *SacI* was obtained.

Plasmid c extracted from a *dam*⁻ strain was digested with *ClaI* and the gene containing fragment was purified on a low melting agarose gel. In order to sequence both strands between the *SacI* and *Asp718* sites where the mistakes were, the DNA had to be cloned into both M13mp18 and M13mp19. The *ClaI* fragment was cut with *SacI* and cloned into the *SacI* and *AccI* sites of M13mp19 - sequencing from the *SacI* site. This cloning produced two different products sequencing out in both directions from the *SacI* site. This gave data for the critical region as well as confirming sequence at the beginning of the contig. The *ClaI* fragment was also digested with *SacI* and *EcoRI* and ligated into those sites in M13mp18. Only the internal *SacI-EcoRI* fragment was expected however there were two different sized inserts identified on agarose gel electrophoresis of the single stranded DNA. The larger one was shown to be the result of the *ClaI* sites ligating to each other producing a *SacI-EcoRI* fragment which enabled the sequencing of the first part of the contig on the other strand once again confirming data already collected.

cut within the sequence indicating that alot of refining needed to be done to the sequences.

As the importance of sequencing on both strands had been made clear by the results from the *EcoRI-Asp718* subclone and the need to orientate the *Asp718* fragment another approach was employed.

3.8.4 Sequencing of *Sau3A* partial digests

The *AgeI* fragment was purified on a low melting agarose gel. This fragment is ± 910bp long and covers the *EcoRI* junction of subclones. It also spanned the area of the *EcoRI-AgeI* fragment that was producing multiple bands. The purified fragment was digested with *Sau3A* a 4 base cutter and inserted into the phosphatased *BamHI* site of M13mp18. Twenty four plaques were picked and the DNA sequenced from these. The sequences were aligned by eye. It proved useful to have a frame work from which to work in aligning the sequences. However the digestion with *Sau3A* was more complete than partial. This was seen by several different *Sau3A* fragments from different places being cloned together. For this reason the sequence following each GATC (the recognition sequence of *Sau3A*) junction was never assumed as being the sequence that really followed the preceding sequence. These sequences were verified by the frame work sequence provided by the original subclones.

The *BclI-EcoRI* fragment, that included the *Asp718* fragment, was purified on low melting agarose and d'gested with *a diluted concentration of Sau3A to achieve partial digestion*. These were ligated into M13mp18 at the *BamHI* site. This fragment was chosen as the subclone junctions of three of the fragments were encompassed and the other strands of both the large 750bp *BclI-Asp718* and the *Asp718* might be sequenced. A further twenty four clones were sequenced and the sequences aligned by eye. These sequences allowed for the contig to be arranged. However when the sequence was analyzed, using a program designed espacially for *Streptomyces* (Frame Program by Gary Kleman, Department of

labelling reactions. Sequenase Version 2.0, in the presence of Mn^{++} ions, reduces the average length of DNA synthesised at the termination step, intensifying bands corresponding to sequences close to the primer. As the fragment was ± 365bp almost all of it overlapped and confirmed the sequence.

The *BclI-Asp718* fragment was too long (± 750bp) to achieve any reliable overlap. Reading the sequence at over 300bp was more difficult with the bands closer together. There were also stops present. This fragment of DNA contained an *XmaI* site ± 200bp in from the *BclI* site. An *XmaI-EcoRI* fragment was ligated into the same sites of M13mp18. Several of the insert containing phages were sequenced and more data obtained.

The *EcoRI-AgeI* sequence could be read clearly from the *AgeI* site inwards, however from the *EcoRI* site there were multiple bands at each level of the sequencing ladder, some darker than others but not significant enough to give certainty to the sequence. DNA was extracted several times and cleaned more intensely, dITP reactions were also run however there was not much increased resolution. A possibility could have been that there was more than one phage present in the stock carrying a different sequence.

Another factor that seemed to affect the clarity of the sequence was the width of each lane on the gel. The combs supplied with our apparatus produced very narrow wells with the result that the sample filled most of the wells and the concentrating of it in a small area seemed to produce bands that were a bit fuzzy. Later when combs that were slightly thicker (0,4mm versus 0,25mm) and nearly twice as wide were used the bands became much sharper and the sequence data more reliable.

On examining the sequence data that had been collected thus far several restriction sites were identified. To test the accuracy of the sequence data these fragments in the pUC clones were digested with some of the enzymes. Of eight enzymes used only two

elucidate compressions in the DNA. In order to obtain a maximum number of tracks on a gel and still facilitate easy reading of the sequences reaction sets were loaded alternately. For example, the first ACGT set was loaded into the first four wells on the gel. The next four lanes were skipped and the third reaction set was loaded. When all the odd-numbered samples were loaded with the 4 lanes left open between them, these samples were electrophoresed into the gel for 5 minutes. The remaining even-numbered samples were then loaded into the open wells and the electrophoresis completed. This produced a distinct gap between each reaction set making it easier to read (Nelson and Krawetz, 1994). In order to increase the number of bases read off a gel, wedges of 4cm of additional spacer were used. These were placed on top of the existing spacers at the bottom of the gel.

Each of the 4 subclones were sequenced from both ends as they had been cloned into M13mp18 and M13mp19, except for the *Asp718* fragment. This fragment could not be directionally cloned as it had the same sites on each end. Thus cloning into both M13mp18 and M13mp19 would not increase the chances of both orientations. For this reason I used the better phosphatased M13mp18 for cloning and decided to screen many of the white plaques in order to find both orientations. This procedure was repeated twice and 24 plaques were screened each time. These phages either contained no insert or just the fragment in consistently the same orientation. The only known restriction site in this fragment was a *NotI* site which had been mapped to the one end. This site is 100% GC rich - GCGGCCGC. As there were several compressions and stops in this sequence initially this site could not be identified. Running a dITP reaction in parallel to the normal reaction resolved some of the compressions but not all. This was when the subcloning of the *Asp718* fragment was repeated for the second time in order to get either the other strand or the same strand form the opposite end.

The *EcoRI-AspI* fragment was sequenced on both strands. In order to determine the sequence close to primers Mn^{++} was added to the

modified dr.

Analysis of these products using TLC showed the unmodified rifamycin SV and the rifampicin moving off the origin when a solvent system of 4:1 chloroform:methanol was used. However the modified products remained on the origin. Alteration of the solvent system to 9:1 methanol:chloroform (Quan S, unpublished) moved the products off the origin.

The rifamycin SV products migrated together. The decolorised rifampicin product was hardly detected. The products using phosphorylated rifampicin migrated together. The ribosylated product that had not been exposed to cells but just to medium ran slower than those extracted from broth containing cells. The others however did not run differently.

It appears as if the *iri*$^{+}$ DNA produces the most significant phenotypical effect on rifampicin. It is puzzling that it does not decolorise the other inactive forms as they have been modified on the ansa bridge and not on the chromaphore. This may be the result of the steric effect of the modification which alters binding of the enzyme. Since rifampicin is not the natural substrate of this enzyme it may have a weaker affinity for it. Thus slight changes in the shape of the drug may have greater affects on substrate binding or substrate-enzyme interaction. These results can be seen in the figure below.

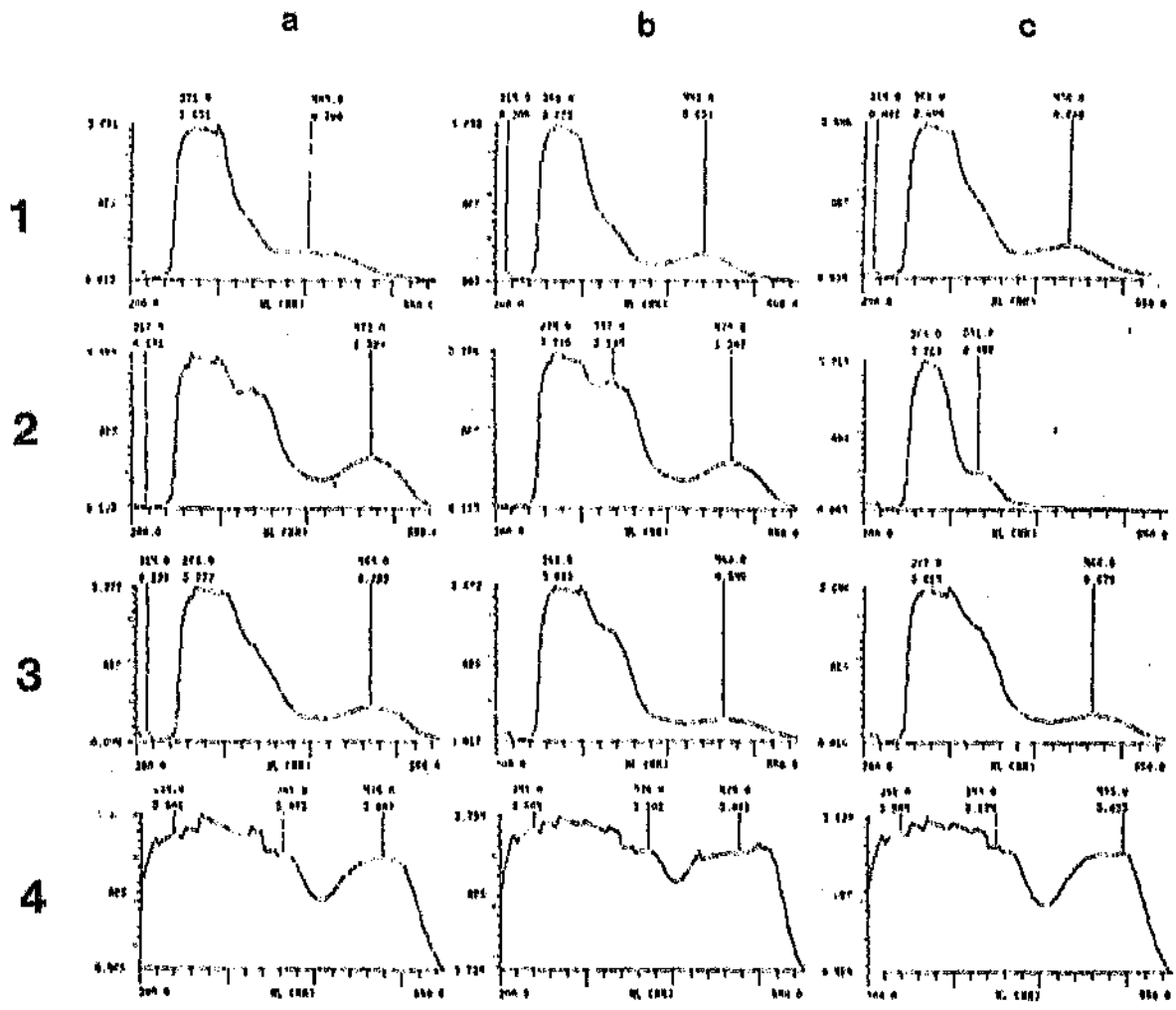


Figure 3.34 Absorption spectra of rifamycin compounds. 1. Rifamycin SV, 2. Rifampicin, 3. Rifampicin-phosphorylated, 4. Rifampicin-ribosylated, a) from medium, b) from *iri*$^-$, c) from *iri*$^+$

Rifamycin SV showed no significant change in absorption spectrum. This was tested for inactivation and was found to be mostly inactivated. Rifampicin showed the loss of its 475nm peak (Dabbs, 1987). There was no change in the spectra for the other two

Table 3.34 Decolorisation of rifamycin compounds by Iri

Compound	Decolorisation
Rifamycin SV	NO
Rifampicin	YES
Rifampicin-phosphorylated	NO
Rifampicin-ribosylated	NO

Rifampicin was the only compound to be decolorised. As the modified products were inactivated already assays to this effect would have been redundant. In order to ascertain if the *iri* gene product was affecting the drug in a place other than the chromaphore the following experiments were done.

The products of these and similar cultures were extracted with ethyl acetate. The ribosylated form of the antibiotic could not be extracted in this way so the supernatant was lyophilised and the resuspended in ethanol, in which this compound was soluble. Scans to determine changes in their absorption spectra were undertaken.

used for the assay included TE, pH 8,0 and a phosphate buffer pH 7,3 both with DTT. This phosphate buffer was used in the previous *Pseudomonas* work. Two other hydroxylases had been characterised from *Rhodococcus* (Armstrong and Patel, 1993; Straube G, 1987) - a phenol hydroxylase and a resorcinol hydroxylase with pH optima of 7,9 and 7,0 respectively. Later a Tris, pH 9,0 buffer was used, this also allowed inactivation without inhibition.

Next the effect of methimazole on *Rhodococcus* was investigated. Cultures containing recombinant plasmids were grown into log phase before the addition of the inhibitor. Between 15 and 30 minutes later 30µg/ml of rifampicin was added. A range of concentrations of methimazole were studied. Rifampicin was decolorised in the presence of 50mM of the inhibitor although the growth rate was retarded. At 200µg/ml growth was inhibited. Indicating that other vital flavin- dependant enzymes had been inhibited. In each case a control strain just bearing the vector was used.

3.9.10 Effect of Iri on rifamycin derivatives

The effect of the *iri* gene product were tested on the following compounds for which the structures were known: rifamycin SV, rifampicin, rifampicin that had been phosphorylated (Dabbs et *al*, 1995b) and rifampicin that had been ribosylated (Dabbs et al, 1995a). The antibiotics were assayed for decolorisation, change in absorbance over the range of 200-550nm and altered mobility using thin layer chromatography.

Cultures of Ri8 containing either pDA71 or the *BclI-ClaI* inserted vector were grown into log phase before being challenged with 30µg/ml of the different antibiotics.

inactivated 10µg/ml rifampicin while the insoluble fraction inactivated some of the antibiotic relative to the control. This was probably the effect of incomplete disruption of all *Rhodococcus* cells present. The cell wall structure of these bacteria makes them far more difficult to disrupt than *E.coli*.

3.9.9 Inhibition studies with methimazole

At the time this work was done the sequence that showed the greatest homology on BLAST scanning was a pentachlorophenol-4-monooxygenase from *Flavobacterium* (Orser CS *et al*, 1993). This enzyme contained the FAD binding site and also required NADPH for catalysis.

Tomasi *et al*, (1995) proposed the use of methimazole (MMI) as an inhibitor of bacterial monooxygenases. This compound was known to have a high affinity for mammalian flavin-containing monooxygenases and to be selectively oxidised by this enzymatic system in the presence of cytochrome P-450 (Ziegler, 1993). These authors used methimazole to inhibit the metabolism of polychlorinated phenols in *Pseudomonas cepacia*. The inhibitor was added at a concentration of 0,5mM and incubated with the cells for 15 min at 28°C before the addition of the substrate.

In vitro assays were carried out as before with a 15 minute preincubation of 0,5mM methimazole before the addition of rifampicin. Various combinations of cofactors were also added before the inhibitor: FAD, FAD + NADH and FAD + NADPH. Those containing NADPH were inactivated and the rest were not. At this concentration methimazole had no effect on inactivation. Concentrations were increased as high as 10mM without any effect. The time of adding the cofactors, before or after preincubation, had no effect. Decreasing the amount of enzyme present while increasing the amount of methimazole did not prevent inactivation and neither did preincubating for up to an hour. The *Pseudomonas* work had been done on whole cells rather than extracts, whole cells could not be inhibited either. The buffers that had been

due to a contaminant the cultures were checked for contamination. There was only *E.coli* growin the cultures. Thus there may have been no contaminant or an *E.coli* contaminant of differing phenotype to CSR603.

3.9.7 Cofactors required by Iri for function

Preliminary DNA sequence data had indicated the presence of an FAD binding region within the protein. The sequence also confirmed the presence of an aspartate within the binding motif at position 278. This has been conserved in FAD binding proteins and is responsible for forming hydrogen bonds with the 0-3 of the ribityl moiety of the bound FAD cofactor (Eggink G *et al*, 1990).

To confirm this data *in vitro* rifampicin inactivation assays were done on *E.coli* extracts of *iri* containing strains in the presence of various cofactors. They were assayed using the plate method.

Table 3.33 Cofactor requirements of Iri

Plasmid	Cofactors	Inactivation
pDA71±	-	-
BclI-ClaI	-	-
BclI-ClaI	0,05mMFAD	-
BclI-ClaI	0,05mMFAD + 0,5mM NADH	-
BclI-ClaI	0,05mMFAD + 0,5mM NADPH	YES

These results were reproducible with up to 20µg/ml rifampicin.

3.9.8 Intracellular location of enzyme

The sonicated cell extract was separated into soluble and insoluble phases by centrifugation. Each phase was examined for its ability to inactivate the drug. The soluble phase carried the enzyme. Enzyme prepared from *Rhodococcus* carrying an *iri* plasmid was also separated into soluble and insoluble phases. Assaying both fractions it was shown that the soluble phase totally

autoradiographs showed that all the cellular proteins had been labelled. Energy levels were increased to 80 and 120J/m^2 and still all the proteins were labelled. It seemed possible since the irradiative burst was happening over just a few seconds that some of the cells were being protected in the aqueous film by those overlying them. Cells were then exposed to 5 successive bursts of 20J/m^2 with agitation of the cells in between. All the proteins were still labelled. As the chromosome was clearly not being damaged sufficiently another look at the sensitivity of the strain to UV damage was required.

Cells were grown for 3 hours before being exposed to successive bursts of 50J/m^2 with agitation in between. Samples were removed and plated after 0, 5, 10, 15 and 20 bursts.

Table 3.32 Sensitivity of CSR603 to successive bursts of 50J/m^2

Number of bursts	CFUs/100µl
0	$7,6 \times 10^7$
10	± 2400
15	± 672
20	± 29

This revealed a 0,00038% survival after 20 bursts of 50J/m^2. As this seemed to be knocking out most of the cells it would therefore prevent most background protein expression off the chromosome from occurring. Since there were still viable cells present even after 20 bursts the next experiment exposed cells to either 20 or 40 bursts. The autoradiograph showed that a state had been reached when there wasn't any protein expression at all. It was now possible to find the place in between where the plasmid was expressed and the chromosome not. Exposure to 7 bursts showed that all proteins were being labelled. Subsequently exposure to 14 bursts produced all labelled proteins in the control and no protein in the experiment. As this could have been

interest can be visualised after being separated on a gel and autoradiographed.

BclI-ClaI in pDA71 and pDA71 containing at tiny insert, to prevent production of a functional endonuclease, were transformed into CSR603. The transformants were patched onto selective plates to check for phenotypic expression of the *iri* gene. Once this had been confirmed these transformants were used in order to optimise UV conditions.

The transformants were grown in K medium for 3 hours before being serially diluted onto LA plates and exposed to different energies of UV light. These were incubated overnight to determine survival. A range of energies from 0-70J/m^2 in increments of 10 were used to narrow down the range. At dilutions of 10^{-2}, 10^{-4} and 10-6 there was growth only on the 0 plates. This was repeated at 0, 10 and 20J/m^2 at dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10-4. There was evidence of slight growth on the 10^{-1} plate of 10J/m^2 and none on the others. Thus 10J/m^2 was selected as the first energy level to irradiate the cells with for the maxicell experiment.

The maxicell experiment was conducted according to the protocol of Sancar *et al*, (1979). More bands than could account for just plasmid encoded proteins were present. This indicated that a higher level of irradiation should be used. The protocol also called for all incubations to occur at 37°C while expression of the *iri* gene has been optimised at lower temperatures. So this procedure was repeated with exposure to 20J/m^2 and the incubation of cells after exposure at room temperature. Autoradiographs still showed too many proteins. Freshly labelled methionine arrived (that used previously was quite old). I tried doing all procedures at 28°C in stead of 37°C with and without low levels of rifampicin present - 1µg/ml (in case the protein needed to be upregulated). However the growth was extremely slow. Next the cells were grown at 37°C until the cycloserine was added one hour after irradiation this time with 40 or 60J/m^2. After 30 minutes 0,2µg/ml rifampicin was added for the overnight incubation. The

Table 3.31 Expression of *iri* genes in *E.coli*

Plasmid	LA+Amp	LA+Amp+20μg/ml Rif
pDA71	+++	
BclI-ClaI	+++	+++
ClaI-ClaI	+++	
#16	+++	
#39	+++	

where +++ indicates growth

This indicated that neither the *R.equi* clone nor the *R.erythropolis* clone was being expressed off its own promoter in *E.coli*. However the plasmid with the smallest insert which was inserted in the correct orientation for gene expression was being expressed off the lambda promoter. Later expression was shown to be temperature sensitive rather than IPTG inducible. Since it had now been shown that the *iri* gene was now expressed in *E.coli* and previous experiments had shown that this construct did not show an overexpressed protein on a gel a maxicell system was employed.

3.9.6 Protein expression using maxicells

The maxicell system utilises a DNA repair deficient host strain, CSR603. The strain is transformed with the appropriate plasmids and the subjected to DNA damage with ultra violet irradiation. The chromosome is unable to repair itself. However as the plasmids are so much smaller and numerous per cell these are not as easily damaged. Since the protein machinery of the cell is still functional, plasmid replication can continue. By adding a radiolabelled amino acid after starving the cells, the only proteins transcribed will be plasmid encoded. Thus by performing this on a transformant that contain the vector alone and another that contains the same vector that now contains the gene of interest that is expressed in the host, the polypeptide of

For the purposes of checking all the reading frames this plasmid was digested with *XmaI* to linearise it. Portions of this digestion were either subjected to treatment with Klenow enzyme which would fill in the overhang and thus shift the reading frame or treated with Mung bean nuclease which would cut off the overhang thus shifting the reading frame in the opposite direction. These were then ligated and transformed into *E.coli*. Plasmids were extracted from the transformants and screened for loss of the *XmaI* site by digestion with isoschisomer *SmaI*. Only the Klenow treatment had succeeded in introducing a frame shift. These constructs were analyzed by PAGE as described previously. No overexpression of the fusion protein was seen. Optimisation of mung bean nuclease function was required. This was carried out in various restriction enzyme buffers as well as ligation buffer. Fresh enzyme was obtained as the other aliquot had expired. The buffer suggested by the manufacturers was made up and tried, efforts were also made to use short treatments of *Bal31*. None of these were successful. Some of these were screened by patching onto LA plates containing 100μg/ml ampicillin and 10μg/ml B1 with and without 20μ/ml rifampicin. These were grown at 28°C as this had been shown to enhance phenotypic expression of the *BclI-ClaI* fragment in pDA71 (Dabbs ER, personal communication). This had been alluded to in a previous experiment that showed a slight increase in resistance with this plasmid. However none of these had increased resistance to rifampicin.

3.9.5 Enhanced expression of *rri* in *E.coli*

To check for expression leading to increased resistance of other constructs in pDA71 the following plasmids were transformed into rifampicin sensitive MM294-4 and grown at 28°C on plates containing B1.

of interest and *lacZ* did not prevent transcription of the gene of interest after induction with IPTG.

The *BclI-ClaI* fragment was prepared from plasmid c and inserted into the *BamHI* and *AccI* sites of pGEM3(f)+. This would place it under the control of the *lac* promoter. The map of pGEM3(f)- indicated that insertion into the *BamHI* and *AccI* sites would place the fragment into the orientation where it would not be expressed for this reason it was inserted into pGEM3(f)+.

E.coli was transformed with this construct and the control of pGEM3(f)+ alone. At an OD_{540} of 0,5 0,03mM IPTG was added and induction continued for 2 hours. Cells were then harvested and resuspended in loading buffer and separated on a 10% polyacrylamide gel. Pre-induction samples were also loaded on the gel. The only extra bands that were seen were those indicating host β-galactosidase. On checking the map for pGEM3(f)+ it became evident that the differences between the two vectors was not the orientation of the multiple cloning site (as in the case of the pUC vectors), but the phage origin which affected which strand would be sequenced if these vectors were used for that purpose.

On analysis of the multiple cloning sites of the pUC vectors the fragment needed to be cloned into pUC118. A construct already existed of the *ClaI* fragment inserted into this vector. If it was correctly orientated deletion of the *XmaI* fragment would produce the required construct. However *XmaI* and *EcoRI* digestions revealed that it was inserted in the other direction. Although the direction was incorrect the plasmid could still be used to extract the *XmaI* fragment. This was purified on low melting agarose. Insertion of this fragment into the *XmaI* site of the multiple cloning site would not be directional it would also prevent future manipulations that may need to be done to alter the reading frame. So this fragment was cloned as an *XmaI-BamHI* fragment, where the *BamHI* site was found in the multiple cloning site on the *XmaI* fragment. The fragment was verified by the presence of the *Asp718* fragment.

was no phenotypic difference between these. Mung bean nuclease was used to shift the reading frame in the other direction with a paralled lack of success. It could have been the case that the gene was being transcribed and translated but lacked modification to make it functional in *E.coli*. Thus protein was prepared from *E.coli* carrying plasmid with the gene in both orientations and the vector alone. These strains had also been exposed to rifampicin in case that induced expression of the gene. Cells were lysed with SDS and the contents separated on a 10% polyacrylamide gel. No distinctive band was present in either of the *iri*$^+$ containing cells.

3.9.3 Checking for overexpression in *Rhodococcus*

As the gene is definitely expressed in *Rhodococcus* this was used as the next host for these plasmids. The plasmids were introduced into these rifampicin resistant cells by electroporation. The transformants were tested for their ability to inactivate rifampicin. The *iri*$^+$ plasmid containing cells decolorised 30μg/ml of rifampicin while the pDA71 containing cells were unable to decolorise any of it. These bacteria were then grown in TYG with rifampicin. The stationary phase cultures were harvested. One millilitre of culture was pelleted and resuspended in 0,5ml Tris-sucrose with 5mg/ml lysozyme. These were incubated at 37°C for 30 minutes. The cells were harvested again and resuspended in 10mM Tris, pH 8,0, that also contained 1% SDS and traces of RNAse and DNAse I. This was also incubated for 30 minutes at 37°C. Sample loading dye was added and they were boiled for 5 minutes before being loaded on the polyacrylamide gel. No distinct band was seen indicating the presence of the Iri polypeptide.

3.9.4 Construction of further plasmids for overexpression

By this time the orientation of the gene on the *BclI-ClaI* fragment had been determined and the start codon seemed to be internal to the *XmaI* site. It seemed to be the experience of others in our laboratory that upstream sequence between the gene

transpose.

Insertion elements are more well described in the mycobacteria. One of these, IS6110, which is specific to the *M.tuberculosis* complex is used in DNA fingerprinting for epidemiological studies (Cave *et al*, 1991). Analysis of isolates from some patients have shown the acquisition of more IS elements through the course of an infection (Warren *et al*, 1995). Insertion sequences have been associated with virulence in *M.avium* (Kunze *et al*, 1991). Martin *et al* (1990) were the first to describe the transposition of an antibiotic resistance element in mycobacteria. They cloned and sequenced a fragment of DNA responsible for sulphonamide resistance in *M.fortuitum*. This region also contained an open reading frame homologous to a site specific integrase of the Tn21 family. The mycobacterial resistance element was flanked by repeated sequences of 880bp similar to the insertion elements of the IS6 family. It was also shown to transpose to different sites in *M.smegmatis*. This data indicates that transposable elements are present in this group of bacteria, that their ability to transpose has been proven and that they have been shown to transpose genes that confer antibiotic resistance. Examination of *R.equi* isolates from patients with rifampicin resistance could be tested for transposition by Southern blotting with the *iri* gene.

The evidence seems to suggest that low level antibiotic resistance to rifampicin may be possible and significant in nocardioform bacteria.

4.3 RIFAMPICIN INACTIVATION

The search for the gene responsible for rifampicin inactivation had been initiated after the discovery that rifampicin was inactivated and decolorised by *Rhodococcus erythropolis*. This led to the screening of all available strains for their ability to inactivate and decolorise the ansamycins. It was noted that

site of infection in the body it is at low concentration. Therefore any mechanism that allows the bacteria to overcome these low concentrations of the drug is an antibiotic resistance. These become more significant with long term infections. Recently a low level rifampicin-resistance gene was cloned from *M.smegmatis* (Dabbs and Quan, unpublished). This gene allows for the inactivation of rifampicin by ADP-ribosylation of the drug. Other mechanisms of rifampicin inactivation have been described in *Nocardia*: glucosylation (Yazawa *et al*, 1993) and phosphorylation (Yazawa *et al*, 1994). Inactivation caused by degradation of the drug was also found in *Nocardia* (Yazawa *et al*, 1993). None of these genes have been cloned.

The presence of a single copy of one of these genes probably does not confer resistance to these bacteria unless it is a repressed gene and a mutation occurs in the repressor. However, as it has been shown in this work, the presence of multiple copies of the gene (in this case, plasmid encoded) may give rise to increased resistance. A resistance that is able to cope with the concentrations of the drug received *in vivo*.

A possible mechanism for this would be inclusion of one of these genes into a transposon. The presence of antibiotic resistance genes in transposons is well documented. Transposons that carry genes encoding drug resistance range in size from 2,5 to 23kb. Some transposons with terminal inverted repeat sequences are capable of independent [illegible]ment and are called insertion sequences (Lupski, 1987). Insertion sequences have been described in most of the nocardioforms. Denome and Young (1995) have identified two putative insertion sequence (IS) elements in *Rhodococcus*. In the strain studied 4 copies of the insertion sequence were found. A mutant of the strain was found to have 5 copies which indicated an ability to transpose. Jager *et al* (1995) isolated an element from *R.fascians* as well as from *Arthrobacter*, *Brevibacterium* and *Corynebacterium*. Yao *et al* (1994) describe an IS element in *Nocardia asteroides* in which 9 copies were found in the genome. They showed its ability to

4.2 RIFAMPICIN RESISTANCE

Rifampicin resistance in most cases of bacterial resistance has been associated with the mutational alteration of the β-subunit of RNA polymerase. The entire *rpoB* genes of *M.leprae* (Honore *et al*, 1993) and *M.tuberculosis* (Miller *et al*, 1994) have been sequenced, and several mutations associated with rifampicin resistance in both species have been identified (Honore and Cole, 1993; Telenti *et al*, 1993). In a recent study, Williams *et al* (1994), analyzed a 305bp fragment of the *rpoB* gene from 110 rifampicin resistant and 10 rifampicin susceptible strains of *M.tuberculosis* from different parts of the world. In 102 of 110 resistant strains 16 mutations effecting 13 amino acids were observed. No mutations were observed in susceptible strains. These authors observed that 8 of their resistant strains showed no mutation in this area. Telenti *et al* (1993) showed that 2 of 66 resistant isolates also lacked mutations in this region. This seemed to indicate that other mutations outside of the 305bp region may have been responsible. Two other mutations to resistance were known to occur outside this region in *E.coli* (Jin and Gross, 1988). Sequencing of one of these regions in *M.tuberculosis* showed no mutational alteration, the other region had not been sequenced at the time of publication. This does hint at another possible mechanism of resistance to rifampicin. This possibility has been postulated by Mizuguchi *et al* (1995), who analyzed high level, intermediate level and low level resistance to rifampicin in *M.tuberculosis*. In their analyses they found that in more than 50% of the intermediate or low level resistant strains there was no mutation in *rpoB*. Guerrero *et al* (1994) report that the most frequent mechanism of resistance to rifampicin among clinical isolates of *M.avium* and *M.intracellulare* does not involve alterations of the β-subunit of RNA polymerase. They propose that an alterative mechanism must be responsible for resistance.

It would not be true to say that only high level resistance is clinically significant. By the time the antibiotic reaches the

„e's that the combination of erythromycin and rifampicin or imipenem and teicoplanin were the most effective treatments of *R.equi* infections. Emergence of rifampicin resistance was noted by Clave *et al* (1991). Recurrent pneumonia due to a rifampicin-resistant *R.equi* was identified in a patient infected with HIV (Nordmann *et al*, 1992).

The apparent increase in the incidence of rhodococcal infections that has developed over the past decade could be due to several factors. It is most likely the result of the HIV epidemic but could also be the result of better diagnostic methods for identifying gram positive bacteria in the clinical environment or an enhanced awareness of opportunistic gram positive pathogens by medical personnel. It would be difficult to determine the actual increase in real terms. It is interesting to see that all the statistics concerning *R.equi* infection come from first world countries. It is probable that the incidence is greatly increased in third world communities, since this organism is found in the soil or obtained from animals. The incidence of HIV infection is also greater in these parts of the world. It is also possible that since infection resembles tuberculoid disease it could make up a percentage of patients diagnosed with tuberculosis. This is more likely in places where diagnosis is made on clinical symptoms ather' than by microbiological investigation. Consultation with the tuberculosis diagnostic facility of the South African Institute for Medical Research confirms that this scenario is quite possible. As the drug regimens that are being recommended for *R.equi* include only one antimycobacterial drug, rifampicin, it is possible that treatment of *R.equi* with antituberculoid drugs would be equivalent to monotherapy with rifampicin. In turn rifampicin monotherapy is a protocol for the development of resistance to this antibiotic. Thus an accurate determination of *R.equi* especially in the third world is necessary. Following on from this identification of rifampicin resistance and the mutation involved will help in the effective treatment of infected individuals.

Of these patients 6 also had bacteraemia and 3 had died of disseminated *R.equi* infection. Less than 5% of the isolates were resistant to erythromycin, rifampicin, tetracycline and trimethoprim-sulfamethoxazole.

Further analysis of 15 isolates confirmed as *R.equi* from 13 patients showed 11 different rRNA gene patterns on ribotyping. There was no discernable difference observed in isolates from HIV and non-HIV patients (Lasker *et al*, 1992). There was also no geographical clustering of the ribotype patterns.

Because of the low number of patients diagnosed with *R.equi*, there had not been much opportunity to develop effective treatment regimens. *In vitro* studies were done with several strains of *R.equi* on numerous antibiotics. Two of these studies came to the following conclusions: Clave *et al* (1991) found imipenem and ceftriaxone to be the most effective beta-lactams studied. The lowest MICs were noted with vancomycin, teicoplanin, erythromycin, clarithromycin, rifampicin, gentamycin and doxycycline. A longitudinal survey, including three strains isolated from the same patient, showed the emergence of rifampicin resistance and a marked increase in the MIC of imipenem. Nordmann and Ronco (1992) showed the most active antibiotics *in vitro* were amikacin, gentamycin, netilmicin, erythromycin, clarithromycin, roxithromycin, ciprofloxacin, sparfloxacin, rifampicin, vancomycin, teicoplanin, doxycycline, minocycline, imipenem, meropenem and trimethoprim/ sulphamethoxazole. Four combinations were synergistic: rifampicin-erythromycin, rifampicin-minocycline, erythromycin-minocycline and imipenem-amikacin.

In vivo studies using a nude mouse model found vancomycin, imipenem and rifampicin to be most effective in monotherapy and suggested that the most useful combinations would be those including vancomycin (Nordmann *et al*, 1992). They also showed that the presence of functional T-cells was necessary for clearing the infection. The experience of Mascellino *et al* (1994)

Of these patients 6 also had bacteraemia and 3 had died of disseminated *R.equi* infection. Less than 5% of the isolates were resistant to erythromycin, rifampicin, tetracycline and trimethoprim-sulfamethoxazole.

Further analysis of 15 isolates confirmed as *R.equi* from 13 patients showed 11 different rRNA gene patterns on ribotyping. There was no discernable difference observed in isolates from HIV and non-HIV patients (Lasker *et al*, 1992). There was also no geographical clustering of the ribotype patterns.

Because of the low number of ratients diagnosed with *R.equi*, there had not been much opportunity to develop effective treatment regimens. *In vitro* studies were done with several strains of *R.equi* on numerous antibiotics. Two of these studies came to the following conclusions: Clave et al (1991) found imipenem and ceftriaxone to be the most effective beta-lactams studied. The lowest MICs were noted with vancomycin, teicoplanin, erythromycin, clarithromycin, rifampicin, gentamycin and doxycycline. A longitudinal survey, including three strains isolated from the same patient, showed the emergence of rifampicin resistance and a marked increase in the MIC of imipenem. Nordmann and Ronco (1992) showed the most active antibiotics *in vitro* were amikacin, gentamycin, netilmicin, erythromycin, clarithromycin, roxithromycin, ciprofloxacin, sparfloxacin, rifampicin, vancomycin, teicoplanin, doxycycline, minocycline, imipenem, meropenem and trimethoprim/ sulphamethoxazole. Four combinations were synergistic: rifampicin-erythromycin, rifampicin-minocycline, erythromycin-minocycline and imipenem-amikacin.

In vivo studies using a nude mouse model found vancomycin, imipenem and rifampicin to be most effective in monotherapy and suggested that the most useful combinations would be those including vancomycin (Nordmann et al, 1992). They also showed that the presence of functional T-cells was necessary for clearing the infection. The experience of Mascellino et al (1994)

4.1 *Rhodococcus equi*

The focus of this work was *Rhodococcus equi*. As this is primarily an animal pathogen it has been an object of investigation for veterinary medicine. Because of the relatively high mortality rates in foals from chronic bronchitis attempts have been made to develop vaccines. Crude whole cell extracts have been used which have had limited success. The efficacy of such vaccines has been uncertain. Several possible virulence factors have been investigated. Mycolic-containing glycolipids of *R.equi* have been introduced into mice resulting in granuloma formation and liver damage (Gotah *et al*, 1991). Studies of virulent and avirulent strains suggested a role for 15- and 17-kilodalton antigens (Takai *et al*, 1991). These authors subsequently went on to show that these antigens were plasmid encoded and preliminarily mapped onto the 85kb plasmid (Kanno *et al*, 1993).

R.equi was first described as a respiratory pathogen in live stock in 1923. Since then there have been reports of clinical illness in humans. Most report cavitating pulmonary invasion resulting in pneumonia and lung abscesses (Emmons *et al*, 1991). Non-pulmonary infections such as endophthalmitis, osteomyelitis, and brain, kidney and pelvic abscesses have been reported (van Feta *et al*, 1983). *R.equi*, like the mycobacteria, is a facultative intracellular organism that has been isolated from alveolar macrophages (Mascellino *et al*, 1994). Recently it has been implicated as a cause of pneumonia, bacteraemia and disseminated infection in HIV patients (Emmons *et al*, 1991). Analysis of isolates received by the CDC, Atlanta, over the period of January 1973 through to December 1990 was carried out by McNeil and Brown (1992). Evaluation was of 107 isolates (101 patient and 7 animal) from blood (32 isolates), sputum (30 isolates), lung tissue (13 isolates) and 32 from other sites. Before 1983, when the first *R.equi* isolate from an HIV infected patient was received, CDC received a total of 52 patient isolates. In the final two years of the study 29 out of 35 isolates were from HIV infected patients with *R.equi* pneumonia.

4.0 DISCUSSION

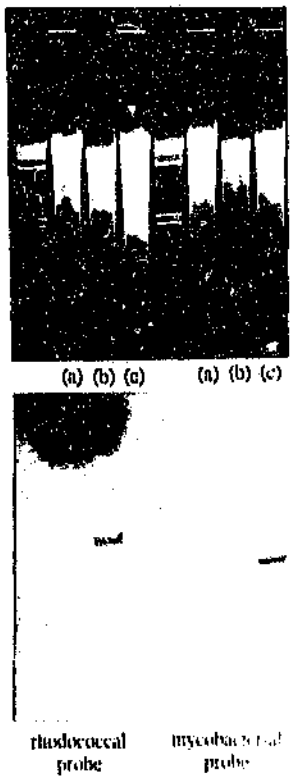


Figure 3.36 Southern blot analysis for homology with *iri* and the rifamycin producer organism.
(a) *A.mediterranei* DNA
(b) *R.erythropolis* DNA
(c) *M.smegmatis* DNA
Rhodococcal probe: *iri* from *R.equi*
Mycobacterial probe: ADP-ribosylating DNA
(Dabbs and Quan)

Under the conditions used here there was no binding of either probe to the *A.mediterranei* DNA. The rhodococcal probe did bind to the *R.erythropolis* DNA. If the gene responsible for rifampicin inactivation had arisen from the producer organism a relatively high level of homology would be expected, however this is not seen. This suggests another origin for a rifampicin inactivating gene or that rifampicin is an alternative substrate for a *Rhodococcus* enzyme that usually has a different function within the cell.

Subsequent experiments with the antibiotic in methanol still gave growth on the no carbon source control. Since the agar was also a potential carbon source, the following experiments were carried out in liquid minimal medium.

The plasmid containing Ri8 strains were grown in rich broth and washed before inoculation into the minimal medium that contained various concentrations of the following potential carbon sources: glucose, catechol, phenol and p-hydroxybenzoate. Naphthalene could not be used as it only soluble in DMSO which is a carbon source. The concentrations used were 0,01%, 0,05% and 0,1%. After more than 2 weeks the only growth observed was in the glucose containing cultures and there was no difference as a result of the *iri* gene. These did not appear to substrates for the *iri* gene product.

3.9.12 *A.mediterranei* and *iri*$^+$ DNA

The final BLAST searches have revealed another set of enzymes that iri has greater homology to than the phenol hydroxylases. These are hydroxylases involved in antibiotic biosynthetic pathways. Although it had already been shown that the rifamycin producer organism did not inactivate rifampicin this did not preclude the existence of a mutated or non-functional or repressed gene. To determine whether the *iri* gene was a functional homologue of an *A.mediterranei* gene or whether that organism was not responsible for an evolutionary ancestor homology was tested by Southern blotting.

Chromosomal DNA was extracted from *A.mediterranei* and *R.erythropolis* and digested with *BglII*. *R.erythropolis* had been shown to be in a similar genomic context which suggested a common ancestor. Therefore it would make a good control. After the DNA had been separated by electrophoresis it was Southern blotted onto a nitrocellulose membrane. The blot was probed with the 5kb *BamHI-BglII* fragment of R.equi that had been cold labelled with DIG.

3.9.11 Effect of Iri on various aromatic compounds

At the time these experiments were undertaken homology searches indicated the highest hits with phenol hydroxylases. It seemed probable that rifampicin was just an alternative substrate for an enzyme that had another function within the cell. This function could have been the degradation of some phenolic or other aromatic compound.

Ri8 should be defective in this degradative function. Thus Ri8 containing pDA71 should show less or no growth than Ri8 containing *BclI-ClaI* when these compounds were the sole carbon source.

ATCC12674, from which Ri8 was derived, was not entirely prototrophic. It had minimal requirements for glutamate and vitamin B1. Thus Ri8 and the strain from which it was derived were replica plated onto the following minimal media plates:
1µg/ml glutamate and 1µg/ml B1
0,1µg/ml glutamate and 0,1µg/ml B1
0,01µg/ml glutamate and 0,01µg/ml B1
0,05% p-hydroxybenzoate
0,05% p-hydroxybenzoate 1µg/ml glutamate and 1µg/ml B1
0,05% p-hydroxybenzoate, 0,1µg/ml glutamate and 0,1µg/ml B1
0,05% p-hydroxybenzoate, 0,01µg/ml glutamate and 0,01µg/ml B1
Growth was found on all these plates as well as on naphthalene plates over a range of 0,01% - 0,1%. The reason was soon apparent, the minimal medium used contained sodium citrate that acted as a carbon source. This did imply that B1 and glutamate were not essential for growth.

Replica plating was repeated using plasmid carrying strains of Ri8. There was no sodium citrate present and the carbon sources used included phenol and p-hydroxybenzoate. Growth occurred on the control lacking a carbon source. The selective pressure for maintaining the plasmids was chloramphenicol and this had been dissolved in ethanol. Ethanol was a good carbon source.

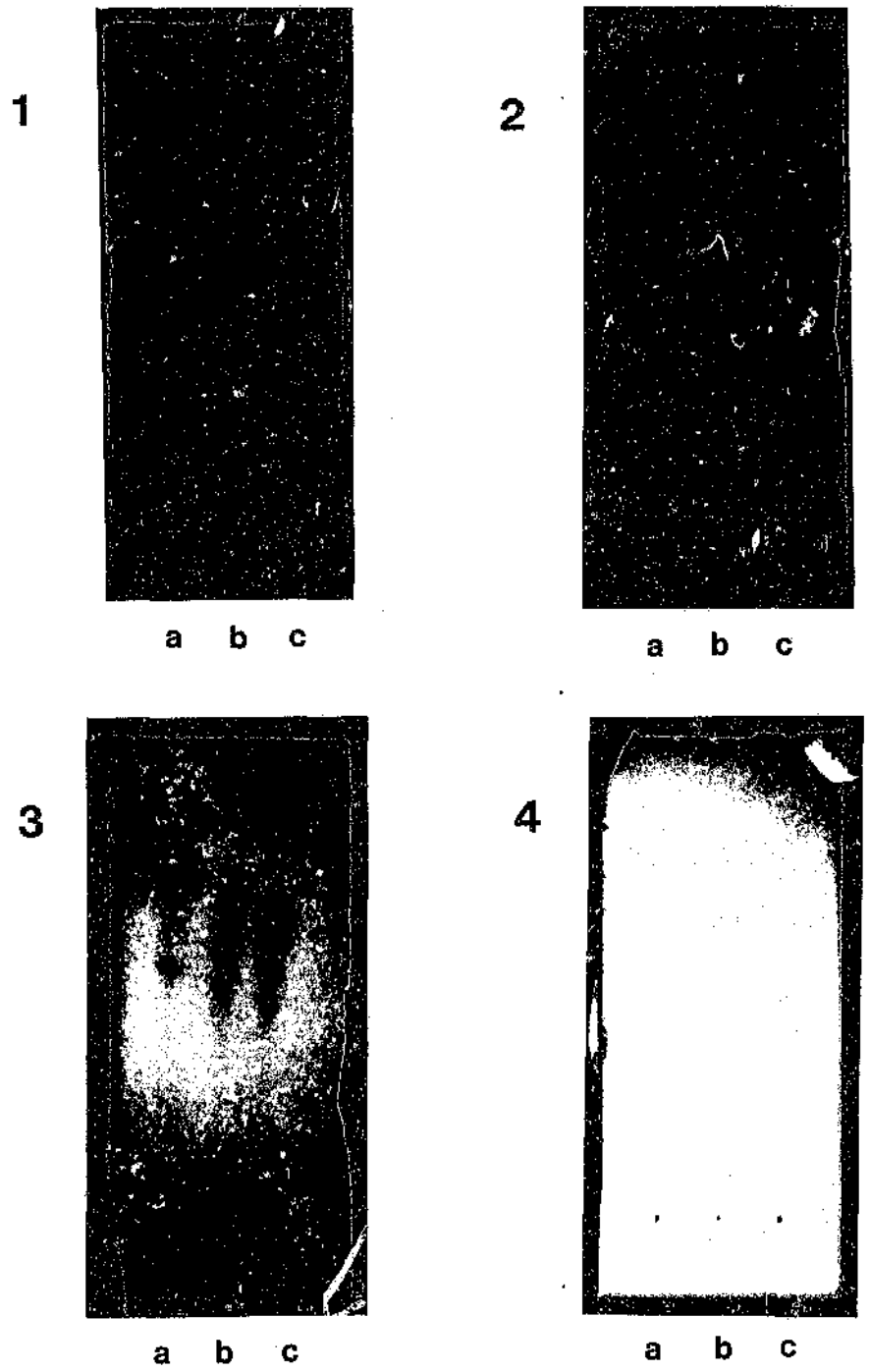


Figure 3.35 TLC analysis of rifamycin compounds. 1. Rifamycin SV, 2. Rifampicin, 3. Phosphorylated rifampicin, 4. ADP-ribosylated rifampicin. Extracted from a) medium, b) *iri*⁻ strain, c) *iri*⁺ strain.

Many of the dioxygenases, which catalyse the incorporation of 2 atoms of oxygen into substrates, are multicomponent enzymes that also require cofactors. These cofactors are capable of reacting with dioxygen. Concerted reactions are spin forbidden between paramagnetic dioxygen with 2 unpaired electron spins, and carbon in organic compounds existing in a singlet state.

In the aerobic degradation of aromatic hydrocarbons, substrates are degraded via the cleavage of the aromatic ring by dioxygenases. Substrates of the aromatic-ring-cleavage dioxygenases possess two hydroxyl groups on the aromatic ring, either *ortho* or *para* to each other. One of the first steps in the degradation of the aromatic compound involves the introduction of one or two hydroxyl groups on the aromatic ring. The introduction of the single hydroxyl groups on the aromatic ring is carried out by monooxygenases. Most hydroxylases fall into a 45-80kDa range. Some monooxygenases incorporate a new hydroxyl group *para* to an existing OH but most catalyse *ortho* hydroxylation.

The strongest sequence conservation is observed in and adjacent to the FAD-binding regions. The local sequence similarities between the hydroxylases are observed in two regions. In the first of these, at the amino-terminal region, a conserved amino acid sequence pattern forms an ADP-binding fingerprint associated with a $\beta\alpha\beta$ fold (Wierenga *et al*, 1986). In p-hydroxybenzoate hydroxylase the conserved amino-terminal binds the ADP portion of FAD (Schreuder *et al*, 1988). The second region of homology among the hydroxylases may be involved in the binding of the flavin moiety of FAD. The 3 dimensional structures of p-hydroxybenzoate hydroxylase show an FAD-binding β-strand (Eggink *et al*, 1990). The sequence homology among the flavoprotein monooxygenases extends beyond this β-strand.

Homology searches of databases constantly produced hits in two regions of the *iri* translated protein. These were in the amino-terminal and in the region that shows homology to flavin binding.

The homology searches were performed using several of the facilities available on the World Wide Web, including BLAST, FASTA and the BCM Search Launcher, all produced matches with hydroxylases that contained conserved FAD binding regions.

4.7 MONOOXYGENASES

The flavoprotein hydroxylases are all monooxygenases; one atom of O_2 is introduced into the substrate as an hydroxyl moiety, the other ends up as water. Many of the enzymes are bacterial in origin and are often induced to high levels by growth of the organism on aromatic substrates as major or sole carbon source. These enzymes are simple flavoproteins in the sense that no other detectible organic coenzymes of transition metal elements are required for catalysis. They do require an NAD(P)H cofactor as a reducing agent. The substrates are aromatic or heteroaromatic and are usually activated by some function such as a pre-existing hydroxyl group. The role of the reduced nicotinamide cofactor is to generate the enzyme-$FADH_2$, which is the reactive species combining with O_2 to form the active hydroxylating agent. The generalised stoichiometry is:

$$S\text{-}H + O_2 + XH_2 \xrightarrow{\text{E-FAD}} S\text{-}OH + H_2O + X$$

where S is the substrate and XH_2 the reducing agent.
The enzyme reaction is separated into 2 steps the oxidation of NAD(P)H to generate 2 reducing equivalents and the hydroxylation of the substrate. Flavoprotein hydroxylases that catalyse the monohydroxylation of the aromatic ring carry out these reactions on a single polypeptide chain. Although most of the monooxygenases are single component enzymes, some multicomponent monooxygenases such as toluene-4-monooxygenase have also been found (Bryne et al, 1995). Other monooxygenases are the mixed function oxidases that include the P-450 cytochromes. These all have a broad substrate specificity and are involved in x-metabolism.

subclone junctions using overlapping fragments. It would allow for easy sequencing of the *iri* gene from Ri8 to determine its exact mutation. It would also allow for sequencing mutants of *iri* that show increased resistance to rifampicin, if such mutants were made.

The approach that was used is the type that has now been used for sequencing the entire genomes of *H.influenza* and *M.genitalium* by The Institute for Genome Research (Fraser *et al*, 1995 and Fleischmann *et al*, 1995). Software capable of aligning shot-gun sequences was developed to cope with constructing the contigs needed. Although the extent of this sequencing was not nearly as great, there was no access to software that was able to align sequences. Alignment was all done by eye which is very time consuming. This approach rather than primer extension sequencing would have been the one to adopt if an automated sequencer was being used. In automated sequencing the cost of the fluorochrome attached to the primer is considerable. However better quality sequence is obtained using labelled primers rather than incorporating label into the extension reactions. Thus using the same universal primer for all sequencing is recommended as long there is software to align fragments.

The change to sequencing single stranded templates was the turning point. Double stranded sequencing of GC rich DNA could be enhanced if a thermostable polymerase was used or single-stranded-binding protein was introduced into the reactions or if glycerol tolerant gels were run. These may have aided in the elimination of secondary structures that seem to be harder to sequence through on a double stranded template than a single stranded template. The use of dITP reaction mixes provided in the Sequenase kit was useful for eliminating compressions. However secondary structures that produced stops across all four lanes were accentuated in dITP reactions. DMSO was found to be the answer to these problems as it seemed to make secondary structures available for sequencing.

subcloning of the *R.equi* gene that the decolorising and inactivation functions were complemented by the same fragment of DNA. It must be said that the *iri* gene may not be responsible for the decolorisation of the drug, it may be responsible for producing the substrate that another enzyme decolorises. These processes would not be distinguished in a mutant that had only an altered *iri* gene.

The transformation of the other mutants had revealed that the level of rifampicin resistance increased above the control indicating that another gene lower down in a cascade could not have been knocked out as the organisms were still protected. It is most likely that the hydroxylated rifampicin is an alternative substrate for a dioxygenase present in the *Rhodococci*. It is also most probable that *iri* and the dioxygenase gene are not found in the same chromosomal context i.e. immediately adjacent to each other on the 5kb fragment.

In addition to increasing the level of rifampicin resistance, the presence of the *iri* gene increases resistance to the other ansamycins but not to representatives of some of the major groups of other antibiotics. A mechanism of resistance, like those found to aminoglycosides, where the drugs have another chemical group attached to them may have had an affect on other antibiotics. Since this mechanism seems to be the result of degradation of the antibiotic due to its aromatic nature this result is not surprising.

4.6 SEQUENCING

The lack of sequencing experience in our laboratory and financial limitations resulted in a less than optimal approach to analysing the *iri* gene. An alternative approach to the one adopted here that may have been more useful. In retrospect, it may have been better to sequence by synthesising primers along the length of the sequence. This would have obviated the need to verify

were hypersensitive but inactivated the drug at the same rate as the parental strain, Ri2, Ri3, Ri4, Ri10, Ri11, Ri12 and Ri13. In spite of the difference in inactivation ability the transformation of these strains with *iri*$^+$ plasmids (from both *R.erythropolis* and *R.equi*) led to restoration of the sensitive phenotype. This may have been due to a gene dosage effect because the gene was encoded on a multicopy plasmid and may not have been because the original mutation had actually been complemented. Hypersensitivity may have occurred at the *rpoB* level but the presence of an inactivating enzyme may have been able to overcome the effect of the low doses of rifampicin. As this restoration of sensitivity occurred in all cases it does not fit the scenario that other genes in a pathway had been mutated as this would not have resulted in the restoration of sensitivity.

The mutant Ri8 was selected as the host for complementation. As was demonstrated later, Ri14 proved to be an equally good host as the same *iri*$^+$ gene fragment (from *R.erythropolis*) was pulled out using both host systems. The slight difference between hypersensitivity and sensitivity of 0,2µg/ml of rifampicin did not hinder the selection of complemented clones. All the transformants that were screened contained the same DNA fragment except for one which was shown later to contain a deletion. This could have been the result of spontaneous mutation to resistance. The deleted plasmid did not code for any inactivating enzyme. The inactivating ability was confirmed to be plasmid borne by the curing of the plasmids and the subsequent loss of activity. By transforming this plasmid into a rifampicin resistant mutant of Ri8 a higher dosage of the drug could be administered without endangering the viability of the cell. After challenging cultures of these organisms with doses of 50µg/ml of the drug inactivation was seen to be simultaneous with the decolorisation of the antibiotic. This indicated that the fragment of DNA that had been cloned encoded both these functions.

Subsequent subcloning confirmed that these two functions were due to the action of a single gene. It was also confirmed by the

Rhodococcus chloramphenicol resistance gene cut it down to half its original size. The process of cutting down increased the number of unique sites that were available for cloning from one to four, *BglII*, *HindIII*, *PstI* and *SfuI*. This vector, still a positive-selection shuttle vector, had an increased copy number and was named pDA71. This was useful for the subcloning and expression of the *R.equi* gene.

4.5 CLONING *iri* GENES

As the possibility existed that a library built from one strain and transformed back into a mutant of the same strain may result in homologous recombination between plasmid and chromosome, DNA was cloned from one *R.erythropolis* ATCC42[illegible]out was transformed into mutants that were derivatives of *R.erythropolis* ATCC12674.

The library of *R.erythropolis* ATCC4277 that was built was comprehensive. It was calculated that there was a 97,5% probability that any gene was present in it. This facilitated the relatively easy selection of mutants that had had their disfunction rectified.

Cloning by complementation of a mutation of known phenotype seemed to be the most appropriate method to follow. Cloning using an expression system was faulted as the protein was unknown. Cloning using degenerate PCR primers was faulted because the gene was unknown. Thus taking the availability of several mutants unable to inactivate rifampicin that were hypersensitive to the antibiotic, complementation seemed the best route.

The Ri (rifampicin hypersensitive) mutants had been made in earlier work, Ri1-8 had been made by E.Dabbs and Ri9-14 by myself (Andersen SJ, 1989) and briefly characterised for my M.Sc.. These mutants fell into three categories: those that did not inactivate the antibiotic, Ri7, Ri8 and Ri14; those that inactivated at a rate slower than wild type, Ri1, Ri5, Ri6 and Ri9; the rest that

thiostrepton resistant transformants in *S.lividans*.

Although positive selection vectors are useful for the construction of genomic libraries, saving time and expense, they do have several draw backs. The number of unique cloning sites are usually limited. The plasmids often lack other desirable features like M13 origins of replication. They also require specialised host cells for maintenance of the plasmids. However once a recombinant plasmid containing the desired insert has been selected this can be cloned into another vector that is user friendly for additional manipulations.

pDA37 was the second *Rhodococcus-E.coli* shuttle vector to be developed. The first was made by Singer and Finnerty (1988). This was made up of a 3,8kb *HindIII* fragment taken from a cryptic *Rhodococcus* plasmid containing an origin of replication. It was cloned into pIJ30, a 6,3kb pBR322 derivative that also contained a *Streptomyces* gene for thiostrepton resistance. There were two potential unique sites for cloning, *BglII* and *SphI*. Similar transformation efficiencies were reported for *R.erythropolis* to those that we have reported (Dabbs *et al*, 1990). The disadvantage of the first shuttle vector is the absence of any function that allows for selection of recombinant plasmids.

pDA37 proved to be very useful in selecting for recombinant plasmids with >79% having an insert of over 200bp. The size of the vector - 14,8kb, seems large yet it did not prevent insertion of relatively large fragments of over 20kb being inserted. It proved to be most effective for our purposes (Andersen and Dabbs, 1991). It has been used by several others to successfully clone *Rhodococcus* genes (Brown, 1990; Goldman, 1991; Heiss *et al*, 1993; Gordhan, 1994). The draw backs of this vector include the use of toxic substance like arsenate and arsenite for selection in *Rhodococcus* and the presence of only one site, *BglII*, in the suicide gene for cloning.

Quan and Dabbs replaced the heavy metal resistances with a

The multipurpose plasmid, pBR322 (Bolivar *et al*, 1977b), has formed the starting material for the construction of several positive selection vectors. One of the original plasmids was pTR262 (Roberts *et al*, 1980). It encoded the tetracycline resistance gene under the control of a lambda promoter. Also present in this vector was the *cI* gene which encodes the lambda repressor. The repressor bound to the promoter and prevented transcription of the antibiotic resistance gene. These clones would not survive in the presence of high levels of tetracycline. However if DNA was inserted into the *cI* gene at the unique *HindIII* or *BclI* sites the repressor would not be transcribed and thus the resistance phenotype would be expressed. In this way selection on tetracycline plates favoured recombinant clones.

Another vector, pVT25 (Vernet *et al*, 1985) used inactivation of the lethal colicin E3 by the insertion of foreign DNA into the *colE3* gene to exert positive selection. This plasmid was maintained in host cells in the presence of another plasmid, pVT26, which carried gene, *imm*, which expresses colicin E3 immunity. Cloning could be done into unique *ClaI* or *BglI* sites.

Other workers used genes that encoded for methylases and restriction endonucleases for positive selection. As previously mentioned it was one of these plasmids, pEcoR251, containing the *EcoRI* endonuclease gene that was used in this work for the construction of pDA37.

A different approach to positive selection vectors was adopted by Elhai and Wolk (1988). This was based on the principle that plasmids containing long palindromic sequences were not viable in most strains of *E.coli*. They transform at very low frequency. If DNA was inserted into the centre of the palindrome they were transformed at a much higher frequency. To maintain these plasmids palindrome tolerant strains were developed. This concept of palindrome based vectors was used by Kieser and Melton (1988). They used a *Streptomyces* broad-host-range plasmid pIJ101 to construct their vector. The vector, pIJ699, gave rise to

4.4 CLONING VECTORS

As the method chosen for cloning was by complementation the need arose for a cloning vector that could be maintained in *Rhodococcus*. At this time only one cloning vector had been proposed, this was the lysogenic actinophage ϕEC. Brownell *et al* (1982) suggested that it could be used to transfect *R.erythropolis* protoplasts. They had previously shown that it could exist either as a plasmid or as an integrated element (Adams and Brownell, 1976). Very few nocardioform plasmids had been reported. Kaswesk *et al* (1982) screened plasmids from *N.asteroides* and could not ascribe phenotypes to them. This hampered their use in the development as vectors. Sensfuss *et al* (1986) identified two large plasmids in a *Rhodococcus* sp. *(N.opaca)* of 110kb and 140kb. Of the 26 antibiotics and 18 heavy metals they screened only one heavy metal resistance was found to be plasmid borne. Dabbs and Sole (1988) described an unstable genetic element in *R.erythropolis* that conferred resistance to arsenate, arsenite, cadmium and chloramphenicol. This they used in the construction of a series of plasmids. One of these would be used in the construction of pDA37.

To facilitate DNA manipulations an *E.coli* origin of replication and a selective resistance gene would increase the usefulness of a vector. Another draw back to many vectors was that the screening of transformants for inserts was often time consuming, even blue-white selection does not give an accurate determination of insertion either arising from frameshift mutations or small deletions. To overcome this problem researchers investigated ways of insuring that transformants contained recombinant plasmids. One of the solutions was of non-recombinant plasmids producing proteins that were lethal to the cell resulting in the non-viability of the cell. DNA could be cloned into the lethal gene and this would prevent transcription and translation of the lethal protein. These vectors were termed positive selection or suicide vectors.

4.4 CLONING VECTORS

As the method chosen for cloning was by complementation the need arose for a cloning vector that could be maintained in *Rhodococcus*. At this time only one cloning vector had been proposed, this was the lysogenic actinophage ϕEC. Brownell *et al* (1982) suggested that it could be used to transfect *R.erythropolis* protoplasts. They had previously shown that it could exist either as a plasmid or as an integrated element (Adams and Brownell, 1976). Very few nocardioform plasmids had been reported. Kaswesk *et al* (1982) screened plasmids from *N.asteroides* and could not ascribe phenotypes to them. This hampered their use in the development as vectors. Sensfuss *et al* (1986) identified two large plasmids in a *Rhodococcus* sp. *(N.opaca)* of 110kb and 140kb. Of the 26 antibiotics and 18 heavy metals they screened only one heavy metal resistance was found to be plasmid borne. Dabbs and Sole (1988) described an unstable genetic element in *R.erythropolis* that conferred resistance to arsenate, arsenite, cadmium and chloramphenicol. This they used in the construction of a series of plasmids. One of these would be used in the construction of pDA37.

To facilitate DNA manipulations an *E.coli* origin of replication and a selective resistance gene would increase the usefulness of a vector. Another draw back to many vectors was that the screening of transformants for inserts was often time consuming, even blue-white selection does not give an accurate determination of insertion either arising from frameshift mutations or small deletions. To overcome this problem researchers investigated ways of insuring that transformants contained recombinant plasmids. One of the solutions was of non-recombinant plasmids producing proteins that were lethal to the cell resulting in the non-viability of the cell. DNA could be cloned into the lethal gene and this would prevent transcription and translation of the lethal protein. These vectors were termed positive selection or suicide vectors.

literature searching it appeared that this was a common problem. This parallel project was thus shelved for a while.

However the hypersensitive, non-inactivating mutant was used in the analysis of *B.subtilis* inactivation by E.Dabbs and Y Mikami. From this it was shown that the mechanism by which the organism inactivates rifampicin was phosphorylation (Dabbs *et al*, 1995b).

Only two strains were found to inactivate the other ansamycin, streptovaricin, under these conditions. Other students in our laboratory have been able to refine the conditions and have been able to show inactivation of this drug by more of these nocardioform bacteria.

An interesting observation was made studying the antibiotic producer organisms. Rifampicin was not inactivated by *A.mediterranei*. This is consistent with the findings of Blanco *et al* (1984) who studied the resistance to inhibitors of RNA polymerase in the strains that produce them. The inconsistency lies in the fact that I was able to show that *S.spectabilis* was capable of inactivating streptovaricin. These workers had been unable to demonstrate the presence of any inactivating enzymes in any of the producer strains. They were able to isolate a mutant of *S.spectabilis* which was sensitive to streptovaricin having a sensitive RNA polymerase. My findings are unusual in the sense that under the conditions I used this strain was capable of destroying its own product. Blanco *et al* cultured the organism in richer media (YEME) and grew the cultures at 30°C where as mine were grown in LB at 28°C. Thus there may have been enzyme induction at lower temperatures or inhibition at higher temperatures that may have been more optimal for antibiotic production.

With the understanding that ansamycin inactivation was more wide spread than had been previously known the focus shifted from the general to the specific - the cloning of one of the genes responsible for inactivation.

several of the strains inactivated but did not decolorise the drug. Subsequent work done by Mikami *et al* at the University of Chiba, Japan shows that several mechanisms of inactivation are operating in the nocardioform bacteria. These include phosphorylation (Yazawa *et al*, 1994) , glucosylation (Yazawa *et al*, 1993) , ribosylation (Dabbs et *al*, 1995) and a decomposition mechanism (Yazawa et al, 1993). The addition of various chemical groups have been shown to be attached to the ansamycin bridge at C21 and C23 in place of an hydroxyl. The additions of these groups does not lead to decolorisation. Similar systems are probably operative in the strains tested here that did not decolorise the antibiotic, *R.australis, S.lividans* and non-nocardioform, *B.subtilis*. It was subsequently demonstrated that the mechanism of inactivation in *B.subtilis* was phosphorylation (Dabbs *et al*, 1995b). *R.rubrapertinctus* showed no ability to inactivate any of the ansamycins. This was confirmed by the sabbatical work of E.Dabbs and Y Mikami (unpublished). Recently this strain has been reclassified as being a member of *Gordona* (quoted in Finnerty, 1992). None of the other strains in this group have been tested for their ability to inactivate rifampicin.

The analysis of the inactivation of the naturally occurring rifamycin SV revealed that in almost every case where rifampicin had been inactivated so was this antibiotic. This indicates that it is not the semisynthetic addition to rifampicin that is the target of inactivation but a section distant from it. The one strain that was not consistent with this was *B.subtilis*. This was able to inactivate rifampicin and streptovaricin but not rifamycin SV.

I began a similar project in parallel with the current work, producing two rifampicin hypersensitive mutants of *B.subtilis* 1A3. I also produced a shuttle vector from pEcoR251 and pC194 that was maintained in both *E.coli* and *B.subtilis* hosts. However a genomic library of an inactivating *B.subtilis* strain showed extensive spontaneous deletions of the inserted DNA. On further

5.0 APPENDICES

the increased intracellular concentration of KRM 1648. These authors plan to sequence the *rpoB* gene from this organism (Hirata et al, 1995).

4.10 CONCLUSION

This work has resulted in the cloning, sequencing and expression of an hydroxylase gene from *Rhodococcus*. This gene is responsible for the hydroxylation of rifampicin which in turn inactivates the antibiotic. This may play an important role in rifampicin resistance in AIDS patients infected with *R.equi*, especially if they have been misdiagnosed with tuberculosis.

The next steps in this project would be to sequence the rest of the cloned DNA. This would identify the chromosomal context of the gene, enabling comparison with homolgous monooxygenase gene contexts. Disruption of the gene by homologous recombination would help in the further characterisation of the gene and biochemical analysis would aid in identifying the natural substrate of the enzyme.

Since the natural substrate of the enzyme has not been determined and rifampicin is used as a substrate by this enzyme it shows a broader substrate range. Engineering of monooxygenases has been accomplished, increasing substrate range (Abril *et al*, 1989). The ability to engineer such modifications may be useful in environmental protection efforts. Recalcitrant pollutants often resemble the natural substrates of microbial enzymes. This has been demonstrated repeatedly in the *Rhodococci*. Thus further investigation into rhodococcal enzymes may not only reveal antibiotic resistances but aid in environmental protection.

These are showing sufficient promise to warrant further evaluation (Dholpe and Ibanez, 1995). They have been found to have MICs lower than rifampicin for most of the mycobacteria as well as *S.aureus*, *S.epidermis*, *Strep.pneumonia* and *Strep.pyogenes* (Fujii et al, 1994). Pharmacokinetic studies have indicated that the concentrations of KRM compounds in plasma are lower than that of rifampicin. However the concentrations in certain organs (lung and spleen) are comparable or even greater (Tomioka *et al*, 1992). It has also been shown that KRM 1648 and KRM 1657 exhibit fewer toxic effects than rifampicin in mice (Saito *et al*, 1991).

The benzoxazinorifamycin that seems to have the greatest effect against *M.tuberculosis* and other organisms is KRM 1648. MICs (MIC_{50} and MIC_{90}) determined for rifampicin and KRM 1648 show values of 4 and 128µg/ml for rifampicin using the BACTEC 460 TB System and 0,016 and 2µg/ml for KRM 1648. This study showed there was cross resistance between the two drugs which they believed was occurring at the RNA polymerase level. It was also shown that the KRM 1648 had a markedly increased permeability into the mycobacteria through the cell wall. This indicates that the activity of KRM 1648 is largely dependant upon its increased ability to accumulate at the target sites inside bacteria (Klemens *et al*, 1994).

These drugs were tested against a rifampicin resistant strain that had an MIC of 8µg/ml. The other reference strain was resistant to 128µg/ml. The first strain is exhibiting a low level rifampicin resistance. KRM 1648 was much more effective than rifampicin on sensitive strains of *M.tuberculosis*. The findings also indicated that both KRM 1648 and rifampicin were less effective against the low level resistant strain. KRM 1648 still significantly reduced the incidence of lung lesions in animals infected with this strain, while rifampicin had no effect. KRM 1648 did not have any effect against the high level resistant strain. It could be the case that an inactivation mechanism is present in the low level resistant strain and that the differential effectiveness of the two antibiotics is a result of

rifamycin S. It has been shown to have good *in vitro* activity against most mycobacterial species. In animal models it was more active against both *M.tuberculosis* and *M.leprae* than rifampicin. Some studies have shown it to be active against some rifampicin resistant strains of both species (O'Brien *et al*, 1987). Neither of these strains have yet been shown to have rifampicin hydroxylating enzymes so this ability to overcome resistance using rifabutin may or may not be relevant to *R.equi*.

Other studies carried out in the same year on other derivatives confirmed increased sensitivity to rifabutin as well as to rifapentine, CGP29861, CGP7040 and CGP27557, against rifampicin sensitive and rifampicin resistant strains of *M.tuberculosis* and the *M.avium-intracellulare-scrofulaceum* complex (Dickinson and Mitchison, 1987).

The most recent development are the novel benzoxazinorifamycins, the KRM series.

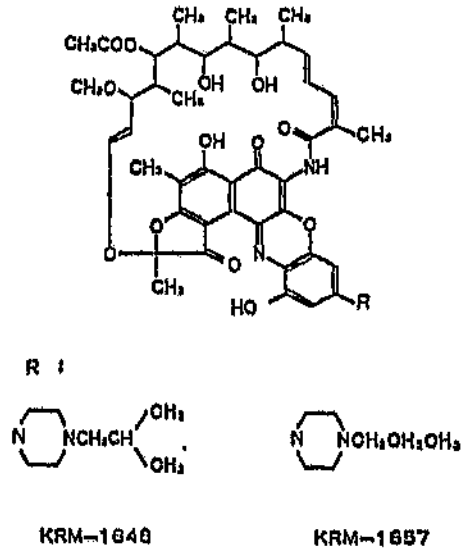


Figure 4.3 Structures of KRM 1648 and KRM 1657

4.9 RIFAMPICIN AND OTHER RIFAMYCINS

The evidence seems to point to rifampicin being hydroxylated in some way, probably on one of the aromatic rings. This may produce a compound that can be used as a substrate by one of the numerous dioxygenases that are found in the *Rhodococci*.

The homologue from *Flavobacterium* has been shown to remove several different groups from aromatic rings and replace them with hydroxyls, these include hydrogen, chloro, nitro, amino and cyano groups at the *para* position in relation to the hydroxyl of phenol. The two adjacent rings on rifampicin have three hydroxyls attached at positions C1, C4, and C8. The possible groups that the enzyme could be acting on are a methyl group at C7; a nitrogen at C2 which attaches the aromatic group to the ansa bridge or the group attached to C3. It is to C3 that the semisynthetic portion of rifampicin is attached, in rifamycin SV this is a hydrogen. Further investigation could be carried out on the ability of *R.equi* to inactivate some of the newer rifamycin derivatives that are in trials at the moment. If the susceptible group has been modified so that it is no longer available to be hydroxylated this drug would most probably be effective against *iri* mediated rifampicin resistance.

The rifamycins are another group of antibiotics which are being modified to produce new generation drugs. Although rifampicin is still the stalwart of anti-mycobacterial therapy new modifications have been introduced resulting in rifabutin, rifapentine, the CGP series and the KRM series.

Bermudez *et al* (1994) state that the majority of rifamycins that have been investigated thus far both *in vitro* and *in vivo* are not active or have reduced activity against the *M.avium-intracellulare* complex. This highlights the reality that although many variations are tried few are effective.

Rifabutin is a semisynthetic spiropiperidyl derivative of

A database search of Genbank for other hydroxylases/ monooxygenases from *Streptomyces, Nocardia, Gordona, Corynebacteria, Mycobacterium* and *Rhodococcus* only identified 2 sequences that have not been previously mentioned. These are a lactate monooxygenase from *M.smegmatis*; which does not appear to have significant homology with this enzyme and an alkene monooxygenase from *R.rhodochrous*. This monooxygenase is one of the multiple subunit types and it has not been published.

Although no other hydroxylases have been cloned and sequenced from *Rhodococcus* species several enzymes have been purified to varying degrees of purity. A phenol-hydroxylase was purified from *Rhodococcus* species P1 that used phenol as sole carbon source (Straube, 1987). *Rhodococcus* species BPG-8 has had a resorcinol hydroxylase (Armstrong and Patel, 1993) and a phloroglucinol hydroxylase (Armstrong *et al*, 1993) partially purified from it. The NADH dependent monohydroxybenzoate monooxygenase was purified from *R.erythropolis* (Suemori *et al*, 1994) and *R.rhodochrous* had a steroid monooxygenase isolated to homogeneity. This enzyme catalysed the oxidation of progesterone to produce testosterone acetate. The enzyme is 60kDa in the monomeric form with a pI of 4,9. The prosthetic group of this enzyme was FAD and the reaction utilised NADPH (Miyamoto *et al*, 1995). The predicted size of the *iri* translated protein is 52kDa with a pI of 5,32.

At this stage there is no evidence that this enzyme can cause the utilisation of phenol, catechol or p-hydroxybutyrate as sole carbon source. So the natural substrate of this enzyme has not been identified. Further aromatic substrates could be tested including pigment precursor molecules. As *Rhodococcus* is known for its ability to degrade cyclic compounds an assumption was made that this enzyme's natural substrate would be degraded. The sequence homologies have introduced the possibility of this enzyme being a biosynthetic enzyme under normal circumstances.

from these strains. It would be expected that enzymes from bacteria from the same grouping would have greater homology to one another than to strains out side of the group. Thus similarity may be a result of conserved binding sites and evolutionary relatedness of the strains rather than on the natural substrates of the enzymes. However further sequencing of the downstream DNA could reveal a cluster that is homologous to the other polyketide synthases. However, if a similar cluster was identified these genes could be used in the production of hybrid polyketides. For this to happen these genes would need to be transformed into *Streptomyces* and that the promoters would be recognised by the host RNA polymerase.

The predicted promoter for the *iri* sequence is based on homology and sequence analysis of promoters in *Streptomyces*. The -10 region (CACACT) is identical to the -10 region of another streptomycete gene *mPIc-p* (Buttner, 1989). It has been shown by analysis of many streptomycete promoters that the T in the 6th position is invariant and therefore critical (Strohl, 1992). The -35 region (TTGCGG) is similar but not identical to other promoters. Analysis shows that the TTG in the first 3 positions occur more than 86% of the time with the G being invariant. The other three bases CGG have all been described at these positions in streptomycete promoters. This sequence formed the apex of a stem-loop structure. These structures have been found in streptomycete promoters. The position of the sequence on the loop would facilitate binding to the promoter. The positions of these sequences, which were -35 and -10 to each other, were identified with the help of transcriptional analysis that had been carried out on the tetracenomycin gene cluster. The transcriptional start site of the *tcmG* gene had been found using S1 nuclease mapping (Decker and Hutchinson, 1993). The consensus sequence for the Shine-Delgarno in *Streptomyces* is a/gGGAGG. A putative Shine-Delgarno sequence AGGAGG was identified -8 to -13 upstream of the start codon of *iri*. This is exactly the same sequence in exactly the same position as the Shine-Delgarno of the *urdE* gene from *S.fradiae* (Decker and Haag, 1995).

rifampicin resistance gene was clone using Ri8 (Quan and Dabbs, unpublished) that inactivates the drug by a totally different mechanism, it cannot be confirmed that the mutation to hypersensitivity is found in the *iri* gene. The probability is high that it is in the *iri* gene as the other obvious possibility would be the *rpoB* gene. Since I have shown that the *iri*⁻ strains can be made spontaneously resistant to high levels of rifampicin, which is indicative of mutation to *rpoB*, and still retain the ability to inactivate the drug it excludes this a the mutation site.

This could be investigated by interrupting the *iri* gene with an antibiotic resistance marker and using this construct to do homologous recombination in the wildtype strain. A double crossover event would effectively exchange the wildtype gene for the inactivated one. A comparison of phenotypes could then be undertaken.

More recent work on these clustered regions have identified further genes. These genes are upstream of the hydroxylase and are transcribed in the opposite direction off divergent promoters. They have been identified in the jadomycin B cluster (Yang *et al*, 199.) and in the daunorubicin cluster (Otten *et al*, 1995). The deduced amino acid sequences of these genes have been shown to be homologous to other known repressors. The JadR, N-terminal region resembles MtrR from *Neisseria gonorrhoeae* and EnvR and AcrR from *E.coli*. These control tandemly linked genes that mediate resistance to structurally diverse agents by an efflux mechanism (Nikaido, 1994). If *iri* was found to be part of a cluster that is repressed, mutations in the repressor could lead to increased rifampicin resistance.

The streptomycetes are studied chiefly because of their antibiotic biosynthetic capabilities. For this reason it is not surprising that, with the exception of the pigment genes, the hydroxylases cloned from this group thus far are involved in that process. No hydroxylases involved in degradation have been cloned

(Grimm *et al*, 1994), urdamycin (Decker and Haag, 1995) and jadomycin B (Han *et al*, 1994) have been cloned. They strongly resemble each other with respect to their genetic organisation and deduced amino acid sequences of the products responsible for the assembly of the polyketide chain (Katz and Donadio, 1993). In addition polyketide synthase genes from different gene clusters can form active enzyme complexes and express hybrid enzymes in a heterologous host. This can lead to the production of novel polyketide compounds (Katz and Donadio, 1993).

All of the gene clusters characterised so far have an hydroxylase as the first ORF in the cluster. The *iri* gene does not seem to have an ORF in the 280bps that have been sequenced upstream of the start codon. This may indicate that this is the first gene in a cluster as there appears to be a second ORF downstream of the gene. This has been partially sequenced and no identification has been made on data base searches.

Other polyketide substances that have been characterised in *Streptomyces* are spore pigments. The *whiE* cluster from *S.coelicolor* (Davis and Chater, 1990) and the *sch* cluster from *S.halstedii* (Blanco *et al*, 1992; Blanco et *al*, 1993) both contain a core set of homologues of 4 genes characteristic of polyketide synthases. Blanco *et al* (1993) examined the upstream regions of these clusters only to find an ORF whose deduced amino acid sequence resembled sequences of aromatic hydroxylases. They were able to show that disruption of these genes affected the spore colour. In *S.halstedii* normally green spores became lilac. *Rhodococcus* species do produce pigments, *R.erythropolis* and *R.equi* are salmon pink in colour and *R.fascians* is bright yellow. Thus the *iri* gene could be involved in pigment production. This could be substantiated by sequencing the down stream region of DNA. Alternatively degenerate PCR primers designed from conserved regions of other cluster genes could be used to amplify sequences from the 8,3kb fragment. Interestingly the *iri*⁻ mutants are no different in colour from the wildtype. However it has not been established what the mutations in these mutants are. As another

were three other regions that produced homology with the other streptomycetes and flavobacteria. These were in the regions of residues 86-136; residues 140-160, which contained mostly identities, and 444-458, which also contained many identities. However no functional significance has been discovered for these sequences at this stage.

4.8 POLYKETIDE HYDROXYLASES

The homologues identified include hydroxylases involved in the biosynthesis of polyketide antibiotics (Bibb *et al*, 1989; Decker and Haag, 1995; Han *et al*, 1994), polyketide pigments (Davis and Chater, 1990; Blanco *et al*,1992) and the degradation of phenolic compounds (Orser *et al*, 1993).

Polyketides form a large group of natural products synthesised especially by bacteria, fungi and plants. Many important antibiotics such as tetracyclines, erythromycin, adriamycin, monensin, rifamycin and avermectins are polyketides. In their biosynthesis multifunctional synthases catalyse condensation of thio-esters derived from acetate, propionate or butyrate to yield aliphatic chains of varying length and carrying various alkyl substituents. Subsequent modifications, including aromatic or macrolide ring closure of specific methylations of glycosylations, generate further chemical diversity.

The greatest homology was found with an hydroxylase used in urdamycin synthesis. The urdamycins are aromatic polyketides produced by *S.fradiae* that exhibit antitumour and antibacterial activity. They belong to a large group of anguacycline antibiotics which comprise more than 100 natural compounds with many pharmacological activities (Rohr and Thiericke, 1992).

Polyketide synthase genes responsible for the production of actinorhodin (Fernandez-Moereno *et al*, 1992), tetracenomycin (Bibb *et al*, 1989), granaticin (Sherman *et al*, 1989), doxorubicin

Figure 4.2 Comparison of deduced amino acids from the flavin binding region of the *iri* gene

```
ident   *       ***  *   *      *    *  *                 *  *
simil   **  ********** * * **** * * *** ********   * *   *****  **
IRI 267 YRRDRVFLAGDAAHIHPPMGGQGLNLGVQDAFNLGWKLAAEINGWAPVGLLDTYESERR
URD 266 YRRGRVLLAGDAAHIHLPAGGQGMNTGIQDAVNLGWKLAAVLRGTASESLLDSYHSERH
SAU 252 YRLGRVLLAGDAAHIHLPAGGQGLNLGFQDAVNLGWKLAAVVRGHGTEELLDSYCRERR
SVE 184 YRDGRVLWAGDAAHQQMPIGGQALNLGLQDAVNLGWKLAAVVRGTAPDGLLDTYHDERH
PCP 289 YRKGNVFLAGDAAHCHSPSGGSGMNVGMQDAFNLGWKIAMVERGEAKPDLLDTYHTERT
TCM 313 YRSGRVFLAGDAAHVHPPAGAFGANGGIQDAHNLAWKLAAVLKGTAGDALLDTYEQERL
OTC 279  RDGRVLLAGDACHIHLPAGGQGLNLGFQDAVNLGWKLGATIAGTAPPELLDTYEAERR
SCH 304  RAGRVFLAGDSAHEMSPTGAFGSNTGIQDAHNLAWKLAAVLGGWAGDGLLDTYDAERR
TFD 302  QQGRVFCAGDAVHRHPPTNGLGSNTSIQDSFNLAWKIAMVLNGTADESLLDTYTIERA
PHE 320  QKGRVCCAGDAIHKHPPSHGLGSNTSIQDSYNLCWKLACVLKGQA[illegible]PELLETYSTERA
PHY 349  KDERVFIAGDACHTHSPKAGQGMNTSMMDTYNLGWKLGLVLTGRA[illegible]DILKTYEEERH
HBH 277  QHGRLFLAGDAAHIVPPTGAKGLNLAASDVSTLYRLLLKAYRE.GRGELLERYSAICL
```

The aspartate (D) 277 forms hydrogen bonds with the O-3 ribityl moiety of the bound FAD cofactor. This residue is conserved through different classes of flavin binding enzymes including reductases and dehydrogenases from both prokaryotes and eukaryotes (Eggink *et al*, 1990). This residue is also present in the protein under investigation.

Site directed mutagenesis of p-hydroxybenzoate hydroxylase introducing an N300D mutation changes the catalytic properties of the enzyme (Palfey *et al*, 1994). This asparagine is highly conserved (Figure 4.2). In the wildtype enzyme residue 300 is in contact with the isoalloxazine ring of the active-site FAD. In the mutant this side chain has moved about 5 angstroms altering the protein structure. The flavin of the p-hydroxybenzoate hydroxylase is reduced by NADPH in the first of the catalysis. The mutation decreased this rate 330-fold, apparently by affecting the reactive c[illegible]ation of the isoalloxazine and pyridine rings. As a resul[illegible]oxylation was 50-fold slower in the mutant than in the wild type. This residue is also present in the protein translated from *iri*.

Thus it can be shown that the protein under investigation does contain all the essential residues that have been identified this far for the binding of FAD. Expression of this protein in *E.coli* showed that FAD and NADPH are needed for enzyme function. There

Most of the high scoring hits were with hydroxylases/ monooxygenases.

Figure 4.1 Comparison of deduced amino acid sequences of the aminoterminal of the *iri* gene

```
ident          *    *      *
simil     ***** *****  *  *        **   ** *        *
IRI     4  VIIVGAGPTGLMLAGELR.....LQGVDVVVVDKDEEPTQFVR   41
URD     5  VIVAGAGPTGLMLAGELR.....LAGVDVIVLDRLAERTGEGR   42
SAU     1  MVVAGAGPTGLALACELA .....LGGARAVVVERRREPEKHSK   38
OTC     6  VVIAGAGPTGLMLACELR.....LAGARTLVLERLAERVDFSK   42
PCP    17  VLIVGGGTTGLIAANELL.....RRGVSCRMIDRLPVAHQTSK   54
TCM    19  VLIVGGGLTGLSAALFLS.....QHGVSCRLVEKHRGTTVLTR   56
SCH    17  VLVVGGSLVGLSTSVFLG.....RLGVRHMLVERHAGTSVHPR   54
WHI    23  VLVVGGSLVGLSTSVFLG.....RLGVRHTLVERHAGTSIHPA   60
DNR    10  VLVVGGGLGGLSTALFLA.....RRGARVLLVERHAGTSVHPR   47
TFD    10  VLVVGTGPAGASAGALLA.....RYGVRTMLINKYNWTAPTPR   47
PHE    38  VLTVGSGPAGSSAAMFLS.....TQGISNIMITKYRWTANTPR   75
PHY    17  VLIVGAGPAGLMAARVLSEYVRQKPDLKVRIIDKRSTKVYNGQ   53
HBH     5  VAIIGAGPSGLLLGQLL.....HKAGIDNVILERQTPDYVLGR   42
```

where ident are identities and simil are similarities in most of the sequences examined:

IRI - hydroxylase from *R.equi*

URD - hydroxylase from *S.fradiae*

SAU - hydroxylase from *S.aureofaciens*

SVE - C-16 hydroxylase from *S.venezuela*

OTC - anhydrooxytetraclycline monooxygenase from *S.rimosis*

PCP - pentachlorophenol-4-monooxygenase from *Flavobacterium*

TCM - tetracenomycin hydroxylase from *S.glaucescens*

SCH - pigment hydroxylase from *S.halstedii*

WHI - pigment hydroxylase from *S.coelicolor*

DNR - hydroxylase duanorubicin biosynthesis from *S.peucetius*

TFD - 2,4-dichlorophenol hydroxylase *Alcaligenes eutrophus*

PHE - phenol monooxygenase from *Pseudomonas*

PHY - phenol hydroxylase from *Trichosporon cutaneum*

HBH - 4-hydroxybenzoate hydroxylase from *P.fluorescens*.

Ribonuclease	Boehringer-Mannheim
Rifampicin	Boehringer-Mannheim
Rifamycin SV	Sigma
SDS	BDH
Sequenase version 2.0 DNA sequencing kit	USB
Spectinomycin	Unmarked
Streptolydigin	Upjohn
Streptomycin	Boehringer-Mannheim
Streptovaricin	Upjohn
Sucrose	Saarchem
T4 Ligase	Boehringer-Mannheim
TES	Sigma
Tetracycline	Boehringer-Mannheim
Thiamine chloride	Merck
Tris	BDH
Tryptone	Oxoid
Tween-80	Merck
Urea	ICN
X-gal	Sigma
Yeast extract	Oxoid

APPENDIX C

Formula for calculating number of clones for a genomic library

$$N = \frac{\ln(1-P)}{\ln(1-a/b)}$$

N = number of clones required
P = probability (e.g.a 95% probability that any gene is present)
a = average size of DNA fragment inserted into vector
b = total size of genome

Hybond-C	Amersham
p-Hydroxybutyrate	Sigma
IPTG	Sigma
Isoniazid	Sigma
Isopropanol	Saarchem
Kasugamycin	Boehringer Mannheim
K_2HPO_4	Merck
KH_2PO_4	BDH
K_2SO_4	BDH
K acetate	Merck
K chloride	Saarchem
Low gelling agarose	FCM Bioproducts
Lysozyme	Boehringer-Mannheim
Maleic acid	Merck
Methanol	BDH
^{35}S-methionine	Amersham
$MgCl_2$	Merck
$MgSO_4$	Saarchem
Molecular weight markers	Boehringer-Mannheim
	Biorad
Na acetate	Searle Company
Na arsenate	Saarchem
Na arsenite	Sigma
NaCl	Saarchem
NADH	Boehringer-Mannheim
$NADPH_2$	Boehringer-Mannheim
NaOH	Saarchem
Na taurocholate	BDH
Naladixic acid	Sigma
Phenol	BDH
Polaroid film 667	Polaroid
Polyethylene glycol (PEG)	Sigma
Proteinase K	Boehringer-Mannheim
pUC sequencing kit	Boehringer-Mannheim
Restriction enzymes	Boehringer-Mannheim
	New England Biolabs
	Amersham

Acrylamide	Merck
Agar	Oxoid
	Merck
Agarose	FCM Bioproducts
Ammonium chloride	Merck
Ampicillin	Boehringer-Mannheim
^{35}S-dATP	Amersham
Bis-acrylamide	BDH
Boric acid	Sigma
Butanol	BDH
$CaCl_2$	Merck
Calf intestinal phosphatase	Boehringer-Mannheim
Casaminoacids	Difco
Catechol	Sigma
Chloramphenicol	Boehringer-Mannheim
Chloroform	ACE
Coomassie blue R250	Saarchem
D-cycloserine	Sigma
DDS (Dapsone)	Sigma
Dichlorodimethylsilane	Merck
Dimethyl formamide	AnalytiCals
Dimethyl sulphoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Boehringer-Mannheim
DNA labelling & detection kit	Boehringer-Mannheim
EDTA	Sigma
Erythromycin	Boehringer-Mannheim
Ethambutol	Sigma
Ethanol	Several sources
Ethidium bromide	Boehringer-Mannheim
Ethyl acetate	Saarchem
FAD	Sigma
Fe chloride	Merck
Fusidic acid	Unmarked
Glucose	BDH
Glycerol	BDH
Glycine	BDH
HCl	Saarchem/BDH

5g NaCl
10g NH_4Cl
pH to 7,5 with NaOH
Water to 1 litre and autoclave

Hershey's salts
5,4g NaCl
3,0g KCl
1,1g NH_4Cl
15mg $CaCl_2.2H_2O$
0,2g $MgCl_2.6h_2O$
0,2mg $FeCl_3.6h_2O$
87mg KH_2PO_4
12,1g Trizma base, adjust pH to 7,4 with HCl, water to 1 litre

Hershey's Medium
100ml Hershey's salts
10mg threonine
10mg leucine
20mg proline
20mg arginine
20µl vitamin B1 (5mg/ml)
After autoclave add
0,8ml 50% glucose

Cycloserine solution
10mg/ml D-cycloserine in 0,1M phosphate buffer pH 8,0 prepared freshly.

APPENDIX B

Sources of Media and Chemical Reagents

Acetic acid	Associated Chemical Enterprises
Acetone	SMM Chemicals

Extraction of single stranded DNA

20% PEG

2,5M NaCl

3,0M sodium acetate

pH5 2 with glacial acetic acid

Denaturing double stranded DNA

0,2M NaOH

0,2mM EDTA pH8,0

Polyacrylamide gels

33,6g urea

42ml 38:2 acrylamide:bis-acrylamide

16ml TBE 5x

24ml water

Running buffer

TBE 1x

Maxicell solutions

K medium

1g	Casaminoacids
0,02ml	Vitamin B1 (5mg/ml)
10ml	10x M9 stock
90ml	water
Add after autoclaving	
0,2ml	1M $mgSO_4$
50%	Glucose
0,02ml	0,5M $CaCl_2$

10x M9 stock

151g	$Na_2HPO_4.2H_2O$
30g	KH_2PO_4

0,035% TEMED
25mM ammonium persulphate

Separating gel
10% acrylamide
0,38M Tris pH 8,8
0,1% SDS
0,035% TEMED
25mM ammonium persulphate

Electrode buffer
25mM Tris
0,2M glycine
0,1% SDS

Sample loading buffer
63mM Tris -HCl pH 6,8
2% SDS
10% glycerol
5% β-mercaptoethanol
0,001% bromophenol blue

Stain
0,1% coomassie blue R250
5:4:1 methanol : water : acetic acid

Destain
5:4:1 methanol : water : acetic acid

Sequencing solutions

Blue/white colour selection
2% X-gal in dimethyl formamide (30μl/plate)
100mM IPTG in water (30μl/plate)

1mM EDTA

20x SSC
3M NaCl
0,3M sodium citrate
pH 7,0

Washing solutions

^,1% SDS
2x SSC

0,1% SDS
0,1x SSC

Non-radioactive labelling

Buffer 1
0,1M Tris pH 7,5.
0,15M NaCl

Buffer 2
0,5% (w/v) blocking reagent in buffer 1
Prepared 1 hour before use

Buffer 3
0,1M Tris pH 9,5
0,1M NaCl
0,05M $MgCl_2$

Polyacrylamide gel electrophoresis

Stacking gel
6% acrylamide (stock : 30% acrylamide and 0,8% bis-acrylamide)
0,176M Tris pH 6,8
0,1% SDS

TE with 10% SDS

4,5M sodium acetate pH 5,6

Transformations

In *E.coli*

50mM $CaCl_2$
10mM Tris-HCl pH 8,0

0,1M $MgCl_2$

In nocardioforms

Protoplast buffer
The following basal solution was made up in 87,5ml water
10,3g sucrose
0,025g K_2SO_4
0,202g $MgCl_2.6H_2O$
After autoclaving 10ml of 0,25M TES pH7,2 was added
Aliquots of 9,75ml were stored frozen at -20°C
Just before use the following were added to each aliquot:
0,25ml 1M $CaCl_2$
0,1ml 0,5% KH_2PO_4

Southern blotting

Denaturing solution
1,5M NaCl
0,5M NaOH

Neutralising solution
1,5M NaCl
0,5M Tris pH 8,0

Running buffer
180ml water
20ml 5x TBE
15µl ethidium bromide (10mg/ml stock in water)

Loading buffer
30% glycerol in TE
0,025% bromophenol blue

Solutions for plasmid preparations of *E.coli*

Solution 1
50mM glucose
10mM EDTA
25mM Tris-HCl pH 8,0
A 20% glucose stock solution was autoclaved separately and added afterwards

Solution 2
0,2M NaOH
1% SDS

Solution 3
5M potassium acetate pH 4,8
Dissolved in 88,5ml water
11,5ml glacial acetic acid added

Ribonuclease
10mg/ml in TE or 3mM Tris or water
Diluted 1:10 for working solution
Boiled for 10 minutes before use

DNA preparations from nocardioforms

10mM Tris-HCl pH 8,0
10% sucrose
5mg/ml lysozyme - freshly added

Protoplast regeneration plates

0,9g NaCl
3,0g tryptone
1,5g yeast extract
35g sucrose
Dissolved in 200ml water and the volume made up to 280ml
4g agar was added
After autoclaving the following were added
6ml 1M $CaCl_2$
10ml 0,25M TES pH 7,2 (N-tris(hydroxymethyl)methyl-2amino ethanesulfonic)
Each regeneration plate contained 22ml of media

Buffers and solutions for agarose gel electrophoresis

TE

10mM Tris pH 8,0
10mM EDTA

TBE 5x

0,89M Tris
0,089M boric acid
0,002M EDTA pH 8,0

TAE

0,04M Tris-acetate
0,001M EDTA

Agarose gels

0,8g (0,4%) or 1,6g (0,8%) or 2,4g (1,2%) agarose
180ml water
20ml 5x TBE
25ml of the above were used per gel
1,5µl ethidium bromide (10mg/ml stock)

TYA
TY
1,5% agar

T2
TY
3mM $CaCl_2$
3mM NaCl
10mM $MgSO_4$

Luria-Bertani Broth (LB)
TY
0,5% NaCl

LA
LB
1,5% agar

1/3 LA
LB
0,5% agar

TYG
TY
1% glycine

TYGS
TY
2% glycine
10,3% sucrose

2xYT
1,6% tryptone
1% yeast extract
0,5% NaCl
pH 7,6 with NaOH

APPENDIX A

Stock Solutions

Antbiotic Stock Solutions

Ampicillin	:	100mg/ml in 1:1 ethanol:water
Chloramphenicol	:	4mg/ml in ethanol
DDS (Dapsone)	:	100mg/ml in 1:1 ethanol:water
Erythromycin	:	1mg/ml in ethanol
Ethambutol	:	10mg/ml in 1:1 ethanol:water
Fusidic acid	:	10mg/ml in ethanol
Isoniazid	:	100mg/ml in 1:1 ethanol:water
Kanamycin	:	100mg/ml in water (filter sterilised)
Kasugamycin	:	100mg/ml in water (filter sterilised)
Naladixic acid	:	10mg/ml in ethanol
Rifampicin	:	10mg/ml in methanol
Rifamycin SV	:	10mg/ml in methanol
Spectinomycin	:	100mg/ml in water (filter sterilised)
Streptolydigin	:	10mg/ml in methanol
Streptomycin	:	100mg/ml in water (filter sterilised)
Streptovaricin	:	10mg/ml in methanol
Tetracycline	:	10mg/ml in methanol

Arsenical Solutions

2M sodium arsenate in water (autoclaved)

3M sodium arsenate; 0,5M sodium arsenate in water (autoclaved)

10% Tween 80

1ml Tween 80

4ml water

5ml ethanol

Media

TY

1% tryptone

0,5% yeast extract

Jager W, Schafer A, Kalinowski J, Puhler A (1995) Isolation of insertion elements from gram-positive *Brevibacterium, Corynebacterium* and *Rhodococcus* strains using the *Bacillus subtilis sacB* gene as a positive selection marker. *FEMS Microbiol Lett* 126:1-6

Jawetz E, Melnick JL, Adelberg EA (1982) *In:* Review of medical microbiology (15th Ed.) Lange Medical Publications, California. pp 219-225, 302-303

Jin DJ and Gross CA (1988) Mapping and sequencing of mutations in the *Escherichia coli rpoβ* gene that leads to rifampicin resistance. *J. Mol. Biol.* 202:45-58

Kalin M, Neujahr HY, Weissmahr RN, Sejlitz T, Johl R, Fiechter A, Reiser J (1992) Phenol hydroxylase from *Trichosporon cutaneum*: Gene cloning, sequence analysis, and functional expression in *Escherichia coli*. *J. Bacteriol.* 174:7112-7120

Kanno T, Asawa T, Ito H, Takai S, Tsubaki S, Sekizaki T (1993) Restriction map of a virulence-associated plasmid of *Rhodococcus equi*. *Plasmid* 30:309-311

Katz L and Donadio S (1993) Polyketide synthesis: prospects for hybrid antibiotics. *Annu. Rev. Microbiol.* 47:875-912

Kaweseck KL, Little ML, Bradley SG (1982) Plasmids in mating strains of *Nocardia asteroides*. *Devel. Ind. Microbiol.* 23:279-286

Kersten H and Kersten W (1974) *In:* Molecular Biology Biochemistry and Biophysics 18: Inhibitors of nucleic acid synthesis - biophysical and biochemical aspects. p 108-122, Springer- erlag

Kieser T and Melton RE (1988) Plasmid pIJ699, a multicopy positive-selection vector for *Streptomyces*. *Gene 65:83-91*

Harayama S and Kok M (1992) Functional and evolutionary relationships among diverse oxygenases. *Ann. Rev. Microbiol.* 46:565-601

Hart DHL, Peel MM, Andrew JH, Burdon JGW (1988) Lung infection caused by *Rhodococcus*. *Aust. NZ J. Med.* 18:790-801

Harvey RL and Sunstrum JC (1991) *Rhodococcus equi* infection in patients with and without human immunodeficiency virus infection. *Rev. Infect. Dis.* 13:139-45

Heiss GS, Gowan B, Dabbs ER (1992) Cloning of DNA from a *Rhodococcus* strain confering the ability to decolorize sulfonated azo dyes. *FEMS Microbiol. Lett.* 78:221-226

Hirata T, Saito H, Tomioka H, Sato K, Jidoi J, Hosoe K, Hidaka T (1995) *In vitro* and *in vivo* activities of the benzoxazinorifamycin KRM 1648 against *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 39:2295-2303

Honore N, Bergh S', Chanteau S, Doucet-Populaire F, Eiglmeir K, Garnier T, Georges C, Laonois P, Lumpiaboon I, Newton S, Niang K, del Portillo P, Ramasesh GR, Reddi P, Ridel R, Sittisombut N, Wu D, Hunger S, Cole ST (1993) Nucleotide sequence from the first cosmid from *Mycobacterium leprae* genome project: structure and function of the Rif-Str regions. *Mol. Microbiol.* 7:207-214

Honore N and Cole ST (1993) Molecular basis of rifampin resistance in *Mycobacterium leprae*. *Antimicrob. Agents Chemother.* 37:414-418

Horn AS and De Ranter CJ (Eds) (1984) *In*: X-ray crystallography and drug action. p 394-404, Oxford Science Publications.

Goodfellow M and Minnikin DE (1981) The genera *Nocardia* and *Rhodococcus In:* The Prokaryotes Vol II. Eds: MD Starr, H Stolp, HG Truper, A Balows and HG Schegel, Springer-Verlag, New York.

Gotah K, Mitsuyama M, Imaizumi S, Kawanana I, Yano J (1991) Mycolic-acid containing glycolipid as a possible virulence factor of *Rhodococcus equi* for mice. *Microbiol. Immunol.* 35:175-185

Grimm A, Madduri K, Ali A, Hutchinson CR (1994) Characterisationof the *Streptomyces peucetius* ATCC29050 genes encoding doxorubicin polyketide synthase. *Gene* 151:1-10

Grosset JH (1989) Present status for chemotherapy for tuberculosis. *Rev. Infect. Dis.* 11:5347-5352

Guerrero C, Stockman L, Marchesi F, Bodmer T, Roberts GD, Telenti A (1994) Evaluation of the *rpoβ* gene in rifampicin-susceptible and -resistant . *Mycobacterium avium* and *Mycobacterium intracellulare*. *J. Antimicrob. Chemother.* 33:661-663

Haider K, Jugnow G, Kohnen R, Lim SV (1981) Degradation of chloronated benzenes, phenols, and cyclohexane derivatives by soil bacteria under aerobic conditions. *In:* Decomposition of toxic and non-toxic organic compounds in soil. Ann Arbor Science, Ann Arbor, p 207-226

Halling SM, Burtis KC, Doi RH (1977) Reconstitution studies show that rifampicin resistance is determined by the largest polypeptide of *Bacillus subtilis* RNA polymerase. *J. Biol. Chem.* 252:9024-9031

Han L, Yang K, Ramalingam E, Mosher RH, Vining LC (1994) Cloning and characterisation of polyketide synthase genes for Jadomycin B synthesis in *Streptomyces venezuela* ISP5230. *Microbiology* 140:3379-3389

Fraser CM *et al* (30 co-authors) (1995) The minimal gene complement of *Mycoplasma genitalium*. *Science* 270:397-403

Fujii K, Tsuji A, Miyazaki S, Yamaguchi K, Goto S (1994) *In vitro* and *in vivo* antibacterial activities od KRM-1648 and KRM-1657, new rifamycin derivatives. *Antimicrob. Agents Chemother.* 38:1118-1122

Gaffney DF, Cundliffe E, Foster TJ (1981) Chloramphenicol resistance that does not involve chloramphenicol acetyltransferase encoded by plasmids from Gram negative bacteria. *J. Gen. Microbiol.* 125:113-121

Glaz ET (1974) Rifampicin in co-oncogenic experiments. *Lancet* ii:404

Goldman A (1991) Towards the molecular characterisation of the genes involved in lignin degradation in nocardioform bacteria. M.Sc. Dissertation, University of Witwatersrand.

Golob H K-H (1989) Fusidic acid inactivation in nocardioform bacteria, identification of the gene/s involved in this mechanism. M.Sc. Dissertation, University of the Witwatersrand.

Golub B, Falk G, Spink WW (1967) Lung abscess due to *Corynebacterium equi*. Report of first human infection. *Ann. Intern. Med.* 66:1174-1177

Goodfellow M and Cross T (1984) Classification *In:* The Biology of the Actinomycetes, p 7-164, Eds: M Goodfelloow, M Mordarski and ST Williams, London, Academic Press.

Goodfellow M and Minnikin DE (1977) Nocardioform bacteria. *Ann. Rev. Microbiol.* 31:159-180

Elhai J and Wolk CP (1988) A versatile class of positive selection vectors based on the non-viability of palindrome-containing plasmids that allow cloning into long polylinkers. *Gene* 68:119-138

Emmons W, Reichwein B, Winslow DL (1991) *Rhodococcus equi* infection in patients with AIDS: Literature review and report of an unusual case. *Rev. Infect. Dis.* 13:91-96

Ferguson JA and Korte F (1981) Transformation of aldrin by soil microorganisms. *Appl. Environ. Microbiol.* 34:7-15

Fernandez-Moreno MA, Martinez E, Boto L, Hopwood DA, Malpartida F (1992) Nucleotide sequence and deduced functions of a set of cotranscribed genes of *Streptomyces coelicolor* A3(2) including the polyketide synthase for the antibiotic actinorhodin. *J. Biol. Chem.* 267:19278-19290

Ferreira NP, Robson PM, Bull JR, van der Walt WH (1984) The microbial production of 3α-H-4α-(3'-propionic acid) 5α-hydroxy-7αβ methylhexahydro-indan-1-one-lactone from cholesterol. *Biotechnol. Lett.* 517-522

Finnerty WR (1992) The biology and the genetics of the genus *Rhodococcus*. *Annu. Rev. Microbiol.* 46:193-218

Fleischmann RD *et al* (40 co-authors) (1995) Whole-genome random sequencing of *Haemophilis influenza* Rd. *Science* 269:496-512

Foster TJ (1983) Plasmid determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiol. Rev.* 47:361-409

Franklin TJ and Snow GA (1975) The problems of resistance to antimicrobial drugs. *In*: Biochemistry of antimicrobial action. p188-199. Chapman and Hall.

Decker H and Haag S (1995) Cloning and characterisation of a polyketide s[illegible]e gene from *Streptomyces fradiae* Tu2717, which carries the genes for biosynthesis of the angucycline antibiotic urdamycin A and a gene probably involved in its oxygenation. *J. Bacteriol.* 177:6126-6136

Decker H and Hutchinson CR (1993) Transcriptional analysis of the *Streptomyces glaucescens* tetracenomycin C biosynthesis gene cluster. *J. Bacteriol.* 175:3887-3892

Dennis SM and Bamford VW (1966) The role of corynebacteria in perinatal lamb mortality. *Vet. Rec.* 79:105-108

Denome SA and Young KD (1995) Identification and activity of two insertion sequence elements in *Rhodococcus* sp.strain IGTS8. *Gene* 161:33-38

Desomer J, Dhaese P, van Montagu M (1990) Transformation of *Rhodococcus fascians* by high voltage electroporation and development of *R.fascians* cloning vectors. *Appl. Environ. Microbiol.* 56:2818-2825

Dholpe AM and Ibanez MA (1995) The *in vitro* activities of novel benzoxazinorifamycins against *M.leprae*. *J. Antimicrob. Chemother.* 35:463-471

Disotell TR, Zehr SM, Ruvolo M (1992) Double-stranded dideoxy sequencing from "dirty" DNA - done in a day. *Biotechniques* 13:48-49

Eggink G, Engel H, Vriend G, Terpstra P, Witholt B (1990) Rubredoxin reductase of *Pseudomonas oleovorans* - Structural relationship of other flavoprotein oxidoreductases based on one NAD and two FAD fingerprints. *J. Mol. Biol.* 212:135-142

Ehrlich P (1959) Chemotherapy. In: The collected papers of Paul Ehrlich. Vol III. p 505-518. Pergamon Press, London.

Clave D, Archambaud M, Rouquet RM, Massip P, Moati N (1991) *In vitro* activity of twenty antibiotics against *Rhodococcus equi*. *Pathol. Biol. (Paris)* 39:424-428

Cross T, Rowbotham TJ, Mishustin EN, Tepper EZ, Antione-Portaels F, Schaal KP, Bickenback H (1976) *In:* The Biology of the Nocardiae, Eds: M Goodfellow, GH Brownell, JA Serrano pp337-371, Academic Press, London.

Dabbs ER (1987) A generalised transducing bacteriophage for *Rhodococcus erythropolis*. *Mol. Gen. Genet.* 206:116-120

Dabbs ER (1987) Rifampicin inactivation by *Rhodococcus* and *Mycobacterium* species. *FEMS Microbiol. Lett.* 44:395-399

Dabbs ER, Gowan B, Andersen SJ (1990) Nocardioform arsenic resistance plasmids and construction of *Rhodococcus* cloning vectors. *Plasmid* 23:242-247

Dabbs ER and Sole G (1987) Plasmid borne resistance to arsenate, arsenite, cadmium and chloramphenicol in a *Rhodococcus* species. *Mol. Gen. Genet.* 211:148-154

Dabbs ER, Yazawa K, Mikami Y, Miyaji M, Morisaki N, Iwasaki S Furihata K (1995) Ribosylation by mycobacterial strains as a new mechanism of rifampin inactivation. *Antimicrob.Agents Chemother. 39:1007-1009*

Dabbs ER, Yazawa K, Tanaka Y, Mikami Y, Miyaji M, Andersen SJ, Morisaki N, Iwasaki S, Shida O, Takagi H, Kadowaki K (1995) Rifampicin inactivation by *Bacillus* species. *J. Antibiot.* 48:815-819

Davis NK and Chater KF (1990) Spore colour in *Streptomyces coelicolor* A3(2) involves the developmentally regulated synthesis of a compound biosynthetically related to polyketide antibiotics. *Mol. Microbiol.* 4:1679-1691

F'anco G, Pereda A, Brian P, Mendez C, Chater KF, Salas JA (1993) A hydroxylase-like gene product contributes to synthesis of a polyketide spore pigment in *Streptomyces halstedii*. *J. Bacteriol.* 175:8043-8048

Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heynekar HL, Boyer HW, Crosa JH, Falkow S (1977b) Construction and characterisation of new cloning vehicles. II. A multipurpose cloning system. *Gene* 2:95

British Pharmacoepia (1973) Addendum (1978) Her Majesty's Stationery Office.

Brownell GH, Saba JA, Denniston K, Enquist LW (1982) Development of a *Rhodococcus* actinophage cloning system. *Devel. Ind. Microbiol.* 23:287-298

Buttner MJ (1989) RNA polymerase heterogeneity in *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* 3:1653-1659

Byrne AM, Kukor JJ, Olsen RH (1995) Sequence analysis of the gene cluster encoding toluene-3-monoxygenase from *Pseudomonas picketti* PK01. *Gene* 154:65-70

Cave MD, Eisenach KD, McDermott PF, Bates JH, Crawford JT (1991) IS6110: conservation of sequence in the *Mycobacterium tuberculosis* complex and its utilisation in DNA fingerprinting. *Mol. Cell. Probes* 5:73-80

Chang Y and Wu GE (1992) A simple and rapid procedure for pouring sequencing gels. *Biotechniques* 12

Cheng S-C and Modrich P (1983) Positive selection cloning vehicle useful for overproduction of hybrid proteins. *J. Bacteriol.* 154:105-108

Benatar SR (1991) The evolution of antituberculosis chemotherapy. *In:* A Century of Tuberculosis - South African Perspectives. Eds: HM Coovadia and SR Benatar, Oxford University Press.

Benatar SR (1991) Tuberculosis in adults. *In:* A Century of Tuberculosis - South African Perspectives. Eds: HM Coovadia and SR Benatar, Oxford University Press.

Bermudez LE, Kolonoski P, Young LS, Inderlied CB (1994) Activity of KRM 1648 alone or in combination with ethambutol or clarithromycin against *Mycobacterium avium* in beige mouse model of disseminated infection. *Antimicrob. Agents. Chemother.* 38:1844-1848

Bibb MJ, Biro S, Motamedi H, Collins JF, Hutchinson CR (1989) Analysis of the nucleotide sequence of the *Streptomyces glaucescens tcmI* genes provides key information about the enzymology of polyketide antibiotic biosynthesis. *EMBO* 8:2727-2736

Blanco MG, Hardisson C, Salas JA (1984) Resistance to inhibitors of RNA polymerase in actinomycetes that produce them. *J. Gen. Microbiol* 130:2883-2891

Blanco G, Brian P, Pereda A, Mendez C, Salas JA, Chater KF (1993) Hybridisation and DNA sequence analyses suggest an early evolutionary divergence of related biosynthetic gene sets encoding polyketide antibiotics and spore pigments in *Streptomyces* spp. *Gene* 130:107-116

Blanco G, Pereda A, Mendez C, Salas JA (1992) Cloning and disruption of a DNA fragment of *Streptomyces halstedii* involved in the biosynthesis of a spore pigment. *Gene* 112:59-65

Abril M-A, Michan C, Timmis K, Ramos JL (1989) Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of substrate range of the pathway. *J. Bacteriol.* 171:6782-6790

Adams JN and Brownell GH (1976) Genetic studies in *Nocardia erythropolis*. *In*: The Biology of the *Nocardia*, eds M Goodfellow, GH Brownell and JA Serrano. Academic Press.

Andersen SJ (1989) Development of techniques for molecular biological characterisation of the novel rifampicin inactivation mechanism in nocardioform bacteria. M.Sc. Dissertation, University of the Witwatersrand.

Andersen SJ and Dabbs ER (1991) Cloning of nocardioform DNA confering the ability to inactivate rifampicin. *FEMS Microbiol. Lett.* 79:247-250

Arai T, Kuroda S, Watanabe I (1981) Biodegradation of acrylamide monomer by a *Rhodococcus* strain. *In:* Actinomycetes 261 Bakt Suppl II. Eds KP Schaal and G Pulver, p 297-308, Gustav Fischer, Stuttgart.

Armstrong SM and Patel TR (1993) Characterisation of the *Rhodococcus* sp. BPG-8 resorcinol hydroxylase. *J. Basic Microbiol.*33:75-81

Armstrong SM, Patel TR, Monacha S (1993) Partial purification and characterisation of phloroglucinol hydroxylase from *Rhodococcus* species BPG-8. *Microbios* 76:93-103

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K Eds. (1994) Current Protocols in Molecular Biology. Wiley, USA pp 19.3.29-19.3.37

6.0 REFERENCES

Formula for calculating DNA concentration from agarose gels

lambda : $\frac{\text{fragment size}}{\text{genome size}}$ x amount of DNA per lane = y

plasmid : $\frac{\text{plasmid size}}{\text{fragment size}}$ x y = amount of plasmid DNA lane

Sizes of molecular weight markers

λII :	λIII :
23.0 kb	23.0
9.4 kb	5.0
6.6 kb	4.2
4.4 kb	3.5
2.2 kb	2.0
2.0 kb	1.9
	1.6
	1.3
	1.0
	0.8
	0.6

Wieringa RK, Terpstra P, Hol WGJ (1986) Prediction of the occurence of the ADP-binding 6$\alpha\beta$-fold in proteins, using an aminoacid fingerprint. *J. Mol. Biol.* 187:101-107

Williams DL, Waguespack C, Eisenach K, Crawford JT, Portaels F, Salfinger M, Nolan CM, Abe C, Sticht-Groh V, Gillis TP (1994) Characerisation of rifampicin resistance in pathogenic mycobacteria. *Antimicrob. Agents Chemother.* 38:2380-2386

Wilson MM (1955) A study of *Corynebacterium equi* in a study of thoroughbred horses in Victoria. *Aust. Vet. J. 31:175-181*

Yang K, Han L, Vining LC (1995) Regulation of jadomycin B production in *Streptomyces venezuelae* ISP5230: Involvement of a repressor gene, *jadR*$_2$. *J. Bacteriol.* 177:6111-6117

Yao W, Yang Y, Chiao J (1994) IS204: an insertion sequence from *Nocardia asteroides (mexicana)* YP21. *Plasmid* 32:262-269

Yazawa K, Mikami Y, Maeda A, Akao M, Morisaki N (1993) Inactivation of rifampin by *Nocardia brasiliensis*. *Antimicro. Agents Chemother.* 37:1313-1317

Yazawa K, Mikami Y, Maeda A, Morisaki N, Iwasaki S (199) Phosphorylative inactivation of rifampicin by *Nocardia otitdis-caviarum*. *J. Antimicrob. Chemother.* 33:1127-1135

Zabeau M and Stanley KK (1982) Enhanced expression of *cro*-β galactosidase fusion proteins under the control of the P_R promotor of bacteriophage lambda. *EMBO J* 1:1217-1244

Zhang Y, Heym B, Allen B, Young D, Cole C (1992) The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature* 358:591-593

Tomasi I, Artaud I, Bertheau Y, Mansuy D (1995) Metabolism of polychlorinated phenols by *Pseudomonas cepacia* AC1100: Determination of the first two steps and specific inhibitory effect of methimazole. *J. Bacteriol.* 177:307-311

Trivedi SS and Desai SG (1988) Primary antituberculosis drug resistance and aquired rifamicin resistance in Gujarat, India. *Tubercle* 69:37-41

Tsukamura M (1972) The pattern of resistance development to rifampicin in *Mycobacterium tuberculosis*. *Tubercle* 53:111-117

van Etta LL, Filice GA, Ferguson RM, Gerding DN (1983) *Corynebacterium equi*: a review of 12 cases of human infection. *Rev. Infect. Dis.* 5:1012-1018

Vernet T, Lau PCK, Narang SA, Visentin LP (1985) A direct selection vector derived from pColE3-CA38 and adapted for foreign gene expression. *Gene* 34:87-93

Wade A (Ed) (1979) Martin Dale: The Extra Pharmacoepia 27ed. p1596-1601, The Pharmaceutical Press.

Walsh C (1979) Flavin- and pterin-dependant monooxygenases *In:* Enzymatic reaction mechanisms. WH Freeman and Company, San Fransisco. pp406-420

Walter U, Beyer M, Klein J, Rehm H-J (1991) Degradation of pyrene by *Rhodococcus* sp UWI. *Appl Microbiol. Biotechnol.* 34:671-676

Warren R, Richardson M, Sirgil F, Victor T, Schaaf S, van Helden P (1995) *Mycobacterium tuberculosis*: Strain Evolution. Abstract: Glaxo Action TB Scientific Conference and Workshops.

Wehrli W and Staehelin M (1971) Actions of the rifamycins. *Bact. Rev.* 35:290-309

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517

Stevis PE and Ho NWY (1987) Positive selection vectors based on xylose utilization suppression. *Gene* 55:67-74

Straube G (1987) Phenol hydroxylase from *Rhodococcus* sp. P1. *J. Basic Microbiol.* 4:229-232

Strebel PM and Seager JR (1991) Epidemiology of tuberculosis in South Africa. *In:* A Century of Tuberculosis - South African Perspectives. Eds: HM Coovadia and SR Benatar, Oxford University Press.

Strohl WR (1992) Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. *Nucleic Acids Res.* 20:961-974

Suemori A, Nakajima K, Kurane R, Nakamura Y (1994) Inactivation of three monohydroxybenzoate monooxygenases from *Rhodococcus erythropolis*: The role of an arginine residue in the substrate binding domain of p-hydroxybenzoate 3-hydroxylase. *FEMS Microbiol. Lett.* 120:177-182

Takai S, Koike K, Ohbushi S, Izumi C, Tsubaki S (1991) Identification of 15-kilodalton to 17-kilodalton antigens associated with virulent *Rhodococcus equi*. *J. Clin. Microbiol.* 29:439-443

Telenti A, Imboden P, Marchesi F, Lowrie D, Cole ST, Colston MJ, Matter L, Schoolfer K, Bodmer T (1993) Detection of rifampicin resistance mutants in *Mycobacterium tuberculosis*. *Lancet* 341:647-650

The Pharmaceutical Codex 11th Ed incorporated with the British Pharmaceutical Codex (1979) The Pharmaceutical Press.

Shaw WV (1967) The enzymatic acetylation of chloramphenicol by extracts of R factor-resistant *Escherichia coli*. *J. Biol.Chem.* 242:687-693

Sherman DH, Malpartida F, Bibb MJ, Kieser HM, Hopwood DA (1989) Structure and deduced function of the granaticin producing polyketide synthase gene cluster of *Streptomyces violaceoruber* Tu22. *EMBO* 8:2717-2725

Siddiqi SH, Aziz A, Reggiardo Z, Middlebrook G (1981) Resistance to rifampicin and isoniazid in strains of *Mycobacterium tuberculosis*. *J. Clin. Pathol.* 34:927-929

Silver S and Keach D (1982) Energy-dependent arsenate efflux: the mechanism of plasmid mediated resistance. *Proc. Natl Acad. Sci. USA* 79:6114-6118

Sinclair R and Bibb MJ (1989) Transcriptional analysis of the repressor gene of the temperate *Streptomyces* phage phi C31 *Gene* 85:275-282

Sippel A and Hartmann G (1970) Rifampicin resistance of RNA polymerase in the bianry complex with DNA. *Europ. J. Biochem.* 16:152-157

Skold O (1976) R factor-mediated resistance to sulfonamides by a plasmid-borne, drug resistant dihydropteroate synthase. *Antimicrob. Agents Chemother.* 9:49-54

Smith JE (1966) *Corynebacterium* species as animal pathogens. *J. Appl. Bacteriol.* 29:119-130

Sorkhoh NA, Ghannoum MA, Ibrahim AS, Stretton RJ, Radwan SS (1990) Crude oil and hydrocarbon-degrading strains of *Rhodococcus rhodochrous* isolated from soil and marine environments in Kuwait. *Environmental Pollution*. 65:1-17

Rohr J and Thiericke R (1992) Angucycline group antibiotics. *Nat. Prod. Rep.* 9:103-137

Roland S, Ferone R, Harvey RJ, Styles VL, Morrison RW (1979) The characteristics and significance of sulfonamides as substrate for *Escherichia coli* dihdropteroate synthase. *J. Biol. Chem.* 254:10337-10345

Romero E, Riva S, Fietta AM, Silvestri LG (1971) Effect of R-factors on rifampicin resistance in *E.coli*. *Nature New Biology* 234:56-58

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a laboratory manual (2nd edition). Cold Spring Harbour Laboratory Press.

Sane DC and Durack DT (1986) Infection with *Rhodococcus equi* in AIDS. *N. Engl. J. Med.* 314:56-57

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci.* 74:5463-5467

Schaal KP and Lee H-J (1992) Actinomycete infections in humans - a review. *Gene* 115:201-211

Schreuder HA, Hol WGJ, Drenth J (1988) Molecular modeling reveals the possible importance of a carbonyl oxygen binding pocket for the catalytic mechanism of p-hydroxybenzoate hydroxylase. *J. Biol. Chem.* 263:3131-3136

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

Otten SL,Ferguson J, Hutchinson CR (1995) Regulation of daunorubicin production in *Streptomyces peucetius* by the *dnrR2* locus. *J. Bacteriol.* 177:1216-1224

Palfey BA, Entsch B, Ballou DP, Massey V (1994) Changes in the catalytic properties of p-hydroxybenzoate hydroxylase caused by the mutation Asn300Asp. *Biochemistry* 33:1545-1554

Perkins EJ, Gordon MP, Caceres O, Lurquin PF (1990) Organisation and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. *J. Bacteriol.* 172:2351-2359

Perlman D (1977) *In*: Structure-activity relationships among semi-synthetic antibiotics. Academic Press.

Prelog V and Oppolzer W (1973) Ansamycine, eine neuartige klasse von mikrobiellen stoffwechselprodukten. *Helv. Chim. Acta.* 56:1179-1187

Pulido D, Zalacain M, Jiminez A (1988) The *hyg* gene from *Streptomyces hygroscopicus*: a novel form of *Streptomyces* promoters. *Biochem. Biophys. Res. Comm.*151:270-274

Quan S and Dabbs ER (1993) Nocardioform arsenic resistance plasmid characterisation and improved *Rhodococcus* cloning vectors. *Plasmid* 29:74-79

Roberts TM, Swanberg SL, Poteet A, Riedel G, Backman K (1980) A plasmid cloning vehicle allowing a positive selection for inserted fragments. *Gene* 12:123-127

Rebollo A, Gil JA, Liras P, Asturias JA, Martin JA (1989) Cloning and characterisation of a phosphate-regulated promoter involved in phosphate control of candicidin biosynthesis. *Gene* 79:47-58

Morisaki N, Iwasaki S, Yazawa K, Mikami Y, Maeda A (1993) Inactivated products of rifampicin by pathogenic *Nocardia* spp.: Structures of glycosylated and phosphorylated metabolites of rifampicin and 3-formylrifampicin SV. *J. Antibiot.* 46:1605-1610

Murakami T, Holt TG, Thompson CJ (1989) Thiostrepton-induced gene expression in *Streptomyces lividans*. *J. Bacteriol.* 171:1459-1666

Nayer-Weidner M and Reiners-Schramm L (1988) Highly efficient positive selection of recombinant plasmids using a novel *rglβ*-based *Escherichia coli* K-12 vector system. *Gene* 66:269-278

Nelson JE and Krawetz SA (1994) Easy read DNA sequencing gels. *Biotechniques* 17:416-417

Nikaido H (1994) Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* 264:382-388

Nordmann P, Chavanet P, Caillon J, Duez JM, Portier H. (1991) Recurrent pneumonia due to rifampicin-resistant *Rhodococcus equi* in a patient infected with HIV (letter). *J.Infect.* 24:104-107

Nordmann P, Kerestedjian J-J, Ronco E (1992) Therapy of *Rhodococcus equi* disseminated infections in nude mice. *Antimicrob. Agents Chemother.* 36:1244-1248

Nordmann P and Ronco E (1992) *In vitro* susceptibility of *Rhodococcus equi*. *J. Antimicrob. Chemother.* 29:383-393

O'Brien RJ, Lyle MA, Snider DE Jr (1987) Rifabutin (ansamycin LM 427): a new rifamycin-S derivative for the treatment of mycobacterial diseases. *Rev. Infect. Dis.* 9:519-530

Orser CS, Lange CC, Xun L, Zahrt TC, Schneider BJ (1993) Cloning, sequence analysis and expression of the *Flavobacterium* pentachlorophenol-4-monooxygenase gene in *E.coli*. *J. Bacteriol.* 175:411-416

Morisaki N, Iwasaki S, Yazawa K, Mikami Y, Maeda A (1993) Inactivated products of rifampicin by pathogenic *Nocardia* spp.: Structures of glycosylated and phosphorylated metabolites of rifampicin and 3-formylrifampicin SV. *J. Antibiot.* 46:1605-1610

Murakami T, Holt TG, Thompson CJ (1989) Thiostrepton-induced gene expression in *Streptomyces lividans*. *J. Bacteriol.* 171:1459-1666

Nayer-Weidner M and Reiners-Schramm L (1988) Highly efficient positive selection of recombinant plasmids using a novel *rglβ*-based *Escherichia coli* K-12 vector system. *Gene* 66:269-278

Nelson JF and Krawetz SA (1994) Easy read DNA sequencing gels. *Biotechniques* 17:416-417

Nikaido H (1994) Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* 264:382-388

Nordmann P, Chavanet P, Caillon J, Duez JM, Portier H. (1991) Recurrent pneumonia due to rifampicin-resistant *Rhodococcus equi* in a patient infected with HIV (letter). *J.Infect.* 24:104-107

Nordmann P, Kerestedjian J-J, Ronco E (1992) Therapy of *Rhodococcus equi* disseminated infections in nude mice. *Antimicrob. Agents Chemother.* 36:1244-1248

Nordmann P and Ronco E (1992) *In vitro* susceptibility of *Rhodococcus equi*. *J. Antimicrob. Chemother.* 29:383-393

O'Brien RJ, Lyle MA, Snider DE Jr (1987) Rifabutin (ansamycin LM 427): a new rifamycin-S derivative for the treatment of mycobacterial diseases. *Rev. Infect. Dis.* 9:519-530

Orser CS, Lange CC, Xun L, Zahrt TC, Schneider BJ (1993) Cloning, sequence analysis and expression of the *Flavobacterium* pentachlorophenol-4-monooxygenase gene in *E.coli*. *J. Bacteriol.* 175:411-416

Mascellino MT, Iona E, Ponzo R, Mastroianni CM, Delia S (1994) Infections due to *Rhodococcus equi* in three HIV infected patients: microbiological fingings and antibiotic susceptibility. *Int. J. Clin. Pharmacol. Res.* 14:157-163

McMurry l, Petrucci RE, Levy SB (1980) Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. *Proc. Natl Acad. Sci. USA* 77:3974-3977

McNeil MM and Brown JM (1992) Distribution and antimicrobial susceptibility of *Rhodococcus equi* from clinical specimens. *Eur. J. Epidemiol.* 8:437-443

Miller HJ, Janse JD, Kamerman W, Muller PJ (1980) Recent observations on leafy gall in Liliaceae and some other families. *Neth. J. Plant Pathol.* 86:55-68

Miller LP, Crawford JT, Shinnick FM (1994) The *rpoβ* of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 38:805-811

Miyamoto M, Matsumoto J, Iwaya T, Itagaki E (1995) Bacterial steroid monooxygenase catalysing Baeyer-Villiger oxidation of C21-ketosteroids from *Rhodococcus rhodochrous*: the isolation and characterisation. *Biochim. Biophys. Acta* 1251:115-124

Mizuguchi Y, Taniguchi H, Nikaido Y, Yamasaki T (1995) Molecular basis of rifampicin resistance in *Mycobacterium tuberculosis*. Symposium Abstracts: Dialogues of resistance between clinical bacteria and antibiotic producers, Tokyo, Japan.

Moola S (1993) Molecular characterisation of the genes involved in nylon monomer degradation and catechol utilisation in nocardioform species. M.Sc. Dissertation, University of the Witwatersrand.

Mascellino MT, Iona E, Ponzo R, Mastroianri CM, Delia S (1994) Infections due to *Rhodococcus equi* in three HIV infected patients: microbiological fingings and antibiotic susceptibility. *Int. J. Clin. Pharmacol. Res.* 14:157-163

McMurry l, Petrucci RE, Levy SB (1980) Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. *Proc. Natl Acad. Sci. USA* 77:3974-3977

McNeil MM and Brown JM (1992) Distribution and antimicrobial susceptibility of *Rhodococcus equi* from clinical specimens. *Eur. J. Epidemiol.* 8:437-443

Miller HJ, Janse JD, Kamerman W, Muller PJ (1980) Recent observations on leafy gall in Liliaceae and some other families. *Neth. J. Plant Pathol.* 86:55-68

Miller LP, Crawford JT, Shinnick FM (1994) The *rpoβ* of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 38:805-811

Miyamoto M, Matsumoto J, Iwaya T, Itagaki E (1995) Bacterial steroid monooxygenase catalysing Baeyer-Villiger oxidation of C21-ketosteroids from *Rhodococcus rhodochrous*: the isolation and characterisation. *Biochim. Biophys. Acta* 1251:115-124

Mizuguchi Y, Taniguchi H, Nikaido Y, Yamasaki T (1995) Molecular basis of rifampicin resistance in *Mycobacterium tuberculosis*. Symposium Abstracts: Dialogues of resistance between clinical bacteria and antibiotic producers. Tokyo, Japan.

Moola S (1993) Molecular characterisation of the genes involved in nylon monomer degradation and catechol utilisation in nocardioform species. M.Sc. Dissertation, University of the Witwatersrand.

Le Chevalier HA (1986) Nocardioforms. *In*: Bergey's manual of systematic bacteriology, Vol II Ed. PHA Smith, Williams and Wilkins.

Lipsky BA, Goldberg AC, Tompkins LB, Plorde JJ (1982) Infections caused by nondiptheria corynebacteria. *Rev. Infect. Dis.* 4:'220-1235

Lowbury EJL and Ayliffe GAJ (1974) *In*: Drug resistance in Antimicrobial therapy, (from the preface), Charles D Thomas publishers, Springfield, Ill.

Lupski JR (1987) Molecular mechanisms for transposition of drug resistant genes and other movable genetic elements. *Rev. Infect. Dis.* 9:357-368

MacDonald CR, Cooper DG, Zajic J (1981) Surface active lipids from *Nocardia erythropolis* grown on hydrocarbons. *Appl. Environ. Microbiol.* 41:117-123

Maggi N, Pasqualucci CR, Ballotta R, Sensi P (1966) Rifampicin: a new orally active rifamycin. *Chemotherapy* 11:285-292

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning: a laboratory manual. Cold Spring Harbour, New York.

Margalith P and Beretta G (1960) Rifampicin XI. Taxonomic study on *Streptomyces mediterranei* nov. sp. *Mycopathol. Mycol. Appl.* 13:321

Martin C, Timm J, Rauzier J, Gomez-Lus R, Davies J, Gicquel B (1990) Transposition of an antibiotic resistance element in mycobacteria. *Nature* 345:739-743

Koch AL (1981) Evolution of antibiotic resistance gene function. *Microbiol. Rev.* 45:355-378

Koch AL and Gross GH (1979) Growth conditions and rifampicin susceptibility. *Antimicrob. Agents Chemother.* 15:220-228

Korzyloski T, Kowszyk-Gindifer Z, Kurytowicz W (1978) *In:* Antibiotics: Origin, nature and properties. Vol 1, American Society for Microbiology p 865-892, 300-303

Kossiakoff MG (1887) De la propriete que possedent les microbes de s'accommoder aux milieux antiseptiques. *Ann. Inst. Pasteur Paris* 1:465-476

Kradolfer F (1968) Uber eine neue klasse von baktericid wirkenden speziellr beruck sichtigung von rifampicin. *Schweiz. Med. Wschr.* 98:622-627

Kumar S, Ganguly NK, Kohli KK (1991) Interaction of rifampicin with non-protein thiols in *Mycobacterium smegmatis*. *Biochem. Internat.* 23:1041-1047

Kunze ZM, Wall S, Appelberg R, Silva MT, Portaels F, McFadden JJ (1991) IS901, a new member of a wide spread class of atypical insertion sequences, is associated with pathogenicity in *Mycobacterium avium*. *Mol. Microbiol.* 5:2265-2272

Lancini C (1983) Ansamycins *In:* Biochemistry and genetic regulation of commercially important antibiotics, Vining LC (Ed), Addison-Wesley Publishing Company. pp 198-212

Lasker BA, Brown JM, McNeil MM (1992) Identification and epidemiological typing of clinical and environmental isolates of the genus *Rhodococcus* with use of digoxygenin-labeled rDNA gene probe. *Clin. Infect. Dis.* 15:223-233

Ziegler DM (1993) Recent studies on the structure and function of multisubstrate flavin-containing monooxygenases. *Ann. Rev. Pharmacol. Toxicol.* 33:179-199

Author: Andersen Susan Jean.
Name of thesis: Molecular biological characterisation of the novel Rifampicin inactivation mechanism in Nocardioform bacteria.

PUBLISHER:
University of the Witwatersrand, Johannesburg
©2015

LEGALNOTICES:

Copyright Notice: All materials on the University of the Witwatersrand, Johannesburg Library website are protected by South African copyright law and may not be distributed, transmitted, displayed or otherwise published in any format, without the prior written permission of the copyright owner.

Disclaimer and Terms of Use: Provided that you maintain all copyright and other notices contained therein, you may download material (one machine readable copy and one print copy per page)for your personal and/or educational non-commercial use only.

The University of the Witwatersrand, Johannesburg, is not responsible for any errors or omissions and excludes any and all liability for any errors in or omissions from the information on the Library website.