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

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ABSTRACT

Nocardioform bacteria are aetiological agents in several human diseases including tuberculosis, leprosy and nocardiosis. Tuberculosis is of major epidemiological importance in South Africa as it is a major cause of death. There has been a rise in resistance of *Mycobacterium tuberculosis* to the antibiotic *rifampicin*. Rifampicin is one of the most effective drugs used in the treatment of tuberculosis. Recently a novel inducible rifampicin inactivation mechanism has been described in a strain closely related to *M.tuberculosis*, this is *Rhodococcus erythropolis* strain ATCC 12674. With the aim of ultimately cloning the gene(s) responsible for the inducible rifampicin inactivation mechanism by complementation, the conditions for this have been optimised.

Mutants have been made which lack the inducible rifampicin inactivation mechanism. These mutants will be used as recipients of DNA from a genomic library containing the gene(s) for the inducible rifampicin inactivation mechanism. The DNA isolated from clones in which the inductible rifampicin inactivation ability is restored should contain genes of the inducible inactivation mechanism.

Transformation conditions in nocardioform bacteria have been optimised, resulting in 15 000 transformants per microgram of plasmid DNA.

A genomic library from a closely related strain ATCC 4277 which inactivates rifampicin in a similar way has been formed after the transformation conditions for *E.coli* strain MM 294 had been optimised.

DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Master of Science in Biotechnology in the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination in any other university.

JPAnderson

28 day of February, 1989.

This is dedicated to the M.Sc Biotechnology class of 1988 -

Anne Daffey, Katrina Downing, Harald Golob and Bhavna Gowan.

This is dedicated to the M.Sc Biotechnology class of 1986 -

Anne Daffey, Katrina Downing, Harald Golob and Bhavna Gowan.

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1.0 INTRODUCTION

1.1 NOCARDIOFORM BACTERIA AND DISEASE

Nocardioform bacteria are the cause of several human diseases, the most well known being tuberculosis. *Tuberculosis* is of major epidemiological importance and arises as a result of infection with *Mycobacterium turberculosis*. In South Africa tuberculosis is a major cause of death with ten people dying every day of this condition.[1] On average 60 000 new cases are reported annually and over 200 000 patients are undergoing treatment at any one time.[1] In a publication of the WHO commemorating the centenary of the discovery of the *tuberculosis* bacterium by Robert Koch in 1882, it was revealed that tuberculosis is the cause of at least three million deaths every year, the world over. Each year there are four or five million new, highly contagious cases and, on average two out of three patients die within two years. The health statistics compiled from third world countries tend to seriously underestimate the prevalence of all diseases, including tuberculosis. The total number of cases of tuberculosis has doubled in the last three decades, due to the doubling of the population.[2]

M.tuberculosis almost always gains access to the body through inhalation or ingestion. Characteristically tubercles form in the tissues where they may grow and caseate or regress and heal. The most common primary site of infection is the lung, but other organs or tissues may be involved.

The disease may run an acute course but is usually a chronic process.[3] Other mycobacterial species have been implicated in human disease. *M.leprae* is the causative organism in leprosy and *M.kansasii*, *M.intracellulare*, *M.scrofulaceum*, *M.marinum*, *M.ulcerans* and *M.chelonei* have been isolated from patients with systemic disease and superficial lesions. The organs most commonly infected are the lungs, lymph glands and skin.[3]

Nocardiosis is primarily a pulmonary infection that frequently disseminates by way of the blood stream and lymphatics to other parts of the body. It may also be a primary disease of the central nervous system, kidneys, heart, eyes and other organs. Clinically and pathologically nocardiosis may be extremely variable presenting as pyogenic infections, encapsulated abscesses or even granulomas. The strains commonly associated with nocardiosis are *Nocardia asteriodes* and *Nocardia brasiliensis*.[4]

1.2 NOCARDIOFORM BACTERIA

Nocardioform actinomycetes are branching bacteria whose reproduction takes the form of fragmentation of the hyphae into bacillae and coccoid elements.[5] It has been recognised that the genus *Nocardia* had been poorly defined and contained bacteria that had little in common.[7] Much effort has been directed in the past few years towards establishing a better classification of actinomycetes. There has been revision and tighter definitions given to established genera and new genera have been proposed. It is into one of these newly defined genera that the bacteria of this study have been classified. The genus is *Rhodococcus*. The proposed mini definition of *Rhodococcus* is Gram positive, aerobic, non-sporulating

actinomycetes that are pleiomorphic but often form a primary mycelium that soon fragments into rod and coccoid elements. A secondary mycelium is not produced but strains contain mycolic acids and have a wall of chemotype IV, and the G+C content of their DNA ranges from 59-69%.[8]

1.3 CHEMOTHERAPY IN TUBERCULOSIS

In the past 25 years the treatment of tuberculosis has been revolutionised by the advent of potent antibacterial drugs. In fact modern chemotherapy is almost always capable of curing the condition if the disease is recognised sufficiently early and effective drug regimens are prescribed and taken long enough. The secret to the success of this treatment has been due to the prevention of the emergence of drug-resistant tubercle bacteria by the use of combined chemotherapy, in which more than one drug is given to the patient, at least in the original months of the regimen.[10] The most widely used antituberculous drugs at present are isoniazid (isonicotinic acid hydrazide), rifampicin, ethambutol and streptomycin. Other drugs including ethionamide, pyrizinamide, viomyin and cycloserine are less frequently employed because of their pronounced side effects.[3]

The development during the last decade of short-course regimens has greatly reduced the period of treatment necessary to obtain bacteriologically quiescent status.[11] The most important of these drugs is rifampicin, which is excellent both as a sterilising drug which kills the last few semidormant organisms in the lesion and in preventing growth of organisms throughout the lesions and thus the emergence of resistance to a companion drug. Pyrazinamide is almost as good as a sterilising drug, but is much less effective than rifampicin in preventing the emergence

of resistance. *Ethambutol* appears to be of no value as a sterilising drug, but is moderately effective in preventing resistance. Clinical cures can now be achieved in 6-12 months. Rifampicin is usually administered as a single dose of 450-600 mg daily.

1.4 RIFAMPICIN RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS

In the treatment of mycobacterial disease, drug resistance can emerge to all of the antibacterial drugs used. *Resistance occurs* most commonly when a drug is *given alone and* when the viable bacterial population in lesions *is large*. In tuberculosis, it first appears after an interval of at least two weeks but more commonly one to four months after the commencement of chemotherapy. The development of drug resistance is widely thought to be the result of the overgrowth of sensitive organisms by mutant resistant bacteria present in wild strains before they ever *come into contact* with the drug concerned. *In the fluctuation* test of Luria and Delbruck the *mutation rate in M.tuberculosis* were found to be about 2×10^{-8} for resistance to isoniazid and streptomycin, 2×10^{-10} for rifampicin and 1×10^{-7} for ethambutol.[11] In a more recent study the following probabilities of the incidence of drug resistant mutants was published:[12] Rifampicin - 10^{-8}

isoniazid, streptomycin, ethambutol, *kanamycin and* p-aminosalicylate - 10^{-6}, *ethionamide*, enviomycin, cycloserine, capreomycin, viomycin and thiacetone - 10^{-5}

It is in the bacterial chromosomes that mutation to drug resistance is thought to occur. The isolation of plasmids from mycobacteria has been described. [13,14] Aminoglycoside resistance *has been associated* with these plasmids, but the *evidence is not* conclusive. It has seemed reasonable

to assume that resistance to isoniazid, rifampicin, pyrazinamide, dapsone and other non-aminoglycoside drugs is always chromosomal and that resistance to streptomycin and other aminoglycosides in tubercle bacilli is probably also chromosomal. In the treatment of tuberculosis the pattern of emergence of resistance suggests little linkage between unrelated drugs, even among aminoglycosides, though there are several well established patterns of cross-resistance between closely related drugs. Those species of mycobacteria well adapted to a parasitic roll in man, *M.tuberculosis*, *M.africanum*, *M.bovis* and *M.lepraе*, all exist within closed lesions in human or animal hosts and do not come into contact with other bacteria including other strains of mycobacteria, which might transmit plasmids to them. So a plasmid mediated resistance system could not readily be exploited by these bacteria to increase their genetic variability.[15]

Rifampicin is considered to be the most valuable and effective chemotherapeutic agent after isoniazid and is widely used in the first line of antituberculous drugs. It was noted that rifampicin, having a different chemical stucture from isoniazid, has an independant bacteriocidal action, and in wild strains of *M.tuberculosis*, the incidence of rifampicin resistant mutants was appreciably lower, while the serum level attained on standard oral dosage was much higher than isoniazid.[35] It was previously stated that the pattern of resistance developed to rifampicin of tubercle bacilli is an obligatory "single-step" pattern. Only one phenotype to resistance to rifampicin was described. Tubercle bacilli were described as being either susceptible or resistant to this antibiotic.[26] This obligatory "single-step" pattern has been observed in tubercle bacilli in respect to rifamycin SV, ethambutol and capreomycin.[36]

In the past decade there has been a significant increase in the prevalence of primary antituberculosis drug resistance,[18],[37] especially for isoniazid and streptomycin.[37] Primary resistance to rifampicin and

pyrazinamide has only recently been described.[36] The salient finding in this report was the increase in primary drug resistance to rifampicin in the last period of study (1981-1984) as compared to the previous three study periods (1969-1980). It has also been noted that the prevalence of rifampicin resistance among treatment failure and relapse cases of pulmonary tuberculosis increased significantly. In a recent study rifampicin resistance in Gujarat, India increased from 2.8% in 1980 to 37.3% in 1986. In approximately 95% of the rifampicin resistant strains there was also resistance to isoniazid or streptomycin, or both, resistance to isoniazid was detected in more than 90% of the strains.[37] This resistance to isoniazid in rifampicin resistant strains has been found by other workers,[38] where all the rifampicin resistant strains (55 resistant strains) that were studied were found to be isoniazid resistant.

1.5 RIFAMPICIN

The rifamycins are a group of closely related antibiotics, produced by *Streptomyces mediterranei*.[12] They were originally isolated in 1959 and showed preferential activity against prokaryotes, particularly Gram positive bacteria including *M.tuberculosis*. Of the natural rifamycins, rifamycin B has proved to be most interesting. In aerated aqueous solutions it undergoes inactivation as the result of the formation of a derivative called rifamycin S, which is more active than rifamycin B. During the process rifamycin B is oxidised to rifamycin O, which is in turn hydrolysed to rifamycin S. Rifamycin S can easily be reduced to rifamycin SV. (Fig.2.1) Rifampicin is a semisynthetic, highly active derivative of rifamycin B. It is one of 500 derivatives prepared by Ciba of Basel and Lepetit of Milen in collaboration.[12]

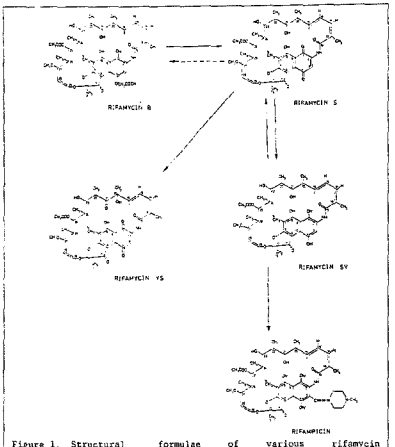

Figure 1. Structural formulae of various rifamycin derivatives. Symbols: —, chemical reactions; ---, biosynthetic pathways.

The rifamycins together with the streptovericins and polypomycins form *an unusual class* of antibiotics. All of them are ansa compounds containing an aromatic ring system which is spanned by an aliphatic bridge. Rifamycins can be described as derivatives of an α-aminonaphthohydroquinone.[14]

Rifampicin is a tasteless reddish-brown crystalline powder that is only very slightly soluble in water but freely soluble in chloroform, ethyl acetate and methenol.[16]

Rifampicin is bactericidal against Gram positive bacteria and *M.tuberculosis*[16,17] and against some Gram negative bacteria including *Neisseria gonorrhoeae*. It has no effect on fungi, but is active at high concentrations against some viruses.[18] There is a variance in minimum inhibitory concentrations with the medium used, but Gram positive organisms are usually inhibited by between 0,002 and 0,5µg/ml, mycobacteria by 0,005 to 2,0µg/ml, and Gram negative organisms by 1,0 to 10,0µg/ml. It has been found that simultaneous administration with other antibiotics may enhance the bactericidal activity of rifampicin. As with other antituberculous compounds resistant mycobacteria rapidly emerge if rifampicin is used alone. However there does not appear to be cross-resistance apart from that between rifampicin and other rifamycins. Resistant gonococci, meningococci and staphylococci have been reported.[15]

Rifampicin is used for the initial treatment or retreatment of pulmonary tuberculosis in association with other antituberculous agents and in the elimination of meningococci from carriers. It has also been used in a variety of conditions caused by other susceptible organisms especially leprosy and gonorrhoeae. The antibiotic has also been shown to have an immunosuppresive action.[19,20]

This drug penetrates polymorphs from patients with chronic granulomatous disease, having a bactericidal effect on the bacteria inside the polymorphs. Used in combination with tetracycline it has a beneficial effect on acne. Other conditions that have been effectively treated with rifampicin include atypical mycobacterial cervical adenitis, bronchitis, brucellosis, endocarditis and gastroenteritis caused by *Salmonella*.[15]

Rifampicin is usually well tolerated. It appears as if the schedule of administration influences the incidence of side effects. Gastrointestinal side effects include nausea, vomiting, diarrhoea and anorexia and have sometimes been severe enough to necessitate withdrawal. This antibiotic produces abnormalities of liver function. Jaundice is usually associated with the simultaneous use of other agents, especially isoniazid, and fatalities have been reported in patients with pre-existing liver disorders or who have taken other potentially hepatotoxic agents.[21] Influenza-like symptoms and febrile reactions have also been reported.[22] Alteration in kidney function and renal failure have occurred and are considered to be due to hypersensitivity.[22,25] Skin rashes,[21,25] urticaria,[21] eosinophilia,[16] leucopenia, [15] thrombocytopenia,[22, 27] purpura, [28] and haemolysis[28] have been reported. Other side effects include confusion, drowziness, weakness, ataxia, dizziness, peripheral neuropathy, blurred vision, transient hearing loss and menstrual irregularities. [15] In a test for carcinogenicity, high doses of rifampicin given to mice were not carcinogenic.[29] Hyperlipidaemia has been reported as a complication of rifampicin [30]

It is important that patients with jaundice should not be given rifampicin and patients with impaired liver function should not have the drug administered in combination with *isoniazid*. It has been found to induce microsomal liver enzymes and to decrease the effectiveness of corticosteroids, coumarin anticoagulants, oral contraceptives and tolbutamide. As a result of administration of rifampicin faeces, saliva, sputum, sweat, tears and urine may be coloured orange-red.

1.6 MECHANISM OF ACTION OF RIFAMPICIN

Rifampicin inhibits the synthesis of cellular RNA in susceptible bacteria while DNA synthesis remains unaffected.[59] It forms relatively stable complexes with RNA polymerase.[61] In studies of the subunits of RNA polymerase from both sensitive strains and resistant mutants, it was found that reconstitution of the insensitive β subunit from resistant strains with α and β' subunits of the sensitive enzyme resulted in a core enzyme which did not interact with the antibiotic. These results indicated that the β subunit is inactivated by the interaction with rifampicin. Analysis of the rifampicin molecule and its interaction with RNA polymerase revealed that changes in the aromatic part of the molecule have little effect whereas modifications of the aliphatic bridge influence the inhibitory potencies. Changes leading to an altered stereochemistry on the ansa ring may impair the fit of the molecule to the acceptor site on the enzyme. With further study it was concluded that the correct shape of the ansa ring and the substituents in positions 20 and 21 (fig.1) seem to be the most important for the potency of the binding enzyme.[62] The nature of the bonds holding together the the rifampicin-enzyme complex is unknown. The binding although tight, is reversible and thus no covalent linkage is involved. The hypothesis is that the oxygens at 1,8,21 and 23 are in some way involved in the formation of hydrogen bonds with specific enzyme functional groups in agreement with known data[14]. The assumption that the side of the molecule where the oxygens at carbons 1,8,21 and 23 lie is involved in the attachment to the enzyme explains very well why modifications at positions 3 and 4, which are on the opposite side of the aromatic ring, almost invariably give active compounds. The nature of the substituents at these positions, however, modulates the antibacterial activity on intact cells, within wide limits. It is generally assumed that transport through bacterial wall and membrane is the main thing influenced by these substitutions. In fact, rifamycins apparently enter bacterial cells by passive diffusion[16] and thus their physiochemical properties,

especially lipophillicity, are expected to be major factors in the ability to cross cell envelopes. In the study of three rifampicin resistant mutants of *Bacillus subtillis* the results suggested that the mutants possess an altered resistant RNA polymerase and that the location of a probable structural gene for this enzyme is close to the resistance markers for the 30s ribosome unit.[43]

The RNA polymerase reaction can be roughly divided into four phases: 1. the binding of the enzyme to the DNA; 2. the initiation phase; 3. the elongation phase, 4. the termination phase. Studies on the individual steps of enzymic RNA synthesis suggest that once chain initiation has been accomplished, RNA synthesis becomes resistant to rifampicin.[44] Rifampicin does not prevent the binding of RNA polymerase to DNA but rather inhibits the binding of purine ribonucleoside triphosphate, which forms the 5'-terminus of the nascent RNA chain. It has been postulated that rifampicin only binds to free enzyme or to a transient enzyme-DNA complex before a conformational change to an activated enzyme-DNA complex has taken place, which causes tight binding of the enzyme to the DNA template and allows RNA chains to be initiated.[45] The investigations on the dependance on rifampicin binding to RNA polymerase on the conformation of the enzyme led Lill and Hartman[46] to assume that during chain elongation the conformation of RNA polymerase is altered in such a way that it is no more accessable to the antibiotic.

1.7 RIFAMPICIN INACTIVATION BY NOCARDIOFORM BACTERIA

R.erythropolis strain ATCC 12674 possesses the ability to overcome the inhibitory effects of rifampicin by inactivating the drug.[47] Other

nocardioforms have been identified as possessing a similar capacity, although to a lesser extent than strain ATCC 12674.[7]

The ability to inactivate rifampicin became apparent when cultures of strain ATCC 12674 had been challenged with rifampicin at a concentration of between 0,05 and 2,0µg/ml. The cultures showed a cessation of growth, but after a lag of 6-8 hours growth resumed. Single colonies isolated from these cultures showed the same level of rifampicin resistance, irrespective of whether they had been challenged with rifampicin or not. With this and the shortness of time before growth resumed after the challenge of the antibiotic, it was suggested that the resumption of growth was unlikely to be the result of the selection of rifampicin resistant mutants. Instead it seemed possible that this organism possessed some sort of inducible resistance mechanism. The possibilities included reduced cell wall permeability, modification of RNA polymerase to avoid inhibition, and conversion of the active drug to the inactive derivative. The last would be distinguishable from the former mechanisms by reduction in rifampicin concentration in cultures of strain ATCC 12674 before resumption of growth.

This was indeed found to be the case, during the log phase rifampicin was inactivated. This suggested that this activity was inducible rather than constitutive. Thus protein synthesis was blocked in a culture of the strain, it was challenged with the antibiotic and the result was that the culture did not act on the rifampicin molecule in manner previously observed. Other experiments ruled out the possibilities of cell wall permeability or a modification in the RNA polymerase mechanism, as being the cause of rifampicin-resistance.

Several other *Rhodococcus* species have been tested for their response to rifampicin. Similar results were obtained, although the rates of inactivation were invariably lower than those found with ATCC 12674. *M.smegmatis* was also tested and behaved in a similar manner to the species

of the genus *Rhodococcus*, although in accordance with the slower growth rate of the organism the time scale was several times greater.

It has been due to the absence of suitable cloning vectors that research in this field has been hampered. However a resistance plasmid has been isolated from *Rhodococcus erythropolis* strain ATCC 12674 carrying genes conferring resistance to sodium arsenate and arsenite, cadmium chloride and chloramphenicol.[8]

The plasmids were large, migrating more slowly than the chromosomal DNA in agarose gels. These plasmids have been engineered to be smaller and with further development will function as cloning vectors, greatly enhancing the quality of research into these organisms.

1.8 THE AIMS OF THIS PROJECT

At present the components of the mechanism of inducible rifampicin inactivation (iri) have not been established. The purpose of this work is to develop the techniques that will be required in an attempt to clone the gene(s) involved in this system. The approach I decided to adopt was to attempt to isolate mutants which had lost the ability to inactivate rifampicin, since the presence of the inducible inactivation system greatly increases the effective level of resistance to this antibiotic. It is anticipated that mutants which had lost the ability to inactivate rifampicin would reveal the phenotype of increased sensitivity to the antibiotic. Attempts to clone the gene(s) involved would therefore be pursued on the basis of restoration of wild type levels of resistance. Thus the primary aim of this work were to produce hyper-sensitive mutants for complementation; to attempt to charactorise these mutants; to in-

crease the efficiency of transformation of *plasmid* DNA both into *E.coli*, for the construction of a library, and into nocardioform bacteria so that ultimately restoration of inducible rifampicin inactivation mechanism will be achieved.

2.0 MATERIALS AND METHODS

2.1 STRAINS

Both the *nocardioform strains*, *Rhodococcus erythropolis* ATCC 12674 and ATCC 4277, were obtained originally from N.Ferreira. CW 22 was obtained from E.Dabbs as a mutant of strain ATCC 12674 that had been "cured" of *resistance* to sodium arsenate and sodium arsenite, cadmium chloride and chloramphenicol. *Arthrobacter oxydans* strain C1 was obtained by screening contaminants for high sensitivity to rifampicin; it was identified by the DSM, the German Collection of Microorganisms. *Escherichia coli* strain NM 294 was obtained from E.Dabbs; it lacks the *E.coli* DNA restriction system. A λ lysogen of this strain was developed by E.Dabbs for this work.

Resistant mutants made from these strains in the course of this work had an additional number "1" added to the designated number. They are CW 22-1 which is streptomycin resistant, ATCC 4277-11 which is acrylamide resistant, and NM 294-1 which is rifampicin resistant. The rifampicin hypersensitive mutants were assigned with the designation Rif * where * was the number of the mutant.

2.2 MUTAGENESIS

2.2.1 N-METHYL-N'-NITRO-N-NITROSO-GUANIDINE MUTAGENESIS IN BUFFER

Mutagenesis was carried out using two differing sets of buffer conditions. Strain CW 22 was grown to exponential growth phase in 5ml of T2 medium containing 1% tryptone (Oxoid,U.K.), 0,5% yeast extract (Oxiod,U.K.), 3mM sodium chloride, 3mM calcium chloride and 10mM magnesium chloride. A 1ml aliquot of the cells was pelleted and resuspended in 0,9ml of one of two buffers, Tris-HCl pH 8,0 or Tris-acid maleate-sodium hydroxide pH 4.8, and 0,1ml of a 1mg/ml stock solution of N-methyl-N'-nitro-N-nitroso-guanidine (NTG) dissolved in the same buffer, yielding a final concentration of 100µg/ml NTG. The cells were incubated for two hours at 37°C after which they were washed in the original buffer. The mutagenised cells were outgrown in T2 media at 28°C. The cells were sonicated for 5s using an MSE ultrasonic power unit with a medium tip, to reduce clumping before being diluted to 10^{-6} in sterile distilled water. 0,1ml of the 10^{-6} dilution was spread onto TYA plates containing 1% tryptone, 0.5% yeast extract and 1.5% bacteriological agar (Merck,Darmstadt) to obtain single colonies. The single colonies obtained were patched onto respective media plates to obtain the various mutants as well as auxotrophs.

2.2.2 N-METHYL-N'-NITRO-N-NITROSO-GUANIDINE MUTAGENESIS OF A GROWING CULTURE.

Bacteria of the strain CW 22 were grown in T2 medium for 3 hours before NTG was added to a final concentration of 100µg/ml. The cells were grown for a further 2 hours. The cells were washed in the aforementioned Tris-HCl pH 8,0 buffer. When these had grown up in T2 medium they were diluted to single colonies and plated as before.

2.3 SELECTION OF MUTANTS HYPERSENSITIVE TO RIFAMPICIN.

All the single colonies from the mutagenesis experiment that grew up were patched onto TYA plates containing 0,1µg/ml rifampicin (Boehringer-Mannheim,West Germany) as well as plain TYA plate and a minimal media plate (see Appendix 1). These were incubated for several days at 28°C. Where no growth occurred on the rifampicin place but growth occurred on the TYA plate, cells were streaked to single colonies before a single colony was taken for replica plating onto TYA plates containing 0,1µg/ml, 0,2µg/ml and 0,3µg/ml rifampicin as well as a TYA plate. Those colonies that grew up on the TYA plate but not on the rifampicin containing plates were considered to be hypersensitive mutants.

Cultures of CW 22 and its derivatives were grown up and spread on TYA plates containing 100µg/ml rifampicin. Rifampicin resistant mutants grew up and were streaked on a rifampicin plate to purify.

2.4 RIFAMPICIN INACTIVATION ASSAYS.

The ability of the different mutants to inactivate rifampicin was assayed using two related techniques. Both the techniques began in the same way. A preculture of each strain was grown in TY medium (1% tryptone and 0,5% yeast extract) for 24 hours or until it reached stationary phase. An aliquot of 200µl of this preculture was inoculated into 5ml of fresh TY medium and an optical density (OD) reading taken. These cultures were allowed to grow for 4 hours at which stage they had more than doubled their OD and were in exponential phase. The OD was determined at 540nm. The ODs were adjusted by removing some of the culture and replacing it with the same volume of TY medium so that each culture had the same OD. At this stage the antibiotic was added. For hypersensitive strains 2,0µg/ml rifampicin was added and for the resistant strains 20µg/ml rifampicin was added. The time when the antibiotic was added was taken as time zero and assays were carried out at various time intervals after that. It is here where two different methods were employed.

2.4.1 RIFAMPICIN INACTIVATION ASSAY USING ASSAY PLATES.

A streptomycin resistant derivative of *A.oxydans*, C1S, was used as an assay organism, as it is sensitive to rifampicin within the required range of concentrations. A 100µl aliquot of this organism which had been grown in TY medium was spread on a plate containing 1% tryptone, 0,5% yeast extract, 0,5% bacteriological agar (this is less than the usual concentration allowing for more even diffusion of the rifampicin) and 200ug/ml streptomycin. Wells were cut into the agar with the blunt end of a pasteur pipette. These wells were each filled with 60µl of culture and the plates

incubated for two days at 28°C when the zones of inhibition became apparent.

2.4.2 QUANTITATIVE RIFAMPICIN INACTIVATION ASSAY.

At the time of the assay 1ml of the culture was harvested, the OD determined at 590nm and the 1ml aliquot pelleted in a Hagar microfuge. The supernatants were removed and stored at -20°C until an assay using aliquots from each time interval could be undertaken. The supernatants were then thawed and each added to a different test-tube containing 4ml TY medium with 200ug/ml streptomycin and a 1 in 500 dilution of C1S. A set of standards were also organised in the same way containing known concentrations of rifampicin in place of supernatant. The turbidity of the cultures was measured by determining the OD at 590nm over the next three days. The OD was determined using a Spectronic 601 spectrophotometer. A standard curve was constructed of the OD of strain C1S versus rifampicin standards and the concentrations of rifampicin in the supernatants of the experimental strains was determined by reading off the curve.

2.5 PRELIMINARY STUDY OF SUPERNATANT PROTEINS

The parental strain ATCC 12674 was grown in T2 medium. An aliquot was taken from the preculture and inoculated into 30ml of T2 medium. When the culture was in exponential phase the antibiotic was added at a concen-

tration of 1µg/ml. At time intervals after that of 24 hours, 48 hours and 72 hours, 10ml aliquots were pelleted at 15 000 rpm for 15 minutes in a Beckman J2-21 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 750 µl 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.

Separating gels containing 10% acrylamide and stacking gels containing 6% acrylamide were prepared from a stock solution of 30% acrylamide and 0,8% N,N'-bis- methylene acrylamide which had been filtered. The final concentrations in the separating gel were as follows: 0,375M Tris pH 8,8 and 0,1% sodium dodecyl sulphate (SDS). The final concentrations in the stacking gels were 0,126M Tris pH 6.8 and 0,1% SDS. The gels were polymerised by the addition of 0,035% by volume of N,N,N',N' tetramethyl ethylene diamine (TEMED) and 0,025N ammonium persulphate. Ten centimetre separating gels were poured of thickness 1,5mm with a stacking gel of 2,5cm. The electrode buffer contained 0,025M Tris, 0,192M glycine and 0,1% SDS. The sample loading buffer was made up of 0,0625M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% B-mercaptoethanol and 0,001% bromophenol blue (Saarchem, South Africa) as a dye. The sample loading buffer was added as an equal volume to the sample. 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 in a 5:1:4 solution of methanol : acetic acid : distilled water.

2.6 DNA EXTRACTION

2.6.1 EXTRACTION OF PLASMID DNA FROM *E.COLI*

An overnight culture of strain MM 294 containing pDA 27 (*see fig. 7*) the putative shuttle vector formed from pECOR 251 and the nocardioform plasmid pDA 22 was grown in 250ml of Luria broth (LB) containing 1% tryptone, 0,5% yeast extract and 0,5% sodium chloride as well as the antibiotic ampicillin with a final concentration of 50µg/ml. The stationary phase culture was pelleted at 6 000 rpm for 10 minutes. The pellet was resuspended in 10ml of cold TE (10mM Tris, 10mM EDTA, pH 8,0) and pelleted as before. The cells were then resuspended in 2ml of a cold 25% sucrose, 50mM Tris-HCl pH 8,0 solution to which 0,25ml of a fresh lysozyme solution (10mg/ml) lysozyme was added and swirled gently on ice for 15 minutes. Afterwards 0,25ml of 0,5M EDTA pH 8,0 was added and swirled for a further 5 minutes on ice. To promote lysis 2,5ml of a cold detergent solution containing 0,2% Triton x 100 (Sigma, St. Louis, MO), 62,5mM EDTA pH 8,0 was added and swirled for an additional 10 minutes until partial clearing was achieved. This was centrifuged at 18 000 rpm for 45 minutes before the supernatant was decanted and the volume measured. Caesium chloride was added at 0,95g/ml of supernatant. Ethidium bromide (Boehringer-Mannheim, West Germany) in a 10mg/ml solution in water was added at 0,07ml per ml of cleared lysate. The refractive index, measured on a refractometer (Atago Co. Japan), was adjusted to 1,392 before loading the sample into Beckman quickseal ultracentrifuge tubes. These samples were centrifuged for 16 hours at 40 000 rpm in a V.Ti 65,2 vertical rotor in a Beckman L7-55 ultracentrifuge. Plasmid bands were visualised under UV light and extracted using a syringe. The ethidium bromide was removed by mixing with TE saturated with butanol in equal volumes (1:1). The plasmid DNA was diluted three fold with TE and stored at -20°C.

2.6.2 PREPARATION OF CHROMOSOMAL DNA FROM NOCARDIOFORMS

Organisms were grown for 2-3 days in 250ml of TYG medium (TY medium with 1,5% glycine). Harvesting of the organisms was achieved by centrifugation at 6 000 rpm for 15 minutes. Cells were resuspended in 10ml of 10mM Tris pH 8,0, 10% sucrose to which 50-100mg of lysozyme in 5ml of Tris-sucrose was added. This was incubated at 37°C for 2 hours. The cells were pelleted at 12 000 rpm for 15 minutes and resuspended in 8ml of TE with 1-2mg proteinase K (Boehringer-Mannheim) dissolved in it. To this was added 0,8ml TE containing 10% SDS or as much as was needed to achieve partial clearing. Following a 2 hour incubation at 37°C the lysate was centrifuged in a 50Ti rotor in a Beckman L5-50 ultracentrifuge at 35 000 rpm for 30 minutes. DNA was decanted off and 1g/ml caesium chloride was added and dissolved. A clearing centrifugation was performed at 18 000 rpm for 15 minutes and the DNA carefully poured out from under the scum. Ethidium bromide was added and the refractive index adjusted to 1,390 before the Beckman quickseal ultracentrifuge tubes were loaded, sealed and placed into the V.Ti 65,2 vertical rotor and centrifuged for 14 hours at 45 000 rpm. After extracting the chromosomal band, the DNA was decolourised using a 1:1 solution of TE : butanol solution. The DNA was dialysed against TE for a minimum of 4 hours whereafter the concentration of DNA was determined by spectrophotometric measurement at 260nm (OD_{260} of 1,0 = 50ug/ml dsDNA).

2.6.3 EXTRACTION OF PLASMID DNA FROM NOCARDIOFORMS

This was performed in a similar way to the extraction of chromosomal DNA except that the refractive index was adjusted to 1,392 and the process continued as before.

2.7 DNA CUTTING AND LIGATIONS

Both chromosomal DNA and shuttle vector DNA which had been stored at -80°C in TE was ethanol precipitated, dried and resuspended in Medium buffer (Boehringer-Mannheim, West Germany). Chromosomal DNA was digested with 0,09 units of Bgl II (Boehringer-Mannheim, West Germany) per microgram of DNA for 3 hours at 37°C while shuttle vector DNA was digested with 9 units of Bgl II per microgram of DNA for 1,5 hours at 37°C. The digested DNA was treated with TE-saturated [illegible] and chloroform : isoamyl alcoholed (24:1) to remove the restriction enzyme before [illegible] ug precipitated, dried and resuspended in ligation buffer (20mM Tris, [illegible] mM magnesium chloride, 10mM dithiothreitol and [illegible]). In each ligation 0,15µg of Bgl II digested shuttle vector was [illegible] to multiple of 0,17µg of chromosomal DNA extracted from [illegible]. This was made up to a volume of 50µl with ligation buffer, to [illegible] unit in 1 µl of T4 ligase (Boerhinger-Mannheim, West Germany [illegible]). The ligation mixture was then incubated at 14°C overnight.

2.8 TRANSFORMATIONS

2.8.1 TRANSFORMATION OF DNA INTO E.Coli

100ml of LB was inoculated with 1ml of an overnight culture derivative of NM 294-1. The cells were grown with vigorous shaking at 37°C to a density of approximately 5 x 10^7 cells/ml or an OD reading at 540nm of 0,2 (this usually took 2 hours). The culture was chilled on ice for 10 minutes before being centrifuged at 6 000 rpm for 10 minutes at 4°C in a precooled rotor. The cells were resuspended in half the original culture volume of an ice-cold, sterile solution of 50mM calcium chloride and 10mM Tris-HCl pH 8,0. This cell suspension was placed in an ice water bath for 15 minutes and then centrifuged at 6 000 rpm for 10 minutes at 4°C. The supernatant was discarded and the cells resuspended in 1/15 of the original volume of the Tris-HCl calcium chloride solution. This was dispersed into 0,2ml aliquots into pre-chilled eppendorf tubes where it was kept for an hour on ice. The DNA in ligation buffer or TE was added and gently mixed in with the cells whereafter it was transfered to a water bath preheated to 42°C for one minute. One millilitre of LB was added to each tube and incubated for one hour without shaking. The cells were then pelleted and the supernatant discarded. The cells were resuspended in the residual LB and then spread extremely gently onto Luria agar plates (LB + 1% agar) containing 60ug/ml ampicillin. These plates were placed at 37°C for no longer than 18 hours before the colonies were counted. The colonies were washed off the plate with LB containing 60ug/ml ampicillin and stored in a container at -80°C.

2.8.2 TRANSFORMATION OF PLASMID DNA INTO NOCARDIOFORM BACTERIA

A preculture of CM 22 was grown in TYG medium from which 1ml was taken and inoculated into 100ml of TYG medium. When the culture reached a post exponential prestationary phase it was divided into 1ml aliquots and frozen at -80°C until it was needed. The cells were thawed slowly by placing at 4°C. They were pallated and resuspended in 1ml of P buffer (See Appendix 1) with 5mg of lysozyme dissolved in it. This was incubated at 37°C for one hour without agitation. The cells were then washed with 1ml of P buffer and gen resuspended in 0,5ml of P buffer. The cells were dispensed into 75µl aliquots to which 0,1µg of plasmid pDA 30 was added. A 50% weight per volume solution of Polyethylene glycol 1000 in P buffer was added (80µl) mixed and left for 4-5 minutes. Before being gently spread on protoplast regeneration plates (0,33% sodium chloride, 1% tryptone, 0,5% yeast extract, 12,5% sucrose and 1% agar) and incubated for 5-10 days at 28°C. Eight hours after the spreading of the plates 0,5ml of 3M arsenate (Searchem, SA) 0,5M arsenite (BDH Chemicals, Poole, UK) which had been filter sterilized was underlaid. After diffusion in the 25ml of plate medium this gave final concentrations of 60mM arsenate and 10 mM arsenite.

2.9 PLASMID SCREENS

2.9.1 PREPARATION OF PLASMID FROM E.COLI

Single colonies were inoculated into 5ml of LB containing 60µg/ml ampicillin and incubated overnight at 28°C with aeration. 1ml of overnight culture was pelleted in a microfuge and cells resuspended in 100µl of solution 1 (50mM Tris pH 8,0, 10mM EDTA). This remained at room temperature for 5 minutes before 200µl of solution 2 (0,2N sodium hydroxide, 1% SDS) was added and gently mixed. This stood on ice for 5 minutes whereafter 150µl of a precooled solution 3 (5M potassium acetate pH 8,0) was gently mixed in. After 5 minutes on ice this mixture was centrifuged for 1 minute and the supernatant removed to a sterile eppendorf to which an equal volume of isopropanol was added. This was left to stand at room temperature for 5 minutes. ' was precipitated by centrifuging for 5 minutes. The DNA was washed with cold 96% ethanol by spinning for 5 minutes. The pellet was dried and resuspended in 12µl TE containing 1mg/ml ribonuclease (Boehringer-Mannheim, West Germany). To this 2µl of sample loading buffer (0,25% bromophenol blue, 40% (W/V) sucrose) was added to be loaded on an agarose gel. Agarose gels were prepared using Seachem HGT agarose at 0,4% weight for volume in TBE buffer (0,089M Trisborate, 0,089M Boric acid and 0,002mM EDTA pH 8,0) and 0,6µg/ml ethidium bromide. These gels were set in the gel apparatus of the Biorad Minisubmarine Agarose Gel Unit. The electrode buffer used was TBE made from a 5x stock with 0,075µg/ml ethidium bromide added. Electrophosis was carried out at a constant voltage of 80V until the tracking dye reached the end of the gel (1,5-2 hours) before the bands were visualised by placing the gel on an Ultraviolet Products transilluminator model HE 33. Photographs were taken using a camera with Polaroid E655 film. To determine the size of the inserts in the plasmids this procedure was followed through. The difference

came in the resuspending of the DNA however. The DNA was resuspended in 15µl of a stock solution of 175µl sterile water, 10µl 10x medium buffer, 5µl Bgl II and 1µl ribonuclease. This was left to resuspend and digest at 37°C overnight before it was loaded with sample loading buffer on a gel.

2.9.2 PREPARATION OF PLASMID FROM NOCARDIOFORMS

Strains were grown in TY medium supplemented with 1,5% glycine. Aliquots of 1ml were then resuspended in the same volume of 0,5M sucrose, 100mM Tris, pH 8.0, to which 5mg of lysozyme had been added, and incubated for 1 hour at 37°C with shaking. They were then pelleted and resuspended in 280µl of TE, after which 4,0µl of 10% SDS in the same buffer was added. Mixing with gentle inversion. After 15 minutes at room temperature, 40µl of 4,5M sodium acetate, pH 6.0 was added, and mixed in the same manner. Samples were placed on ice for 1 hour then centrifuged in a microfuge for 20 minutes. The supernatant was extracted once with phenol and once with 24:1 chloroform : isoamyl alcohol, then the nucleic acid was ethanol precipitated. After air drying it was redissolved in 20µl of TE buffer containing 1mg/ml ribonuclease. Mixed with loading buffer, samples were analysed by electrophoresis at 80V in 0,4% agarose gels buffered with TBE.

3.0 RESULTS

With the aim of cloning the gene(s) of the novel, inducible rifampicin inactivating mechanism (iri) it has been necessary to prepare basis of this work. This included making the mutants that will be necessary for cloning by complementation, studying their inactivation as well as optimising the conditions for an acceptable level of transformation efficiency.

3.1 THE PRODUCTION OF MUTANTS.

Mutants are not produced in nocardioform bacteria as readily as they are in organisms, such as *Escherichia coli*. It has been shown that auxotrophs can be produced in *E.coli* at a frequency of 60-80% after NTG mutagenesis (E.Dabbs, personal communication). However in this work, the frequency of auxotroph production in nocardioform bacteria was approximately 2%. It had been still lower but various steps were taken to increase the efficiency. The difference in mutagenic efficieny could be due to the absence of an error prone repair mechanism in *nocardioforms*, and also because these bacteria are *mycelial* and form syncytia. Thus colonies that grow up after mutagenesis may not be colonies of bacteria possessing a single mutation but colonies containing several mutant bacteria.

To reduce the clumping the following steps were taken: the TY medium was supplemented with 3mM sodium chloride, 3mM calcium chloride and 10mM

magnesium chloride, as it was found that this increased the colony forming units per OD reading by between 10 and 50 fold (E.Dabbs, personal communication). In addition to the change in medium some cultures were sonicated for 5s with a medium tip. The pH of the buffers for NTG mutagenesis was altered as this might affect the permeability of the cell wall as a result of different ionisation at differing pH. A pH of 4,8 is most effective for the mutagenesis of Gram negative becteria particularly *E.coli*. It is possible that a pH of 8,0 would be more effective against actinomycetes.[7]

From the NTG mutagenesis undertaken, 14 mutants were found to be hypersensitive to rifampicin at 0,1ug/ml. The first nine mutants were isolated from CW 22 while the following five were isolated from the streptomycin resistant derivative of CW 22, CW 22-1. Eight mutants, Rif 1 to Rif 8, had been isolated prior to the commencement of thus study by E.Leobs. I isolated mutants Rif 9 to Rif 14 after screening 1189 colonies. The conditions under which these mutants were formed is tabulated below.

Mutant	Parent Strain	pH of NTG	Growth Phase of Culture
Rif 9	CW 22	8,0	
Rif 10	CW 22-1	8,0	logarithmic
Rif 11	CW 22-1	8,0	
Rif 12	CW 22-1	4,8	
Rif 13	CW 22-1	8,0	logarithmic
Rif 14	CW 22-1	8,0	stationary

Table 1.1 Conditions that resulted in the production of rifampicin hypersensitive mutants.

Approximately 1000 colonies from the mutagenesis of a related strain *R.equi* ATCC 14887 were screened but no hypersensitive mutants were found.

3.2 RIFAMPICIN INACTIVATION ASSAYS

The first assays that were done, used the plate assay method. In this way it could be easily seen if the culture had inactivated the antibiotic or not, by the presence or absense of the zones of inhibition. Originally these tests were carried out using the hypersensitive mutants of CW 22 and CW 22-1 and the sensitive strains as controls. It was important that the assays were testing the action of the *inducible rifampicin inactivation mechanism and* not that mechanism in addition to the effects of cell wall permeability and other intrinsic factors. For this reason the

rifampicin resistant mutants were made and assayed, so inactivation of the antibiotic would be the result of the inducible inactivation mechanism.

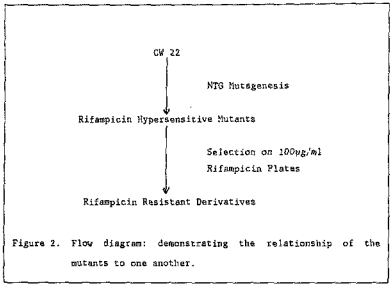

Figure 2. Flow diagram: demonstrating the relationship of the mutants to one another.

Each of the mutants which had been isolated was assayed in this way. The assay organism that was used was a streptomycin resistant derivative of *Arthrobacter oxydans*, C15. The purpose of using a streptomycin resistant derivative was to decrease the possibility of contamination with the advantage that the supernatants did not need to be filter sterilised because CW 22 and its derivatives are streptomycin sensitive. *A.oxydans* was chosen as it showed sensitivity to rifampicin across the range of concentrations of the antibiotic required for this study. The streptomycin resistant derivative of CW 22, CW 22-1 was proposed as a candidate assay organism but was found to be ineffective across the concentration range required.

	6 h	22 h	46 h	74 h
CW 22	*			
Rif 1	*	*		
Rif 2	*			
Rif 3	*			
Rif 4	*			
Rif 5	*	*		
Rif 6	*			
Rif 7	*	*	*	*
Rif 8	*	*	*	*

Table 2.1 A representative rifampicin inactivation assay of rifampicin resistant derivatives of CW 22 and mutants hypersensitive to rifampicin. The * indicates the presence of rifampicin in the supernatants of the cultures. Rifampicin was added to an *initial* concentration of 20µg/ml.

It became apparent that three of the mutants were not inactivating the antibiotic, namely Rif 7, Rif 8 and Rif 14. Four of the mutants namely Rif 1, Rif 5, Rif 6 and Rif 9 were found to inactivate *rifampicin more* slowly than the *parental strain* CW 22. The other mutants seemed to inactivate the antibiotic at a similar rate to the parental strain.

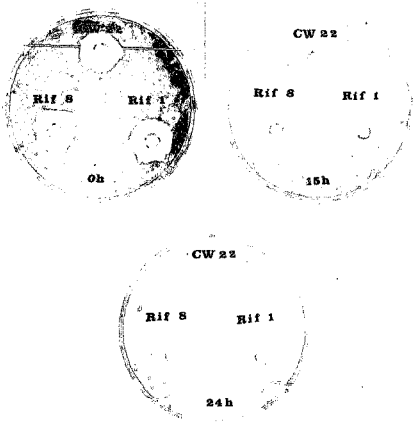

Figure 3. Rifampicin inactivation revealed by zones of inhibition: Rifampicin remaining in the cultures of CW 22, Rif 1 and Rif 8, assayed by zones of inhibition on a lawn of strain C1S. At 0, 15 and 24 hours after addition of 20µg/ml of the drug, 60µl aliquots were removed and placed in wells of a TYA plate containing 200µg/ml streptomycin.

To ascertain if the rate of inactivation varied with *different* rifampicin resistant mutants of a *particular rifampicin* hypersensitive strain, *rifampicin inactivation assays* were carried out with several different colonies of rifampicin resistant mutants of each rifampicin hypersensitive strain.

	16 h	20 h	24 h	40 h
CW 22-1	*			
Rif 13	*			
Rif 13	*			
Rif 13	*			
Rif 14	*	*	*	*
Rif 14	*	*	*	*
Rif 14	*	*	*	*

Table 2.2 A representative rifampicin inactivation assay of rifampicin resistant mutants, where several colonies of each mutant were *assayed* to determine whether the *inactivation pattern was a* function of the colony or of *the mutant itself*. In each case the * indicates the continued presense of *rifampicin* in the supernatants of the cultures revealed by zones o f inhibition.

It was difficult to determine the concentration of *rifampicin in* the supernatants of the cultures using the *plate method as* the standard curves produced, when *varying* known concentrations of rifampicin were placed in the wells, were very steep and would show great inaccuracy in concentration determination. For this reason a liquid assay was developed to quantitatively determine the residual rifampicin concentration in supernatants. This assay also used *A.oxydans*, CIS. It was decided that one mutant from each putative class of *hypersensitive mutants i.e.* one

that did not inactivate rifampicin, one that seemed to inactivate the antibiotic slower than the parental strain and the parental strain itself would be used in the new assay. As a result CW 22, Rif 1 and Rif 8 were used.

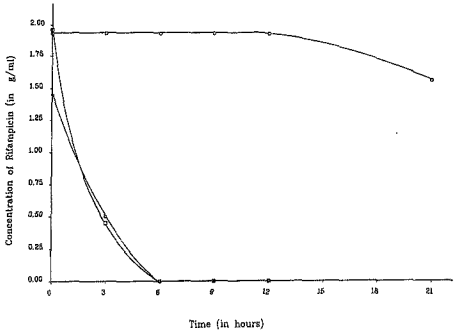

Figure 4. A quantitative rifampicin inactivation assay: Assay of rifampicin hypersensitive mutants of CW 22 and CW 22. Concentration of residual rifampicin remaining in the supernatants of CW 22 (□), Rif 1 (◇) and Rif 8 (○) cultures after 0, 3, 6, 9, 12 and 21 hours. A one tenth dilution of a late logarithmic phase culture was resuspended (at T=0) in fresh TY medium. After observation of increase in turbidity of culture, rifampicin was added to a final concentration of [illegible] g/ml. Cultures incubated at 28°C with agitation and the supernatants assayed using the liquid assay technique.

It is clear from the data presented that both CW 22 and Rif 1 had totally inactivated the rifampicin within in 6 hours of the challenge while Rif 8 had not inactivated the rifampicin.

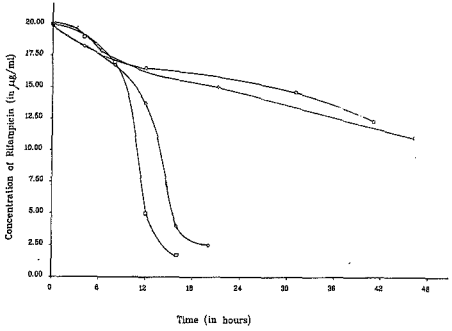

Figure 5. A quantitative rifampicin inactivation assay: Assay of rifampicin resistant derivatives of rifampicin hypersensitive mutants of CW 22 and CW 22. Concentration of residual rifampicin remaining in the supernatants of the cultures of rifampicin resistant derivatives of CW 22 (□), Rif 1 (◇), Rif 8 (○) and a cell free control (◁). A one tenth dilution of a late logarithmic phase culture was resuspended (at T=0) in fresh TY medium. After the observation of the increase on turbidity of culture, rifampicin was added to a final concentration of 20µg/ml. Cultures incubated at 28°C with agitation and the supernatants assayed using the new liquid assay technique.

From the data presented above it appears that Rif 1 does indeed inactivate the antibiotic at a rate slower than CW 22 and that Rif 8 does not inactivate rifampicin. A cell free control was used to determine what effect the media had on rifampicin over a period of time, when this control curve is compared with the curve for Rif 8 there appears to be little difference.

Rif 8 is a candidate mutant to be used as a recipient in the transformation of DNA from a genomic library. The DNA of the genomic library should be of a strain closely related to CW 22. It needs to be slightly different, to prevent reintegration of the library into the host chromosome through recombination. The library DNA should come from a strain that inactivates the drug in the same way as CW 22. Thus the gene for inducible rifampicin inactivation when it is transformed into Rif 8 will restore its ability to inactivate rifampicin. It was found that ATCC 4277 was able to inactivate rifampicin. An acrylamide resistant mutant of ATCC 4277, ATCC 4277-1[1, had been made (B.Gowan) so these two strains were compared for rifampicin inactivation ability.

	15 h	19 h	23 h	40 h
ATCC 4277	☆	☆	☆	
ATCC 4277-1[1	☆	☆	☆	

Table 2.3 A comparison of rifampicin inactivation between ATCC 4277 and an acrylamide resistant derivative, ATCC 4277-1[1. In each case the continued presence of rifampicin in the supernatant is indicated by a ☆ in the table and a zone of inhibition on the plate.

As there appears to be no difference between the ability of ATCC 4277 and its acrylamide resistant derivative to inactivate rifampicin, the

acrylamide resistant derivative was used to construct a genomic library in *E.coli*.

3.3 PRELIMINARY STUDY OF SUPERNATANT PROTEINS

The supernatant proteins from cultures of ATCC 12674 exposed to rifampicin, were extracted by ammonium sulphate precipitation as cell free inactivation of rifampicin had been previously shown. In this step the volume in which the protein was dissolved was reduced from 10ml to 0,5ml. To concentrate the proteins still further the protein that had been resuspended in 0,5ml of water after ammonium sulphate precipitation was lyophilised and resuspended in 60µl of water. Of this 15µl was loaded on polyacrylamide gels. Studies were done varying the times of harvest of the supernatant after addition of the antibiotic. Cultures were harvested after 24, 48 and 72 hours, each with a control to which no antibiotic had been added. Concentrations of rifampicin ranging between 0 and 2µg/ml were added to different cultures and then harvested at the same time. Unfortunately these did not offer a clear result. It did appear however that the bacterial cells could have lysed when treated with 2µg/ml rifampicin for 48 hours. It also appeared as if the rifampicin inactivating protein could have been released into the supernatant after 48 hours.

3.4 THE CLONING VECTOR

A new cloning vector was developed for this work by A. Daffey (unpublished). The vector was constructed from two plasmids, one originating in *E.coli* and the other in *R erythropolis* ATCC 12674. The purpose was to produce a shuttle vector so that cloning could be performed in an *E.coli* background and the cloned DNA transferred to nocardioform background.

The *E.coli* plasmid that was used was the suicide vector pECOR 251. This plasmid is 3346 base pairs large and contains the gene for ampicillin resistance and the gene encoding the restriction endonuclease Eco RI. The *E.coli* origin of replication allows it to replicate in *E.coli*. In the Eco RI gene is a unique Bgl II site. If the suicide vector is cleaved with Bgl II and exogenous DNA is ligated into the site it prevents the Eco RI gene being transcribed. If no exogenous DNA is introduced and the vector self ligates, the Eco RI gene is transcribed and the endonuclease cleaves the host's DNA, killing the host. The Eco RI gene is under the control of a lambda repressor so the plasmid can be maintained in a strain containing a lambda lysogen.

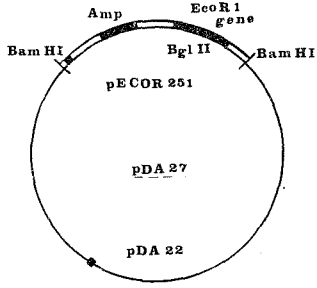

Figure 6. Diagramatic representation of the shuttle vector pDA 27: Amp=Gene for ampicillin resistance; (■)=Origin of replication

The nocardioform plasmid used was pDA 22. This plasmid is approximately 10,5kb in size and contains an origin of replication, genes conferring resistance to both arsenate and arsenite and a single Bam HI site. The shuttle vector was formed by joining pECOR 251 and pDA 22 in their single Bam HI sites and has been named pDA 27.

Spontaneous nocardioform mutants have been obtained which are resistant to either arsenate or arsenite, but not to both. Thus these bacteria transformed with pDA 22 and grown up on selection media containing both arsenate and arsenite would prove to be transformants and not resistant mutants.

3.5 THE CHROMOSOMAL DNA IN THE GENE LIBRARY

The chromosomal DNA used for creating the library was extracted from an acrylamide resistant derivative of *ATCC 4277*, *ATCC 4277-1j1*, which had also been shown to inactivate rifampicin (Table 2.3). This was DNA digested with Bgl II. There are other restriction endonucleases that can be used that will produce cohesive ends having the same consecutive base sequence as Bgl II. These are Bam HI, Bcl I and Sau 3A. These enzymes do not have the same recognition sequence. The recognition sequences are similar in respect to the central four bases but differ at the outer bases. This is the case with Bgl II, Bam HI and Bcl I. Sau 3A is a four base pair cutter and cleaves DNA more often than the six base pair cutters, as the frequency of the recognition sequence is higher. Ligation of DNA cleaved with two different enzymes yielding compatable cohesive ends would occur but the restriction endonuclease recognition sequence would be eliminated. Bgl II was used to cleave both pDA 27 and chromosomal DNA so that the recognition sequence could be maintained. In this way digestion of the construct with Bgl II and subsequent agarose gel electrophoresis would enable the determination of the insert size.

The chromosomal DNA was only partially digested as there may be a Bgl II site in the gene of interest. By usually partially digested DNA, such a site would only be cleaved part of the time. To determine the amount of Bgl II to use, digestions of chromosomal DNA was done with serial dilutions of Bgl II.

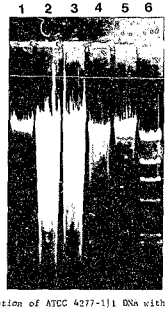

Figure 7. *The digestion of ATCC 4277-1|1 DNA with serial dilutions of Bgl II: 1µg DNA was digested with 9; 0,9; 0,09 and 0,009 units of Bgl II for 3 hours at 37°C (lanes 2-5). 1µg DNA was not digested (lane 1) and lambda II DNA molecular weight markers (lane 6) were loaded to observe the manner of separation of the gel.*

From the above data it was decided to use 0,09 units per microgram of DNA for 3 hours at 37°C.

3.6 TRANSFORMATION INTO E.COLI

In an attempt to increase the efficiency of the calcium chloride transformation of Maniatis *et al*[88] I varied the parameters involved. These included the length of time calcium chloride treated cells were left on ice, the length of time for which the competent cells were heatshocked and the ratio of chromosomal DNA to vector DNA that provided the most transformants. For the first six conditions a lambda lysogen of MM 294 (Dabbs, unpublished) was transformed with the suicide vector of pECOR 251.

Initially a difference was made of heatshocking competent cells at 42°C for 0; 1,5 and 3 minutes. This was simultaneously compared with competent cells that had had DNA added at time=zero and competent cells that had been incubated in a calcium chloride solution on ice for one hour before the DNA was added. It became apparent that heatshock at 42°C and incubation on ice are necessary for increased transformation efficiency. The cells were transformed with 1µg of DNA.

	Heatshock (min)		
Incubation (h)	0	1,5	3,0
0	31	47	29
1	54	132	136

Table 3.1 A comparison of transformation efficiency for cells incubated in the calcium chloride solution for one hour and for those which had not been incubated, varying the time of heatshock between 0 and 3 minutes. The numbers in the table indicate the number of colonies that grew up on Luria Agar plates containing 30µg/ml ampicillin after 16 hours at 37°C.

It had been mentioned by other authors[18] that it was beneficial to incubate competent cells on ice for 24 hours. This was compared with incubation for 1 hour and for 2 hours on ice, with heatshocking at time intervals varying 0 and 3 minutes.

	Heatshock (min)			
Incubation (h)	0	1	2	3
1	39	176	77	46
2	31	66	14	33
24	4	26	13	24

Table 3.2 A comparison of two conditions varied in a calcium chloride transformation of MM 294 with pECOR 251. The one condition was the varied time that the calcium chloride treated cells sat on ice, namely 1, 2 and 24 hours. The other condition was the varied time of heatshock at 42°C of 0, 1, 2 or 3 minutes of the competent cells after the plasmid had been

added. The numbers in the table indicate the number of colonies that grew up on *Luria agar containing* 30µg/ml ampicillin after 16 hours at 37°C.

It is clear from these results that a one hour incubation on ice with a one minute heatshock *yielded the* highest transformation efficiency. The transformations that followed were performed using this new set of conditions.

3.7 LIGATION OF pDA 27 DNA AND ATCC 4277-1|1 DNA

An attempt was made to optimise the ratio of the shuttle vector pDA 27 cons.ructed by A. Deffey (unpublished) and ATCC 4277-1|1 DNA. The competent cells were transformed *with shuttle vector* that contained no chromosomal DNA and ratios of 1:1, 1:2, 1:4 and 1:8 shuttle vector to chromosomal DNA.

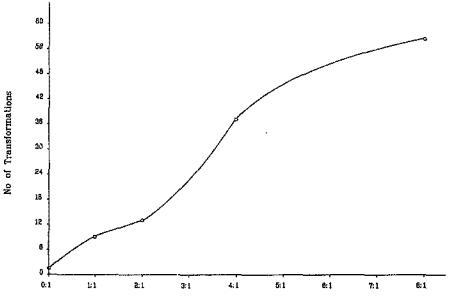

Figure 8. Assay of ligation and transformation efficiency: These results were obtained at increasing ratios of ATCC 4277-1|1 DNA to pDA 27 DNA. In each transformation 0,3µg pDA 27 DNA ligated with 0; 0.3; 0,6; 1,2 and 2,4µg ATCC 4277-1|1 DNA was transformed into MM 294 cells that had been incubated in a calcium chloride solution for 1 hour on ice and heatshocked for 1 min at 42°C. Phenotypic expression occurred by incubation in LB for 1 hour at 37°C. Cells were pelleted and spread on LA plates containing 60µg/ml ampicillin and incubated at 37° for 16 hours.

It can be seen that the ratio of chromosomal to shuttle vector DNA of 8:1 yielded the most transformants. In a subsequent experiment comparing ratios of 8:1 and 16:1, it was found that an 8:1 ratio yielded more transformants than a 16:1 ratio. The experiment was done in triplicate and the 8:1 ratio plates yielded on average 162 transformants while the 16:1 ratio plates yielded an average of 128 transformants.

Plasmid screens were done to determine whether the colonies that grew up contained plasmids with inserts. Plasmid DNA was digested with BglII. The DNA was electrophoresed on 0,4% agarose along with λ II DNA molecular weight markers. The insert sizes were found to be 6,92 on average. A genome size for *R.erythropolis* could not be found so calculations were based on the size of *M.tuberculosis* ($3x10^6$ Daltons),[51] 2344 clones would be needed to yield a probability of 95% that any particular gene will be present in the library. However it appears as if only approximately one third of the transformants have detectable inserts. Thus a library of at least 7032 would be ideal. A library was constructed using the conditions that had been optimised. The library size is at present 3552 clones.

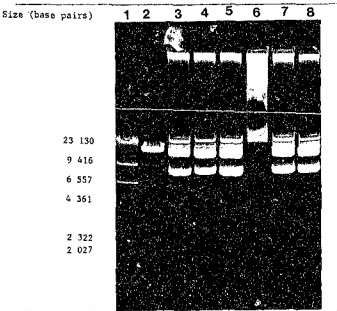

Figure 9. Plasmid screen of NM 294-1 cells transformed with pDA 27: DNA containing Bgl II digested ATCC 4277-III inserts Lambda II DNA molecular weight markers (lane 1) show the manner of separation of the gel. Purified shuttle vector (lane 2), DNA extracted from transformants (lanes 3-8).

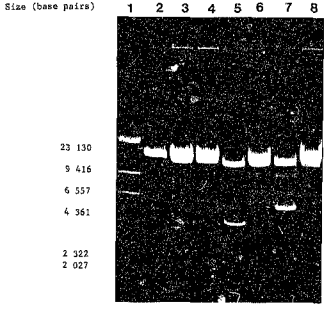

Figure 10. Plasmid screen to determine insert sizes of ATCC 4277-1|1: 0,3µg Shuttle vector having been digested with Bgl II was ligated with 0,3µg of Bgl II partially digested ATCC 4277-1|1 DNA. To excise the inserts the plasmids were extracted from the transformants and digested with 12 units Bgl II (lanes 3-8). Lambda II DNA molecular weight markers (lane 1) for size determination (for MW sizes see Appendix 1). Shuttle vector digested with Bgl II (lane 2) a control with no insert.

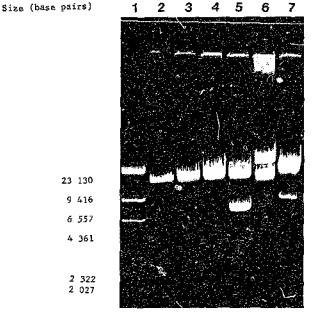

Figure 11. Plasmid screen to determine insert sizes of ATCC 4277-1|1: 0,3µg Shuttle vector having been digested with Bgl II was ligated to 0,3µg (lanes 3, 4) and 2,4µg (lanes 5-7) Bgl II digested ATCC 4277-1|1 DNA and transformed into MM 294-1. The DNA from the transformances was digested with 12 units Bgl II to determine insert size. Lambda II DNA molecular weight markers (lane 1) for size determination (for MW sizes see *Appendix 1*) and purified shuttle vector digested with Bgl II (lane 2) is a no insert control.

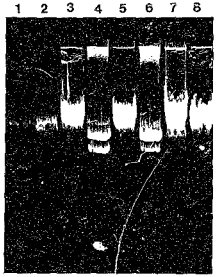

Figure 12. Plasmid screen to determine insert sizes of ATCC 6277-1[1: 0.3µg shuttle vector having been digested with Bgl II was ligated to 2.4µg ATCC 6277-1[1 DNA and transformed into *competent* MM 294-1 cells. The plasmids extracted were digested with Bgl II to determine the insert size. *Lambda II* DNA molecular weight markers (lane 1) (for MW size see Appendix 1) used in si: rmination. Shuttle vector digested with Bgl II (l no insert control. Plasmids digested with Bgl II (3-8).

3.8 TRANSFORMATION INTO STRAIN CW 22

The efficiency of the nocardioform transformation is very low in comparison to that of *E.coli* so several parameters were varied to achieve greater transformation frequency. The recipient strain that was used was CW 22 and the transforming plasmid pDA 30. The plasmid pDA 30 is a derivative of the plasmid originally isolated from ATCC 12674. The parameters that were varied include the growth phase of the bacteria, the presence and absence of agitation of the cells during the lysozyme treatment, the time of the arsenate/arsenite underlay, the concentration of the arsenite, the number of protoplasts and the amount of DNA for transformation.

It was found that agitation of the cells for one hour at 37°C in the presence of 1mg/ml of lysozyme in P buffer was detrimental to the protoplasts as fewer transformants grew up on regeneration plates than on the regeneration plates spread with protoplasts that had not been agitated but had stood at 37°C for 1 hour. Samples had been taken at different stages in the growth of the organism and transformations were performed with these samples. This study was done in collaboration with A. Daffey and H. Golob.

		Agitation	
Time (h)	OD(540nm)	Yes	No
??	1.11	5	7
30	2,76	0	13
28	5,16	4	6
46	3,37	2	10

Table 4.1 Transformation of CW 22 protoplasts *with* 0,1µg pDA 30 DNA with and without agitation of the lysozyme *incubation step*, incubation 1 hour at 37°C. Result taken after 5 *days incubation of* protoplast regeneration plates at 28°C. The numbers *in the* last two columns indicate the number of transformants.

A post-*exponential*, pre-stationary phase culture was found to yield the *greatest* number of transformants. A bulk culture of 100ml of CW 22 was *inoculated* with 1ml of preculture. This was grown for 14 hours at which time twenty 1ml aliquots were pipetted into eppendorf tubes and stored at -80°C. This was repeated every eight hours to 46 hours. One tube of protoplasts from each sampling was thawed slowly at 4°C and transformations performed on protoplasts from different stages of the growth phase. This study was done in collaboration with A. Daffey and H. Golob. The following results were obtained.

Time (h)	OD at 540nm	No. Transformants
14	0,82	0
22	1,94	5
30	3,33	0
38	3,89	20
46	4,15	9

Table 4.2 Transformation of CW 22 protoplasts with 0,1ug pDA 30 at different stages in the growth phase of recipient cells. Results taken after 5 days incubation of protoplast regeneration plates at 28°C.

Arsenite has been found to be approximately ten times as toxic as arsenate so it was decided to vary the concentration of arsenite, from a final concentration of 10mM to a final concentration of 20mM, to observe if there was any difference in the number of transformants that grew up. Simultaneously the time at which the arsenate/arsenite (final concentration of 60mM arsenate) solution was underlayed was varied to find the time that yielded the maximum number of colonies. The arsenate/arsenite solution is underlayed to allow time for cell wall re-formation from protoplasts and for phenotypic expression of resistance genes.

	Time of underlay (hours)				
Arsenite	4	8	12	16	20
10mM	4	3	4	1	1
20mM	3	3	3	1	0

Table 4.3 Transformation of CW 22 protoplasts with 0,1µg pMA 30 varying the time and concentration arsenate/arsenite underlay. The protoplast regeneration plates were underlayed with 0,5ml of a solution containing 3M arsenate and either 0,5M arsenite or 1,0M arsenite at times 4, 8, 12, 16 and 20 hours. Result taken after 5 days incubation of protoplast regeneration plates at 28°C. The numbers in the table indicate the number of transformants.

There appeared to be no difference in the numbers of transformants with the concentrations of arsenite used. It did however appear as if underlays after 12 hours were less effective. The regeneration plates were incubated for a further 5 days as other colonies grew up after 10 days. Colonies from both phases of this biphasic growth were found to be transformants when patched on TYA plates containing 60mM arsenate and 10mM arsenite.

	Time of underlay (h)		
Arsenite	4	8	12
10mM	30	76	33
20mM	47	35	66

Table 4.4 The increased number of transformants obtained from the protoplast regeneration plates of Table 4.1 after a further 5 days of incubation at 28°C is indicated in the table.

Reflecting on the results in Table 4.4 it was decided that a stock solution of 3M arsenate and 0,5 arsenite would be used to underlay regeneration plates giving a final concentration of 60mM arsenate and 10mM arsenite. It was also apparent that the optimal time for underlay was between 8 and 12 hours after spreading the protoplasts on regeneration plates.

Previously 75µl of protoplasts from the stock had been used for each transformation. To examine if this was an optimal number of protoplasts different volumes were aliquoted and made up to a standard volume of 100µl with P buffer. To each 0,1µg pBA 30 was added. The number of transformants using 75µl of cells was significantly more than with other volumes after 5 days.

µl Protoplasts	12	25	50	75	100
No. Transformants	4	11	10	28	13

Table 4.5 The number of transformants of GW 22 produced when 0,1µg of pDA 30 was transformed into vc umes of cells varying as 12, 25, 50, 75 and 100µl and incubated at 28°C for 5 days is indicated in the table.

The regeneration plates were incubated for a further 5 days to allow expression of the biphasic transformants. There was a very great increase in the number of transformants. These were checked by patching on 60mM arsenate 10mM arsenite plates and all were found to be transformants.

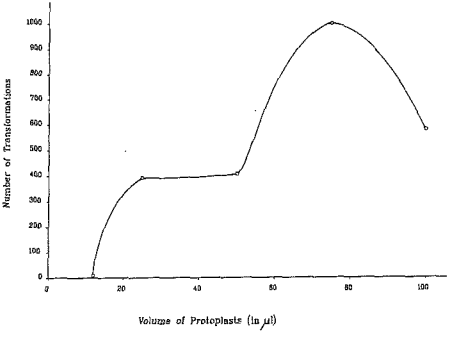

Figure 13. Transformation varying protoplasts: 0,1µg pDA 30 DNA was transformed into different volumes, 12, 25, 50, 75 and 100µl of *Cw* 22 protoplasts and the number of transformants assayed by determination of the number of colonies on the protoplast regeneration plates underlayed with 0,5 ml of 3M arsenate, 0,5M arsenite after 8 hours, after incubation at 28°C for 10 days.

As the amount of DNA can also be varied, it was decided that differing quantities of DNA would be added to a constant volume of cells. Thus to 50µl of cells from 0,0 to 0,2µg of DNA was added. After 5 days of incubation it appeared as if 0,1µg of DNA was an optimal amount.

DNA in µg	0,0	0,05	0,1	0,15	0,2
No. Transformants	4	7	23	15	16

Table 4.6 Transformation of pDA 30 DNA in varying amounts of 0; 0,05; 0,1; 0,15 and 0,2µg was introduced into constant volumes of 50µl of recipient CW 22 protoplasts. The number of transformants is determined by the number of colonies on protoplast regeneration plates after 5 days at 28°C.

To allow for the biphasic colonies to grow up the regeneration plates were incubated for a total of 10 days. The transformants that grew up in the latter 5 days were tested and found to be true transformants, both through patching on 60mM arsenate 10mM arsenite plates and by a plasmid screen (Fig.14). The transformation efficiency obtained here is 15 000 transformants per microgram of plasmid DNA (Fig 13). This is a dramatic increase resulting from the optimising of conditions.

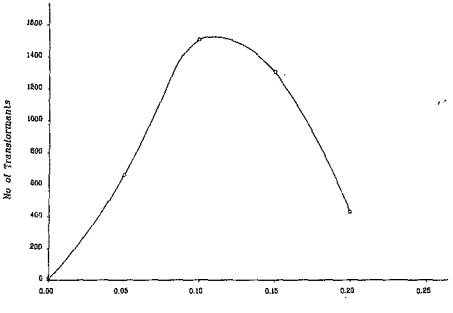

Figure 14. Transformation varying DNA concentration: The volume of recipient CW 22 protoplasts remained constant at at 50µl. The amount of pDA 30 DNA added was 0; 0,05; 0,1; 0,15 and 0.2µg. The number of transformants was determined by the number of colonies on the protoplast regeneration plates underlayed with 0,5ml of 3M arsenate, 0,5M arsenite after 8 hours, after incubation for 10 days.

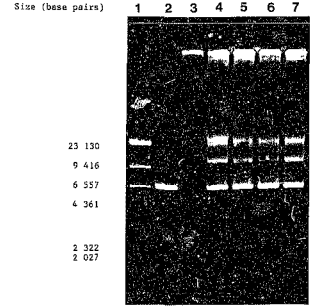

Figure 15. Plasmid screen of CW 22 transformed cells: Transformants selected from plates (results recorded in Fig.14) were subjected to extraction of their DNA. Lambda II DNA molecular weight markers (lane 1) show the manner of separation of the gel. The purified plasmid is in lane 2. DNA of CW 22 that was not transformed is in lane 3. CW 22 transformed with 0,1µg DNA is lanes 4 and 5. CW 22 transformed with 0,15µg DNA in lanes 6 and 7.

The efficiency of the transformation has been increased to approximately 15 000 transformants per microgram DNA from approximately 25 transformants per microgram. A genomic library of ATCC 4277-1|1 DNA is being produced. Rifampicin hypersensitive mutants of CW 22 that will be used as recipients for cloning the gene(s) for the inducible rifampicin inactivation mechanism have been made. Thus conditions seem to have been optimised for gene cloning.

4.0 DISCUSSION

Nocardioform bacteria have not been the subject of such intense research as have organisms, such as *E.coli* and *B.subtilis* . Numerous very clear experimental protocols for *E.coli* and *B.subtilis* exist, but not nearly as many for work with nocardioform bacteria. This has necessitated the optimisation of conditions for many of the undertakings of this work.

Mutagenesis of *E.coli* has been very clearly defined and optimised specifically in respect to concentrations of mutagens with respective pH and temperature considerations.[42] Much of the work on nocardioform bacteria has been based on the conditions used with *Streptomyces* by Hopwood *et al* (1985).[43] NTG has been found to cause cluster lesions in DNA in various organisms and very often to induce additional unwanted mutations. This effect is not well documented in *Streptomyces*. The effect of a chemical mutagen such as NTG depends on a complex interaction between time and conditions of treatment, with respect to pH, temperature and concentration of the mutagen. Hopwood *et al*[43] used a pH of 8,0 for mutagenesis with NTG.

Work done in our laboratory confirms that for nocardioforms a pH 8,0 is preferable to pH 4,8 and that mutagenesis conducted on a growing culture in exponential phase yields a higher proportion of auxotrophs (H. Golob and B. Gowan).

CW 22 is sensitive to rifampicin, that is, it has a sensitive β subunit *of RNA polymerase (rpoβ+) and the inducible rifampicin* inactivation mechanism (iri+). The rifampicin resistant derivative of CW 22 is rpoβ- and iri+. The hypersensitive mutants that were produced, for example Rif

7 and Rif 8 are rpoβ+ and iri-. These were assayed for their ability to inactivate rifampicin. Then rifampicin resistant derivatives of these hypersensitive mutants which are rpoβ- and iri- were assayed for their ability to inactivate the antibiotic.

The purpose of using these mutants for cloning by complementation would be to restore the iri function to an iri- mutant.

Cultures needed to be inoculated from a fresh preculture in order to obtain reproducible results. It was also necessary to standardise the OD of the cultures at the time of the antibiotic challenge to within 0.01 of each other in order to obtain meaningful comparisons. Several of the mutants were excluded from further study in this work. Rif 2 showed a much greater tendency to clump than the wild type or any other of the mutants and Rif 5 proved to be an very slow grower.

The assaying for rifampicin inactivation using the liquid technique was found to be far more reproducible and accurate than the plate assay and also allowed for easier quantitation of the residual rifampicin concentration in the supernatants of the cultures.

Although numerous assays were carried out only some representative assays have been included in this dissertation. Each mutant was assayed several times to obtain reproducible results and then several (3 or 4) rifampicin resistant derivatives were assayed from each mutant.

The problem encountered with the study of the supernatant proteins was not found in the isolation of the proteins, but rather in the preparation of the polyacrylamide gel. These gels were not fractionating optimally as even the molecular weight markers were not showing sufficiently sharp bands. This continued to occur even after new solutions had been made and filtered and advice sought from staff and students who perform polyacrylamide gel electrophoresis regularly.

Bulk plasmid DNA preparations were carried out using standard techniques for *E.coli*. The plasmids extracted were pECOR 251 which was used in the optimisation of conditions for transformation into *E.coli* and for construction of the shuttle vector pDA 27 which was also extracted in the same way. The chromosomal DNA of the nocardioform bacteria was extracted using a method modified by E.Dabbs. This protocol included the growth of bacteria in the presence of glycine and incubation with proteinase K, primarily because of the cell wall structure of nocardioforms. The glycine interferes with the synthesis of gly-gly cross-links, weakening the cell wall, and proteinase K enzymatically digests the cell walls components. Ribonuclease was added to decrease DNA contamination with RNA.

Both chromosomal donor DNA and vector DNA had been digested with Bgl II and ligated with T4 ligase for transformation into MM 294. In ds DNA, T4 ligase catalyses the repair of single strand nicks with adjacent 3' hydroxyl and 5' phosphoryl termini. As a consequence the enzyme will *catalyse the joining or ligation of vector and donor DNA fragments with* complementary staggered or blunt ends to form recombinant DNA molecules. The efficiency of a ligation reaction depends greatly on the concentration of DNA termini in the reaction mixture. The termini may undergo inter- or intra-molecular ligation to produce oligomeric linear or circular ligation products respectively. The relative yields of these structurally different products depends on the total concentration of DNA termini. For this reason I assayed the ligation efficiency varying the quantity of chromosomal DNA and keeping the quantity of vector DNA constant. It was found that when 8 times as much chromosomal DNA was added to vector DNA the greatest number of transformants grew up.

The calcium chloride treatment generally used with *E.coli* permits one to obtain competent cells which yield over 10^6 transformants per microgram of pBR 322 DNA[56]. A more complex procedure involving treatment of cells with rubidium, in addition to calcium chloride, has been reported,[56] in

which over 10^7 transformants were obtained per microgram of DNA. The method used in this work was a modification of Maniatis *et al* (1982)[54]. The modifications that were made in direct variance with the findings of other workers. Cells were found to be most competent after a one hour incubation on ice in a calcium chloride solution, this contrasts the findings of Dagert and Ehrlich (1979)[56] who found *E.coli* cells to have improved competence after 24 hours on ice. My findings showed that heatshock at 42°C increased the transformation efficiency whereas some other authors[59] have found that transformation efficiency decreased with heatshock.

Several authors have investigated the parameters influencing the transformation efficiency in Streptomycetes[59,66] Of special importance are the growth phase of the mycelium at the time of protoplasting, the temperature at which the mycelium and regenerating protoplasts are incubated, the number of protoplasts used per transformation, the PEG concentration, the dryness of the regeneration plates and of course the composition of the medium.

In this work I tested several parameters. Initially it was important to discover the best stage in the growth phase for transformation. This was established to be a prestationary-postexponential stage. Once this had been recognised a bulk preparation of aliquots of cells for protoplasting was performed and the cells stored at -80°C. These same cells were used in all subsequent experiments. The conditions originally used were a modification by E. Dabbs of the method of Bibb *et al* (1978)[61]. It became apparent that no agitation of the cells during incubation with lysozyme was an improvement on the technique. The time allowed for phenotypic expression of the arsenate/arsenite resistance conferred by the plasmid pDA 22 was also varied. It was found that a period of 16 hours or longer was not useful as few transformants grew up, but that a time between 8 and 12 hours maximized the protoplast regeneration efficiency. Changing the concentration of the arsenite did not change the transformation ef-

ficiency. In determining the relative concentration of DNA to protoplasts an optimal proportion of 0,1µg of pOA 22 DNA to 50µl of cells was determined. This produced an efficiency of 15 000 transformants per microgram of DNA. This result was achieved after prolonged incubation of the protoplast regeneration plates where a biphasic production of transformants became apparent.

Several other experiments were conducted simultaneously with this work. One was an attempt to amplify a stretch of DNA that spanned resistance determinants for rifampicin, fusidic acid and erythromycin (conducted in collaboration with H. Golob), another involved an attempt to isolate phages from soil to other related nocardioform bacteria, in the hope that these phages would be of use in phage typing and in the development of cloning vectors. However no phages were found highly specific to the bacteria used in this study.

This work has been to further the frontiers of the molecular biology of nocardioforms. It has in effect provided a springboard for this work to be carried through into my Ph.D. It is hoped that this work will ultimately lead to greater knowledge of rifampicin resistance in *Mycobacterium tuberculosis* and from there to a greater ability to combat this disease that causes so much death and destruction not only in South Africa but world wide.

5.0 APPENDIX

5.1 ANTIBIOTIC SOLUTIONS

Rifampicin was made up in methanol in stock solutions of 10, 1,0 and 0,1ug/ml.

Streptomycin was dissolved in water and filter sterilised. It was stored as a stock solution of 100ug/ml.

Fusidic acid was dissolved in ethanol at a concentration of 10ug/ml.

Erythromycin was dissolved in ethanol at a concentration of 10ug/ml.

5.2 MINIMAL MEDIA

A-N Buffer Stock:	final concentration in g/l
458,5g $K_2HPO_4.3H_2O$	9,17
134,0g NH_4PO_4	3,0
25,0g Tri-sodiumacetate.$2H_2O$	0,5
5,0g $MgSO_4.7H_2O$	0,1

-dissolve in 5l distilled water(*pH 7,0-7,1*) - *do not* autoclave

-keep sterile by the addition of 50ml chloroform

100ml A-N stock		500ml water
1g NH_4Cl	+	15g *agar*
400ml water		5g glucose

-*autoclave* separately then mix

-add supplimants before panning

5.3 *P* BUFFER

Make up the following basal solution:

Sucrose	103,00g
K_2SO_4	0,25g
$MgCl_2.6H_2O$	2,02g
Distilled water to	800 ml
TES buffer(5,73% p.7 ?)	80 ml

Dispense in 80ml aliquots and autoclave

Before use, add to each flask:

KH_2PO_4(0.5%)	1 ml
$CaCl_2.2H_2O$(3,68%)	10 ml

5.4 LAMBDA II MOLECULAR WEIGHT MARKER

Band on gel	Size (base pairs)
*	23 130
*	9 416
*	6 557
*	4 361
*	2 322
*	2 027

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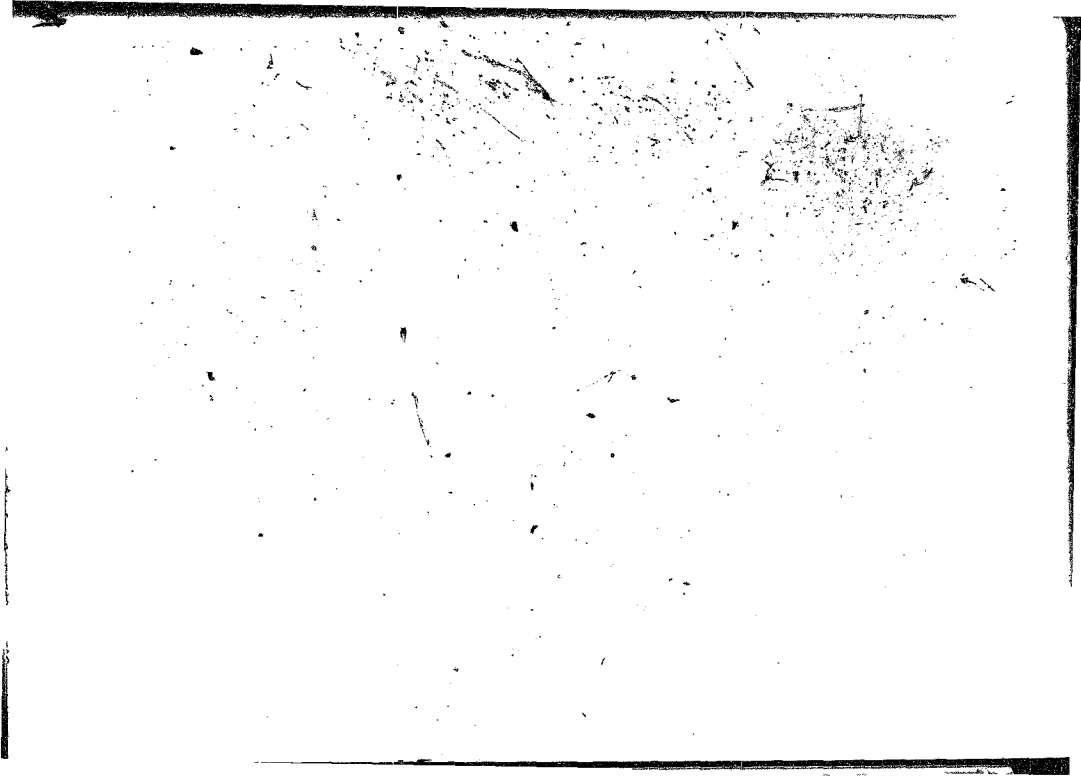

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