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INVESTIGATING THE GENES FOR BILE ACID METABOLISM IN NOCARDIOFORM BACTERIA

SHARON TERESA BROWN

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in partial fulfilment of the requirements of the Degree of Master of Science in the field of Biotechnology.

FEBRUARY 1991

To all peacemakers, may their hopes be realized,
and their dreams become reality.

DECLARATION

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



ST Brown

28th day of February 1991

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr Eric Dabbs, for his advice and guidance throughout the course of this study.

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1.0 ABSTRACT

Nocardioform bacteria were studied for their ability to interconvert bile acids. From the studies of utilisation and resistance curves, the most suitable donor and recipient strains for complementary gene cloning, were Arthrobacter oxydans strain C1 and Rhodococcus erythropolis strain ATCC 4277-1 respectively. Sodium deoxycholate was used as the selective bile acid. A sodium deoxycholate and sodium taurocholate hypersensitive recipient was created by NTG mutagenesis, and designated Rhodococcus erythropolis strain 4277-1.063. A donor strain, Arthrobacter oxydans C1.070 was created by spontaneous mutation, and was able to utilise high concentrations of the bile acids. The genomic library of Arthrobacter oxydans C1.070 was built up in Escherichia coli, using the shuttle vector plasmid pDA37. The inserts in plasmid pDA37 were confirmed to be of the genomic library, by nonradioactive DNA labelling and detection. Due to complementary gene cloning, Rhodococcus erythropolis 4277- 1.063 was enabled to utilise 20mg/ml of sodium deoxycholate.

Further studies would still have to be performed to verify that the ability to utilise the sodium deoxycholate was due to gene cloning. Elucidation of the metabolic pathway could aid in the nocardioform interconversion of bile acids becoming industrially applicable.

2.0 INTRODUCTION

Steroids are a diversified class of oxygenated tetracyclic isoprenoid derivatives, characterised by a cyclopentanoperhydrophenonthrene ring system. The sterols e.g. cholesterol, and bile acids (derivatives of cholesterol) are the two most important groups of steroids. Steroids are vital to eukaryotic life, as they are the precursors which are essential for membrane stability and cell growth. Bile salts are essential for lipid digestion and absorption. Sterols are crystalline alcohols containing 27-30 carbon atoms. They possess a 3 β-hydroxy group and an endocyclic double bond, usually in the 5,6 position, and have a side chain which exhibits various degrees of branching and unsaturation, as diagrammised in Figure 1.

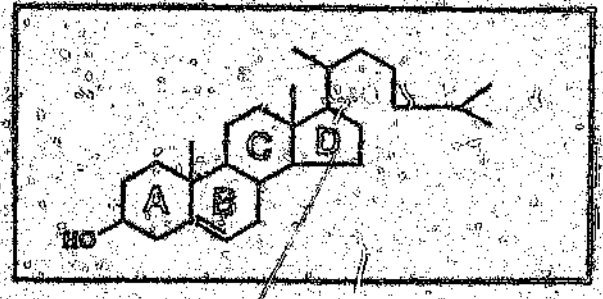


Figure 1. Diagrammatic representation of cholesterol.

Bile acids have 24-28 carbon atoms, the nucleus of which contains hydroxylic substituents and

part or all of the side chain of 5 β-cholestane (Whiting, 1986). They contain hydroxyl groups substituted at positions C3, C7 and C12 of the steroid nucleus. The three major bile acids are cholic acid, chenodeoxycholic acid and deoxycholic acid, as diagrammised in Figure 2.

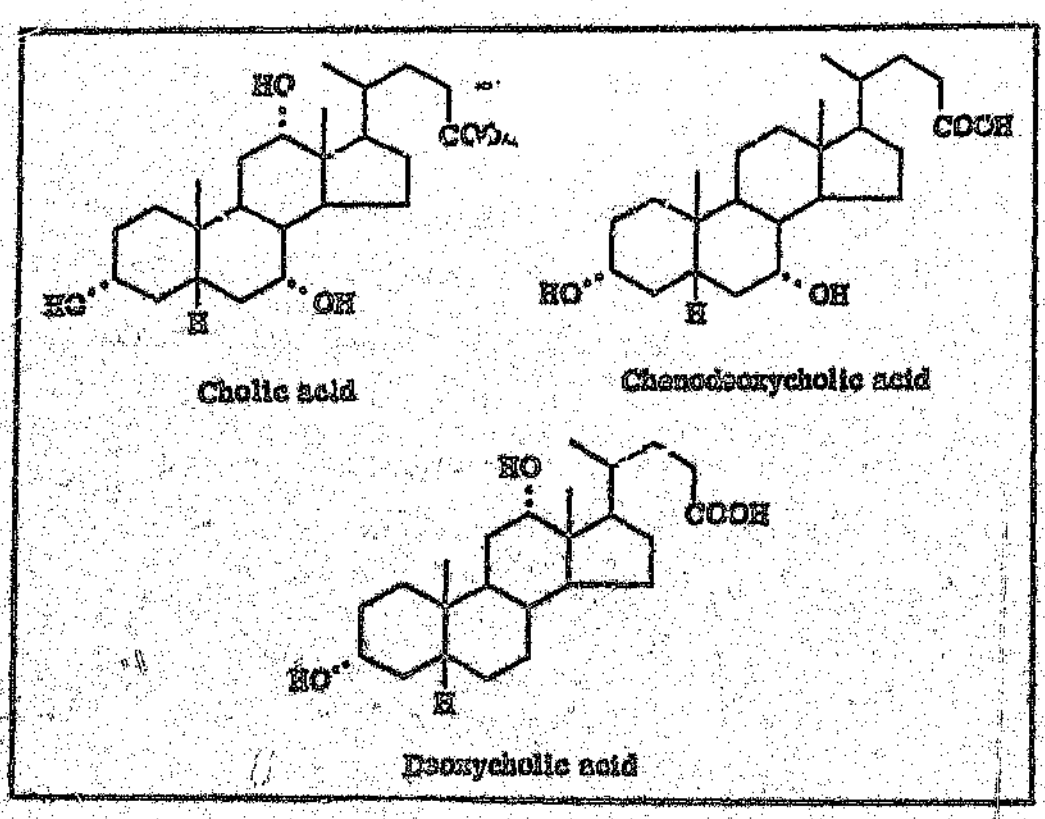


Figure 2. Diagrammatic representation of some bile acids

Whiting, M.J (1986). Bile Acids. Ad. Clin. Chem. 25:169-232.

Bile acids in humans, are usually enzymatically conjugated with either glycine or taurine, to make six major bile acids, namely the glycine and

chenodeoxycholic acid and deoxycholic acid.

In principle, two methods can be employed for the synthesis and production of pharmacologically active steroids. Namely, total synthesis by chemical methods or partial synthesis from naturally occurring steroids, which already contain the cyclopentanoperhydrophenanthrene structure. Since the discovery that the side chain of various abundant, naturally occurring steroids such as cholesterol (wool grease), β-sitosterol or campesterol can be degraded selectively by microorganisms, this conversion method has attracted much attention and several useful processes have been developed. Selective side chain cleavage of sterols for the synthesis of C19 or C22 steroids and the partial degradation of the steroid ring system to hexahydroindan propionic acid derivatives, can be used for the preparation of steroids with unnatural configuration.

2.1 MICROBIAL STEROL METABOLISM.

Degradation of cholesterol by microorganisms has been a well known phenomenon in natural circumstances. Data (Arima et al, 1969) suggests that cholesterol decomposing microorganisms in

that cholesterol decomposing microorganisms in nature exist not only in Mycobacterium and Nocardia, as was formally discovered, but are widely distributed among actinomycetes and bacteria. Mycobacterium and Nocardia are quite peculiar, in that a number of cholesterol decomposing strains belong to these genera. In other genera, decomposing strains appear rather incidentally and there seems no apparent correlation between the cholesterol decomposing activity of microorganisms and their taxonomical characteristics.

For the removal of a saturated aliphatic C17 side chain by chemical methods, the newly developed technique of remote oxidation is most promising (Schoemer and Martin, 1980). As an alternative to chemical methods, the microbial removal of the aliphatic side chain of phytosterols is most promising. The steroid ring structure and the side chain are metabolised by different mechanisms. These enzymatic reactions do not follow a given order, but occur simultaneously and independently. Thus, if the structure of the side chain is modified so that the enzymes normally involved in the degradation are unable to catalyse the fission, the ring system will be attacked resulting in accumulation of metabolites

with partly oxidised ring structures. On the other hand, in substrates with a modified ring structure blocking the first enzymic reactions in the ring oxidation, the side chain will be degraded, resulting in the formation of 17-ketosteroids.

2.2 DEGRADATION PATHWAY OF THE STEROL SIDE CHAIN

The pathway of the C17 sterol side chain degradation during the microbial conversion of cholesterol to 17-ketosteroid, was elucidated by Sih et al. (1967, 1968). In contrast to mammalian systems where 17-ketosteroids are formed via cleavage of the C20-C22 bond, followed by cleavage of the C17-C20 bond, microorganisms shorten the side chain of cholesterol by a mechanism similar to the β-oxidation of fatty acids. By the successive removal of 3C, 2C and again 3C compounds, microbial degradation results in a C19-steroid formation. This process is diagrammised in Figure 3 (Martin, 1984). Under ordinary conditions, both the steroid nucleus and the hydrocarbon side chain are attacked simultaneously in a series of reactions which eventually lead to carbon dioxide and water.

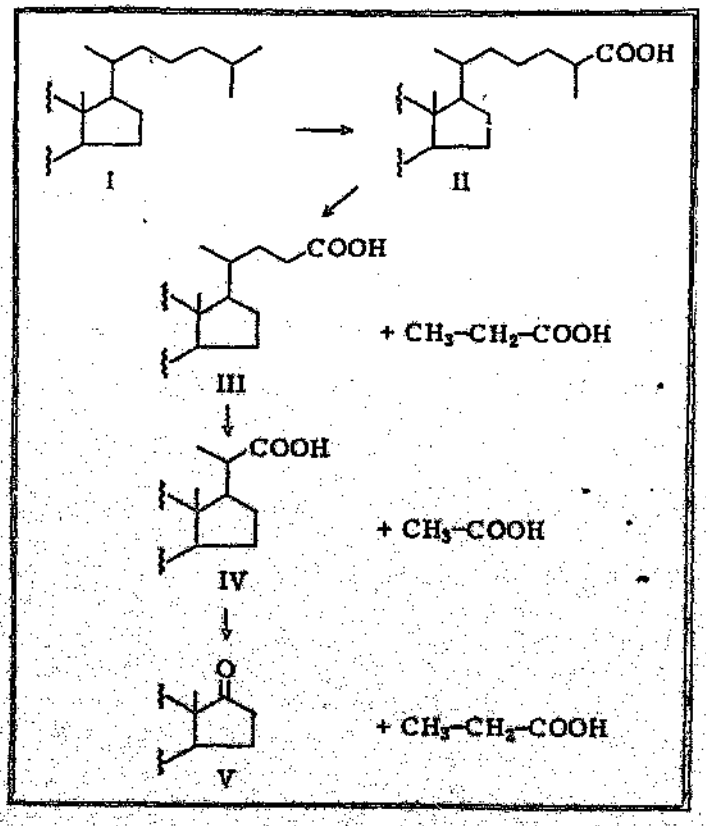


Figure 3. Microbial side chain cleavage of cholesterol.

Martin, C.K.A. (1984) Sterols. Ch.3. In: Biotechnology Vol.6a (Eds.) Rehm, H.J. and Reed, G. (Vol. Ed.) Kieslich, K.. Verlag Chemie, Weinheim.

By interfering with either Δ1-(2)-dehydrogenation or 9-(α)-hydroxylation normal metabolic reactions responsible for the rupture of the steroid nucleus, (the C9 - C10 bond) it is possible to remove the cholesterol side chain selectively. This has been accomplished by exposure of oxygenated derivatives of cholesterol, to microorganisms of the genera Nocardia, Arthrobacter, Mycobacterium and Corynebacterium

(Sih and Wang, 1965 ; Sih et al., 1965) or by the use of metabolic inhibitors such as α,α'-dipyridyl and δ-phenanthroline, which apparently inhibit the 9α -hydroxylase selectively.

Following the C27 -hydroxylation and oxidation of the resulting alcohol to a C27 carboxylic acid, propionic acid, acetic acid and again propionic acid are removed, resulting in the formation of C24 and C22 carboxylic acids as intermediates, and finally in the formation of the C17-keto compounds.

Therefore, this last step involves dehydrogenation and addition of water, followed by aldolytic cleavage to yield the 17-keto function and propionic acid. Phytosterols branched at C24 are degraded via 24-oxo intermediates.

The various abundant naturally occurring sterols, including cholesterol and the phytosterols, β-sitosterol, stigmasterol (soya beans) and campesterol, have long been recognised as potentially valuable sources of starting material for the manufacture of steroid drugs. The major limitation in their usefulness has been the difficulty in selectively removing saturated

aliphatic side chains while maintaining the integrity of the steroid ring nucleus. Thus chemical cleavage of the side chains of these compounds, with the exception of stigmasterol, is unsatisfactory because of the formation of numerous products. While several studies have shown that many microorganisms can degrade sterols, under normal conditions this has entailed decomposition of the steroid nucleus concomitant with side chain degradation (Arima et al.,1969). Only when such fermentations are carried out in the presence of certain inhibiting agents (Nagasawa et al.,1969), or with chemically modified sterol substrates (Sih and Wang, 1965; Sih et al.,1965) has it been possible to effect the selective microbial degradation of the 17-alkyl side chains. However, these processes appear to have limited practical usefulness due to their relative inefficiency or costliness.

2.2.1 SELECTIVE SIDE CHAIN CLEAVAGE OF STEROLS

As complete degradation reactions are of no commercial interest, methods have been developed to selectively cleave the side chain of sterols by microorganisms. The resulting C19 or C22 steroids are important sources for the manufacture of steroid drugs. These processes are

based on the inhibition of the key enzymes for steroid ring degradation, the C-1(2)-dehydrogenase and 9(α)- hydroxylase.

Three different methods have been employed to inhibit one or both of these enzymes:

a) Structural modification of the substrates, thus preventing enzymic attack on the ring system,

b) Inhibition of the 9(α)-hydroxylase by chemical means, thus preventing enzymic attack on the ring system,

c) Mutation of the microorganisms, to inactivate the C-1(2)-dehydrogenase and/or 9(α)-hydroxylase.

The enzyme 9(α)-hydroxylase is a monoxygenase consisting of several proteins forming an electron transfer chain. Some of these proteins contain ferrous ions as essential metal ions. Removal or replacement of these ions results in complete inactivation of the enzymatic activity. Thus iron chelating agents inhibit the ring cleavage enzyme(s) without affecting enzymes responsible for side-chain metabolism (Sih et

al., 1968). Enzyme inhibitors used for the accumulation of 17-ketosteroids have been observed with chemicals classified as: lipophilic chelating agents, metal ions with similar ion radii to Fe^{2+}, inorganic SH-reagents and auto-oxidizable redox dyes. The most effective inhibitors include α,α'-dipyridyl, 1,10-phenanthroline and 8-hydroxyquenoline, however, they are toxic, and can only be added after the culture has grown for some time.

The selective side-chain degradation in bacteria belonging to the genera Nocardia, Mycobacterium, Arthrobacter and Corynebacterium, proceeds through C24 and C22 intermediates to give C19 steroids of the androstane series. Useful products which can be applied in medical steroids' manufacture are C22-acids, C19-steroids-androstene derivatives, as well as products of partial ring structure degradation. The C22 and C19 steroids can be produced either by appropriate mutants of microorganisms, which lose the ability of ring structure degradation or by total sterol degraders in the presence of inhibitors, preventing the enzymic attack on the ring structure. Studies of the Rhodococcus species strain IM58 (Zajaczkowska and Sedlaczek,

1988), showed that the second 3C compound is cleaved less readily, yielding a mixture of C22 and C19 products. The side chain degradation by Rhodococcus species strain IM58 could be strongly directed towards C22-acids by the action of non-steroidal inducers, especially lanolin and lecithin. This type of induced increase in directed enzyme activity is not common in sterol side-chain degrading microorganisms.

2.2.2 MUTANTS DEFECTIVE IN THE TOTAL DEGRADATION OF STEROLS

Mutagenic treatment has been used for the production of organisms capable of selectively degrading the sterol side-chain. Such mutants are biochemically blocked from degrading the nucleus and can be used to efficiently produce steroids from sterols, without the necessity of modifying the substrate or of adding chemical inhibitors. Thus, sterol conversions became much more reproducible.

The generation and isolation of mutants are well established processes in microbial genetics, the mutagens of choice include ultra-violet (UV) light and N-methyl-N'-nitro-N-nitrosoguanidine (NTG). However, after treatment with nitrosoguanidine, a procedure known to induce

multiple lesions, it is possible that isolated mutants, could carry mutations other than those indicated by the accumulated products.

A series of mutants which are blocked at various stages of the sterol degradative pathway have been isolated from the potent sterol degrader Mycobacterium fortuitum ATCC 6842 (Worcha et al.,1978). Sitosterol bioconversions by these mutants result in the accumulation of a number of intermediate compounds, some of which are potentially useful as substrates in the manufacture of medically important steroids. These intermediates include androst-4-ene-3, 17-dione, androsta-1,4-diene,3,17-dione, ring-A degraded tricyclic compounds and various 9(α)-hydroxy-steroids. Ring A degraded tricyclic compounds have been used as starting material for the total synthesis of useful 19-norsteroids.

Other examples include the conversion of androstenedione to testosterone, the synthesis of estrone from androstadienedione and the recent development of some very exciting chemical procedures for putting corticosteroid side-chains into 9(α)-hydroxyandrostenedione

2.2.3 DEGRADATION OF STEROLS TO HEXAHYDROINDAN DERIVATIVES

Besides their use as substrates for microbial C19 or C22 steroid production, sterols are also used as substrates for oxidation to hexahydroindan derivatives, which contain intact steroid rings C and D (see Figure 1). These compounds are also available by microbial degradation of androstanes and pregnanes, however these substances are more expensive than sterols.

2.3 DEGRADATION PATHWAY OF THE STEROID RING SYSTEM

A variety of microorganisms are capable of utilising the cyclopentanoperhydrophenanthrene nucleus as sole carbon source and of oxidising it completely to carbon dioxide and water. The degradative pathway of the steroid ring system is carried out as in Figure 4 (Martin, 1984). Sterols containing the 3β-hydroxy-5-ene configuration II are transformed first to the corresponding 3-keto-4-ene-compounds III. The enzymes involved in this step are either NAD-dependent dehydrogenases or oxidases, requiring only molecular oxygen for their action. Various sterols are substrates for these enzymes, however, the length of the C17 side chain determines the reaction rate. Oxidation of the

3β-hydroxy configuration (II) function is followed by the isomerisation of the Δ^5-double bond, a reaction which has been shown to be catalysed by an isomerase as well as various cholesterol oxidases. This isomerisation step can also occur nonenzymatically.

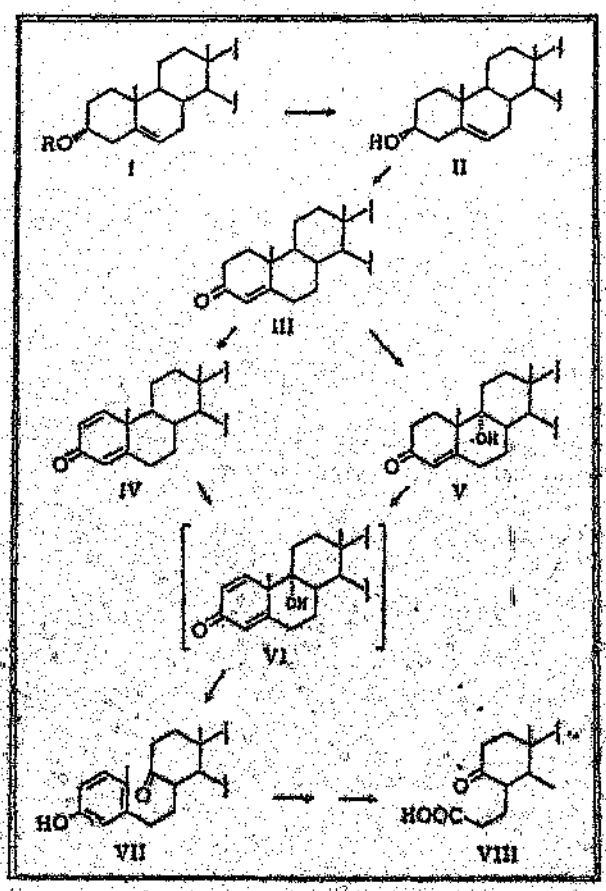


Figure 4, Degradation Pathway of the Steroid Ring System.

Martin, C.K.A, (1984), Sterols. Ch.3. In: Biotechnology Vol.6a (Eds.) Rehm, H.J. and Reed, G. (Vol. Ed.) Kieslich, K., Verlag Chemie, Weinheim.

The subsequent enzymic steps are similar in the degradation of sterols and steroids. Depending on the organism studied, the further metabolism

of the 3-keto-4-ene configuration (III) compounds involves 9(α)hydroxylation followed by C-1(2)-dehydrogenation or vice versa. The resulting metabolite VI undergoes simultaneous aromatization and cleavage of the B-ring, via a nonenzymic reverse aldol type reaction, to produce a 9,10-secophenolic derivative VII. It has been shown that the mechanism of the C-1(2)-dehydrogenase proceeds via a transdiaxial loss of the α,2β-hydrogens. The second enzyme acting at the early stage of ring degradation, the 9(α)-hydroxylase, can be inhibited by complexing agents for ferrous ions. It has been shown (Martin, 1984) to be a monooxygenase, requiring molecular oxygen for action. In subsequent reactions ring A is degraded to yield the hexahydroindan propionic acid derivatives VIII.

2.4 CHOLESTEROL OXIDASE

In spite of the great scientific and practical importance of the microbiological cleavage of the sterol side-chains, little is known about environmental factors which influence the activity of enzymes involved in the complex process. Cholesterol oxidase (E.C.1.1.3.6) which catalyses the initial step in cholesterol

degradation has been isolated from species of Nocardia (Buckland et al., 1976; Cheetham, 1979), Brevibacterium, Corynebacterium and Schizophyllum (Wilmanska and Sedlaczek,1988). The overall and specific activity of cholesterol oxidase synthesised during growth of Arthrobacter species IM 79 was analysed (Wilmanska and Sedlaczek, 1988). The increased activity of the enzyme in cultures which were brought into contact with cholesterol, at the stage of inoculum development, may indicate the inductive nature of the Arthrobacter species IM 79 cholesterol oxidase. Cholesterol oxidase produced by a new strain belonging to the genus Arthrobacter, was shown to be controlled among others, by medium composition, the mode of steroid substrate addition, and the subsequent rate of cholestenone formation, and suitable enzyme induction.

2.5 INDUSTRIAL APPLICATIONS

The world market value in 1980 was over $300 million for steroids and steroid-based products (Kieslich, 1980). The biotechnological processes that have been used for several years, now aim at the production of suitable parent materials for the production of steroids, and at transformations which will yield either intermediates or end products. However,

introduction of new steroids into commerce is limited and so new developments in microbial biotechnology of steroids is restricted. Compounds with the characteristic steroid structure abound in nature, both in plants and animals, but concentrations differ widely. For reasons of costs, only a few naturally occurring compounds can be considered as starting materials. Besides diosegnin (Dioscorea species e.g. yams), the bile acids from cattle gall bladders and stigmasterol (soya beans) are the most important raw materials. However, biotechnological production of whatever substance, will only be attractive economically when the process has a good product-volume-time yield.

As a result of a shortage of diosegnin and an increased demand for steroid drugs, the conversion of plant sterols to intermediates useful for the synthesis of medically important steroid hormones has been investigated intensively. Based upon studies on the pathway of sterol metabolism by certain bacteria, methods for selective cleavage of the 17-alkyl side chain and for partial degradation of the ring structure have been developed on a laboratory scale. Since these processes have to compete with chemical

methods, high conversion efficiencies in the presence of a large substrate have to be obtained.

Some of the processes for the synthesis of 17-ketosteroids, using mutants blocked in their ability to degrade the steroid ring system, are now utilised on an industrial scale. The fermentation products are very useful starting compounds for the synthesis of pharmacologically active steroid drugs : 4-androstene-3,17-dione can be converted to testosterone, and other steroids with androgenic activity, estrogens like estrone are accessible by pyrolytic aromatization of ring A. In addition to 17-ketosteroids, other steroids prepared by oxidative microbial degradation of sterols are of industrial importance. For instance, bisnorcholanic acid derivatives are considered useful starting materials for the synthesis of new corticoids, and hexahydroindan derivatives are precursors for chemical synthesis of retrosterrids or steroids containing hetero atoms.

There are three general processes used to produce finished steroid products;

a) Direct isolation from natural sources, such as the recovery of conjugated estrogenes from horse urine and of cardiotonic steroids from the plant Digitalis.

b) Partial synthesis from steroid raw materials of animal and plant origins. The partial synthesis of steroid hormones and their analogs is the most important process with respect to microbial biotechnology.

c) Total synthesis from non-steroidal materials.

All three methods are commercially operated, but only the two latter methods use microbial transformations (Kieslich, 1980; Smith, 1984). Mutants that selectively degrade sterols due to blocked pathways have been used industrially by Wovcha et al., 1978 ; Hill et al., 1982 ; Nakamatsu et al., 1983 and Ferreira et al., 1984. Whether a certain microbial transformation step will find industrial applications, is not governed by feasibility only. The identification of the best microorganisms for the purpose, the availability of a relatively inexpensive nutrient, and the measures taken to increase concentrations in the broth are important. The microorganism must also not produce any toxins or

toxic compounds. Steroid substances have poor water solubility and micronization. Preparation of suspensions, or emulsion, addition of surfactants and apportioning of substrate addition, can be just as decisive from an economic point of view, as is the volume-time yield, in the case of biotechnological production of compounds.

A factor of prime importance in industrial biotechnology, is the optimisation of fermentation conditions. Due to indispensable use of high quality apparatus, of an expensive measuring and controlled technique, and of frequently extracting methods of product isolation and purification, the costs of biotechnological-process implementation are frequently, but no means generally, higher than those involved in chemical processes.

2.6 NOCARDIOFORM BACTERIA

Nocardioform actinomycetes are branching bacteria that reproduce by fragmentation of all, or more or less accidently involved, parts of their hyphae into bacilli and coccoid elements. The remaining actinomycetes reproduce by spores formed on or in definite parts of the mycelium. Nocardioform bacteria were classified in the genera Nocardia and Actinomyces, or as members of

the "rhodochrous" complex (Goodfellow and Alderson, 1977), which at the time formed the family Actinomycetaceae (Bergey's Seventh Edition). In a classification based primarily on morphological, chemical and spore characters, actinomycetes have since been reclassified into over thirty genera and ten families. On this basis, nocardioform bacteria can be classified into twenty genera and seven families.

The current position is that nocardioform bacteria are aerobic, gram-positive nonsporing actinomycetes, that produce a rudimentary-to-extensive substrate mycelium,which usually fragments into bacillary and coccoid elements. They have a wall of chemotype IV (have meso-diaminopi melic-acid, arabinose and galactose in their walls) and the glycine and cytosine content of their DNA ranges from 59-69%. Extensive aerial hyphae bearing spores may be formed (Nocardia, Micropolyspora, Saccharomonospora, Saccharopolyspora), although arthrospores can also be found on the substrate mycelium (Micropolyspora). Some strains contain mycolic acids (Nocardia, Rhodococcus). On the basis of these properties, bacteria are classified in the family Nocardiaceae, with Nocardia being the type genus. At present ten

species of Rhodococcus can be recognised.

Nocardioforms can degrade many toxic compounds, including acrylamide, phenolic compounds and insecticides; degrade lignin; interconvert steroids; produce such antibiotics as rifamycins and hygromycin. Individual species are the aetiological agents of tuberculosis, leprosy and nocardiosis. However, little is known about the biology of the nocardioforms in natural habitats. Ecological studies have been severely hampered by the lack of suitable selective isolation methods and by poor taxonomy, as there are few simple and reliable tests available for the differentiation of Nocardia, Rhodococcus, Mycobacterium and related taxa. It is generally thought that soil is the primary reservoir of these bacteria. The transformation of steroids by nocardiae can be diagrammised as in Figure 5. It is conceivable on the basis of data supporting the figure, that the conversions are carried out by unspecific enzymes (Tarnok, 1976).

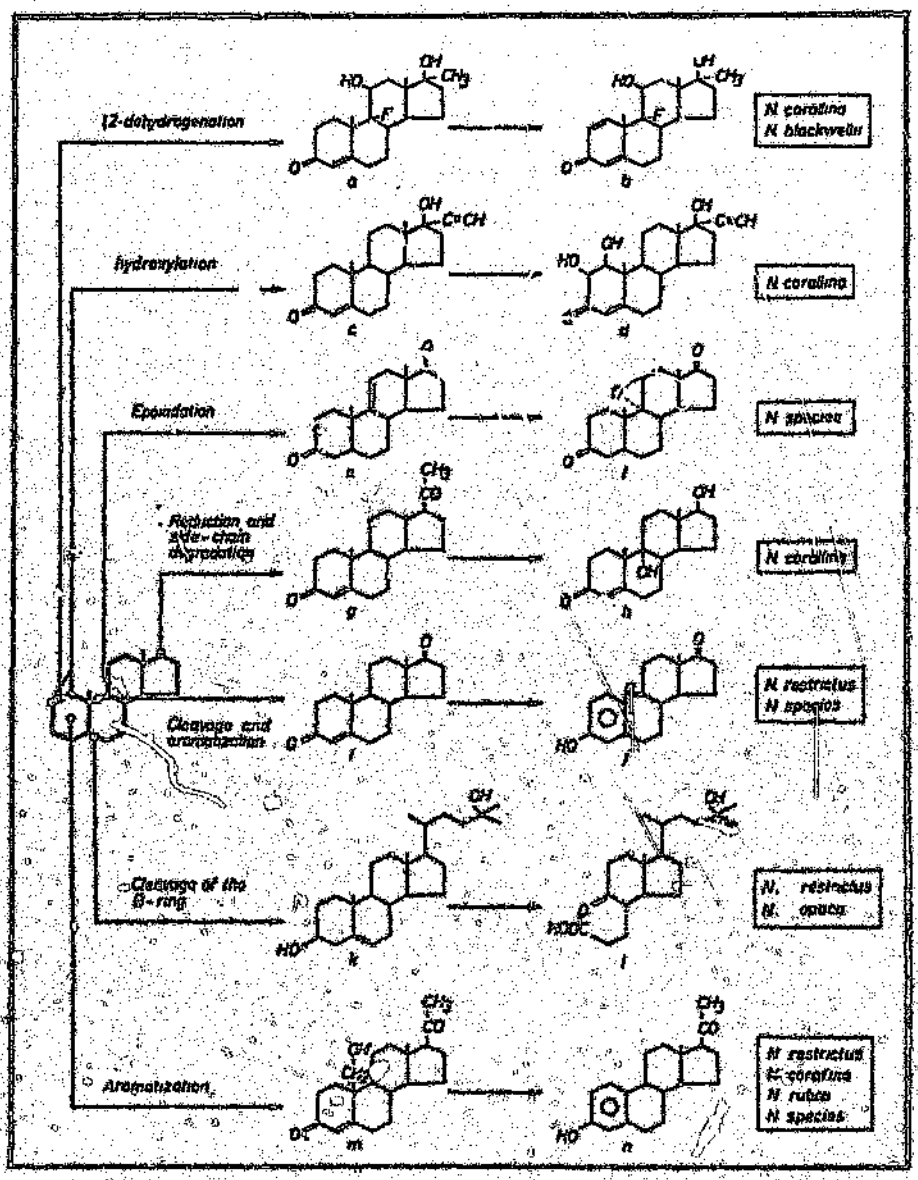


Figure 5. Transformation of steroids by Nocardia.

Tarlok, I. (1976). Metabolism in Nocardiae and Related Bacteria. In: The Biology of the Nocardiae. (Eds.). Goodfellow, M., Brownell, G.H. and Serrano, J.A.. Academic Press, London.

2.7 AIMS OF PROJECT

The aim of this work was to clone the gene(s) for interconverting bile acids in nocardioform bacteria, for eventual use in pharmaceutics. A suitable donor and recipient were to be created by mutagenesis, using *Rhodococcous erythropolis* strain ATCC 4277-1 as recipient, and *Arthrobacter oxydans* strain C1 as donor. Cloning by complementation using the shuttle plasmid vector, pDA37 was to be performed. The complementation of the appropriate mutation was to be observed by growth on the selective plates containing the appropriate bile acid(s).

3.0 MATERIALS AND METHODS

3.1 NOCARDIOFORM STRAINS

STRAIN NO		GENERAL REMARKS	ORIGIN
Rhodococcus erythropolis	ATCC 4277-1	Streptomycin resistant mutant of 4277	B.Gowan
R. equi	ATCC 14887		E.Dabbs
R. australis	ATCC A448		N.Ferreira
R. rubropertinctus	ATCC 25593		N.Ferreira
Mycobacterium smegmatis	DSM 43756		E.Dabbs
Arthrobacter oxydans	C1	Characterised by DSM	E.Dabbs
A. oxydans	C7	Characterised by DSM	E.Dabbs
A. oxydans	C1.070	Spontaneous mutant of C1 Sodium taurocholate resistant	S.Brown
KD1		NTG mutant of 14887-1 Sodium taurocholate mutant	K.Downing
12.7iii		Sodium taurocholate hypersensitive UV mutant of 14887-1	K.Downing
R. erythropolis	4277-1.063	NTG mutant of 4277-1 Sodium chenodeoxycholate hypersensitive	S.Brown
R. erythropolis	4277-1.014	Histidine auxotroph of 4277-1	S.Brown
R. erythropolis	4277-1.001	Spontaneous mutant of 4277-1 Sodium taurocholate hypersensitive	S.Brown
R. erythropolis	4277-1.002	NTG mutant of 4277-1 Sodium taurocholate hypersensitive	S.Brown

3.2 ESCHERICHIA COLI STRAINS

STRAINS	GENERAL REMARKS	ORIGIN
λMM294	Lysogenic form of MM294	E. Dabbs
MM294-1	Restriction -, Recombination + mutant of MM294 (non-lysogenic) Resistant to Rifampicin	E. Dabbs

3.3 VECTORS

VECTORS	GENERAL REMARKS	ORIGIN
pDA30	Previously designated E1B1	E. Dabbs
pDA37	Ligation of pDA30 and pEcoR251 Ampicillin Resistant Shuttle vector Suicide vector	S. Anderson B. Gowan

Plasmid pDA37 was created by the ligation of plasmid pDA30 (cut at a Bgl II site) with plasmid pEcoR251 (cut at Bam HI site). The plasmid pEcoR251 part has the EcoR1 END gene for endonuclease action and has the genes for ampicillin resistance. Plasmid pDA37 was used (see Figure 6) as it was a positive selection vector for ampicillin, a suicide vector and a shuttle vector.

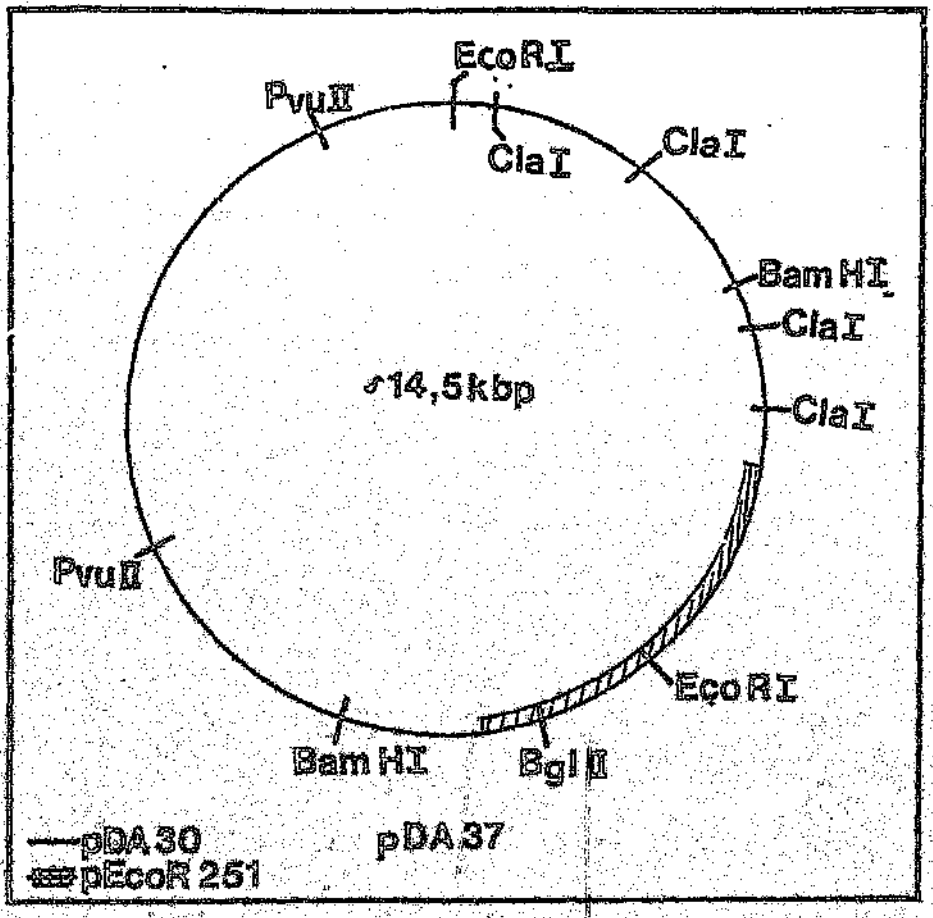


Figure 6. A Diagrammatic Representation of Plasmid pDA37

Gowan, B. (1989). The Development of Techniques for the Molecular Biological Characterisation of Acrylamide Degradation in Nocardioform Bacteria, M.Sc. Dissertation, University of the Witwatersrand, Johannesburg.

3.4 BACTERIAL GROWTH

3.4.1 Broths

Nocardioform strains were grown in TY (Tryptone Yeast Broth) (section 8.A.1). To obtain deoxynucleic acid (DNA), generally 4% TYG (Tryptone Yeast Glycine Broth) (Section 8.A.2) was used, except for A. oxydans C1 and C1.070 where 2% TYG was used. Nocardioform strains used

in mutagenesis were grown in T2 (section 8.A.3). E. coli strain λMM294 was grown in LB (Luria Broth) (section 8.A.5) supplemented with 10mM $MgSO_4$, to obtain the plasmid vector pDA30. Luria Broth was also used to grow MM294-1 and employed in transformations, plasmid screens or extractions. For plasmid screens and extractions, the LB was supplemented with 50 µg/ml ampicillin. Utilisation and resistance curves of a particular bile acid were carried out using both Minimal medium plus glucose (section 8.A.7) (MM+G) and Minimal medium without glucose (section 8.A.8) (MMnoG), supplemented with a concentration of the bile acid.

3.4.2 Agars

TYA (Trypton Yeast Agar) (section 8.B.1) was used as a positive control for all spot tests and for patchings. LA (Luria Agar) (section 8.B.2) was used to grow up cells to single colonies, as was TYA, and was sometimes supplemented with an antibiotic. The transformed MM294-1 colonies were selected for, by using LA+50 µg/ml ampicillin. Top agar (section 8.B.4) was employed for phage typing. Protoplast regenerating medium plates (PRMP)

(section 8.B.5) were used to grow up the transformed nocardioform bacteria, either C1 or C1.070.

3.5 STORAGE OF STRAINS

A 30 % glycerol solution was aliquoted into 1ml sets, then autoclaved at 121°C for 20 minutes. The solution was stored at -80°C. When needed, a vial was thawed and then inoculated with 100 µl of a bacterial strain and stored at -80°C.

3.6 ANTIBIOTIC STOCKS

Solutions of 10mg/ml of ampicillin (Boehringer, Mannheim GmbH, Mannheim, W. Germany, hereafter referred to as "Boehringer") at 10mg/ml of streptomycin (Boehringer) were made up using sterile water and absolute ethanol in a 1:1 ratio. The solution was then stored at 4°C.

3.7 ARSENIC STOCKS

Sodium arsenate and sodium arsenite were used as a positive selection for nocardioform bacteria with the arsenic resistance plasmid.

Solutions of:

2M sodium arsenate (SAARchem, Muldersdrift, South Africa, hereafter referred to as "SAARchem"),

1M sodium arsenite (BDH Chemicals Ltd, Poole, England, hereafter referred to as "BDH"),

2M sodium arsenate; 0.5M sodium arsenite,

were made in sterile distilled water, then filter sterilised using a 0.45 µm filter. All of the solutions were stored at room temperature.

3.3 CARBON SOURCES

Solutions of 10% were made up of:

CARBON SOURCE	SOLVENT	METHOD OF STERILISATION
Stigmasterol (SAARchem)	DMSO	
Cholesterol (SAARchem)	DMSO	
B-sitosterol (SAARchem)	DMSO	
Sodium Taurocholate (SIGMA)	water	Filtered
Sodium Deoxycholate (SIGMA)	water	Filtered
Sodium Chenodeoxycholate (SIGMA)	water	Filtered
Sodium Acetate (BDH)	water	Autoclaved
Sodium Citrate (SAARchem)	water	Autoclaved
Succinic Acid (BDH)	water	Autoclaved
Sodium Pyruvate (SAARchem)	water	Autoclaved
Glycerol (SAARchem)	water	Autoclaved

(Sigma Aldrich Corporation, St Louis, Missouri, hereafter referred to as "Sigma")

All of the solutions were autoclaved at 121°C for 20 minutes. Filtering was performed using 0.45µm filters. All carbon sources were stored at 4°C.

The solvent DMSO (Dimethyl Sulphoxide) (BDH) and absolute alcohol were also tested as carbon sources.

For utilization studies, the varying concentrations (0mg/ml to 200 mg/ml) of different carbon sources were made in MMnoG (section 8.A.7) either as broth or as agar plates. The nocardioform(s) being investigated were either replica plated onto the agar plates, or inoculated into the broths. The controls included a negative (no bacteria) control, and a positive control. Growth was observed by eye on the plates, but in the liquid media was recorded using a spectrophotometer (Beckman), at a wavelength of 540nm (visible light spectrum).

To study the resistance of an organism to a carbon source, the same procedure as for utilisation studies was performed, except that MM+G (section 8.A.8) was used. All of the incubations were kept at room temperature, with liquid cultures being aerated.

3.9 REPLICA PLATING (Spot Testing)

This method allowed for one single colony to be used per organism, for the entire range or concentration of carbon sources, being tested.

Into the wells of the sterile "female" part of the apparatus, one drop or if many tests were performed, two drops, of sterile water were placed. One bacterial colony per well was added and mixed well. The wells were numbered. The "male" part, bearing the steel prongs, was placed after flaming, into the wells. Ensuring that the correct orientation was maintained, the steel prongs were pressed onto the agar. For each different carbon source a new test had to be performed. Each nocardioform was tested in duplicate. The plates were incubated at room temperature or at 26°C, and were observed for growth every three days. The plates were then scored against the positive and negative controls.

3.10 DETERMINATION OF THE RECIPIENT AND DONOR DNA FOR THE GENE CLONING

The donor DNA was determined by observing the bacterial strain which appeared to be the best

utiliser of the bile acids. The recipient DNA was chosen from the strains which could not use the bile acids.

3.11 LIQUID CULTURE FOR UTILISATION AND RESISTANCE STUDIES OF BILE ACIDS

The nocardioforms to be tested included the strains 4277-1, 4277-1.063, 4277-1.001 and 4277-1.002. They were individually precultured in 5ml of MM+G (section 8.A.7) until all had reached stationary phase. Afterwhich 1ml of each culture was then washed twice with sterile distilled water. The test carbon sources were set up in 5ml of media per test tube. In utilisation studies MMnoG (section 8.A.8) was used, and MM+G was used in resistance studies. Only bile acids were tested, and included sodium taurocholate (ST), sodium chenodeoxycholate (SC) and sodium deoxycholate (SD), in concentrations ranging from 0 mg/ml to 200 mg/ml with 10mg/ml intervals.

Each test tube was inoculated with 10µl of pre-culture, except for the no cell control. The test was performed in duplicate. The tubes were then loaded onto a revolving drum, and incubated at room temperature until growth

(turbidity) was observed. Then every tube's optical density at 540nm was recorded, and thereafter every 24 hours, for the next 4-6 days.

3.12 GROWTH CURVES

Growth curves of strains 4277-1, 4277-1.063 and 4277-1.014 were done, to determine the time taken to reach, and the optical density of the cultures, at logarithmic and stationary phase. Test tubes were set up in duplicate, with 5ml of T2 (section 8.A.3). Each tube was inoculated with 10µl of washed pre-culture, then loaded onto the revolving drum and incubated at room temperature. The turbidity was recorded at either hourly or 6 hourly intervals, at 540nm, until it had reached a peak and were unchangeable, or decreased.

3.13 N-METHYL-N'-NITRO-N-NITROGUANIDINE (NTG) MUTAGENESIS

Solutions of 1.25 mg/ml of NTG (Sigma) was made up in sterile distilled water and filter sterilised using a 0.45 µm filter, then stored in the dark at 4°C.

The conditions for NTG-mutagenesis of 4277-1 were optimised by B. Gowan (1989).

Strain 4277-1 was grown up in T2 (section 8.A.2) until logarithmic or stationary phase. A 1ml aliquot of the culture was washed using 1ml of 10mM Tris HCl, pH8 (section 8.C.2) then resuspended in 0.9ml of 10mM Tris HCl, pH8, and 0.1ml of NTG to a final concentration of 125µg/ml. The cells were placed at 37°C for 2 hours, with agitation, after which they were washed with sterile distilled water, and resuspended in 100 µl of T2. The cells were added to 5 ml aliquots of T2. A concentration of 50µg/ml streptomycin was added to prevent contaminant growth, and the culture was allowed to reach stationary phase at 26°C (room temperature) with aeration. A 1ml aliquot was then sonicated for 5 seconds to reduce clumping, and diluted to 10^{-8} in T2. An aliquot of 100µl of the dilution was then plated onto TYA plates (section 8.B.1) supplemented with 50µg/ml streptomycin. The plates were then incubated at room temperature, and observed for growth.

3.13.1 Patching

When single colonies had grown, they were replica "patched" onto a series of plates. A total of 112 colonies per plate could be patched. The plates used to assay for mutants were, in order of patching:

1. A Minimal medium plate with no glucose (MMnoG)in order to detect agarose mutants.
2. A Minimal medium plate with no glucose, but with the addition of the appropriate carbon source to detect mutants unable to utilise the carbon source.
3. A Minimal medium plate with glucose to detect auxotroph production, which is a means of assaying the mutagenesis.
4. A TYA plate as a positive control.

3.13.2 The Enrichment Procedure

This procedure was employed to select for auxotrophs and for particular carbon source mutants. It was based on the penicillin selection or auxotroph enrichment procedure as used for E. coli, which depends on the fact that ampicillin kills active, growing bacteria, but not cells that are quiescent.

Strain 4277-1 which had been treated with NTG was allowed to reach stationary phase in T2. Then 1ml of the culture was washed twice with sterile distilled water. An amount of 0.1ml was added to 5ml of MM+G supplemented with 50µg/ml streptomycin. The culture was allowed to grow at room temperature with aeration, until it had reached logarithmic phase. The cells were then washed twice with sterile distilled water and used to inoculate 5 ml of MMnoG supplemented with 50µg/ml of streptomycin, supplemented with one of:

20 mg/ml sodium deoxycholate (SD),

20 mg/ml sodium chenodeoxycholate (SC),

40 mg/ml sodium taurocholate (ST).

The cultures were allowed to incubate overnight at room temperature with aeration, then challenged with 50µg/ml of ampicillin. The culture was then incubated for a further 4 hours, after which 1ml was washed twice with sterile distilled water and diluted to 10^{-6} to 10^{-8}. An aliquot of 100µl of the appropriate dilution was plated onto TYA plates supplemented with 50 µg/ml streptomycin, and incubated at room temperature. The single colonies that grew were then patched onto the four different plates. The selective plate contained the

appropriate enrichment bile used. The enrichment procedure was repeated twice.

Concurrently with the enrichment procedure, a control using T2 as the enrichment broth and MM+G as the selective broth, was performed for the selection of auxotrophs. The selective plate was MM+G.

3.14 TESTING PUTATIVE MUTANTS

The colonies that could only grow on the positive control plates (TYA) were further tested. They were spot tested (section 3.9) onto selection plates of all three bile acids, of different concentration ranges, in an attempt to discover hypersensitive and/or resistant mutants. Putative auxotrophs were spot tested onto MM+G agar, each having been supplemented with 1% of one of the twenty amino acids.

Any putative auxotrophs that could not grow were spot tested onto MM+G agar supplemented with combinations of amino acids or vitamins, which included:

1% Adenine (Sigma),

4.5µg/ml vitamin B1 (from 1mg/ml stock in sterile water) (Sigma),

1% Isoleucine (Merck, Dormstadt, Germany, hereafter referred to as 'Merck') + 1% Valine (SAARchem),

1% Tryptophan (SAARchem) + 1% Tyrosine (SAARchem) + 1% Phenylalanine (SAARchem) + 0.1% Shikimic Acid (Merck).

Those that appeared to be mutants were then designated a code. The controls in the spot tests included the parental strains of 4277-1, C1 and 14887. The mutants were then stored in glycerol (section 3.5) at -80°C.

All of the amino acids were stored in 10% solutions, in absolute alcohol or in sterile distilled water. The solutions were stored at 4°C.

3.15 SPONTANEOUS MUTANTS AND THEIR FREQUENCY

A flask containing 5ml of MM+G (section 8.A.7) was inoculated with 50µl of pre-cultured and washed 4277-1, 14887 and C1. The cultures were then grown overnight at room temperature with aeration, before 1ml of each culture was washed and sonicated for 5 seconds. Then 200 µl of each culture was plated onto MMnoG agar (section 8.A.8), supplemented with 100 mg/ml or 200 mg/ml of each of ST, SD and SC. The plates were then

incubated at room temperature.

The remaining sonicated cells were diluted to 10^{-6}, 10^{-8} and 10^{-10} in sterile distilled water, and 100µl per dilution per strain was plated onto TYA (section 8.B.1), and incubated at room temperature. The number of colony forming units (CFU) per ml was determined from growth on TYA by:

$$\frac{\text{Number colonies per plate} \times \text{dilution factor}}{\text{amount plated}} = \text{CFU/ml per strain}$$

The frequency of spontaneous mutants produced was determined by:

$$\frac{\text{Number CFU mutants on selective plate/ml}}{\text{CFU/ml}} = \text{frequency of mutation}$$

Spontaneous mutations were checked for their ability to utilise or be resistant to high concentrations of bile acid.

3.16 FREQUENCY OF AUXOTROPHS FROM NTG-MUTAGENESIS

The same was performed as for spontaneous mutation rates, except that the frequency was determined by reference to the number of auxotrophs produced. The CFU were determined

from growth during stationary phase of 4277-1 in MM+G.

3.17 CALCULATION OF THE MUTATION REVERSION RATE OF SELECTED MUTANTS

An inoculum of 50µl of eight auxotrophs were each grown up in 5ml of T2. A 1ml aliquot was washed twice, sonicated for 5 seconds and then diluted to 10^{-6}. An amount of 100µl of each auxotroph was plated onto both TYA (section 8.B.1) and MM+G agar (section 8.B.3). The TYA plate determined the number of colony forming units (CFU) per ml, and the MM+G plate determined the number of revertants. The plates were incubated at room temperature, and were observed for growth.

The colonies were counted, and the reversion rate calculated:

CFU/ml as described in section 3.15

$$\text{Reversion rate} = \frac{\text{Number CFU/ml on MM+G}}{\text{CFU/ml on TYA}}$$

3.18 BULK PREPARATION OF NOCARDIOFORM CHROMOSOMAL DNA OF C1.070

3.18.1 Optimisation of C1 Growth in TYG

To be able to rupture the cell wall, and release the chromosomal DNA, glycine was needed. However a concentration of glycine which would produce a significant inhibition of growth, but produced a good yield of chromosomal DNA needed to be determined.

Flasks were set up, with one containing 10ml of TY (section 8.A.2) only, and others of TY supplemented with 1%, 2%, 3%, 4%, and 5% of glycine (section 8.A.2). An aliquot of 100ul of washed C1 cells were inoculated into each of the flasks and then incubated at room temperature with aeration, until stationary phase was reached.

The procedure described for C1 was performed using C1.070

3.18.2 DNA Extraction

A solution of 200 ml of 2% TYG was inoculated with 2ml of C1.070 and incubated overnight at

room temperature with aeration. The culture was harvested using 500 ml bottles and a JA-10 rotor (Beckman) centrifuged at 6000 rpm for 15 minutes. The supernatant was discarded, and the pellet was resuspended in 10 ml of 10mM Tris HCl pH8 (section 8.C.2); 10% (w/v) sucrose. Then 5 ml of 10mM Tris; 10% Sucrose, in which 100 mg of lysozyme (Boehringer) had been freshly dissolved was added, mixed well, and incubated at 37°C for 2 hours. The cells were then pelleted at 12 000 rpm for 10 min in a JA-20 rotor (Beckman).

The supernatant was decanted off and the pellet was drained well. The cells were resuspended in 8 ml of TE (section 8.C.1), with 1-2mg of Proteinase K (Boehringer) dissolved in it (per 250 ml culture). One tenth of the volume (v/v) of TE; 10% SDS (sodium dodecylsulphate) (BDH) was added, mixed well and incubated at 37°C for 2 hours. The solution was then placed into 50 Ti tubes (Beckman), and centrifuged at 18 000 rpm for 45 minutes. The DNA solution was then decanted off into a fresh tube, and its volume was measured.

3.18.3 DNA Purification by Caesium Chloride - Ethidium Bromide Density Gradients

To the DNA solution, an equal weight per volume of caesium chloride (Boehringer) (CsCl), was added, and dissolved.

The solution was centrifuged at 18 000 rpm for 15 minutes. The DNA was decanted out from underneath the scum, its volume was measured and 0.07ml of Ethidium bromide (EtBr) (section A.C.5) solution per ml of DNA was added. The refractive index of the solution was then adjusted to 1.390. For plasmid extractions from nocardioforms the refractive index needed to be adjusted to 1.392. The solution was loaded into Quickseal tubes (Beckman), balanced, sealed and centrifuged in a VTi 65,2 rotor (Beckman) for at least 14 hours at 40 000 rpm. The chromosomal DNA was then extracted by needle extraction, after visualisation of the DNA with ultraviolet (UV) light.

If necessary the bands could be pooled and re-centrifuged to concentrate the yield. The DNA then needed to be decolourised and concentrated.

3.19 DECOLOURISATION AND REMOVAL OF CAESIUM CHLORIDE

The DNA was decolourised by the addition of 200µl of 1:1 TE saturated butanol (SAARchem) (section 8.C.1). The eppendorf tubes containing the DNA were shaken until the TE saturated butanol colourised as much as possible, then centrifuged for 30 seconds and the upper or coloured band was removed. The process was repeated until the DNA was colourless.

The DNA could be stored in CsCl at -80°C, or the CsCl could be removed by dialysing against TE pH8 for a minimum of 4 hours (section 8.C.1). The dialysis tubing was prepared as in section 8.D.3. The DNA was then placed into sterile eppendorf tubes and stored at -80°C.

3.20 FORMULA FOR THE DETERMINATION OR THE SIZE OF THE GENOMIC LIBRARY

The formula used to calculate the number of colonies needed to represent a genomic library of an entire genome, was:

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

Where, N = Number of clones required

P = Probability (95 %) that any given gene is present

a = average size of the DNA fragment inserted into the vector.

b = total size of genome

The size of the genome of C1 was available from the literature, and was extrapolated to C1.070.

3.21 BULK PLASMID ISOLATION OF THE SHUTTLE VECTOR (pDA37) FROM *E. coli* λMM294-1 (Maniatis et al., 1982)

Solution 1 :

50 mM glucose

25 mM Tris HCl (pH8)

10 mM EDTA

The Tris HCl and EDTA were made up as one solution, and the glucose as a separate solution. The solutions were autoclaved at 121°C for 20 minutes. The Glucose then added and the solution was stored at 4°C.

Solution 2 :

0.2N NaOH (SAARchem)

10 % SDS

The solution was autoclaved at 121°C for 20 minutes and then stored at room temperature.

Solution 3 :

5M Potassium Acetate (pH 4.8)

60 ml 5M Potassium Acetate (Merck)

11.5 ml glacial acetic acid (SAARchem)

28.5 ml sterile water

Solution 3 was stored at room temperature.

A flask containing 250 ml of LB (section 8.A.5) supplemented with 50 µg/ml of ampicillin was inoculated with 2 ml of pre-cultured λMM294-1, then incubated at 33°C with aeration, until the culture had reached stationary phase. The culture was harvested using 500 ml bottles and a JA-10 rotor, by centrifugation at 6000 rpm for 15 minutes. The supernatant was discarded.

The pellet was resuspended in 6.5 ml of solution 1 containing 5 mg/ml of freshly dissolved lysozyme. The suspension was then transferred into sterile JA-20 rotor tubes, and left to stand at room temperature for 5 minutes. To the suspension, 13 ml of solution 2 was added, mixed vigorously, and then left to stand on ice for 10 minutes. Then 10 ml of ice-cold solution 3 was added. The suspension was mixed vigorously, and left to stand on ice for a further 10 minutes. The solution was then centrifuged at 20 000 rpm for 20 minutes at 4°C.

The cell DNA and bacterial debris should have formed a tight pellet at the bottom of the tube. Equal quantities of the supernatant was then transferred into separate tubes, and allowed to warm to room temperature. To each tube 0.6

volumes of isopropanol (BDH) per volume of DNA was added, mixed well and allowed to stand at room temperature for 15 minutes.

The plasmid was recovered by centrifugation at 15000 rpm for 30 minutes at room temperature. The pellet was washed with 5ml per sample, of ice-cold absolute ethanol. The ethanol was decanted off and the pellet was drained for 30 minutes in a vacuum desiccator, followed by resuspension in 4ml per sample of TE pH8 (section 8.C.1). The plasmid was purified by centrifugation to equilibrium in CsCl-EtBr density gradients (section 3.18C). Decolourisation and CsCl removal was performed as in section 3.19.

3.22 DETERMINATION OF THE SHUTTLE VECTOR (pDA37) CONCENTRATION

Aliquots of 10µl of pDA37, 10µl of C1.070 chromosomal DNA and 1µl of C1.070 chromosomal DNA were loaded into a 0.4% agarose gel (section 8.E), along with 0.1 µg and 0.5 µg of DNA molecular weight marker II (Boehringer). The gel was electrolysed at a potential difference of 100V and a current of 40 mA for 45 minutes to 1 hour. Determination of the uncut plasmid and

uncut C1.070 chromosomal DNA concentration determined how much DNA to load, once the DNA had been digested.

If they were not already stored in Boehringer Buffer M, the respective DNAs were phenol purified (section 8.D.1), ethanol precipitated (section 8.D.2), and resuspended in their original volume in one tenth (v/v) of Buffer M, by incubation at 42°C for at least 6 hours. Then 15µl of pDA37 were aliquoted into eppendorf tubes, to which 0.1µl of restriction endonucleases Pvu II and Bam HI (Boehringer) were added, mixed well and digested at 37°C for 3 hours.

For the resolution of smaller DNA fragments, electrolysis was performed on the samples using a 1.2% agarose gel, with DNA molecular weight marker λ III (Boehringer). Amounts of 10µl, 3.2 µl and 0.32 µl of digested pDA37, and 1.8 µl and 0.2µl of digested C1,070 DNA were loaded. Amounts of 0.5µg and 0.1µg of λIII were loaded. The gel was electrolysed for 1.5 hours. The gels were viewed under UV light and the concentrations of DNA was determined.

The size of the fragments of pDA37 were determined by reading off the fragment sizes, determined by the distance that they had migrated, from the graph of the size of the λIII fragments (y axis) plotted against the distance that they had migrated (x axis). The concentration of pDA37 was then determined by comparing a fragment of known size of pDA37 with a fragment of λIII of equal band intensity. The concentration was determined by:

λIII:

$$\frac{\text{size of fragment compared}}{\text{size of } \lambda\text{III}} \times \text{amount of DNA loaded} = X$$

pDA37:

$$\frac{\text{size of plasmid}}{\text{size of fragment compared}} \times X = \text{amount of plasmid per lane}$$

The total amount of λIII loaded was known, therefore it could be calculated how much plasmid pDA37 was present.

3.23 DETERMINATION OF C1.070 DNA CONCENTRATION

The concentration of the uncut chromosomal C1.070 DNA, which formed a single band when

electrolyse was determined as in section 3.22, except that the single band was used.

3.24 AVERAGE SIZE OF DONOR FRAGMENT INSERTS (C1.070) IN THE SHUTTLE VECTOR (pDA37)

Both C1.070 DNA and pDA37 had to be digested with Bgl II, then ligated and transformed into MM294-1, before a plasmid screen could be performed to determine the average insert size.

3.24.1 DNA Digestions

Both DNAs were stored in Buffer M (section 3.22). For the digestions, the required volume of each DNA was aliquoted into an eppendorf tube, and 0.1µl, of Bgl II was added, mixed and incubated at 37°C for a minimum of 3 hours for C1.070 DNA and a minimum of 1 hour for pDA37. The ligation protocol was then performed:

3.25 DNA LIGATIONS

Solutions:

1. 1M Tris-HCl pH7.6

   The solution was autoclaved at 121°C for 20 minutes, and stored at room temperature

2. 1M $MgCl_2$ (SAARchem)

The solution was autoclaved at 121°C for 20 minutes, and stored at room temperature

3. 100mM Adenosine triphoshate (ATP) (Boehringer)

The solution was stored at -20°C

4. 1mM Dithiothreitol pH7 (SIGMA)

The solution was filter sterilised (0.45µm) and Stored at -20°C

Ligation Buffer

From the stock solutions 1,2 and 3, the buffer was made up

9.5 ml sterile distilled water		Final concentrations
200µl	1M Tris-HCl	20mM
100µl	1M $MgCl_2$	10mM
60µl	100mM ATP	0.6mM
100µl	1M Dithiothreitol	10mM

After digestion, the volumes of DNA, were made up to 200µl with TE (section 8.C.1). Then phenol purified (section 8.D.1), ethanol precipitated (section 8.C.2), and the pellets were resuspended in the original volume using ligation buffer, and incubated for a minimum of

1 hour at 37°C. The two DNAs were then added together, in the determined ratios, and 1µl of T4 DNA ligase (Boehringer) was added. The ligation mixtures were incubated at 14°C for at least 14 hours (optimal at 20 hours or overnight). The controls for each stage included a plasmid free control, an uncut plasmid control, a C1.070 DNA free control and a T4 DNA ligase free control. An *E. coli* transformation by the calcium chloride method was then performed.

A series of ligations were performed to optimise the pDA37 digestion time, and the ratio of C1.070 DNA: pDA37.

3.26 *E. coli* TRANSFORMATION BY THE CALCIUM CHLORIDE METHOD

(Maniatis et al., 1982)

A flask containing 100ml of LB (section 3.A.5) was inoculated with 1ml of the recipient strain MM294-1, and incubated at 37°C until the culture had reached an optical density (OD) at 550 nm wavelength of 0.2. The culture was then rapidly chilled on ice, and centrifuged at 5000 rpm for 10 minutes in a pre-chilled JA-10 rotor. The supernatant was discarded, and the cells were

gently resuspended in half of their original culture volume, in an ice-cold sterile solution of 50 mM $CaCl_2$, 10 mM Tris (pH8). The cell suspension was then placed on ice for 15 minutes, before being centrifuged at 5000 rpm for 5 minutes. The supernatant was again discarded, and the cells resuspended in one fifteenth of the original volume of $CaCl_2$-Tris buffer. Then 0.2ml aliquots were placed into pre-chilled eppendorf tubes, and placed on ice for 1 hour.

The ligation mixture was then added gently, whilst the tubes were still on ice. The entire mixture was then heat shocked at 42°C for 1 minute in a water bath, followed by the addition of 1ml of LB, and incubated for 1 hour at 37°C without agitation. The cells were centrifuged for 30 seconds and gently resuspended in 100µl of LB, and spread onto LA supplemented with 50µg/ml ampicillin (section 8.B.2). The plates were incubated overnight at 37°C.

3.27 E. COLI PLASMID SCREEN

This was performed to check for the presence of plasmid pDA37 with insert, after transformation.

The buffers described in section 3.21 were used. The selected single colonies of transformed MM294-1 were grown overnight in 5ml of LB supplemented with 50µg/ul ampicillin (section 8.A.5), with aeration. Then 1ml of the culture was harvested by centrifugation for 1 minute, and the pellet was resuspended in 100µl of solution 1 supplemented with 5mg/ml of lysozyme, and left at room temperature for 5 minutes. Then 200µl of solution 2 was added, mixed vigorously, and placed on ice for 5 minutes. An amount of 150µl of pre-chilled solution 3 was added, mixed vigorously and placed on ice for 5 minutes, then microfuged for 1 minute. The supernatant was transferred to a fresh sterile eppendorf tube, and one volume (v/v) of isopropanol (approximately 350µl) was added, and stood at room temperature for 5 minutes. The DNA was then precipitated by centrifuging for 5 minutes at room temperature. The pellet was then washed with 2.5 volumes (v/v) of ice-cold absolute ethanol, and centrifuged for 5 minutes.

The ethanol was decanted off, and the pellet was dried at 60°C for 20 minutes, then resuspended in the required volume of sterile water and preboiled RNAase (1mg/1ml) (Boehringer). The

presence of pDA37 was determined by electrolysing samples using a 0.4% agarose gel (section 8.E), along with a plasmid and a plasmid free control. The percentage of cells with insert were determined, and used to calculate the size of the genomic library.

3.28 STORAGE OF THE GENOMIC LIBRARY IN MM294-1

The transformed MM294-1 cells were washed off the plates with 1-2ml of LB (section 8.A.5) supplemented with 100µg/ml ampicillin. The cells were pooled and poured into a sterile glass shock bottle and stored at -80°C. Samples were also stored in glycerol (section 3.5) at -80°C and -20°C.

3.29 CONCENTRATION OF THE GENOMIC LIBRARY

The stored genomic library (section 3.28) was thawed, mixed well and used to inoculated 100ml of LB (section 8.A.5) supplemented with 50µg/ml ampicillin. The plasmid pDA37 was extracted (section 3.21) in bulk, and its concentration was determined (section 3.22). The pDA37 plasmids with a C1.070 insert were stored in TE pH8 (section 8.C.1) at -80°C. The concentration

of the genomic library was needed to be able to determine the number of transformants of 4277-1.063 necessary per µg of C1.070 DNA, for a representative number of colonies of the entire C1.070 genomic to be achieved.

3.30 OPTIMISATION OF THE 4277-1.063 NOCARDIOFORM TRANSFORMATION

Protoplast Buffer (Okanishi et al., 1974; Hopwood and Wright, 1978)

The following solution was made up to 800 ml with distilled water:

103g sucrose (SAARchem)

0.35g K_2SO_4 (Merck)

2.02g $MgCl_2.6H_2O$ (Merck)

The solution was dispensed into 80ml aliquots, autoclaved at 121°C for 20 minutes and stored at -80°C.

To each aliquot, the following order of solutions were added:

1 ml K_2PO_4 (0.5%) (Merck)

10ml 0.25M $CaCl_2.2H_2O$(3.68 %) (Merck)

10ml 0.25M TES Buffer (5.73 %) pH7.2 (Sigma)

Each solution had been autoclaved separately at 121°C for 20 minutes, and stored at room temperature

Polyethyleneglycol (PEG) 4000

1g PEG 4000 (Merck) dissolved in 1ml of protoplast buffer.

The nocardioform transformation was first performed on 4277-1.063 and 4277-1.014 with plasmid pDA30 (E_1B_1). All conditions except for the optimal growth phase of the nocardioform had been previously optimised by S. Gowan (1989).

The optimal growth phase was determined by inoculating two flasks containing 100 ml of 4% TYG (section 8,A.2), with 1ml of either pre-cultured 4277-1.063 or 4277-1.014 (histidine auxotroph). The flasks were incubated at room temperature with aeration. Once growth was observed, and for every 12 hours afterwards, five sets of 1ml were collected per culture. Of each set of samples, one was used to measure the OD (540nm), the rest were stored at -80°C. Samples were collected until the OD had reached a peak and then decreased (post-stationary phase). The nocardioform transformation was then performed on one sample of each strain, collected from the different growth phases.

3.30.1 Nocardioform Transformation

Separate aliquots of 1ml from cultures of 4277-1.063 and 4277-1.014 in 4% TYG were centrifuged. The cells were resuspended in 1ml of Protoplast buffer, containing 5mg/ml of lysozyme, and incubated at 37°C for 2 hours with agitation. The cells were pelleted, and gently resuspended in 1ml of protoplast buffer, and then aliquoted into 100µl sets in eppendorf tubes. The negative control had 2µl of TE pH8 (section 8.C.1), added, whilst the test samples had 2µl of pDA30 added. Then PEG to a final concentration of 30% was added. After 4 minutes, the contents of each tube were carefully spread onto prptoplast regenerating medium plates (section 8.B.5), and incubated at room temperature. After 12-16 hours, 0.5ml of 3M arsenate, 0.5M arsenite (section 3.7) underlay was added and the plates were further incubated until single colonies grew.

As 50ng of pDA30 per transformation was used, the number of transformants per µg of pDA30 was calculated, to be able to determine the growth phase (OD reading), which resulted in the greatest efficiency of transformation.

Using the results of the transformation with pDA30, and from the determination of the concentration of pDA37 plasmid with an insert (section 3.29), the number of colonies needed for the transformed cells, to be representative of the entire C1.070 genome was calculated. However the pDA37 was found to be too dilute, as only a maximum of 20µl could be used per transformation. Thus the pDA37 had to be concentrated by ethanol precipitation (section 8.D.2) and resuspended in half of the original volume.

3.31 SCREENING FOR CLONING BY COMPLEMENTATION ON SELECTIVE PLATES

Once the transformed 4277-1.063 and 4277-1.014 colonies had grown, they were washed off the plates with 1ml of TY (section 8.A.1) supplemented with 40 mM sodium arsenate, per plate. The colonies were pooled and used to inoculate 100 ml of TY supplemented with 40 mM arsenate. The cultures were incubated at room temperature with aeration, until the ODs were comparable. The non-transformed strains of 4277-1.063 and 4277-1.014 were also grown, but

without the arsenate supplements. Then 1ml aliquots of all four cultures were washed with sterile distilled water and 100µl of each was spread onto the relevant selective plates. Transformed 4277-1.063 and non-transformed 4277-1.063 cells were spread onto MMnoG (section 8.B.3) and MMnoG supplemented with 40mg/ml ST and MMnoG supplemented with 20 mg/ml SD, as well as onto MM+G, and MM+G supplemented with 40mg/ml ST, and MM+G supplemented with 20mg/ml SD. The 4277-1.014 and transformed 4277-1.014 cells were spread onto MM+G, and TYA plates. Then all the plates were incubated at room temperature and observed for growth.

3.32 NOCARDIOFORM PLASMID SCREEN

All of the solutions were as described in section 5.18.

From the culture of transformed 4277-1.063, 100µl was spread onto TYA (section 8.B.1) supplemented with 40mM arsenate, and incubated at room temperature until single colonies grew. Fourteen colonies were then plasmid screened. Each colony was grown overnight in 5ml of 4% TYG supplemented with 40 mM arsenate, at room

temperature and with aeration. From each culture 1ml was harvested by centrifugation for 30 seconds, resuspended in 800µl of Tris- HCl; 10% sucrose with 3mg/ml of freshly dissolved lysolyme, and shaken for 1 hour at 37°C. The cells were then pelleted for 1 minute, drained well and resuspended in 280µl of TE. Then 40µl of TE; 10% SDS was added. The suspension should become translucent as the cells lyse, after which they were incubated at 60°C for fifteen minutes.

To the lysed cells, 35µl of 4.. ..ium acetate, pH6 was then added, mixed and the cells were placed on ice for 30 minutes. The debris was pelleted by centrifugation in the cold for 30 minutes. The upper layer was decanted off into a fresh sterile tube, and phenol extracted (section 8.D.1), ethanol precipitated (section 8.D.2), and finally resuspended in 30µl of TE containing freshly boiled ribonuclease (1mg/ml), for 1 hour at 42°C. Aliquots of 10µl were taken per sample, to which 2 µl of loading buffer (section 8.A) was added, and the samples were loaded onto a 0.4% agarose gels.

For enzymic digestions, 13.5µl of sample was taken, to which 1.5µl of Buffer M was added, and 0.1 µl of Bgl II was mixed in. Digestion occurred overnight at 37°C after which the samples were electrolysed and run on 0.4% agarose gels, for 1.5 hours. The gels were viewed by UV light before being photographed.

3.33 VERIFYING THE SOURCE OF CLONED DNA

All of the solutions were made up to 1 litre with distilled water and autoclaved at 121°C for 20 minutes. The solutions were stored at 4°C.

3.33.1 Southern Transfer

Denaturing Solution:

1.5M NaCl

0.5M NaOH

Neutralising Solution:

1.5M NaCl

0.5M Tris.

0.001M EDTA

pH 7.2

20 x SCC

3M NaCl

0.3M Na-citrate

pH7.0

The DNA of 4277-1.070 and MM294-1 were resuspended in TE. Volumes of 5µl, 1µl and 0.1µl of both DNAs were electrolysed using a 0.4% agarose gel (section 8.E.) for 1 hour. This was to determine the relative concentrations of the two DNAs so that equal concentrations were used in the Southern Transfer. The relevant volumes, were digested with 0.1µl of Bgl II overnight, and electrolysed using a 0.4% agarose gel. The gel was viewed under UV light to check that the bands were of equal intensity.

The gels (three in total, to accommodate seven sets of 4277-1.070 and MM294-1 DNA) were incubated in 0.25M HCl for 15 minutes, then placed in in denaturing solution. This was repeated for a duration of 30 minutes each. The gels were then transferred into neutralising solution, which was repeated, each fo 30 minutes. The excess liquid was then blotted off the gels.

Nitrocellulose membranes (Hybond-C, Amersham U.S.A.) were wet in distilled water and then soaked in 2 x SCC. The transfer apparatus was set up, and the wick (filter paper) was soaked in 20 x SCC and was positioned. The transfer buffer (20 x SCC) was poured into the buffer reservoir. The gels were inverted onto the wick, their orientation was marked, and all air bubbles were gently pressed out. The pre-soaked nitrocellulose membranes were placed over the gels, marked for orientation and the position of the wells, and air bubbles were gently removed. A further piece of soaked filter paper was placed over the nitrocellulose membrane, and then topped with 4-5 cm of absorbent paper, and covered with a glass plate and a weight. The whole set was sealed, and left for transfer to occur overnight.

After transfer, the nitrocellulose membranes were removed and the gels were checked using UV-light to ensure that transfer had occurred. The membranes were then washed in 2 x SCC, to remove any excess agarose, air dried and then baked in a vacuum oven for 2 hours at 80°C.

3.33.2 DNA LABELLING AND DETECTION

The Non-radioactive DNA labelling and Detection Kit of Boehringer Mannheim, Catalogue Number 1093657 was used. The principle of the method was that the DNA was labelled by random primed incorporation of digoxigenin labelled deoxyuridine-triphosphate (dUTP).

The dUTP was linked via a spacer-arm to the steroid hapten digoxigen (Dig-dUTP). After hybridisation to the target DNA, the hybrids were detected by enzyme - linked immunoassay using an antibody-conjugate (anti-digoxigenin alkaline phosphatase conjugate, <Dig>AP) and the subsequent complex was colour reacted with 5 bromo-4 chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT) (see Figure 7).

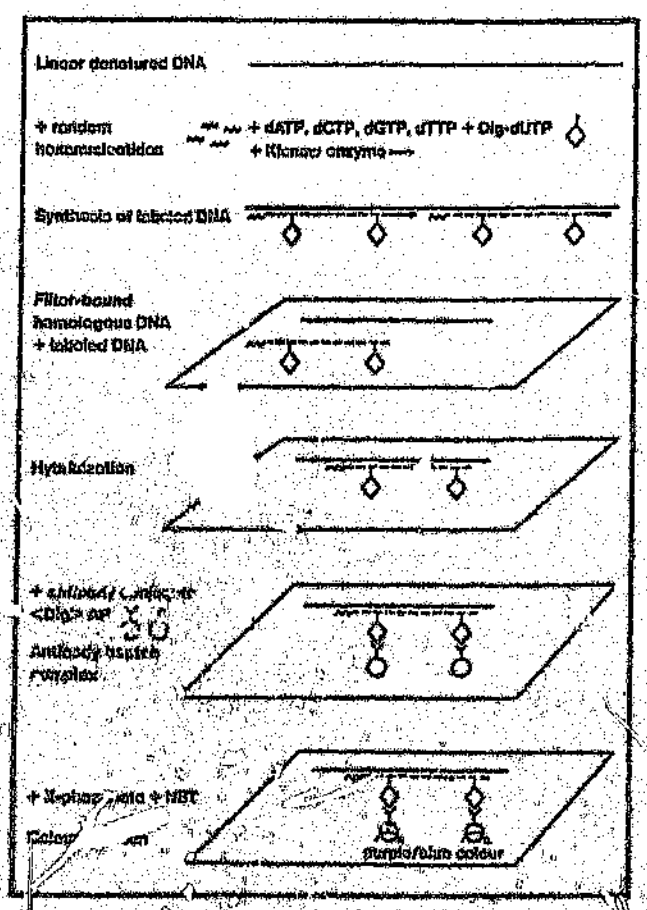


Figure 7. Nonradioactive DNA labelling and detection. (Boehringer Mannheim GmbH, Mannheim, W.Germany. Nonradioactive DNA labelling and detection kit, Catalogue Number 1093657).

The pDA37 cells with inserts (samples 1-6) had to be extracted from MM294-1, as described in section 3.27 and resuspended in Buffer M. Their respective concentrations were determined as in section 3.22. Once the concentration of each fragment had been determined, 15µl of Bgl II digested pDA37 cells with an insert were loaded

onto a 0.8% low melting agarose gel (Sea Kern). The procedure was the same as described in Section 8.E., except that the electrolysis was performed at 4°C. A potential difference of 80V and a current of 35mA was applied for 3.5 hours, after which the gel was placed onto the UV transilluminator, and the inserts were cut out of the gel. A band of pDA37 was also cut out of the gel to act as a labelling control. All of the isolated bands were stored at -20°C in the agarose.

i) DNA Labelling

0.2M EDTA (pH8) made up to 1 litre and the pH was adjusted. The solution was autoclaved and stored at room temperature.

TE

10mM Tris pH8

1mM EDTA

The solution was made up to 1 litre and the pH was adjusted. The solution was autoclaved, and stored at room temperature.

N-laurolysarcosine - Na salt (Boehringer)

A solution of 10 % (w/v) was made up in 10ml of distilled water then autoclaved and

stored at room temperature.

10 % (w/v) SDS

The solution was made up in 10 ml of distilled water, then autoclaved and stored at room temperature.

LiCl (Merck)

A 4M LiCl solution was made up to 1 litre, then autoclaved and stored at room temperature.

The samples were heated at 60°C for 30 minutes to melt the agarose. The DNA was phenol extracted and ethanol precipitated (section 8.D), and resuspended in sterile distilled water for 1 hour at 42°C. All of the samples were then denatured by heating in a water bath at 95°C for 10 minutes, and then rapidly chilled on ice. The samples were then used immediately.

Volumes of DNA equivalent to 1µg of DNA were placed in eppendorf tubes, to which was added; in succession:

2µl of hexanucleotide mixture

2µl of dNTP labelling mixture

The final volumes were made up to 19µl with sterile distilled water, and 1 µl of Klenow enzyme was added.

The mixtures were then incubated overnight at 37°C. The reaction was stopped by the addition of 2µl of 0.2M EDTA. By using 2.5µl of LiCl and 75µl of ice cold ethanol, after mixing well, and being left for 30 minutes at -70°C, the cells were precipitated by centrifugation for 20 minutes. The pellets were washed with ice-cold absolute ethanol, centrifuged for 5 minutes, and dried at 60°C for 20 minutes. The pellet was then dissolved in 50µl of TE for 2-3 hours at 42°C.

ii) Hybridisation

Hybridisation Solution:

5 x SCC

0.5 % (w/v) blocking reagent

0.1 % N-lauroylsarcosine Na-salt

0.02 % (w/v) SDS

The solution was incubated at 60°C to dissolve the blocking reagent. The solution was prepared at least 1 hour prior to use.

The nitrocellulose filters were pre-hybridised in hybridisation solution at 60°C for 1 hour, then cut into their individual strips so that there was one lane each of C1.070 DNA and MM294-1 DNA per sample probe. The hybridisation solution was replaced with 15ml of fresh hybridisation solution containing 260ng of labelled, freshly denatured DNA sample probe. The labelled vector (pDA37) served as a control probe. They were then incubated overnight at 60°C.

After incubation the filters were washed twice for 5 minutes with 2 x SCC; 0.1 % (w/v) SDS, followed by another two 15 minute washings at 60°C with 0.1 x SCC; 0.1 % (w/v) SDS.

iii) Immunological Detection

Buffer 1:

0.1N Tris

0.15M NaCl

pH7.5

The buffer was made up to 1 litre, then autoclaved and stored at 4°C

Buffer 2:

0.5 % (w/v) Blocking reagent in Buffer 1

The blocking reagent was dissolved at 60°C

Buffer 2 was prepared 1 hour before use.

Buffer 3:

0.1M Tris

0.1M NaCl

0.05M $MgCl_2.6H_2O$

pH9.5

The buffer was made up to 1 litre, then autoclaved and stored at 4°C

Colour Solutions

45µl NBT-solution

35µl X-Phosphate solution

The solution was made freshly each time, in 10 ml of Buffer 3

NBT (nitroblue tetrazolium salt, 75mg/ml in dimethylformamide, 70% (v/v),

X-phosphate (5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt, 50mg/ml in dimethylformamide).

All incubations were performed at room temperature and except for the colour reaction, with shaking. The membranes were briefly washed (1 minute) in Buffer 1, then incubated for 30

minutes with Buffer 2. The membranes were washed briefly in Buffer 1. Antibody-conjugate (polyclonal sheep anti-digoxigenin Fab-fragments, conjugated to alkaline phosphatase) was diluted 1 in 5000 in Buffer 1, and each membrane was incubated for 30 minutes with 20 ml of the solution. The unbound antibody-conjugate was removed after two washings of 15 minutes each with Buffer 1. Each membrane was equilibrated for 2 minutes in 20 ml of Buffer 3, and then incubated with 10 ml of the colour solution, and placed in the dark. The colour reaction started to form within a few minutes, and was completed after one day. The reaction was stopped by storing the membranes in TE pH8.

3.34 TESTING FOR CONTAMINANTS

As one mutant appeared to be a contaminant, the similarities and differences between the parental strain 4277-1 and the mutants 4277-1.001 and 4277-1.063 were tested, by means of phage typing, streptomycin resistance and studying the pattern of the ribosomal proteins.

3.34.1 Phage Typing

If a phage was specific for a particular microorganism, it would invade the cell and lyse

it, causing zones of clearing or plaques in a lawn of bacterial growth.

Cultures were grown in 5 ml of TYC (section 8.A.8). Samples of 1ml were centrifuged, and resuspended in 1ml of TYC. The following components were then added to a sterile glass test tube in succession:

Test	Control
2ml TYC	2ml TYC
20ul Bacterial culture	20ul Bacterial culture
20ul phage 41 (specific for 4277-1)	
↓	↓
mixed	mixed
↓	↓
Add 2ml Top Agar (section 8.B.4)	Add 2 ml Top Agar
↓	↓
mixed	mixed
↓	↓
Poured onto TYCA plates (section 8.B.1)	Poured onto TYCA plates

The plates were incubated at room temperature, and observed for plaques.

i) Isolation of Phage for Typing

The isolation was performed before the plates containing plaques became overgrown. The top agar was melted at

42°C for 2 hours, then collected and centrifuged at 12 000 rpm for 10 minutes. The phage lysate 41, was recovered in the supernatant, filtered (0.45µm) and stored at 4°C.

3.34.2 Streptomycin Resistance

Plates of TYA (section 8.B.1) supplemented with 50 µg/ml of streptomycin were streaked with 4277-1, 4277-1.001 and 4277-1.063 to single colonies. The plates were incubated at room temperature and observed for their pattern of streptomycin resistance.

3.34.3 Isolation of Ribospmes

i) Preparation of Ribosomes

10mM Tris, 500mM NH_4Cl, 10mM $MgCl_2$, pH7.5

A solution of 10mM Tris and 500mM NH_4Cl was made up in 900 ml of distilled water. The pH was adjusted and then 10mM $MgCl_2$ was added. The solution was made up to a final volume of 1 litre.

150mM Magnesium Acetate in Glacial Acetic Acid

The solution made up in 200 ml of Glacial Acetic Acid (SAARchem)

Cultures of 4277-1 and 4277-1.001 in 100 ml of 1% TYG were incubated at room temperature, until they had reached stationary phase. The cells were pelleted at 6000rpm for 10 minutes, and then resuspended in 8ml of Tris, NH_4Cl, $MgCl_2$ buffer. The solution was then poured into small glass bottles and chilled on ice. The cells were then sonicated for 15 seconds, after which they were centrifuged at 15000rpm for 15 minutes. The supernatant was poured into 50Ti tubes and centrifuged at 45000rpm for 2 hours. The pellet was resuspended in 0.5ml Tris, NH_4Cl, $MgCl_2$ buffer using magnetic fleas for 20 minutes. The solution was poured into eppendorf tubes and centrifuged for 45 seconds. The supernatant was pooled into a large centrifuge tube per strain, and 1ml of Magnesium Acetate in glacial acetic acid was added, whilst stirring, and stirred for a further 15 minutes. The samples were centrifuged at 15 000 rpm for 10 minutes. The supernatant was poured into wide (1cm) dialysis tubing (section 8.D.3) and dialysed against 2 % glacial acetic acid for 2 hours, and further dialysed overnight with fresh solution. The samples were poured into small glass bottles and lyophilised overnight. If not directly used, the samples were stored at 4°C.

ii) Running of one-dimensional polyacrylamide gels

1D separating polyacrylamide gel (5% gel)

25ml sterile water, to which was added:

18g Urea (SAARchem)

2.5g acrylamide (SAARchem)

0.09g bisacrylamide (SAARchem)

0.4g Na_2 EDTA (Sigma)

1.6g boric acid (Sigma)

2.4g Tris (Boehringer)

The gel was made up to 49.5ml with sterile water.

1D sample polyacrylamide gel

25ml sterile water, to which was added:

24g urea

2g acrylamide

0.1g bisacrylamide

0.04g Na_2 EDTA

0.16g boric acid

The gel was made up to 49.5ml with sterile water

1D electrode buffer

216g Urea

2.88g Naz EDTA

5.76g boric acid

8.73g Tris

The solution was made up to 600ml with sterile water.

7% Ammonium Persulphate

7g ammonium persulphate (SAARchem) in 100 ml of sterile water was made.

20 % Glycerol Solution (lubricant)

20 ml glycerol in 80 ml of sterile water.

For five samples, 11ml of separating gel to which was added, 100µl of 7% ammonium persulphate was degassed on ice with stirring for 5-10 minutes. Then 30µl of TEMED (BDH) was added. The 1D gel tubes were sealed at the bottom, and loaded to the marked level. Then tapped to dispel air bubbles, and the open side was gently overlaid with water, to form a straight polyacrylamide meniscus, and then left to set for 1 hour at 40°C (exothermic reaction). When set, the water from the polymerised 1 D tubes was absorbed, and the tubes were placed

into the 1D electrophoresis apparatus, such that they did not touch the bottom of the apparatus. The buffer was added, submerging both ends of the tube. Then 150µl of sample gel was mixed gently with each sample and loaded onto the top of the tubes. The apparatus was sealed and electrolysed for 25-30 hours at a potential difference of 100V and a low current, in the cold room.

iii) Running of two-dimensional polyacrylamide gels

2D separating gel (Filtered if necessary)

210g urea

105g acrylamide

2.7g bisacrylamide

30.4ml glacial acetic acid

5.6 ml 5M KOH (Merck)

The gel was made up to 250 ml in sterile water.

Bottom gel

14g acrylamide

0.34g bisacrylamide

The gel was made up to 200 ml in sterile water.

2D Buffer

To 2 litres of distilled water, was added:

28g glycin

3ml glacial acetic acid

(The buffer was chilled before being used).

To the bottom gel, 10ml of 7% ammonium persulphate per 200 ml of gel was added and degassed on ice. Then 1ml of TEMED was added, and the gel was poured into the bottom plate and the 2D gel chamber was positioned on top and left to polymerise for 1 hour.

The 1D gels were switched off, removed from the tubes using 20 % glycerol as a lubricant, and placed along the top slots of the 2D gel chamber, with the orientation noted. To 200 ml of 2D separating gel, 7ml of 7% ammonium persulphate was added, the gel was degassed, and then 1ml of TEMED was added. About 30 ml of separating gel was added to each chamber underneath the 1D gel, and left to polymerise for 30 minutes. The bottom gel was then removed and the 2D buffer was poured into the chamber. The apparatus was sealed, and electrolysed for 2 hours at a potential difference of 75V, in the cold, with a very high current.

iv) Staining

Amido Black

25ml glacial acetic acid

1g amido black (Boehringer)

The solution was made up in 475ml of distilled water.

After completion of the electrolysis, the gels were removed from the apparatus and placed in amido black stain for 3 to 4 minutes. Then washed in running water for 1 - 1.5 hours. The gels were transferred to 2% glacial acetic acid and destained overnight. The destaining procedure was repeated until the spots were clearly visualised, and the gels were then photographed.

3.35 RESTRICTION ENDONUCLEASES

All of the enzymes were obtained from Boehringer

Enzyme	Incubation Buffer	Recognition Sequence
Bgl II	M	A↓GATCT
Cla I	H	AT↓CGAT
Pvu II	M	CAG↓CTG
Bam HI	B	G↓GATCC
Eco RI	H	G↓AATTC
Pst I	H	CTGCA↓G

The buffers were supplied in 10 X concentrations and were diluted to a 1 X concentrate before the enzyme was added. In 1µl of enzyme there were 8 units, and as one unit was the enzyme activity that completely cleaved 1µg of λDNA in 1 hour, at optimal temperature in the incubation buffer, 0.1 µl of enzyme was ample for digestions.

In double digestions, if the two restriction endonucleases did not have the same buffer necessary for 100 % activity, one digestion was performed alone, the DNA was purified, and then the second digestion was performed.

3.36 PHOTOGRAPHY

All photographs, except for the Southern blots, were taken with a Fotodyne Incorporated Polaroid camera, at the widest aperture of 4,5, and an f stop of either 1 or 2. The Southern blots were photographed by Central Graphics, University of the Witwatersrand.

4.0 RESULTS

4.1 UTILISATION OF SOLE CARBON SOURCES

The utilisation of various carbon sources, including the bile acids, by the nocardioform bacteria was the basis for the choice of the donor and recipient bacteria for the gene cloning. The results of the preliminary spot tests were as in Table 1. The concentrations were from the literature.

Table 1. Utilisation of glucose and other carbon sources by nocardioform bacteria.

STRAINS	42277-1	14987	A448	25593	KDI	DSM43756	12.7181	C1	C7
YEA	+++	+++	+++	+++	+++	+++	+++	+++	+++
MM+G	+++	+++	+++	+++	+++	++	+++	+	++
MM-G	+/-	+	+	+		+	+/-	+	+/-
2% DMSO	+	++	+++	+++		++	+	+	+
100ug/ml cholesterol in DMSO	+	+++	+++	+++		++	+	++	++
100ug/β sitosterol in DMSO	+	+++	+++	+++		++	+	++	+
100ug/ml stigmasterol in DMSO	+	++	++	++		++	+	++	+
20ng/ml ST	+	++	++				+	++	
2% ethanol	+	++	++	++		++	+	+	+
0,1% sodium acetate	+	+	++	+		+	+	+	+
0,1% sodium citrate	+	+	++	+		+	+	+	+
0,1% succinate	+	+	++	+		+	+	+	+
0,05% glycerol	++	++	+	+	++	++	++	++	+
0,1% glycerol	++	+++	+	+	++	++	+++	+++	+
0,05% sodium pyruvate	+	+	+	++		++	+	++	+
0,1% sodium pyruvate	+	+	++	++		++	+	++	+

Key: ± slight growth

\+ moderate growth

++ good growth

+++ very good growth

From the results of Table 1, it can be seen from growth on MMnoG that 14887, A448, 25593, DSM43756 and C1 utilised agar as a sole carbon source. The rest of the strains utilised agar poorly with strain KD1 not utilising it at all. As both A448 and 25593 utilised the 2% DMSO much more than the other strains, these results were checked in liquid cultures. The results of the liquid cultures were as shown in Table 2.

Table 2. Comparison of the growth of nocardioform bacteria on agar and in liquid cultures supplemented with DMSO.

STRAINS	AGAR		BROTH	
	MMnoG	MMNOG + 2% DMSO	MMnoG	MMnoG + 2% DMSO
A277-1	+/-	+	NO GROWTH	NO GROWTH
A448	+	++		
25593	+	++		
DSM 43756	+	++		
C1	+	+		

Key:

± slight growth

+ moderate growth

++good growth

The cultures were left to incubate for five weeks. Due to nocardioforms being slow growing bacteria, oxygen was not limiting. However, no

growth was observed.

Utilisation of the cholesterol was similar to utilisation of the DMSO, and as the cholesterol was dissolved in DMSO the result was taken to indicate that cholesterol could not be utilised. The results for β-sitosterol and stigmasterol were comparable, and follow the same pattern of non-utilisation as cholesterol. For these reasons, these steroids were no longer pursued in this study.

The results of the utilisation of succinic acid, sodium citrate and sodium acetate, did not show much difference from the utilisation of agar, and these compounds were no longer pursued in the study. There was evidence that ethanol could be used as a sole carbon source, but its evaporation would always be a problem in ensuring that the correct concentration was achieved, thus it too was not studied further.

The utilisation studies of glycerol showed that growth was better overall, and that KD1 could utilise it. The glycerol was utilised as a carbon source, as growth on the plates were better than the growth on the agar plates. All of the strains increased their utilisation of the glycerol with

time and concentration, except for A448, 25593 and C1.

The studies of pyruvate showed poor growth for all strains, except for 25593, DSM 43756 and C1. There was no increase in pyruvate utilisation with concentration or time of incubation. From the preliminary studies of utilisation of sodium taurocholate (ST) it appeared that the concentration of ST was too high at 100mg/ml, which had been taken to be the optimal concentration for utilisation by K.Downing (1989). A range of concentrations was then studied. The bile acids were further studied as they differed minimally in their side chain structures and all of them were water soluble.

4.1.1 Sodium Taurocholate

At first autoclaved ST was used in the utilisation studies, but the results were found to vary significantly from test to test. Then filter sterilised ST was used, and a comparison made, as in Table 3, as to which was the better and more stable method of sterilization. The results from the filter sterilised ST utilisation studies were found to be more similar from test to test. All of the further studies were carried out using filter sterilised bile acids.

Table 3. Sensitivity to autoclaved versus filter sterilised sodium taurocholate by nocardioform bacteria.

STRAINS	M H NO G	FILTERED ST (mg/ml)					AUTOCLAVED ST (mg/ml)				
	MH NO G	1	5	10	15	20	1	5	10	15	20
4271-I	+/-	+/-	+	+	+	+	+/-	+	+	+	+
14887	+	+	+	+	+	+	+	+	+	+	+
A448	+	+	+	+	+	+	+	+	+	++	++
25593	+	+	*	+	+	+	+/-	+/-	*	*	*
KD1											
DSM43756	+	+	+	+	+	+	+/-	+/-	+/-	*	*
12,7iii	+/-	+	+	+	+	+	+/-	+/-	+/-	+/-	+/-
C1	+/-	+	+	++	++	++	+	+	+	++	++
C7	+/-	+/-					+/-				

Key: * secondary growth on metabolic by-products

± slight growth

+ moderate growth

++ good growth

The autoclaved ST induced the same pattern as for filtered ST up to 10mg/ml concentration, except for 25593, DSM 43756 and 12.7iii, which were significantly inhibited from 1mg/ml. Strain KD1 did not utilise ST at all. From 10mg/ml of ST onwards the other strains showed slightly less growth, except for the growth of A448 which seemed to have been enhanced. A more comprehensive utilisation and resistance study of ST was performed, as in Table 4.

Table 4. Sensitivity and resistance to sodium taurocholate by nocardioform bacteria.

STRAINS	MM NO G	ST (ug/ml) 1	5	10	15	20	25	30	35	40	45	50	MM + G	ST (ug/ml) 1	5	10	15	20	25	30	35	40	45	50
4277-1	+/-	+/-	+	+	+	+	+	+	+	+	+/-		+(+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
14087	+	+	+	+	+	+	+	+	+	+	+/-		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A449	+	+	+	+	+	+	+	+	+	+	+		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
25593	+	+	+	+	+	+	+	+	+	+	+/-		+++	+++	+++	+++	+++	+++	++	+	+	+/-	+/-	
KD1													+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
DSM 43756	+	+	+	+	+	+	+	+	+	+	+/		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+
12.7111	+/-	+	+	+	+	+	+	+	+	+	+/-		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
C1	+	+	+	++	++	++	++	++	++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
C7	+/-	+/-											++	++										

Key: ± slight growth
+ moderate growth
++ good growth
+++ very good growth

Over a time period of six days, only the growth of 4277-1 increased on the utilisation plates. All of the growth was considered poor to moderate except for C1 which showed good growth, but as it was a slower growing organism, an increase in growth with time was not significantly observed. The resistance of the microorganisms (MM+G plates) to ST, was considered high for all, except C7. However, of the strains that could grow on 50mg/ml of ST, none could grow on 100mg/ml or more of ST.

4.1.2 SODIUM DEOXYCHOLATE

Autoclaved and filter sterilised SD were tested, and the growth patterns were found to be similar for all strains. Only at concentrations of 20mg/ml and above were there differences. The filter sterilised SD was better utilised and the strains had more resistance to it, but this was found to be the case with the strains that were most sensitive to SD. The results of SD as the sole carbon source are shown in Table 5. All secondary growth was characterised by satellite colonies which grew on the metabolised bile acids.

Table 5. Sensitivity and resistance to sodium deoxycholate by nocardioform bacteria.

STRAINS	SD (mg/ml)															
	MH NO G	1	5	10	15	20	25	30	MH + G	1	5	10	15	20	25	30
4277-1	+/-	+/-	+	+	+	+	+		+++	+++	+++	+++	+++	+		
14887	+/-	+/-	+	+	+	++	++		+++	+++	+++	+++	+++	++	+/-	
A448	+/-	+/-	+/-	+/-	+/-				+++	+++	+++	+++	+++	+/-		
25593	+	+	+	+	+	+			+++	+++	+++	+++	+	+		
XD1									+++	+++	+++	+++	+++	++	+	
DSM 43758	+/-	+/-	+/-	+/-	*	*	*		+++	+++	+++	+++	++	+		
12,7iii	+/-	+/-	+	+	+	+	++		+++	+++	+++	+++	+++	+++	++	+
C1	+	+	+	++	++	++	++		+++	+++	+++	+++	+++	+++	++	++
C7	+/-	+/-							++	++	*	*	*			

Key: * secondary growth on metabolic by-products

± slight growth

\+ moderate growth

++ good growth

+++ very good growth

On the no carbon source plates, poor growth all round was observed, which illustrated that these strains could not utilise agar very well. Strain KD1 did not grow at all. With the supplementation of SD, the growth was not improved of A448, 25593, DSM 43756 and C7. The other strains, 4277-1, 14887, 12.7iii and C1 showed an enhancement of growth, but after a certain concentration, particular for each strain, inhibition occurred. All growth was found to be inhibited at 30mg/ml of SD. With time, only the growth of A448 and C1 was found to have increased slightly.

The SD resistance studies showed very good growth all round. Inhibition of growth was only started at 15mg/ml of SD, with the exception of C7, which showed growth inhibition at 5mg/ml. By a concentration of 20mg/ml, drastic growth inhibition was observed for all other strains, with the exception of 12.7iii and C1, where inhibition occurred at 35mg/ml of SD. With time, all growth increased, and followed the same pattern of resistance. The strains 12.7iii and C1 were found to be able to utilise, and be most resistant to SD, although C1 grew very slowly.

Strain C7 was found to be hypersensitive to SD.

4.1.3 SODIUM CHENODEOXYCHOLATE

The patterns of utilisation of and resistance to SC were generally found to follow those of SD, as seen in Table 6.

Table 6. Utilisation of and resistance to sodium chenodeoxycholate by nocardioform bacteria.

STRAINS	SC (mg/ml)															
	MM NO G								MM + G							
	MM NO G	1	5	10	15	20	25	30	MM + G	1	5	10	15	20	25	30
4277-1	+/-	+/-	+	++	++	++	++	+/-	+++	+++	+++	+++	+++	+++	+++	
14887	+	+	+	++	++	++	++	+/-	+++	+++	+++	+++	+++	+++	++	+++
A449	+	+	+	+	+				+++	+++	+/+	+++	*			
25593	+	+	+	+/-					++	++	++					
KD1									+++	+++	+++	+++	+++	+		
DSM 43756	+	+	+	*	*	*			+++	+++	+++	++	++			
12.7111	+/-	+/-	+	++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	++
C1	+	+	+	++	++	++	++		+++	+/+	+++	++	++	++	++	
C7	+/-	+/-							++	+						

Key: * secondary growth on metabolic by-products

± poor growth.

+ moderate growth

++ good growth

+++ very good growth

For both utilisation and resistance studies, at concentrations of 35mg/ml and above, no more growth was observed for any of the strains. At a

concentration of 10mg/ml, most strains seemed to have been stimulated and grew well. In the resistance studies, all of the strains grew well, except C7, and most were suddenly inhibited from growing at a particular concentration of SC. Strain 12.7iii was observed to be the most resistant to, and be able to utilise SC the most.

4.1.4 SUMMARY OF THE RESISTANCE AND SENSITIVITY OF NOCARDIOFORM BACTERIA TO BILE ACIDS.

From the results of Tables 4 to 6, the resistance and sensitivity to the bile acids were summarised in Table 7.

Table 7. The concentrations of maximum resistance and sensitivity of nocardioform bacteria to bile acids.

STRAINS	ST (ug/ml)		SD (ug/ml)		SC (ug/ml)	
	RESISTANCE	SENSITIVITY	RESISTANCE	SENSITIVITY	RESISTANCE	SENSITIVITY
4277-1	50	45	20	25	25	30
14887	50	45	25	25	30	30
A448	50	45	20		15	15
25593	45	45	20	20	15	15
XD1	50		25		20	
DSM 43756	50	45	20		15	5
12.7iii	50	45	30	25	30	30
C1	50	50	30	25	25	25
C7	1	1	1	1	1	1

From the results of Table 5, it appeared as if A448 and DSM 43756 could not utilise SD as the sole carbon source. Except for C7, all of the

strains had a higher level of resistance and a lower level of sensitivity to ST than for the other bile acids. Strain KD1 could not utilise or was sensitive to all of the bile acids.

4.2 CHOICE OF RECIPIENT AND DONOR DNA FOR GENE CLONING

Sodium deoxycholate was chosen to be the bile acid from which the recipient and donor strains were determined, from the utilisation and resistance studies, as the results were generally midpoint of ST and SC. From Table 5, C1 was considered to be the most resistant to, and have the best utilisation of SD, and thus was chosen as the source for donor DNA. Strain 4277-1 was chosen as the recipient strain, as 14887 has been previously studied by K. Downing (1989).

The strains 25593 and DSM 43756 gave no evidence that they grew better on SD than on agar only. The strains KD1 and 12.7iii were derivatives of 14887, and apart from the strains C1 and 4277-1, most of the other strains had stringent amino acid requirements for growth. Strain DSM 43756 was included as a reference, as it was very closely related to the Rhodococcus strain.

The strains A448 and 14887 were chosen as alternate sources of DNA for ST and SD

utilisation respectively. To ensure that they could use bile acids as sole carbon sources, liquid culture utilisation tests were performed. The results were seen in Table ..

Table 8. Utilisation of the bile acids in liquid culture, by the strains to be used as alternative sources of DNA.

STRAINS	M U NO G			
	MM NO G	SD(15mg/ml)	ST (15mg/ml)	SC (15mg/ml)
A448	+	++	++	++
DSM43756	++	+++	+++	+++

Key: + moderate growth

++ good growth

+++ very good growth

The results s' wed that both A448 and DSM 43756 could utilise SD, ST and SC as sole carbon sources.

4.3 UTILISATION OF AND RESISTANCE TO BILE ACIDS BY 4277-1

Broth was used to establish utilisation and resistance curves, which excluded agar as an extra carbon source. For these studies filter sterilised bile acid was used, based on the findings of Table 3.

Once 4277-1 had been chosen as the recipient strain, it was necessary to determine the optimal concentrations of the bile acids for the resistance and utilisation studies. After numerous tests, the most representative graphs were plotted to fit the best curve. Utilisation of and resistance to ST (Figure 8) showed correlation to the plates (Table 4), where maximum resistance was found to be at 75mg/ml, and utilisation at 55mg/ml, which were much greater concentrations than for the other two bile acids.

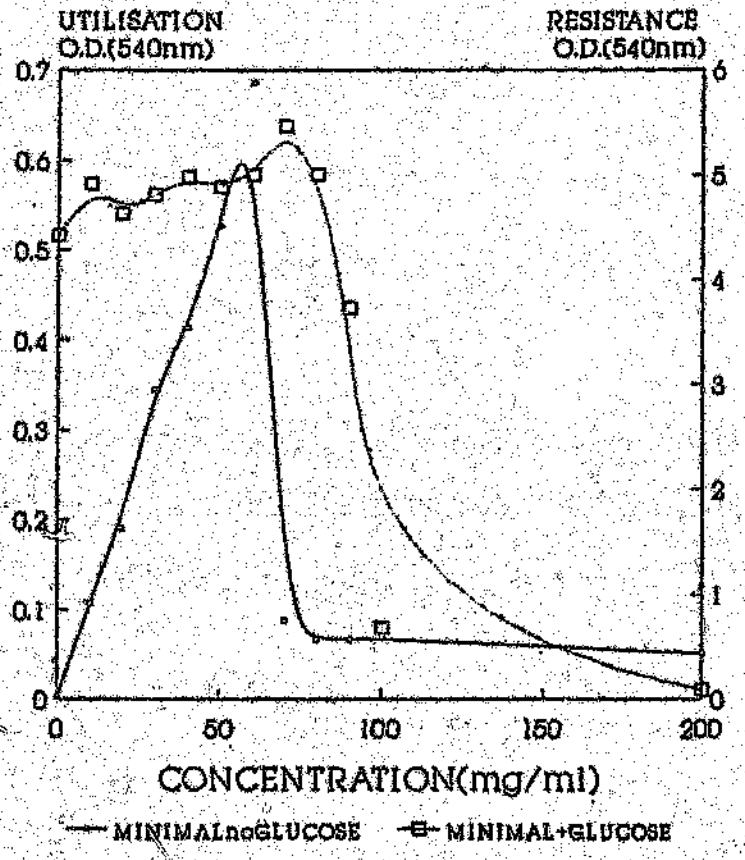


Figure 8. Utilisation and resistance of 4277-1 to varying concentrations of sodium taurocholate, as determined by optimal density at 540nm.

Once 4277-1 had been chosen as the recipient strain, it was necessary to determine the optimal concentrations of the bile acids for the resistance and utilisation studies. After numerous tests, the most representative graphs were plotted to fit the best curve. Utilisation of and resistance to ST (Figure 8) showed correlation to the plates (Table 4), where maximum resistance was found to be at 75mg/ml, and utilisation at 55mg/ml, which were much greater concentrations than for the other two bile acids.

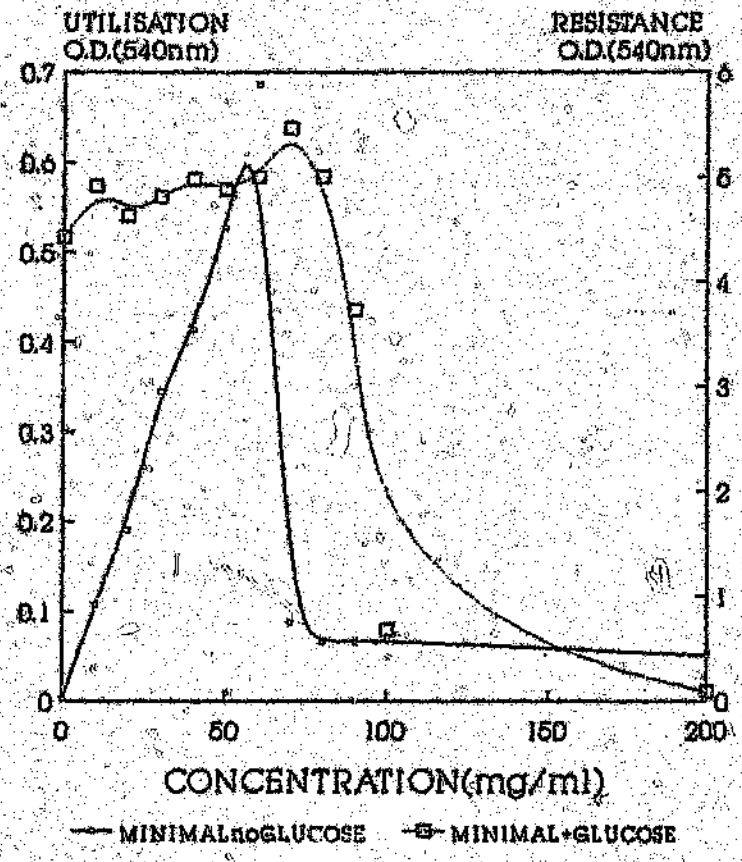


Figure 8. Utilisation and resistance of 4277-1 to varying concentrations of sodium taurocholate, as determined by optimal density at 540nm.

The utilisation of SD correlated with Table 5, as maximum utilisation of SD was at a concentration of 10mg/ml, and after 20mg/ml of SD, 4277-1 was no longer resistant to SD. (Figure 9).

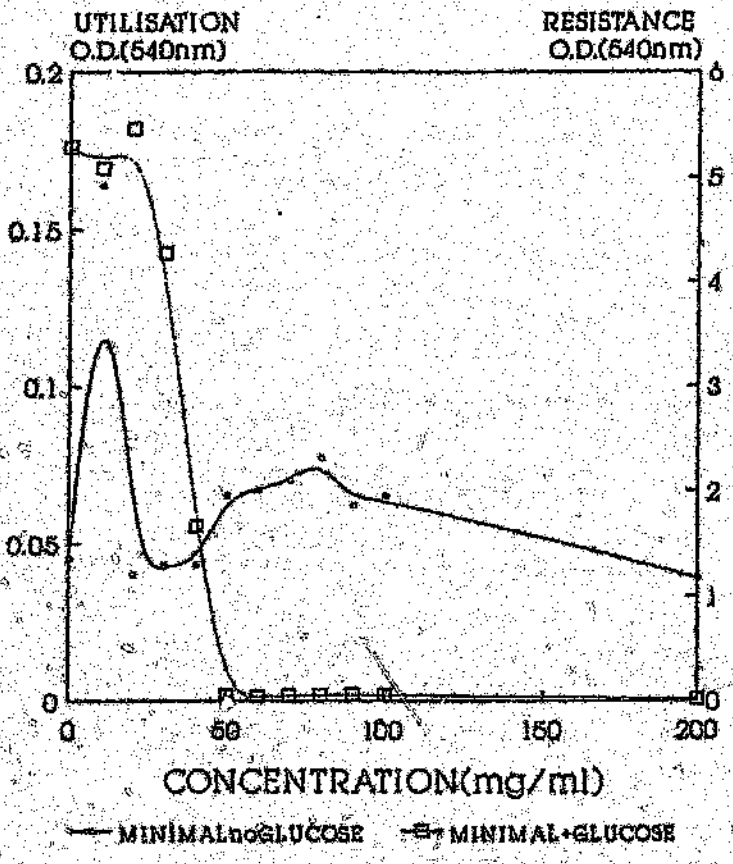


Figure 9. Utilisation and resistance of 4277-1 to varying concentrations of sodium deoxycholate, as determined by optimal density at 540nm.

The patterns of utilisation and resistance of 4277-1 to SC were comparable to the results of Table 6 (Figure 10), where maximum utilisation of SC was at 20mg/ml, and after 20mg/ml of SC, 4277-1 was no longer resistant to SC.

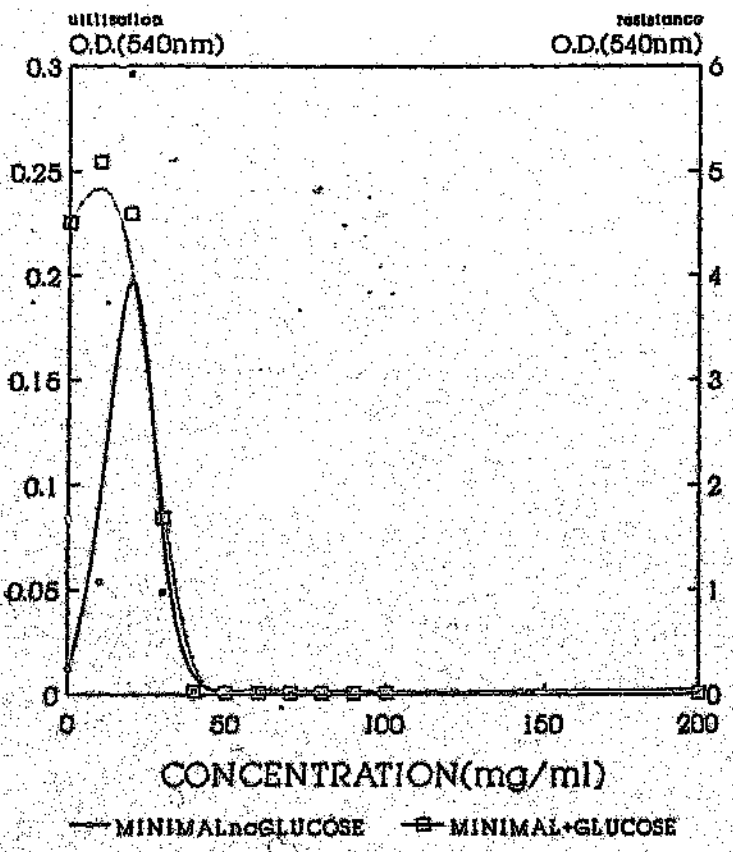


Figure 10. Utilisation and resistance of 4277-1 to varying concentrations of sodium chenodeoxycholate, as determined by optical density at 540nm.

When the utilisation of the three bile acids by 4277-1 was compared (Figure 11), it was found that ST was utilised much more. Sodium chenodeoxycholate was utilised the least, with SD being midway. All three shared a classical utilisation curve.

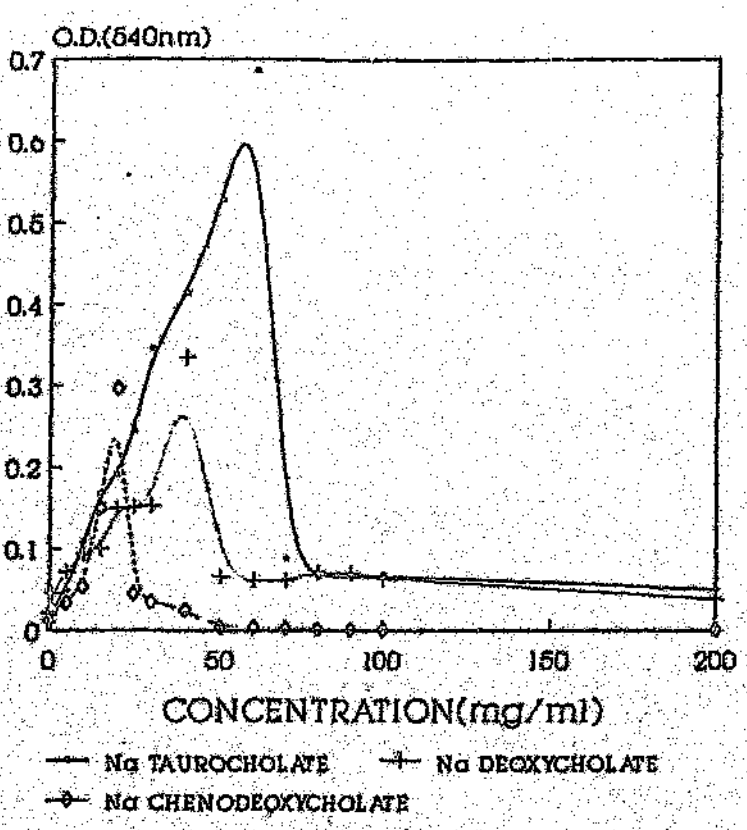


Figure 11. Comparison of 4277-1 utilisation of varying concentrations of sodium taurocholate, sodium deoxycholate and sodium chenodeoxycholate, as determined by optical density at 540nm.

A similar pattern was found for the resistance of 4277-1 to the bile acids (Figure 12).

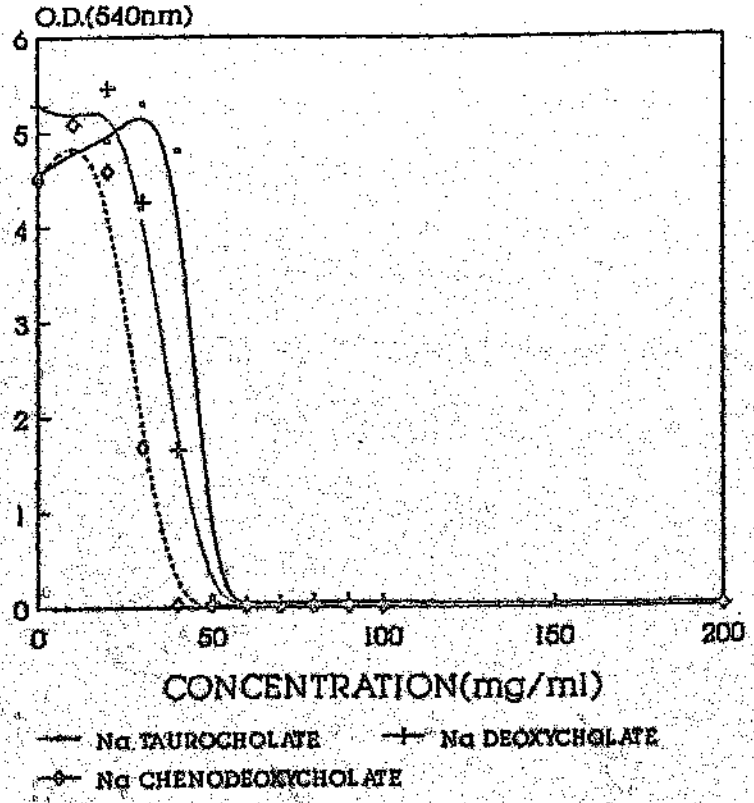


Figure 12. Comparison of 4277-1 resistance to varying concentrations of sodium taurocholate, sodium deoxycholate and sodium chenodeoxycholate, as determined by optimum density at 540nm.

The level of utilisation or sensitivity was proportional to the resistance, that is, the compound that was utilised the least, 4277-1 was least resistant too.

It was found that the SD precipitated out of solution, which gave erroneous results. Studies of the pH of SD determined that 40mg/ml of SD in broth at a pH of 7.19 remained in solution, but when the broth became acidic (pH5.58), the SI

precipitated. By adding one drop of dilute sodium hydroxide, the pH was raised, and SD went back into solution. As the cells utilised the broth, the medium became more acidic, due to the accumulation of cell metabolic by-products. To overcome this problem, one drop of 10% sodium hydroxide was added to the cultures before the OD readings were taken. However, the sodium hydroxide could have lysed the cells.

The resistance and utilisation curves established the optimal concentrations of the bile acids, so that when testing the mutants of 4277-1, it could be determined whether they were hypersensitive mutants or non-utilisers of the bile acids.

The growth of 4277-1 due to stimulation by the bile acid, was represented by the area under the resistance and utilisation curves. Strain 4277-1 appeared to be stimulated by larger concentrations of ST, and thus utilised, and was more tolerant to higher concentrations of ST, than the other two bile acids. The utilisation and resistance patterns were more similar between SC and SD. The low resistance of 4277-1 to SC (Figure 12) could have been due to 4277-1 being hypersensitive to SC.

4.4 NTG-MUTAGENESIS

Before NTG-mutagenesis could be performed, the growth curve of 4277-1 had to be elucidated. Once 4277-1 (a filamentous bacteria), entered the stationary growth phase, as it was in different forms and not single colonies, a reduction in the efficiency of mutagenesis resulted, along with different effects when plated out. The most representative OD of the growth curves of 4277-1 in T2 (Figure 13), enabled the growth phases to be identified.

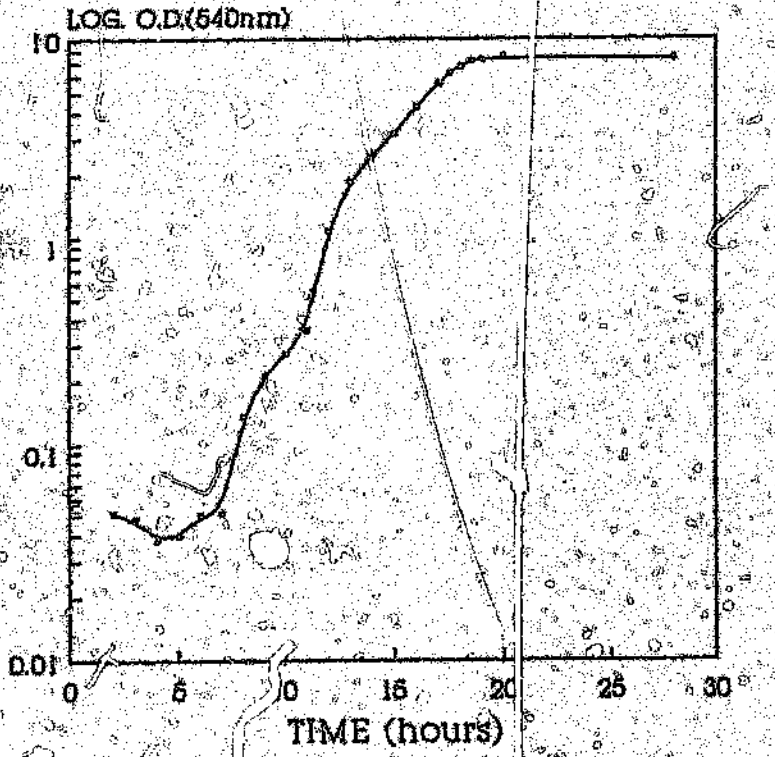


Figure 13. Growth curves of 4277-1 in T2 medium, as determined by optical density at 540nm.

A comparison of the growth pattern of 4277-1 cells mutagenised at logarithmic phase and at stationary phase was performed (Figures 14 and 15).

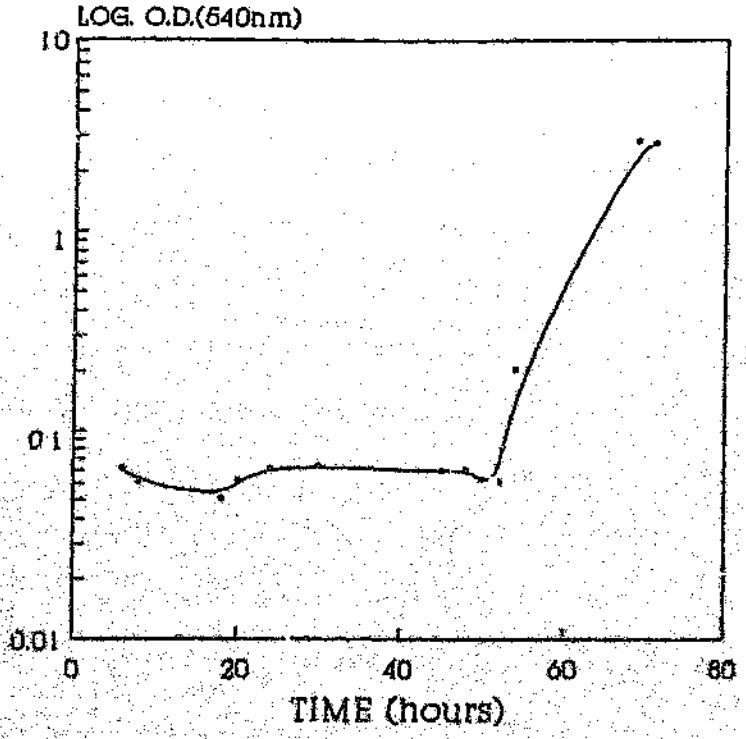


Figure 14. Growth curve of 4277-1 in T2 medium after NTG-mutagenesis during logarithmic phase, as determined by optical density at 540nm.

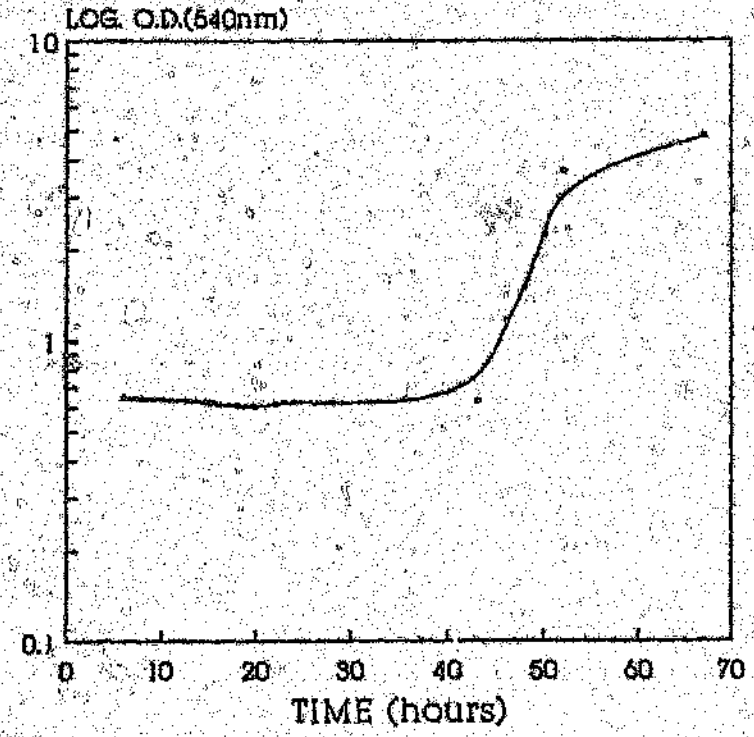


Figure 15. Growth curve of 4277-1 in T2 medium after NTG-mutagenesis during stationary phase, as determined by optical density at 540nm.

In both growth curves, the lag phase was very long, and the OD value of the stationary phase was less than in the non-mutagenised 4277-1 cells, although inoculum density was adjusted so that the same number of cells were used in the two cultures. The cells mutagenised at stationary phase recovered more quickly, and at stationary phase were 1 OD value more than the logarithmic-mutagenised cells. Altnough slight peaks were found in Figure 14 during the lag phase, no visible differences were observed. The growth curves did not show any considerable differences, yet the efficiency of mutagenesis of cells at logarithmic and stationary phase differed considerably, as shown in Table 9.

Table 9. Comparison of NTG-mutagenesis efficiency of an exponentially growing culture and a stationary phase culture of 4277-1.

GROWTH PHASE OF CULTURE	% AUXOTROPHS
Exponentially growing culture	2,16
Stationary phase culture	0,89

The efficiency of mutagenesis was determined by calculating the percentage of auxotrophs produced per number of cells patched. For further mutagenesis, cells in the logarithmic phase were used. Patching had to be done immediately after

the cells had grown into single colonies, as if refrigerated, the cells entered into stationary phase (i.e. filamentous forms) on storage, their viability could not be certain, and they would no longer be single colonies.

4.4.1 THE ENRICHMENT PROCEDURE

Nine rounds of NTG-enrichment mutagenesis was performed from which 67 putative mutants were created. Eight of these mutants were confirmed to be auxotrophs. To enable the treatment with ampicillin to be effective, the cells had to be actively growing, thus the doubling time of the 4277-1 mutagenised cells had to be determined (section 3.12). It was found to be between 7 to 8 hours in T2 medium, and 9 to 10 hours in MM+G.

4.5 SPONTANEOUS MUTAGENESIS

Cultures of the strains 4277-1, C1 and 14887 were plated onto selective utilisation plates of 100mg/ml and 200mg/ml of ST, SC and SD, to select for possible spontaneous mutants that could utilise high concentrations of bile acid. Simultaneously, a spot test of all the nocardioforms onto the same concentrations of bile acids, yielded no growth at all.

From the plated cultures, colonies grew on the ST supplemented plates only. The results were as in Table 10.

Table 10. Spontaneous mutants generated from selected nocardioform bacteria and their mutation frequency.

Strains	Colony forming units/ml from MMnoG +100mg/ml ST	Mutants/ml	Frequency of mutagenesis
C1	1.16×10^9	10	8.6×10^{-9}
4277-1	2.91×10^9	5	1.7×10^{-9}
14887	1.92×10^9	5	7.8×10^{-9}

One of the C1 mutants which was found to grow on 100mg/ml of ST and the other two bile acids, was then used as the donor DNA, and designated C1.070.

4.6 Testing Putative Mutants

The putative mutants which were found to be able to utilise high concentrations, or to be non-utilisers of the bile acids were selected, and spot tested to determine their characteristics. The spot tests checked if the mutants with an increased ability to grow on one bile acid as the sole carbon source, corresponded with an increased ability to grow on the other two bile acids. The mutants and the parental

strains of 4277-1, 14887 and C1 were streaked out to single colonies, to ensure that they were all in the same physiological state. The results were found as in Table 11.

Table 11. Mutants of 4277-1, C1 and 14887 with increased ability to utilise bile acids.

STRAINS	TTA	MM + G	M & no G			
			MMnoG	20mg/ml SD	20 mg/ml SC	40mg/ml ST
4277-1	+++	+++	+/-	+	+	++
4277-1.068	+++	+++	+	+	++	+
C1	+++	++	+	+	+	+
C1.069	+++	++	+	+	++	++
C1.070	+++	+++	+	++	++	++
14887	+++	+++	+/-	+	+	*
14887.071	+++	+	+/-	+/-	*	
14887.072	+++	*	+/-	+/-	*	
14887.073	+++	+				
14887.074	+++	+/-		*		

Key: * secondary growth on metabolic by-products

± slight growth

+ moderate growth

++ good growth

+++ very good growth

The ability to grow on a high concentration of one bile acid, did not necessarily correspond to the ability to grow on high concentrations of the other bile acids as the sole carbon source. Some of the mutants were found to have reverted.

The same was done for the putative mutants which had been isolated for their reduced ability to utilise a bile acid as the sole carbon source. It was determined whether their ability to utilise the other two bile acids was effected. The results were as in Table 12. These mutants were created by NTG-mutagenesis of 4277-1.

Table 12. Mutants of 4277-1 with reduced ability to utilise bile acids.

STRAINS	TYA	MM + G	M M no G			
			MMnoG	20ng/ml SD	20 ug/ml SC	40ng/ml ST
4277-1	+++	+++	+/-	+	+	++
4277-1.001	+++	++	+	*	*	*
4277-1.002	+++	+++	+	+	+	+++
4277-1.003	+++	+++	+	+	+	+++
4277-1.054	+++	++	+/-	*	*	*
4277-1.059	+++	++	+/-	*	*	*
4277-1.063	+++	+++	+	*	*	*
4277-1.084	+++	+	+/-	*	*	*

Key: * secondary growth on metabolic by-products

± slight growth

+ moderate growth

++ good growth

+++ very good growth

From Table 12, 4277-1.002 and 4277-1.003 did not appear to be any more sensitive to the bile acids than 4277-1. Mutant 4277-1.001 appeared to be a non-utiliser of all of the bile acids, yet it had

been isolated as a ST resistant mutant. Its colonies were different, and when further spot tested gave inconsistent results, which suggested that it was a contaminant. Stain 4277-1.001 was then tested in broth supplemented with ST, with 4277-1.002 used as a mutant control. The mutant 4277-1.002 should have had the same pattern of ST utilisation as 4277-1 (Table 12). The mutant 4277-1.001 was found to have died, but 4277-1.002 gave the expected pattern, as seen in Figure 16.

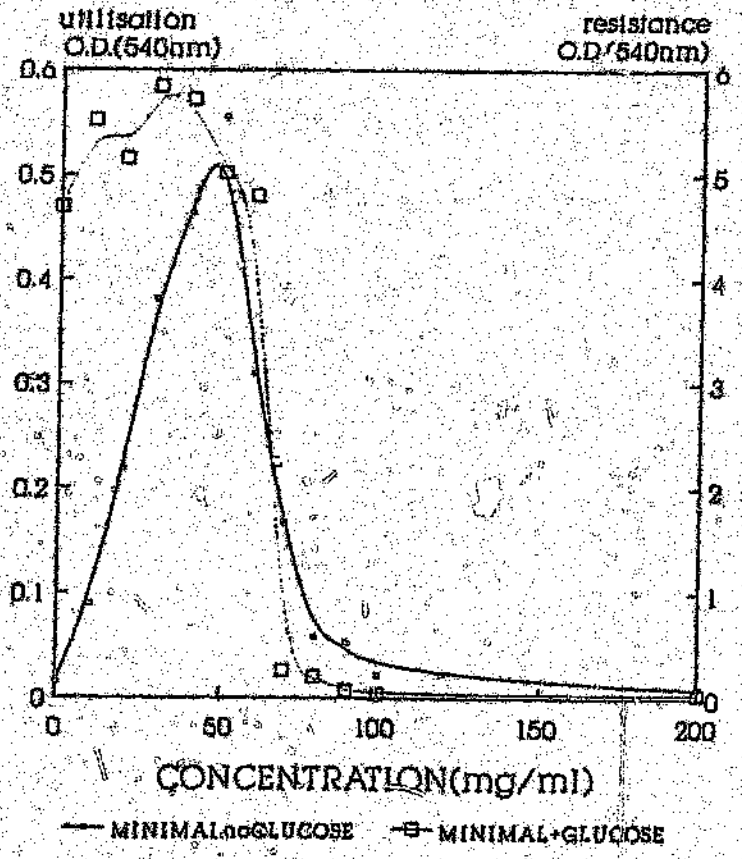


Figure 16. Utilisation and resistance of 4277-1.002 to varying concentrations of sodium taurocholate as determined by optical density at 540nm.

The mutant 4277-1.001 was then tested to confirm if it was a contaminant (section 4.8).

4.6.1 4277-1.063

Due to the mutant 4277-1.001 being a possible contaminant, the next most appropriate mutant appeared to be 4277-1.063 (see Table 12). Growth on MMnoG, indicated that it was not an autotroph. Growth was inhibited on the plates supplemented with concentrations of bile acids. Spot tests of 4277-1, and 4277-1.063 were performed, as in Table 13, which indicated that 4277-1.063 could not utilise SC, SD or ST.

Table 13. Comparison of the ability of 4277-1 and 4277-1.063 to utilise and their resistance to bile acids.

STRAINS	TYA	MM NO G	20ug /ml SC	20ug /ml SD	40ug /ml SD	20ug /ml ST	40ug /ml ST	MM + G	20ug /ml SC	40ug /ml SC	20ug /ml SD	40ug /ml SD	20ug /ml ST	40ug /ml ST
4277-1	+++	*	++	++		++	*	+++	+++		+++		+++	+++
4277-1.063	+++	*						+++	+++		+++		+++	*

Key: * secondary growth on metabolic by-products

± slight growth

+ moderate growth

++ good growth

+++ very good growth

From these preliminary results it appeared that 4277-1.063 had an increased sensitivity to the bile acids. Broth cultures supplemented with ST and SD were used to further investigate the resistance to and utilisation of the bile acids by 4277-1, 4277-1.002 and 4277-1.063. Mutant 4277-1.002 was included as a control. The bile acids SD and ST were the chosen selective plates when screening for the mutants. Figure 17 illustrated that 4277-1.063 was a resistance sensitive mutant, as it could utilise ST, but it was only resistant to concentrations of up to 10mg/ml of ST.

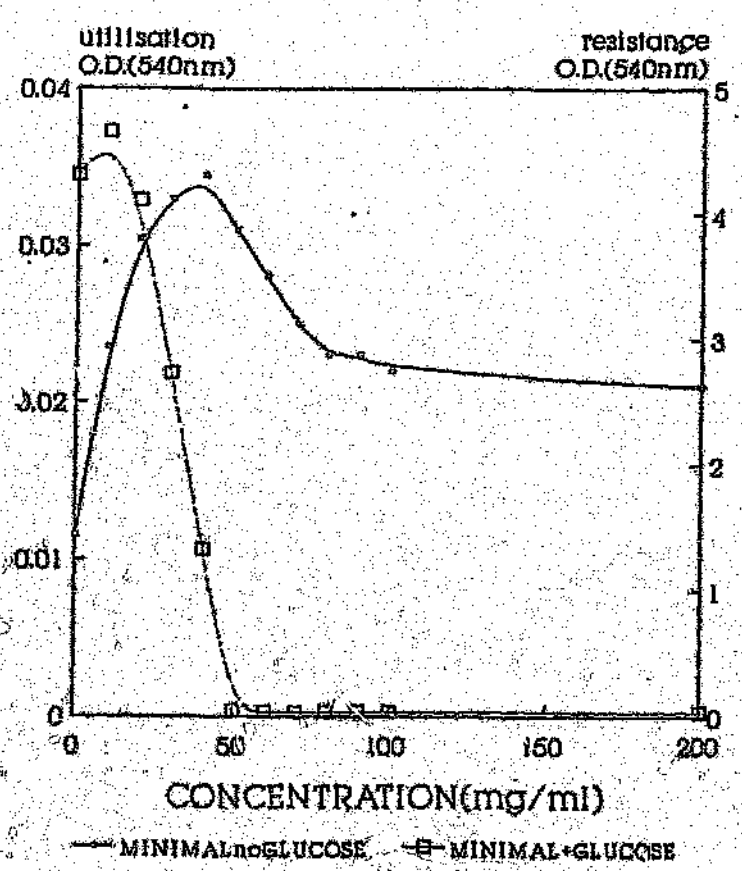


Figure 17. Utilisation and resistance of 4277-1.063 to varying concentrations of sodium taurocholate, as determined by optical density at 540nm.

Thus 4277-1.063 could utilise ST, but was sensitive to it. Figure 18 showed that the pattern of resistance to SD was similar.

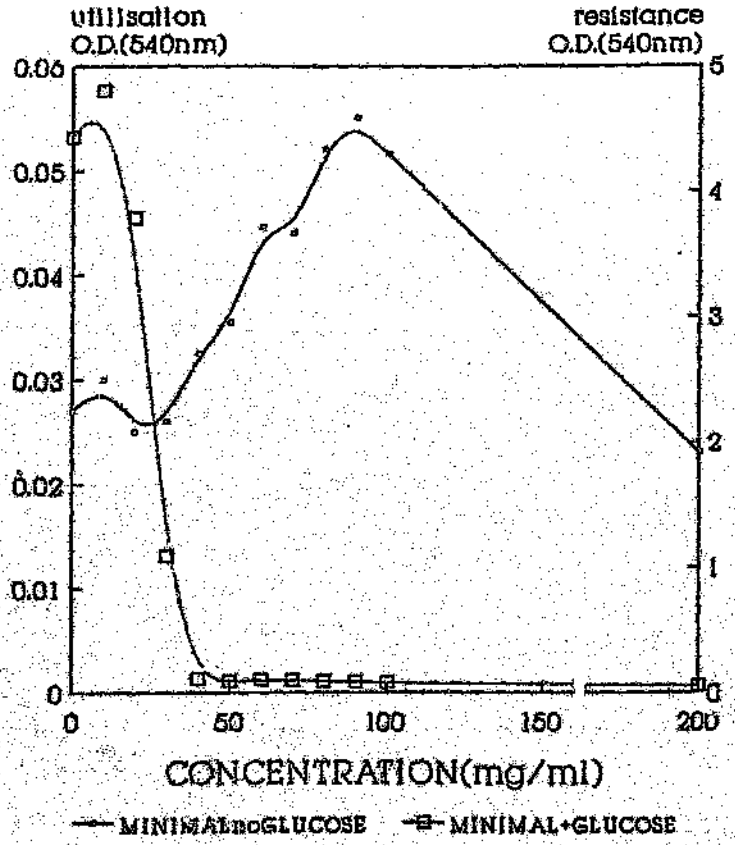


Figure 18. Utilisation and resistance of 4277-1.063 to varying concentrations of sodium deoxycholate, as determined by optical density at 540nm.

Mutant 4277-1.063 appeared to be less resistant to SD, as its growth was inhibited by 20mg/ml of SD. The utilisation curve was very different. The gradient to the point of maximum utilisation was not as great as for ST, but the inhibition in utilisation was much more dramatic. This could have been due to the effects of addin odium hydroxide to the cultures supplemented with SD.

In comparison to the parental strain 4277-1, mutant 4277-1.063 could not utilise SD very well (Figure 19).

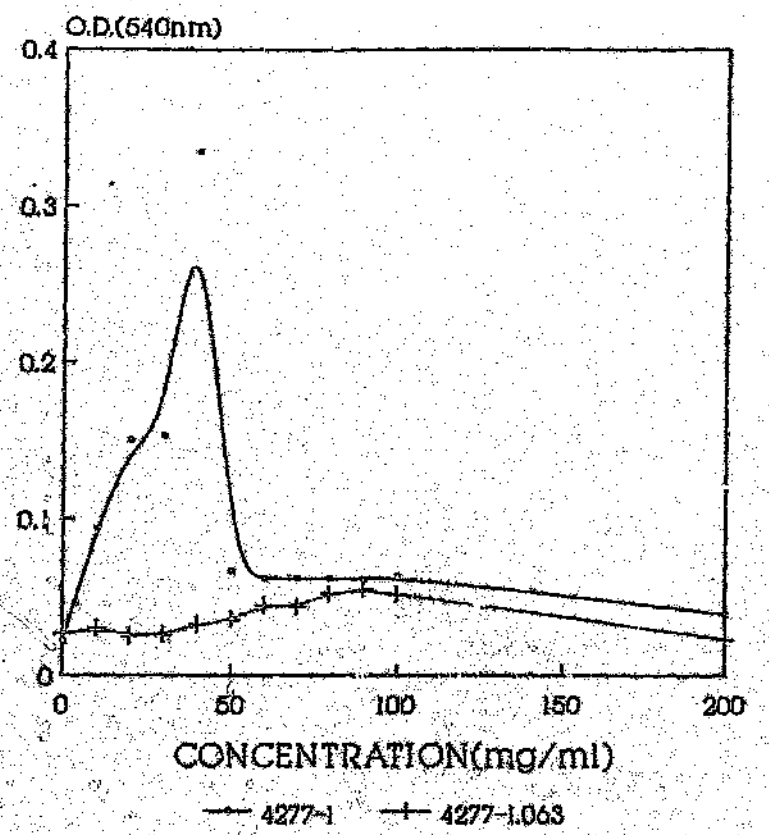


Figure 19. Comparison of the utilisation of varying concentrations of sodium deoxycholate by 4277-1 and 4277-1.063 as determined by optical density at 540nm.

From the pattern of resistance, 4277-1.063 was much less resistant to SD than 4277-1. Mutant 4277-1.063 was a much slower grower than 4277-1. The decrease in growth, once inhibition was effected, was much greater for 4277-1.063 than for 4277-1. (Figure 20).

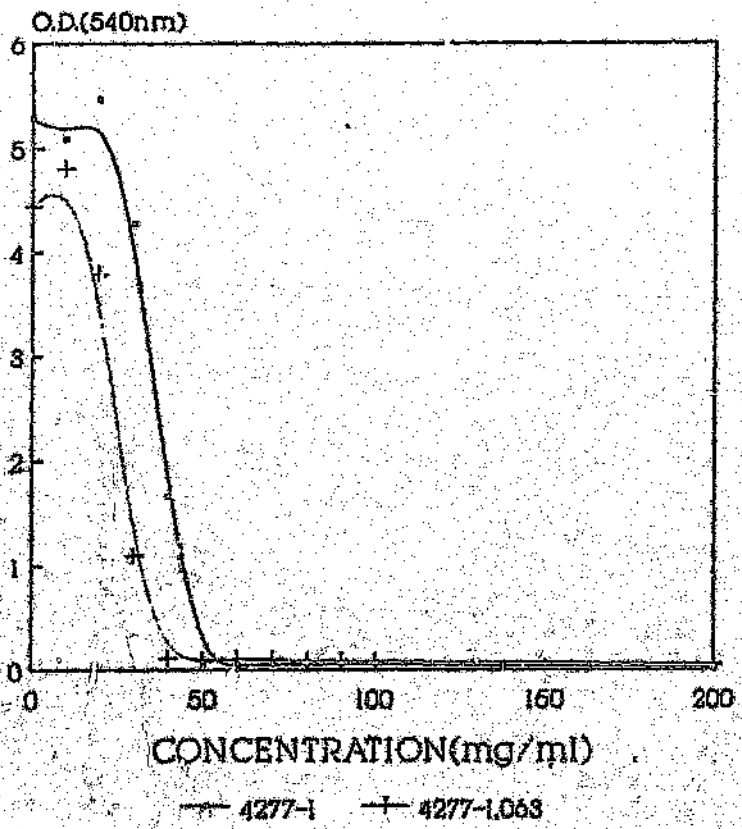


Figure 20. Comparison of the resistance to varying concentrations of sodium deoxycholate by 4277-1 and 4277-1.063, as determined by optical density at 540nm.

These results suggested that 4277-1.063 was a hypersensitive mutant of SD. A comparison of utilisation of and resistance to ST confirmed this. Strain 4277-1.002 was included as a control in the ST studies. Figures 21 and 22 illustrated that 4277-1.002 had a utilisation and resistance pattern similar to that of 4277-1.

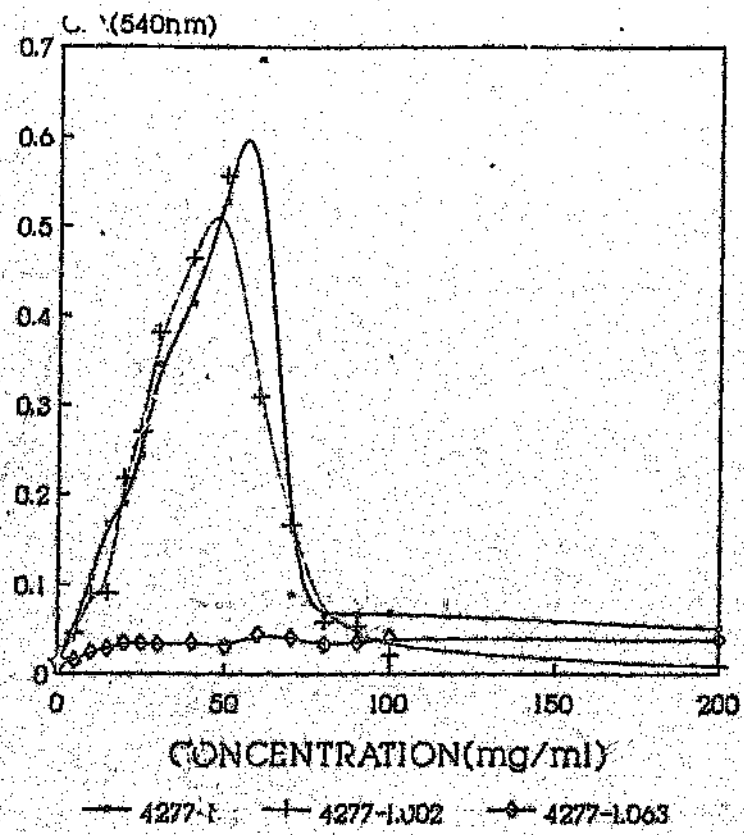


Figure 21. Comparison of the utilisation of varying concentrations of sodium taurocholate by 4277-1, 4277-1.002 and 4277-1.063 as determin d by optical density at 540nm.

From Figure 21, the pattern of utilisation of ST by 4277-1 and 4277-1.063 did not differ signif cantly from the pattern of utilisation of SD (Figure 19), although as expected, higher concentrations of ST could be utilised before inhibition of growth occurred. Although mutant 4277-1.002 could not utilise concentrations of ST as high as 4277-1 could, its pattern of utilisation was similar, and corresponded to the spot tests shown in Table 12.

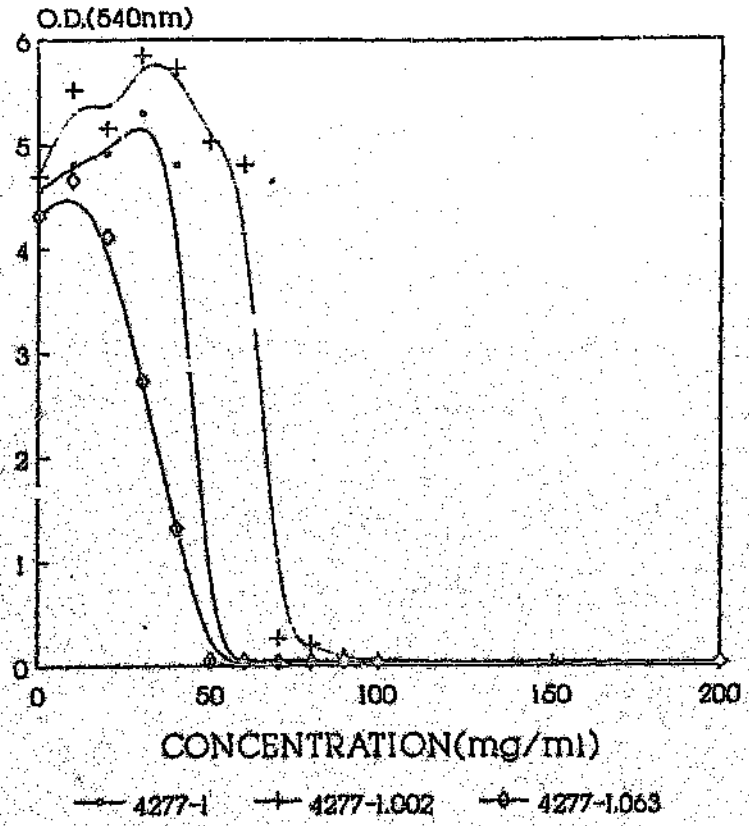


Figure 22. Comparison of the resistance to varying concentrations of sodium taurocholate of 4277-1, 4277-1.002 and 4277-1.063 as determined by optical density at 540nm.

The resistance pattern of 4277-1 showed that at 30mg/ml of ST, growth seemed to have been stimulated, but very soon afterwards was dramatically inhibited. Mutant 4277-1.002 was more resistant than 4277-1, and followed a similar pattern of apparent stimulation at 30mg/ml and dramatic inhibition by 70mg/ml of ST. Mutant 4277-1.063 showed a much less resistant pattern, and a more gradual inhibition of growth. Mutant 4277-1.063's pattern of resistance supported the view that it was a hypersensitive

ST and SD mutant. From these results, the choice of 4277-1.063 as the recipient, hypersensitive strain was supported.

4.6.2 C1.070

In order to obtain C1.070 chromosomal DNA, growth in glycine was necessary. A glycine concentration was needed where the most significant inhibition of growth occurred, which indicated that the glycine-glycine bonds in the cell wall had weakened, and would aid in the disruption of the cell wall. Yet a growth rate high enough to give a sufficient yield of DNA was needed. To determine the correct concentration, the growth of C1 in varying concentrations of glycine in TY was determined, as in Table 14.

Table 14. Optimisation of the concentration of glycine in TY, for maximum yield of C1 DNA.

CONCENTRATION OF GLYCINE	GROWTH OF A. oxydans		
	20 HOURS	30 HOURS	40 HOURS
TY	+++	+++	+++
TY+1% GLYCINE	+++	+++	+++
TY+2% GLYCINE	++	++	+++
TY+3% GLYCINE	+	+	++
TY+4% GLYCINE	+	+	+
TY+5% GLYCINE	+	+	+

Key: + moderate growth

++ good growth

+++ very good growth.

These results were then extrapolated to C1.070. The 2% glycine broth was determined to be the best concentration at which a good yield of DNA could be obtained.

4.6.3 Auxotrophs

A pool of auxotrophs were necessary as a mutant control. The production of auxotrophs created due to a point mutation were a means of determining the efficiency of mutation. All of the auxotrophs were produced from the enrichment procedure, by selection for non-utilisation of the bile acids and not by specific selection for auxotrophs. The auxotrophs and their reversion rates were determined as in Table 15.

The reversion rate was a good indicator of the stability of the mutants generated from the NTG-mutagenesis, as their mutation could be identified. The only auxotroph that did not show reversion was 4277-1.014, which was then used as a control for optimisation of the nocardioform transformation.

Table 15. Auxotrophs produced and their reversion rates.

MUTANT	AUXOTROPH	COLONY FORMING UNITS / ml	REVERSION RATE
4277-1.008	Histidine	3.04×10^9	9.8×10^{-9}
4277-1.013	Cysteine	1.29×10^9	7.8×10^{-9}
4277-1.014	Histidine	6.5×10^8	
4277-1.016	Histidine	2.09×10^9	9.6×10^{-9}
4277-1.036	Histidine	3.35×10^9	1.8×10^{-8}
4277-1.045	Tryptophan	1.66×10^9	3.0×10^{-8}
4277-1.046	Histidine	2.55×10^9	1.2×10^{-8}
4277-1.052	Histidine	1.93×10^9	1.0×10^{-8}

Two putative auxotrophs were found to be "leaky" mutants, and one had reverted. Some metabolic pathways could have requirements for more than one amino acid, e.g. the aromatic amino acids, and needed tryptophan, phenylalanine, tyrosine and shikimic acid. Others could need vitamins, notably vitamin B1, or purine or pyrimidine. It was not important how much of these compounds were used, as long as they were less than the concentration of amino acid. The exact concentration of amino acids, was not critical, but its presence was.

4.7 Maintenance of the shuttle vector pDA37.

Plasmid pDA37 was maintained in the E. Coli λ lysogen strain λMM294. It contained the suicide function and it would self destruct if the λ

promoter was not inactivated. Inactivation would prevent the EcoRI END gene, which encodes for endonuclease, from being activated. The λMM294 was heat sensitive, as the λ promoter was activated at 37°C, therefore the culture was grown at 33°C.

4.8 Testing for Contamination

From the results of spot tests, the unusual colony appearance, and the microorganism's death, 4277-1.001 was tested to determine if it was a contaminant. The tests included phage overlay, streptomycin resistance and studies of the pattern of the ribosomal proteins.

4.8.1 Phage Overlay

Phage 41 was not specific for, but could lyse 4277-1. Thus it could be used to determine whether 4277-1.001 was a mutant of 4277-1. Plates of TYC were used, where the calcium chloride was necessary for phage attachment. The phage overlay was carried out on 4277-1, 4277-1.001 and 4277-1.063, and the results were as in Table 16. The mutant 4277-1.063 served as a control.

Table 16. Phage overlay with phage 41 and 4277-1, 4277-1.001 and 4277-1.063.

MICROORGANISM	PRESENCE OF PLAQUES
4277-1	-
4277-1 + phage 41	+
4277-1.001	-
4277-1.001 + phage41	-
4277-1.063	-
4277-1.063 + phage41	+

It appeared that 4277-1.001 and 4277-1 were not the same organism, but that as expected 4277-1.063 and 4277-1 were. However, the mutation in 4277-1.001 could have effected the outer membrane, disabling phage 41 from recognising, attaching and lysing 4277-1.001.

4.8.2 Streptomycin Resistance.

A second set of testing for strepomycin resistance was performed. Table 17 records the results.

Table 17. Streptomycin resistance of 4277-1, 4277-1.001 and 4277-1.063.

MICROORGANISMS	GROWTH	
	TYA	TYA + 50µg/ml STREPTOMYCIN
4277-1	+	+
4277-1.001	+	+
4277-1.063	+	+

The same streptomycin resistance pattern for all three microorganisms was recorded. The results were not in agreement with the phage overlay, thus a more thorough study, of the ribosomal protein pattern was performed.

4.8.3 Ribosomal Proteins

The ribosomal proteins of 4277-1 and 4277-1.001 were prepared and electrolysed. The results are shown as in Figures 23A and 23B.

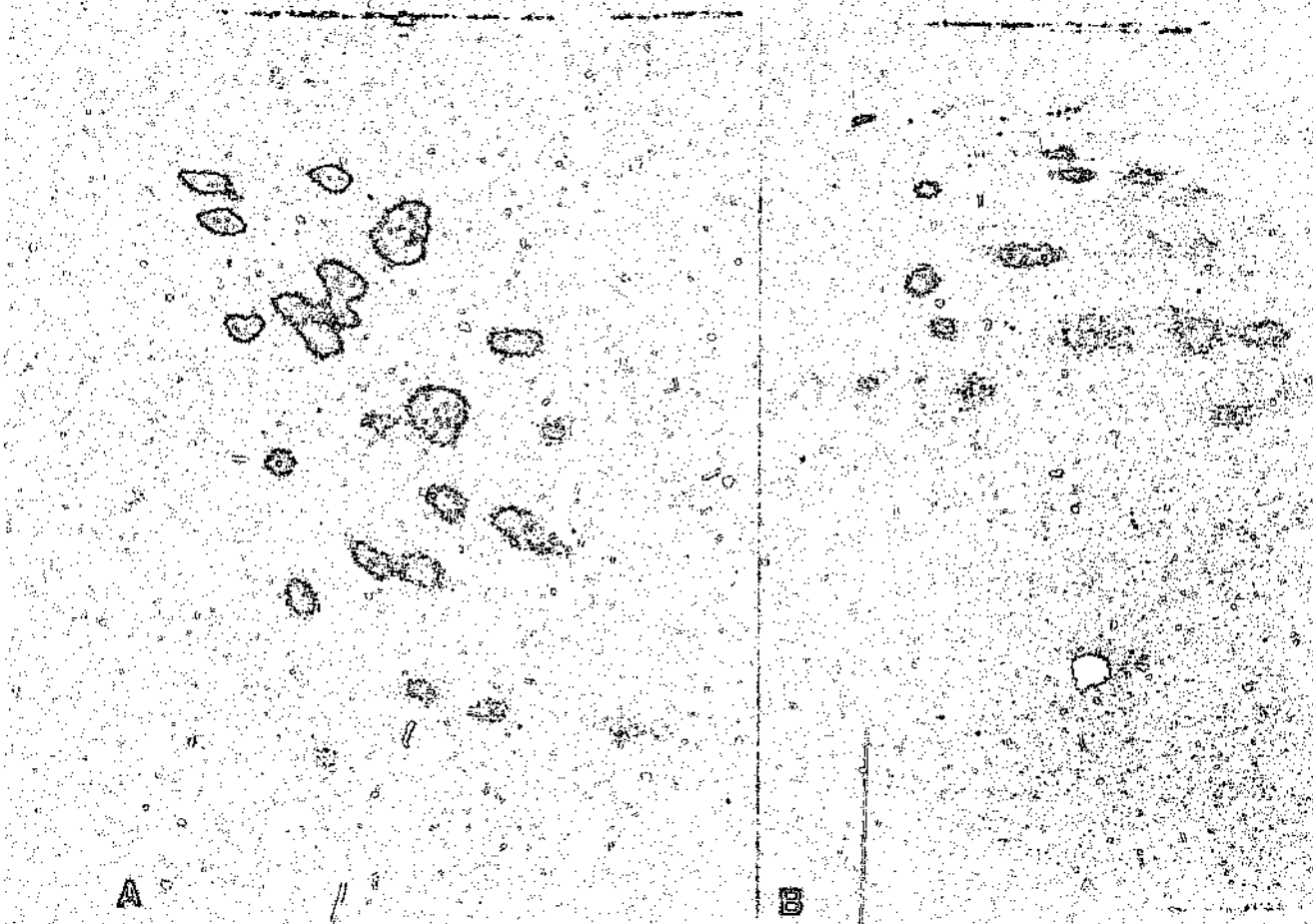


Figure 23. Photographs of the two dimensional polyacrylamide gels of the isolated ribosomal proteins of 4277-1 (A) and 4277-1.001 (B).

No common identity or similarity between the two ribosomal protein spot patterns were observed. It was interpreted that 4277-1.001 must therefore be a contaminant.

4.9 Concentrations of DNA

Only estimates of the uncut chromosomal C1.070 DNA could be made, as the maximum resolution of a 4% agarose gel was 20 kb. Thus the size of the genome was taken to be 20kb, although it was known to be 3030kb. The single chromosomal band was compared with a λ II band of equal intensity (section 3.22), and the concentration was estimated. An amount of 10µl of pDA37 was taken to be the maximum value loaded into the gel, as seen in Figure 24.

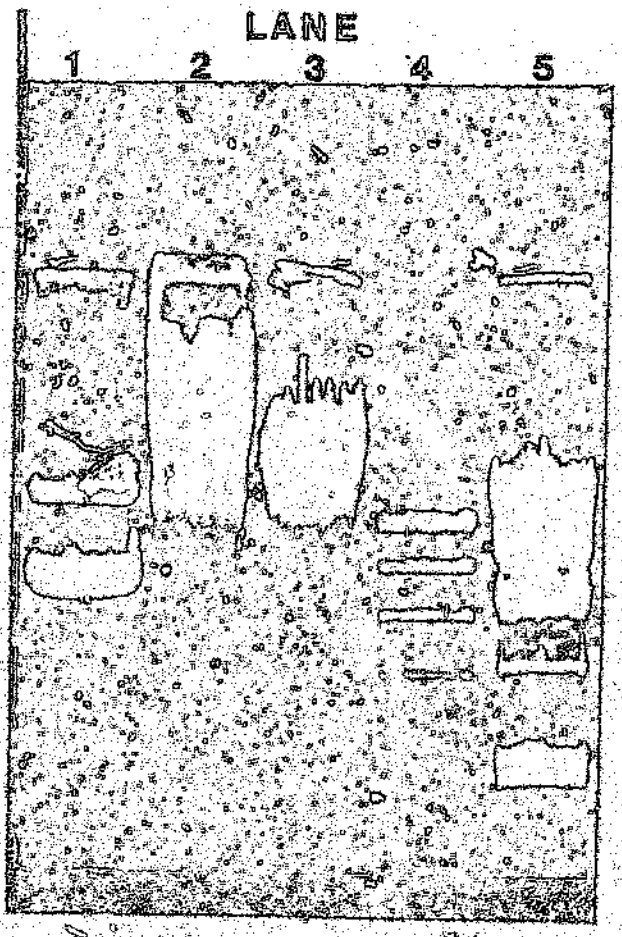


Figure 24. Photograph of a 0.4% agarose gel of 10µl of uncut pDA37 (lane 1), and 10µl (lane 2) and 1µl (lane 3) of C1.070. DNA molecular weight marker λII was loaded in amounts of 0.1µg (lane 4) and 0.5µg (lane 5).

For the digested samples of pDA37, lesser amounts were loaded. After double digestion with Bam HI and Pvu II, the samples were electrolysed using a 1,2% agarose gel, as in Figure 25.

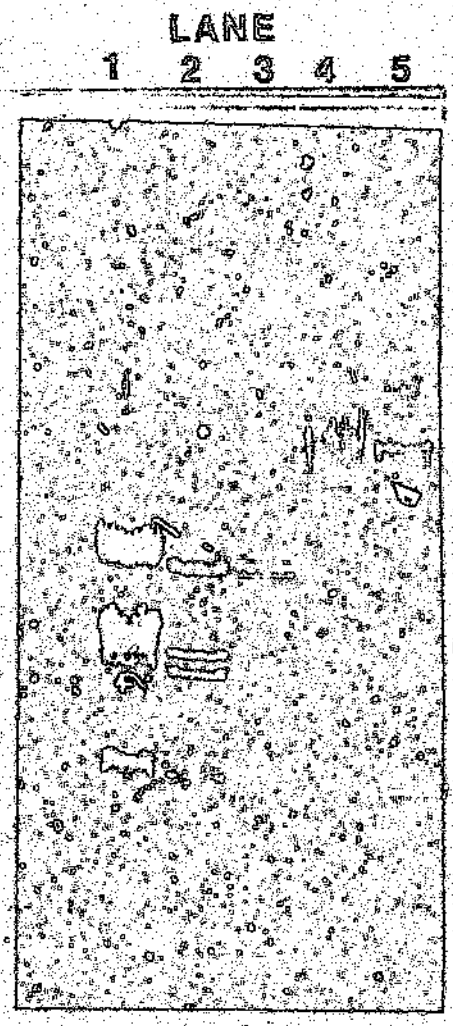


Figure 25. Photograph of a 1.2% agarose gel of PvuII and Bam HI double digested 10µl (lane 1), 3.2µl (lane 2), and 0.32µl (lane 3) amounts of pDA37, and uncut 1.8µl (lane 4) and 0.2µl (lane 5) amounts of 1.070 DNA.

For the concentration calculations, a graph of the calibration curve of DNA molecular weight marker λII was first drawn (Figure 26).

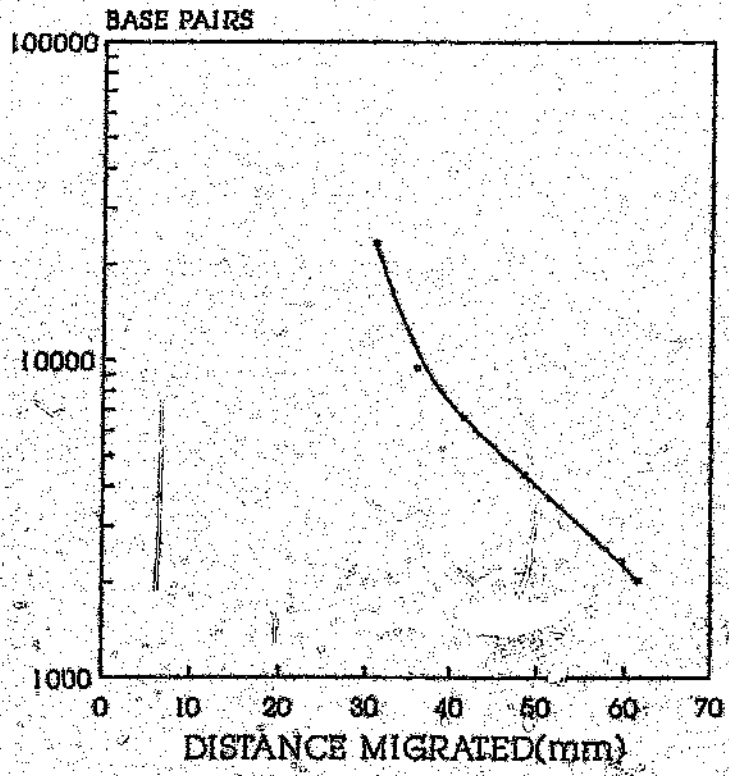


Figure 26. Calibration curve of the DNA molecular weight marker λIII.

The curve represented the distance moved by the different sized fragments. By comparison of the distances moved by the fragments generated after digestion with Pvu II and Bam HI, the fragment sizes were determined. The concentrations were as in Table 18.

Table 18. Calculations of the DNA of pDA37 and C1.070.

DNA	DNA CONCENTRATION ng/µl
pDA37	60
C1.070	130

An approximate concentration of the chromosomal DNA of C1.070 could be determined.

4.10 Optimisation of ligation and transformation of MM294-1

The time needed for the digestion of pDA37, and the ratio of C1.070 DNA to pDA37 DNA had to be optimised. The amount of DNA used to achieve the different ratios, were as in Table 19.

Table 19. The amount of DNA used to achieve different ratios of pDA37 : C1.070 for ligation.

AMOUNT OF C1.070 DNA PER pDA37	AMOUNT OF pDA37(60ng)	AMOUNT OF LIGATION BUFFER TO A FINAL VOLUME OF 50µl
x1 : 60ng = 0.5µl	1µl	48.5µl
x2 : 120ng = 1µl	1µl	48µl
x3 : 180ng = 1.4µl	1µl	47.4µl
x4 : 240ng = 2µl	1µl	47µl
x6 : 360ng = 3µl	1µl	46µl
x8 : 480ng = 4µl	1µl	45µl
x10 : 600ng = 5µl	1µl	44µl

The 1µl (60ng) of pDA37 was used for all the ligations, as it was found to produce ample

colonies. The pDA37 needed to be only minimally digested, thus the ligations and transformations were performed varying both the time of digestion of pDA37 and the ratio of C1.070 DNA to pDA37 DNA. The results were found as in Table 20.

Table 20. Optimisation of the ratio of DNA and the digestion interval, for ligation and MM294-1 transformation.

RATIO OF pDA37 : C1.070 DNA	AVERAGE NUMBER OF TRANSFORMANTS PER PLATE	
	1 HOUR DIGESTION	2 HOURS DIGESTION
No pDA37 control	-	-
Uncut pDA37 control	-	-
1:1 no ligation control	-	-
No C1.070 DNA control	-	-
1 : 1	150	81
1 : 2	84	26
1 : 3	70	13
1 : 4	29	3
1 : 10	-	-

The one hour digestion interval of pDA37 was found to be optimal, as was the 1:1 ratio of the two DNAs.

As the ratio of C1.070 DNA increased, the number of transformants decreased, but the decrease in efficiency was much more pronounced when the pDA37 digestion time was increased. The time of incubation of the transformants was of concern, as if left for too long, secondary growth appeared as satellite colonies utilising the degraded

ampicillin.

4.11 Efficiency and size of inserts of C1.070

Plasmid screens performed on the MM294-1 transformants, determined the efficiency of insertion, and the average size of the fragmented plasmids which had an insert of C1.070 DNA. The efficiency of insertion was determined by using gels, as in Figure 27.

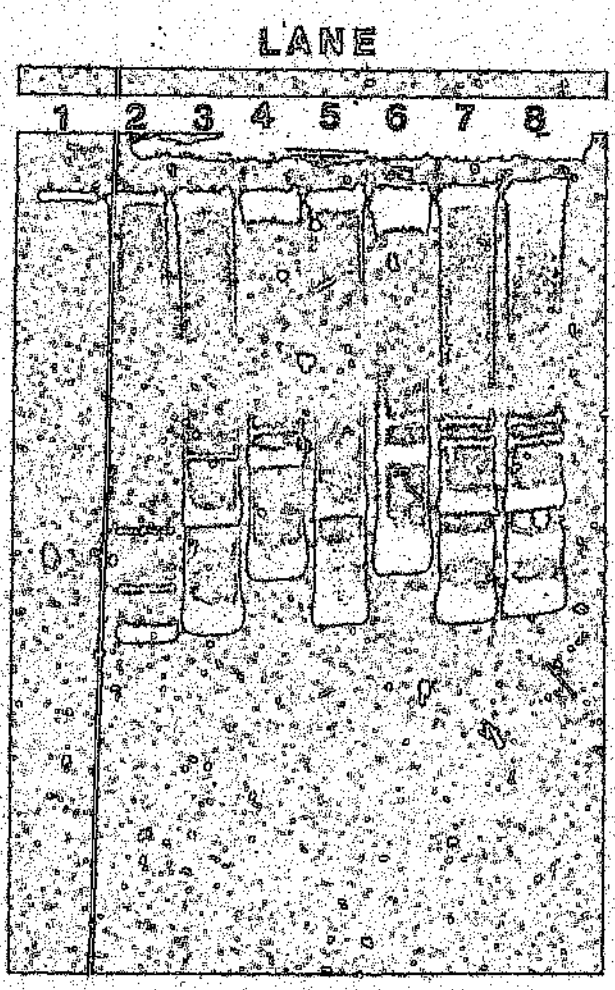


Figure 27. Photograph of a MM294-1 plasmid screen using a 0.4% agarose gel. Amounts of 10ul of six different samples were run in lanes 3 to 8, with a plasmid free control (MM294-1) in lane 1, and a plasmid control (pDA37) in lane 2.

The plasmids with an insert migrated more slowly through the gel. Thus the efficiency of transformation was determined by calculation of the percentage of plasmids with an insert, of the total number of plasmids screened. The efficiency taken over a representable sample number of plasmid screens was found to be 75%.

The pDA37 plasmids with an insert were then digested overnight with Bgl II and electrolysed using either a 0.4% or a 1.2% agarose gel, as in Figure 28.

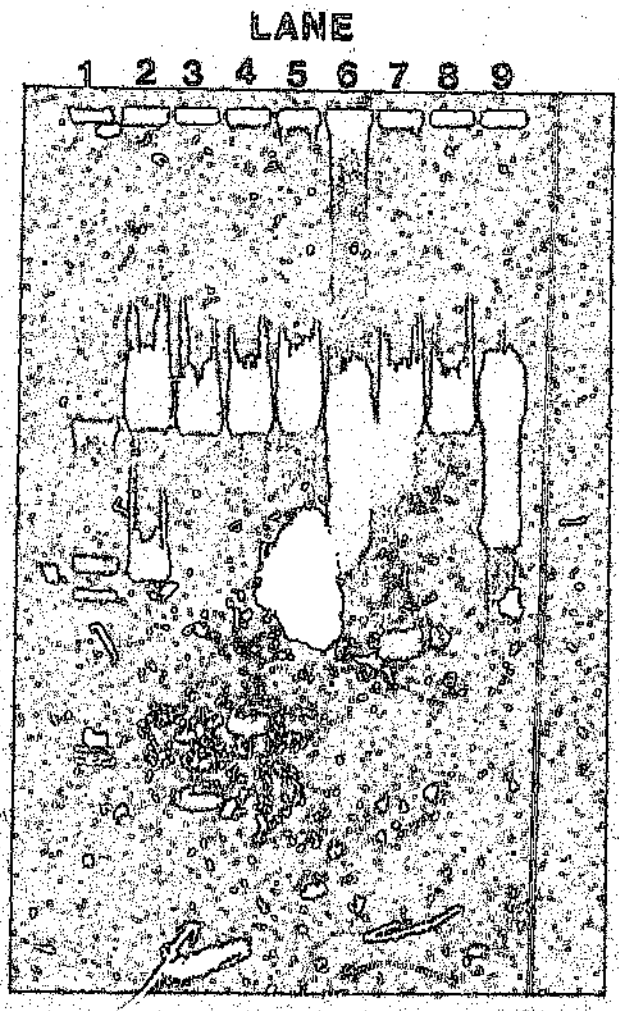


Figure 28. Photograph of a 1.2% agarose gel, of sample of Bgl II digested pDA37 with an insert (lanes 2 to 8). An amount of 0.1ug of DNA molecular weight marker AIII was included (lane 1).

From Figure 28, it was observed that in lanes 2,3,4,7, and 9, the pDA37 had an insert. In lane 6 the pDA37 sample had two fragments, but the pDA37 electrolysed in lane 8 had no insert.

The average size of the inserts was calculated by reading off the size of each fragment, from the

calibration curve of the DNA molecular weight marker λIII (lane 1) (as in Figure 26). The average size of the inserts was found to be 5.7kb, but as the efficiency of transformation and ligation was only 75%, the average size of the insert per transformant was:

$$5.7kb \times 75\% = 4.3kb.$$

Once the average insert size was determined, the size of a representative genomic library was calculated.

4.12 Calculation of a Representative Genomic Library

The *A. oxydans* genomic size was taken to be 2.0×10^9 daltons (Crombach, 1978), where 1 dalton = 660bp, therefore the size of the genome was 3030kb.

From the formula:

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

where P = 95% probability.
a = 4.3kb
b = 3030kb

Therefore for a 75% efficiency of insertion,

$$N = 2110 \text{ colonies}$$

for a representative genomic library to be constructed.

In total 3580 colonies were collected from the MM294-1 transformants.

Once the entire library had been built the nocardioform transformation could proceed.

4.13 Optimisation of Nocardioform Transformation

The growth phase of the cells used for transformation were optimised. As the efficiency of a nocardioform transformation is high, by optimising the conditions, it would be possible to perform one single transformation to achieve the number of single colonies for a representative genomic library.

The conditions for optimal nocardioform transformation were determined using 4277-1.063 (recipient strain) and 4277-1.014 as an auxotroph control (no reversion rate). Their respective growth curves in T2 medium were determined and transformation was performed on the different growth phases using 1µl (25ng) of pDA30 (E_1B_1) per transformation. The results are represented in Figures 29 and 30.

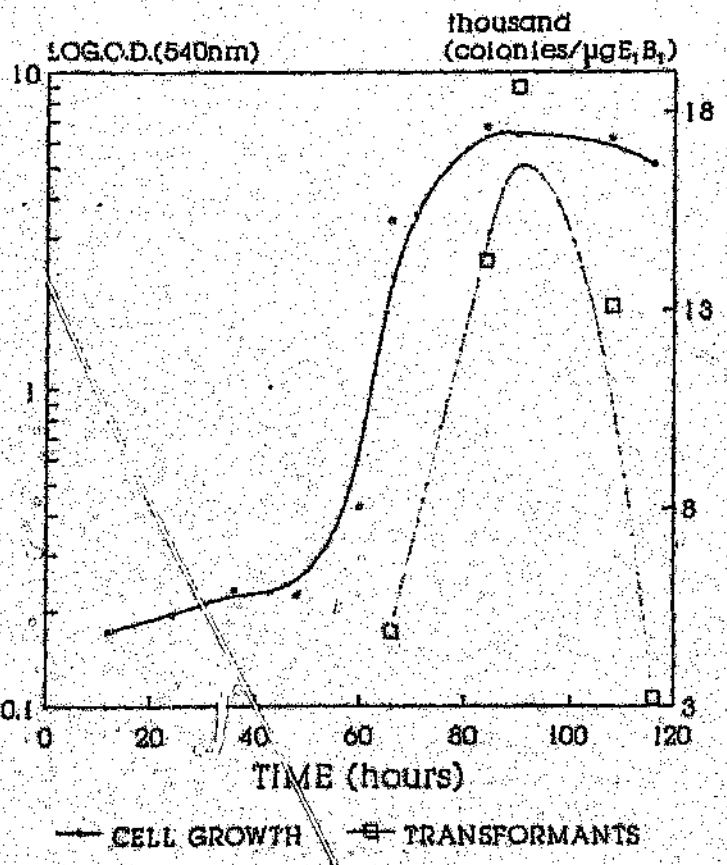


Figure 29. The number of transformants achieved in relation to the growth curve of 4277-1.014 in T2 medium, as determined by optical density at 540nm.

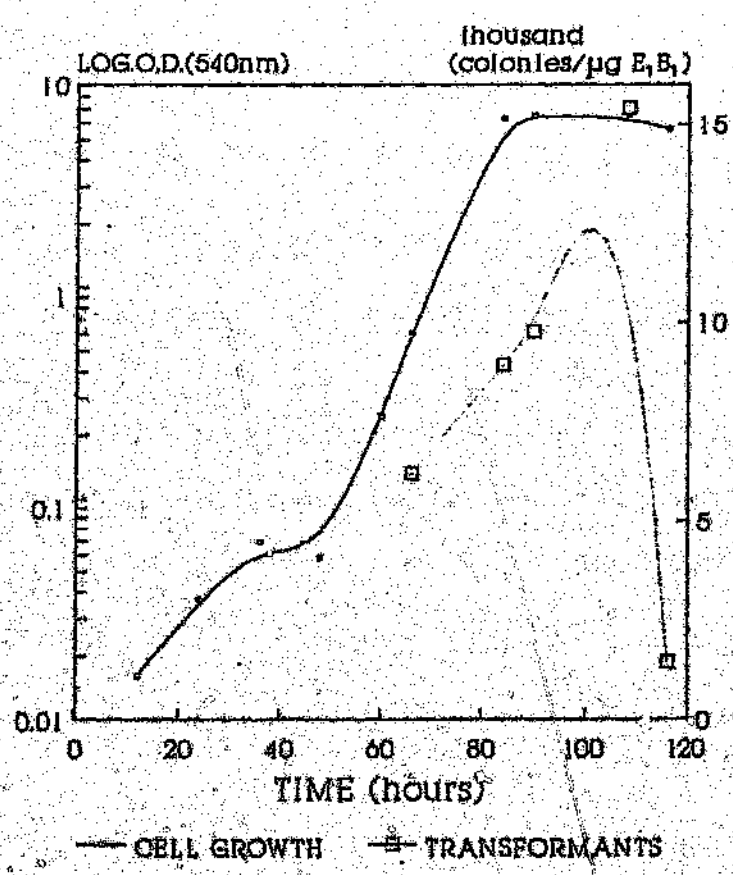


Figure 20. The number of transformants achieved in relation to the growth curve of 4277-1.063 in T2 medium as determined by optical density at 540nm.

The auxotroph 4277-1.014 yielded a better rate of transformation, but both strains showed a very distinct peak in the number of transformants at the OD, of the post-stationary phase.

To check for contamination, samples were taken from both strains, before and after the OD

readings were taken. The samples were streaked to single colonies on TYA supplemented with 50µg/ml of streptomycin, and confirmed that no contaminants were present. The optimal growth phases were taken to be at OD 7.12 for 4277-1.063, and at OD 6.33 for 4277-1.014.

The concentration of the genomic library was determined. After large scale plasmid preparation of pDA37 from MM294-1, and overnight digestion with Bgl II, the concentration of 20ng/µl was determined from the intensity of the fragments. The DNA was concentrated to give a final concentration of 40ng/µl.

It was calculated that in the transformations using pDA30 (E_1B_1), 1µl (25ng) of DNA gave 150000 colonies. However, pDA30 (E_1B_1) was maintained in nocardioforms and was not restricted. Plasmid pDA37, on the other hand, was maintained in E. coli, and was restricted in nocardioforms, which lowered the transformation efficiency by at least a factor of 100. Thus 100 times more pDA37 than pDA30 (E_1B_1) was needed to achieve at least 10000 colonies, to have a true representation of the genomic library of C1.070. Thus 2500ng of pDA37 was needed per transformation. However, a maximum of 20µl of DNA

could be used, thus only 800ng of pDA37 could be used per transformation.

The transformations were performed, plated onto PRMP and underlaid with arsenic. After 7 days of incubation, the results in Table 21 were obtained.

Table 21. The number of colonies achieved after nocardioform transformation.

TRANSFORMATION	NUMBER OF TRANSFORMANTS	TRANSFORMANT PER 25ng OF pDA37
4277-1.063 no pDA37	-	-
4277-1.063 + pDA37	2065	65
4277-1.014 no pDA37	-	-
4277-1.014 + pDA37	1755	55

The number of transformants achieved were much less than those needed for a representation of the entire genome of C1.070. When transformed with pDA30 (E_1B_1), 4277-1.014 yielded a much better transformation efficiency than 4277-1.063, but when transformed with pDA37, this was not found to be true.

4.14 Screening for cloning by complementation

The results of spreading the nocardioform transformants onto the selective plates, are set out in Table 22.

Table 22. Screening for cloning by complementation

TRANSFORMATION	GROWTH					
	MM no G			MM + G		
	MMnoG	40ng/ul ST	20ng/ul SD	MM + G	40ng/ul ST	20ng/ul ST
4277-1.063	-	-	-	+	+	+
4277-1.063+pDA37	-	-	10 colonies	+	+	+
	MM + G					
4277-1.014	-					
4277-1.014+pDA37	+ (>300colonies)					

The preliminary cloning results were successful for 4277-1.014 as the histidine auxotroph could only grow on MM+G when it was transformed i.e. when complemented with the gene for histidine synthesis. The transformed 4277-1.063 only gave results after 16 days of incubation, and then only 10 colonies were found to have grown on the SD selective plates. The colonies were transparent, which reflected their delicate physiological state. Vigorous growth on the resistance plates (MM+G) corresponded to the results of Table 12. The growth on the selective plates were due to cloning by complementation.

4.15 Plasmid Screening of Transformed 4277-1.063

Thirteen transformants were plasmid screened. The result was visualised in Figure 31.

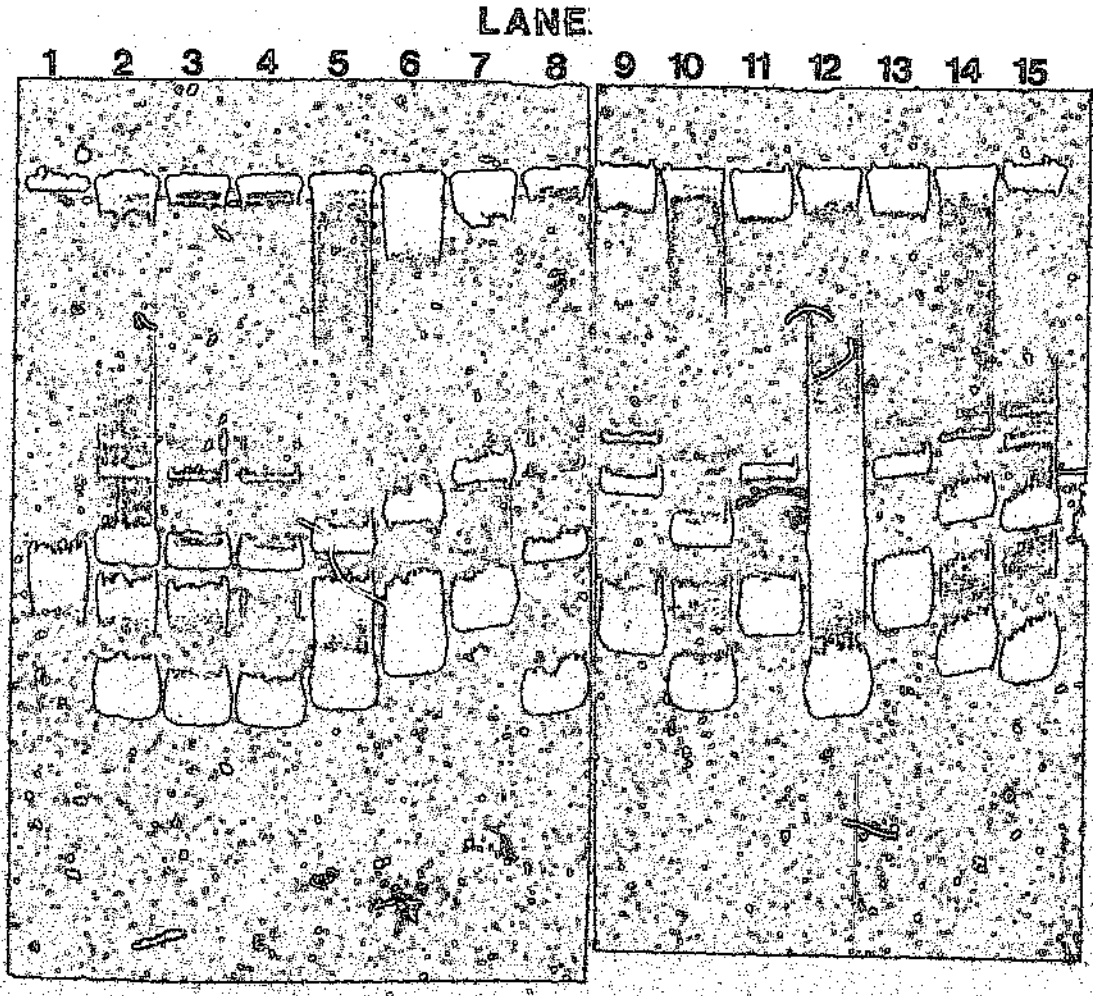


Figure 31. Photograph of a plasmid screen of transformed 4277-1.063, electrolysed using a 0.4% agarose gel. The thirteen transformants (lane 3 to 15) were run with a plasmid free control (4277-1.063) in lane 1, and a plasmid control (pDA37) in lane 2.

From Figure 31, it was observed that all of the transformants contained the plasmid, but not all of them had an insert. With reference to the plasmid control in lane 2, it followed that samples 1, 2, 8 and 10 (lanes 3, 4, 10 and 12 respectively) did not have an insert, but that the remainder did. A further screening was

performed, after overnight Bgl II digestion of the samples thought to have an insert (Figure 32).

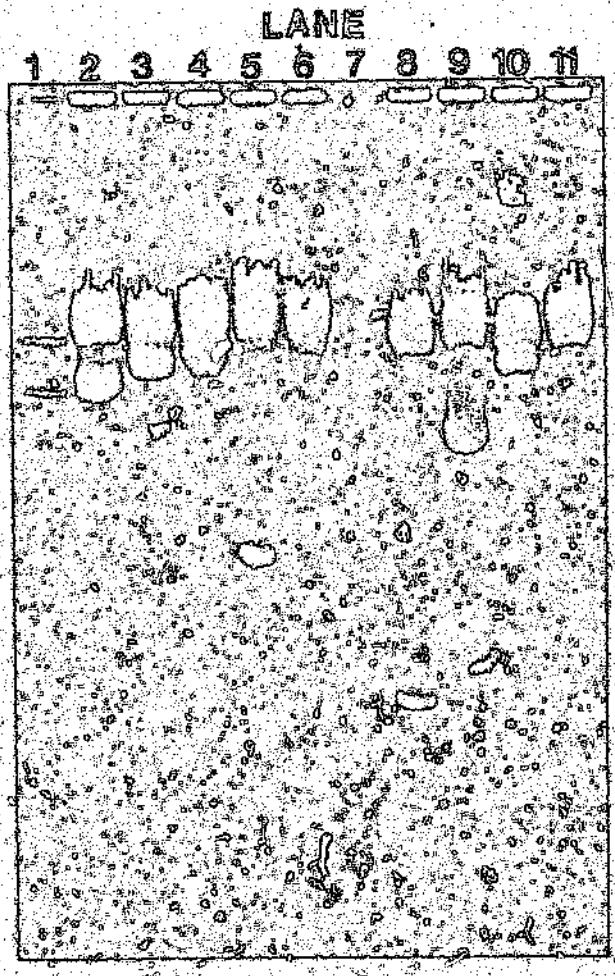


Figure 32. Photograph of Bgl II digested 4277-1.063 transformants on a 1.2% agarose gel. The transformant samples 3,4,5,6,7 were run in lanes 2 to 6 respectively, samples 9,11,12 and 13 were run in lanes 8 to 11 respectively. DNA molecular weight marker AIII was included (lane 1).

Only sample 13 was found not to have an insert on further investigation. Thus the transformation was found to have been successful.

4.16 Non-Radioactive Labelling and Detection

The inserts in pDA37 were labelled and used as probes to test that their source was from the C1.070 genome. Testing was by hybridisation with blots of digested C1.070 and MM294-1. Digested pDA37 was labelled, to act as a negative control. It was established that to have comparable concentrations of C1.070 DNA and MM294-1 DNA, three times as much MM294-1 was needed (15µl) to give equal band intensities as 5µl of C1.070 DNA per lane on a 0.4% agarose gel. The concentrations were determined to be as shown in Table 23.

Table 23. Concentrations of the genomic library inserts used for non-radioactive labelling.

INSERT	CONCENTRATION/µl	CONCENTRATION/15µl
pDA37 (vector)	1875ng	29ng
Insert 1	1875ng	29ng
Insert 2	1875ng	29ng
Insert 3	1875ng	29ng
Insert 4	1875ng	29ng
Insert 5	263ng	4ng
Insert 6	263ng	4ng

The amount of DNA in the 15µl of electrolysed sample was calculated. Only 1µg of DNA was required for labelling, thus only half of the volume per insert was used for labelling. The

volumes were adjusted to obtain 1µg of DNA, as in Table 24.

Table 24. Volumes of the genomic library inserts used to label 1µl of DNA.

INSERT	VOLUME RESUSPENDED IN	CONCENTRATION OF DNA	VOLUME TO GIVE 1µg OF DNA
pDA39 (vector)	58µl	14.5µg	4µl
Insert 1	58µl	14.5µg	4µl
Insert 2	60µl	14µg	5µl
Insert 3	60µl	14µg	5µl
Insert 4	60µl	14µg	5µl
Insert 5	20µl	2µg	10µl
Insert 6	20µl	2µg	10µl

Thus 1µg of each sample of DNA was used for labelling, to yield 260ng of labelled DNA. The results of the DNA probing (by hybridisation) were as shown in Figure 33.

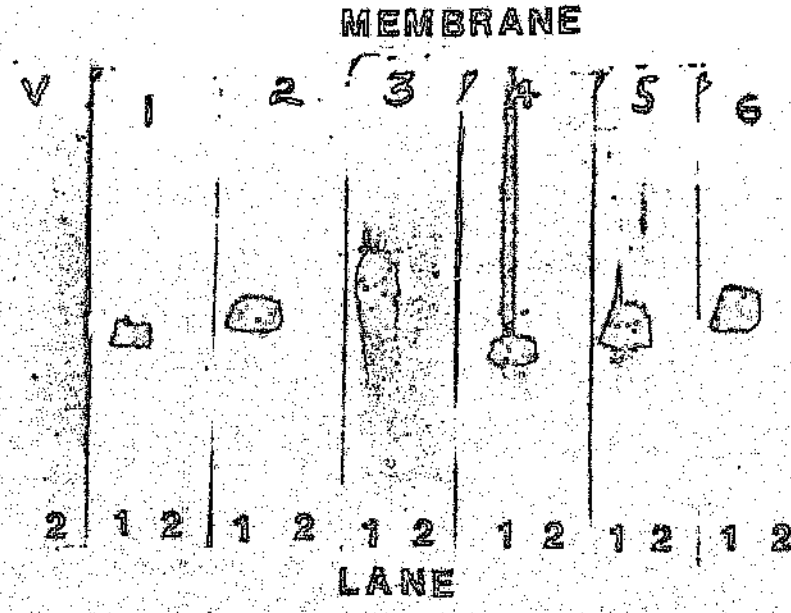


Figure 33. Photograph of the nitrocellulose membranes after hybridisation of the labelled inserts. Each nitrocellulose membrane contained a lane of digested C1.070 DNA (lane 1) and MM294-1 DNA (lane 2). The different C1.070 probes (membranes 1 to 6) showed hybridisation to C1.070 DNA. The shuttle vector, pDA37 (membrane V) did not hybridise.

The hybridisation confirmed that the inserts originated from the C1.070 genome. This verified that the genomic library had been built up, and successfully transferred through pDA37 into MM294-1, without acquiring extraneous DNA.

The negative control of the labelled pDA37 illustrated that the Bgl II digestion site had not been lost during ligation, nor had DNA from MM294-1 been acquired. After digestion, no C1.070 DNA was left attached to the pDA37, which was

demonstrated by the labelled pDA37 not hybridising with C1.070 DNA or MM294-1 DNA.

The hybridisation pattern of the different probes showed some variation. This was consistent with the fact that for the genomic library to have been built, the entire genome had to have been represented, with each fragment originating from a different part of the genome, and being of a different length. Each labelled fragment could hybridise to different parts of the membrane, according to how far their complementary fragments had migrated. This was found to be true from Figure 33. The observed streaks were probably due to degraded labelled DNA, due to nucleus contamination.

5.0 DISCUSSION

5.1 UTILISATION OF SOLE CARBON SOURCES

The strain 4277-1 was shown to grow moderately on Minimal medium and did not need additional growth requirements. Clearly DMSO was utilised when supplemented with agar, but not when in broths. It would be conceivable that the organisms could use DMSO (thus it was not an ideal solvent), but needed some expenditure of energy to utilise the DMSO. This energy was obtainable from agar.

The apparent inability of some nocardioforms to utilise certain carbon sources could have been due to the organisms' inability to metabolise the compound, or due to the concentration not being high enough to stimulate growth. This could have been the case with ethanol, which is a known carbon source of nocardioforms (Downing, 1989). As ethanol is volatile, much of it evaporated, which reduced its concentration in agar significantly. However the three bile acids chosen as the selective agents were water soluble, which avoided the solvent problem.

5.2 RECIPIENT AND DONOR STRAINS

The recipient and donor strains were chosen for their utilisation of SD. They were compatible,

in that the C1 genes could be cloned into 4277-1, and the two were closely related. It has been well documented that the transfer of genes as free DNA (transformation) in microorganisms occurs, but that the efficiency falls dramatically with increasing divergence of the species involved (Bibb et al., 1978). If another bile acid was to be the selective agent, then another combination of recipient and donor strains would probably be more suitable. The observation that higher concentrations of ST could be utilised than SD and SC, before inhibition of growth occurred, could be related to the structure of ST. It was suggested that the presence of the extra hydroxy bond (see Figure 2) meant that the compound was metabolised along a different pathway, which allowed for greater utilisation and tolerance of ST, than SD and SC.

5.3 UTILISATION OF AND RESISTANCE TO BILE ACIDS

The broth cultures were shown to give much more accurate results. The agar plates tended to buffer growth inhibition, making it appear that the nocardiaforms could utilise and be resistant to higher concentrations of bile acid. The inability to grow in the presence of a particular bile acid could be due to the growth curve (area

under the intersection of the utilisation and resistance curves) having been displaced to the right, where stimulation had not yet occurred, or to the left, where inhibition due to hypersensitivity had occurred, but it did not necessarily mean that the nocardioform did not have the genes for bile acid metabolism. If, by the creation of mutants, the growth curve could be shifted to the right, the utilisation of the carbon source would be increased, before inhibition occurred.

The odd peaks seen in the SD utilisation and resistance curves could be due to the addition of the sodium hydroxide, which lysed the cells and thus gave erroneous results.

The relationship between the optical density and the number of viable bacteria per millilitre of culture varied from strain to strain. Thus a calibration curve of the OD at 540nm and the number of bacteria per millilitre of culture was constructed for each new strain used (Maniatis et al, 1982).

5.4 MUTAGENESIS

Nocardioform bacteria being filamentous, tended to clump, therefore for the NTG-mutagenesis, T2

medium was used, as the presence of the ions in the broth reduced clumping. Clumping lowered the mutation efficiencies, as cells in the centre of the clumps would not be subjected to the NTG, and when plated out, single cell colonies would not be formed. Sonication was another method used to reduce the clumping of the cells. The dilution rates when the cultures were plated were of importance, so as to be assured that distinct single cell colonies would result.

The long lag phase of the mutagenised cells was due to much of the DNA having been damaged, which meant that it took longer for the cells to adapt and be stimulated to divide, as repairs had to be made first. If the cells had been kept in the cold room, they became sluggish and contributed to the longer lag time.

The cells that were mutagenised during stationary phase gave a lower percentage of auxotrophs (mutation efficiency), therefore the NTG mutagenesis could not have been very efficient, either due to the cells being in a different (filamentous) form which meant that the cell walls could protect the cell DNA, or due to clumping.

In the enrichment procedure, it was very important that the cells were at a very low concentration when the ampicillin was added. This was so that when the cells were transferred to the enrichment medium, they grew because of the medium and not due to living off the constituents of the killed cells. Thus it was also vital that the ampicillin was not inactivated in any way. Patching should have occurred as soon as the single cell colonies had grown, to prevent the cells from entering stationery phase. The cells could also die if they were stored for too long.

The Enrichment procedure gave mostly auxotrophs whereas it should have given more SD, ST and SC sensitive mutants. This was because ST (which selected for the most mutants) was a detergent. Detergents increase the susceptibility of the cell membrane to ampicillin, by "opening" up the membrane structure. Thus the detergent acted in synergism between ST and ampicillin.

5.5 MUTANT TESTING

Some mutants, in particular 4277-1.002 and 4277-1.001 had been isolated for being ST

sensitive, but on further testing proved to have reverted. This was supported by the reversion rate of the auxotrophs (Table 15), which established that the majority of the tested mutants did have a reversion rate.

Mutant 4277-1.001 was found to have died, either because it was stored for too long, or because it was a contaminant, e.g. a gram-positive sporulater, which if it was not grown under conditions where it could sporulate, it died.

In the study of 4277-1.063, its hypersensitivity to SD and ST was clearly seen in Figures 19 and 21. The growth of 4277-1.063 did not diverge from the basal line, especially with comparison to 4277-1, and was supported by the difference in the pattern of resistance (Figures 20 and 22), where 4277-1.063 was much less resistant to SD and ST than 4277-1. Thus with there being a difference in the resistance patterns, the differences seen in the utilisation patterns could be taken as being true. Possibly a change in 4277-1.063's metabolic pathway, could have resulted in the accumulation of toxic metabolic by-products of ST and SD, which resulted in 4277-1.063's hypersensitivity to the bile acids.

5.6 Plasmid pDA37 AND THE GENOMIC LIBRARY

To maintain the plasmid the lysogenic strain E. coli λMM294 was used. E. coli λMM294 needed magnesium ions or incubation at less than 35°C, as the EcoRI END gene was heat inducible. The magnesium ions bound to the binding site of the EcoRI END gene and repressed it. No glucose was included in the broth (LA) as it would have inhibited the binding to the maltose binding site and would have prevented repression of the EcoRI END gene. Once the DNA was cloned into the EcoRI END gene (cut at the Bgl II site), it inactivated the gene, and together with the ampicillin selection, ensured growth of those cells with an insert in the EcoRI END gene and with the gene for ampicillin resistance.

The strain MM294-1 which was used to build up the genomic library, contained a rifampicin resistance marker. However the marker was incorporated into the chromosome, thus a positive selection was not needed to maintain the strain. It was convenient to have the marker, because if a contamination occurred, treatment with rifampicin would have been sufficient to cure the culture of a rifampicin sensitive contaminant. Rifampicin resistance could also

he - been used as a test for contamination. The strain MM294-1 was non-lysogenic, therefore it did not need magnesium ions, and only needed ampicillin when the plasmid had been transformed into it.

The plasmid pDA37 met the requirements of a good cloning vector, which included: replicon stability maintained in the organism, a means of selection (ampicillin resistance), a means of selecting cloned fragments in the vector (the *E. coli* suicide vector pEcoR251 was digested if DNA was not cloned into the *E. coli* endonuclease gene at the unique Bgl II site. The presence of a λ repressor also turned off the transcription of the endonuclease, preventing the suicide of the plasmid. In the absence of a repressor or a cloned insert, the vector would not survive), and a method of screening the cloned DNA for the desired gene, which involved the bacterial mutants unable to utilise the compound of interest as the sole carbon source.

5.7 BULK DNA PREPARATIONS

In the bulk chromosomal DNA preparations, Proteinase K was used, as it acted on the proteins involved in holding the DNA in its

super-coiled positions, and broke down the cell wall. For the plasmid preparations, lysozyme was used, which destroyed the cell wall, which enabled the plasmid to be released. If ribonuclease or proteinase had been used, it would have resulted in the slacking of the super-coil, or even loss of the plasmid.

Ethidium bromide (EtBr) was used to visualise the DNA bands, as it intercalated with the DNA and fluoresced under UV light. In the density gradient centrifugation, it was used to separate the super-coiled DNA. The EtBr by intercalating caused the DNA to unwind. As linear DNA unwound much more than super-coiled DNA, the two banded at different positions. Caesium chloride (CsCl) was used as the density gradient. Due to its density, when in equilibrium, CsCl formed a density gradient, with the time of centrifugation not being critical. The CsCl bound to the DNA and interchelated with the super-coiled plasmid, but it could nick the DNA to give open circular and linear DNA which migrated together, and separated from the super-coiled DNA. Thus it was vital that the refractive index of the DNA sample was adjusted to 1.392 for plasmid DNA, and to 1.390 for chromosomal DNA. The difference in the

refractive index was due to the chromosomal buoyancy being much lower than plasmid DNA. The refractive index was adjusted so that the bands appeared in the correct position relative to each other. Broad and diffuse bands were due to the EtBr not having fully saturated the DNA. The amount of EtBr was then increased to ensure that all points of the DNA were saturated. In the EtBr - CsCl gradient centrifugation the separation was by structure not size. The linear DNA floated to the top, with the protein and RNA being pelleted on opposite sides of the tube.

5.8 PLASMID SCREENS AND CONCENTRATIONS OF DNA

For the plasmid screens, it was critical that the cells were mixed vigorously to release the plasmid. In determining the concentrations of DNA, agarose gel electrophoresis was used as it was the standard method used to separate, identify and purify DNA fragments (Maniatis, et al., 1982). This was a simple and rapid technique with good resolution.

The electrophoretic migration rate of the DNA was dependent upon five main parameters: the molecular size of the DNA, the agarose concentration, the conformation and base composition of the DNA, the applied current and

the temperature. Super-coiled structures ran the furthest on gels as they were small, compact and offered less resistance, than open circular or linear DNA. The larger the plasmid, the more it would run closer to the linear DNA, due to its floppier structure.

The DNA molecular weight markers could only be used with linear fragments. The marker used, depended on the agarose concentration, as the smaller the marker fragments, the more concentrated was the agarose needed to give good resolution. The molecular weight fragments were present in equimolar amounts. The smaller fragments could only be seen when the gels were overloaded, but which resulted in the other fragments being indistinct. In the DNA molecular weight marker λII, the fourth band (4361 bp) was not representative enough to be used for concentration determination. This was due to the λ having sticky ends, which ligated and ran with the 4361 bp fragments. To prevent sticky ends from ligating (hybridised upon storage), and to obtain the proper fragment pattern, each sample was heated to 65°C for 5 minutes and then rapidly cooled, immediately before electrophoresis.

In the digestion of pDA37 to determine the size of the inserts, Figure 28, lane 6, showed two fragments. This could be explained either by it being two different inserts (especially as they appeared to be large), having been ligated into the pDA37, or more likely, that the inoculum was not taken from a single cell colony, but that lane 6 represented two different transformants, each with a different size of insert.

When the pDA37 plasmids with an insert were first electrolysed uncut, the expected smear effect was produced. This corresponded with the plasmids having different insert sizes, which migrated at different rates. However three distinct bands were observed, which showed that certain sized inserts were more frequently cloned than others.

5.9 ENZYMES

Each restriction enzyme had a set of optimal reaction conditions. The major variables were the temperature of incubation and the composition of the buffer. Although the temperature requirements were fairly strict, the differences between buffers were often slight. To determine how often an enzyme would cut the DNA, to enable the choice of a suitable enzyme, the G + C ratio

of the DNA and the recognition sequence of the enzyme had to be considered. For nocardioform bacteria, with the G+C ratio of 65% and the Bgl II recognition sequence being AGATCT, a cut would be made approximately every 12kb. Thus Bgl II only cut pDA30 (E_1B_1) and pEcoR25I once, which resulted in pDA37 having one Bgl II restriction site.

When the concentration of pDA37 was first attempted, the plasmid was digested with Cla I, but this was unsuccessful. This was due to pDA37 having been maintained in an *E. coli* strain, where the Cla I restriction sites were subjected to methylation in dam+ *E. coli* strains (Vogt-Singer and Finnerty, 1988), thus the restriction sites were not recognised. A double digestion of PvuII and Bam HI had to be used, and the sample was electrolysed using a 1.2% agarose gel, as four fragments of smaller size were produced.

As the Bam HI site of pEcoR25I and the Bgl II site of pDA30 were used for the construction of the shuttle vector pDA37, these sites were subsequently lost on ligation. Thus pDA37 now contained two Bam HI sites and two Pvu II sites (Figure 6). Buffer M was used as both enzymes had 100 % activity in it. The genomic library

was built up using only Bgl II digestions, to prevent the loses of recognition and restriction sites.

5.10 TESTING CONTAMINANTS

The phage overlay and streptomycin resistance could not be taken to adequately identify a contaminant, as the results contradicted each other. The differences could have been due to the mutation. Phage lysis depended on the cell wall constituents, as well as on the internal cell machinery. The cell surface receptors could have been altered due to the mutation, as could the internal machinery. Streptomycin resistance could have been effected, as it is generally by the process of acetylation, phosphorylation, methylation and adenylation that antibiotics are inactivated, all of which were performed by enzymes and could have been effected by mutagenesis.

The fact that 4277-1,001 and 4277-1 showed the same pattern of streptomycin resistance, did not allow for the page overlay results to be conclusive. Thus the study of the pattern of the ribosomal proteins was performed as a more accurate test.

5.10.1 Ribosomal Proteins

Ribosomes are sub-microscopic particles composed of ribonucleic acid (RNA) and protein that have a fundamental role in protein synthesis. They are the sites of protein synthesis, as they serve to bring together a single messenger RNA (mRNA) molecule and charged transfer RNA (tRNA) molecules in the proper position and orientation so that the base sequence of the mRNA molecule is translated into an amino acid sequence. They are composed of a 70S subunit, which dissociates by lowering of the magnesium ion concentration into a 50S and 30S subunit. These subunits are composed of 23S, 5S and 16S RNA respectively.

The ribosomal proteins are distinct for each bacterium, and between related strains there is similarity in pattern. However between 4277-1 and 4277-1.001 there was no similarity, thus they were concluded to be different strains. Even if the mutation had effected the ribosomal proteins, it would be very unlikely that every protein would have been effected. The fact that 4277-1 showed darker, more defined spots (Figure 2) could have been due to more material having been loaded, due to the strain being a faster grower. As ribosomes are the sites of protein synthesis,

cells that are growing more rapidly require faster protein synthesis, and have more ribosomes - up to 40 % of the cell total dry weight (De Robertis and De Robertis, 19[illegible]).

5.11 LIGATION AND *E. coli* TRANSFORMATION

The enzyme T4 DNA ligase was used. It is, a single polypeptide (Mr. 68000) which catalyses the formation of a phosphodiester bond between adjacent 3'-OH and 5'-P termini in DNA. It is used for joining together DNA molecules with compatible cohesive termini. The conditions for optimal enzymic activity have been determined, but it is unclear whether these conditions are optimal for the use of ligase in cloning experiments (King and Blakesley, 1986).

The pDA37 was only minimally digested so that it would not be cut more than once, which if happened, would reduce the transformation efficiency. It was also important that the pDA37 was resuspended and stored in Buffer M so as to prevent nicking of the DNA. The increased ratio of C1.070 DNA to pDA37 DNA, reduced the transformation efficiency, as the C1.070 DNA fragments in competing to ligate with the plasmid, ligated with each other, which prevented ligation with the plasmid. Two different fragments could also have ligated to either end

of the plasmid, which prevented circularization and thus transformation.

For maximum transformation efficiency it was necessary that: the bacterial culture was in the logarithmic phase (OD of 2); the cell density was low at the time of calcium chloride treatment; the cells were maintained at 0°C for 1 hour. During this period, the efficiency of transformation increased from fourfold to sixfold (Maniatis et al., 1980). In logarithmic phase, the cell walls due to their less rigid structure were more conducive to DNA entering the cell, which was helped by the cold treatment. When heat shocked, the DNA was forced into the cells. Only a very small portion of any cells are competent to incorporating plasmid DNA in a stable fashion, and only 1 DNA molecule in approximately 10 000 is successful at transformation. Once inside the bacterium, the plasmid DNA replicates and expresses the drug-resistance markers that allow the transformed cells to survive in the presence of an antibiotic. The ability of bacteria to take up DNA is short-lived. However after exposure to agents that enhance uptake, most strains of bacteria remain in a competent state for only 1-2

days (Maniatis et al., 1982).

Only a portion of the transformed culture of MM294-1 was spread onto a single plate, since the number of transformants obtained did not increase in linear proportion to the volume spread onto a plate. This was perhaps because of toxic substances, released by the cells which had been killed by the antibiotic. Furthermore, when selecting for ampicillin resistance, the density of the plated cells should be low and the plates should be removed from the incubator after 16-24 hours and placed at 4°C. β-lactamase which was secreted into the medium from ampicillin-resistant transformants rapidly depleted the antibiotics in the region surrounding the colonies, thus cells plated at a high density or incubated for long periods, resulted in the appearance of ampicillin-sensitive satellite colonies.

The number of colonies needed for a representative genomic library, had to take into account the percentage of cells without an insert. However all of the cells appeared to have the plasmid. The inserts were found to be of different sizes, which was consistent with the

restriction endonuclease recognition sites being randomly situated in the C1.070 genome.

5.12 NOCARDIOFORM TRANSFORMATION

The plasmid was maintained in TE pH8, as in water it would be nicked and linearised. The transformation was only effective for circular DNA.

High background cell growth was found where the underlay of arsenic did not diffuse through the agar and select for colonies resistant to it. This was due to uneven spreading, or there having been too long an interval before the spreading of the arsenic underlay, during which time the background cells grew.

The colonies could not be simultaneously selected for ST utilisation and arsenic resistance, as a component in Minimal medium precipitated the arsenate. Ampicillin selection could not be used either, as although the plasmid increased ampicillin resistance in E. coli 15 fold, it only affected a 3 fold increase in nocardioforms, which was not considered to be significant. However arsenic resistance was increased 100 fold in nocardioforms, and was selected for, then the ability to utilise SD and ST was selected for.

Due to the point mutation of the histidine auxotroph 4277-1.014, its transformation was expected to be successful. If the 4277-1.063 transformation did not work, then it would be almost certain that the failure was a function of the bile acid utilisation gene(s) not having complemented 4277-1.063, rather than there having been a problem with the transformation procedure. The bile acid metabolism gene(s) could be more complex than those needed to complement an auxotroph. The genes could be clustered or spread throughout the genome, which would make complementation more difficult, than if a single gene controlled the metabolism.

The plasmid pDA30 (E_1B_1)was used to optimise conditions, as it had a higher rate of transformation efficiency, due to it having been maintained in nocardioforms and thus was not restricted. By using pDA30, any differences in transformation were due to growth phase and not restriction of the plasmid. However pDA37 was maintained in an E. coli strain, and was restricted. The amount of DNA being transformed had to be adjusted, to ensure that the same number of colonies were achieved. However this was not possible, due to the volume constraints of

the transformation. Thus not enough transformants were produced to give a representative number of colonies of the C1.070 genome. The slight differences in the number of colonies produced per strain, could be explained by the differences ir strain transformation susceptibility.

Both strains gave the best transformation efficiencies at post-stationery phase, where the cells were quiescent and more susceptible to having DNA introduced into them. The enzymic activity of the cells was also less, thus restriction of the incoming DNA was reduced. For the E. coli transformation however, the logarithmic phase was optimal for transformation, which illustrated the differences in the systems used, and the need to optimise the conditions per strain. The fact that the transformed 4277-1.063 cells took much longer to grow than the transformed 4277-1.014 cells, suggested that the complementation was more complex, which could have been due to more than one gene being involved, or 4277-1.063 could have been a much slower grower due to the extra plasmid load it would then have been carrying. This could also suggest that bile acid metabolism is a complex

process, which was reflected in the slower growth of 4277-1.063.

5.13 NONRADIOACTIVE DNA LABELLING AND DETECTION

The results verified that the cloned DNA had originated from the C1.070 genome, and that the Bgl II site in pDA37 was conserved. The plasmid pDA37 was considered to have proven its worth as a shuttle vector.

The amount of newly synthesized labelled DNA (digoxigenin incorporation every 20-25 nucleotides) depended on the amount and purity of the template DNA. In the standard reaction with 1µg of DNA per assay, approximately 10% of the nucleotides were incorporated into about 260ng of newly synthesized labelled DNA. Reactions with small amounts of template DNA (e.g. 30ng) resulted in a 1:1 ratio of labelled to unlabelled DNA. The amount of labelled DNA generated could be approximated by comparison with the labelled control in hybridisation or direct detection.

If the DNA had not been labelled efficiently, checks could have been done by re-purifying the DNA, labelling for a longer period, and ensuring that the DNA was completely denatured. If the expected sensitivity had not been achieved, the efficiency of labelling could be checked, the

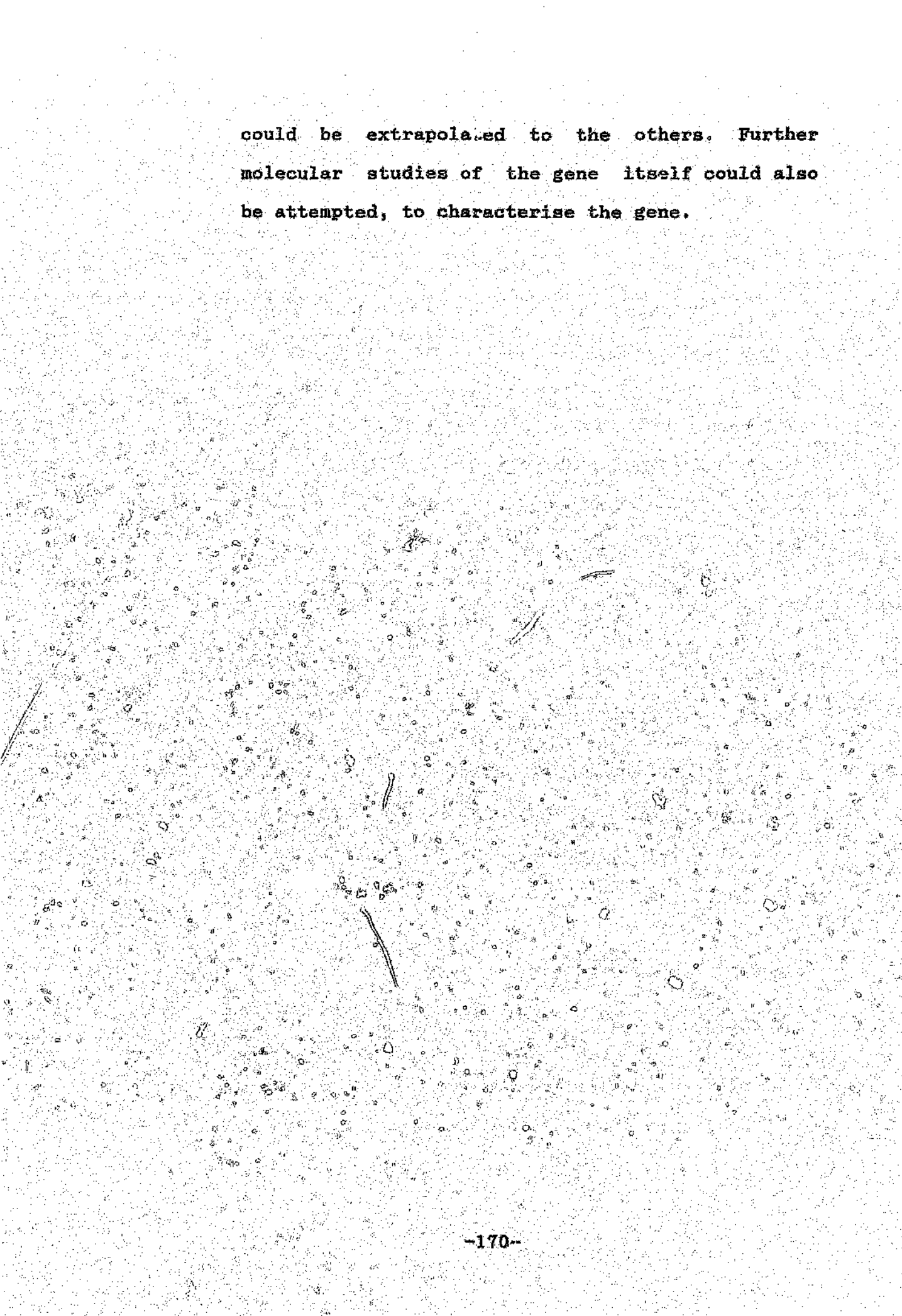
concentration of labelled DNA or the incubation time of hybridisation increased, and the colour development increased up to 3 days. If there had been too much background colour, the amount of labelled DNA in the hybridisation could be decreased, the volume of pre-hybridisation solution could be increased, the type of membrane could be changed and the concentration of the blocking reagent could be increased.

The non-radioactive DNA labelling and detection kit was very sensitive, allowing for the detection of 0.1pg of homologous DNA. Compared to radioactive systems it gave faster results with safer handling, abolishing isotopes. The sensitivity and specificity made it useful for all hybridisation techniques where radioactive labelling and autoradiography were normally required.

5.14 FUTURE PROSPECTS

The transformed 4377-1.063 colonies that could utilise SD would have to be thoroughly tested, for the presence of the insert in the plasmid which was responsible for the SD utilisation. Utilisation of and resistance to SD and other bile acids would need to determined, to ascertain if the increased ability to metabolise one bile acid

could be extrapolated to the others. Further molecular studies of the gene itself could also be attempted, to characterise the gene.



6.0 CONCLUSION

Mutant strains of the donor and recipient strains were created in the form of C1.070 and 4277-1.063 respectively. The mutant C1.070 was selected for its ability to utilise high concentrations of sodium deoxycholate and sodium taurocholate, whereas 4277-1.063 was found to be hypersensitive to these bile acids. The genomic library of C1.070 was built up in MM294-1 and then this library was transformed into 4277-1.063. Cloning by complementation appeared to have been achieved, although the results were preliminary.

Plasmid screens would however, have to be performed to verify that the metabolism of sodium deoxycholate was due to complement y gene cloning. The gene(s) responsible for the metabolism of bile acids would need to be further studied, to characterise the gene(s), and identify the metabolic products. This would allow for the metabolic pathway to be elucidated, so as to understand the mutation which produced the sensitive and resistant mutants, as well as opening up the way for more controlled, and industrially applicable use of nocardioforms in the interconversion of bile acids and steroids.

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8.0 APPENDIX

MATERIALS AND METHODS

All Autoclaving was performed at 121°C for 30 minutes, unless otherwise stated.

A. BROTHS

1. TRYPTONE YEAST BROTH (TY)

1% Tryptone (Oxoid, Basingstoke, England, hereafter referred to as "Oxoid")

0.5% Yeast Extract (Oxoid)

Autoclaved then stored at 4°C

2. TRYPTONE YEAST GLYCINE BROTH (TYG)

TY plus

1% - 4% Glycine (Merck)

If small volumes (10ml) were used the broth was autoclaved for 10 minutes, then stored at 4°C

3. T2 (Dabbs, Personal Communication)

TY plus

3mM NaCl (Saarchem)

3mM $CaCl_2$ (Saarchem)

10mM $MgSO_4$ (Saarchem)

Each solution was made and autoclaved separately, the solutions were then added when they were needed. All of the solutions were stored at room temperature.

4. TRYPTONE YEAST CALCIUM CHLORIDE BROTH (TYC)

TY plus

10mM $CaCl_2$

The solutions were made separately and autoclaved, and then added when needed.

5. LURIA BROTH (LB)

1% Tryptone

0.5% Yeast extract

0.5% NaCl

The broth was autoclaved and stored at 4°C. The broth could be supplemented with preautoclaved 10mM $MgSO_4$, which was added when needed.

6. 10 x A-N STOCK (Modified for Nocardioform Bacteria)

No citrate was used.

91.70g $K_2HPO_4 . 3H_2O$ (Merck)

26.80g KH_2PO_4 (Merck)

1g $MgSO_4 . 7H_2O$ (Merck)

The stock was made up to 1 litre with distilled water. The ph was adjusted to pH7.0. The solution was not autoclaved, instead 10ml/l of chloroform was added. The solution was stored at 4°C.

7. MINIMAL MEDIUM (Hopwood et al.,1985)

The media were made in two Erlenmeyer flasks, which were autoclaved separately, then the contents of one flask was transferred into the other flask.

Flask 1: 50ml A-N Stock (Modified)
0.5g NH_4Cl (Saarchem)
200ml Distilled water

Flask 2: 2.5 g Glucose (Saarchem)
250 ml Distilled water

Makes 500ml of medium.

8. MINIMAL MEDIUM WITHOUT GLUCOSE

Minimal media, except glucose was used.

B. AGARS

All plates were incubated at 37°C overnight before being used, to dry and check for contamination.

1. TRYPTONE YEAST AGAR (TYA) AND VARIATIONS

As for the individual broths, plus 16g/l Agar-Agar (Merck)

The agar was autoclaved and stored at 4°C.

2. LURIA AGAR (LA)

LB plus

16g/l Agar-Agar

The agar was autoclaved, then stored at 4°C

3. MINIMAL MEDIUM

As for the broth, plus

In flask 2: 6-7g Agar Noble (Difco, Detroit, USA)

Makes 500ml of Agar

The media were autoclaved separately, and then mixed, and stored at 4°C.

4. TOP AGAR

1% Tryptone

0.5% Yeast extract

0.5% Agar Agar

The agar was autoclaved, then stored at 4°C

5. PROTOPLAST REGENERATING MEDIUM PLATES

0.9g NaCl

3g Tryptone

1.5g Yeast extract

35g Sucrose (SAARchem)

4g Agar-Agar

The medium was made up to 250ml with distilled water and autoclaved.

The following solutions were autoclaved separately, and added when needed:

6ml 1M $CaCl_2$

10ml 0.25M TES buffer pH7.2 (Sigma) (N-tris(hydroxymethyl)Methyl-2-Amino-ethanesulfonic Acid

Streptomycin was optional.

The plates were poured using 25ml aliquots, then dried at 37°C for 2 days. The medium should be made up fresh.

C. BUFFERS AND SOLUTIONS

1. TRIS (Hydroxymethyl) AMINOMETHANE (T.E.) - ETHYLENEDIAMINETETRAACETIC ACID (EDTA) pH 8.0.

10mM EDTA (SIGMA)

10mM Tris (Boehringer)

The solution was made up to 400ml with distilled water. The pH was adjusted and then the solution

was made up to a final volume of 500ml, autoclaved and stored at room temperature.

2. TRIS HCl, pH 8.0.

10 mM Tris HCl

The solution was autoclaved, then stored at room temperature.

3. TE SATURATED PHENOL

14g Phenol (BDH)

10ml TE pH 8.0

The solution was heated in a water bath until all of the phenol had melted, then stored at - 20°C and thawed when needed.

4. CHLOROFORM: ISO AMYL ALCOHOL

24 parts Chloroform (BDH)

1 part Iso Amyl Alcohol (BDH)

The solution was stored at room temperature.

5. ETHIDIUM BROMIDE (EtBr)

10mg/ml EtBr (SIGMA).

The solution was made up in distilled water and microwaved gently until dissolved, then stored at 4°C.

D. GENERAL METHODS

1. PHENOL PURIFICATION

A volume of DNA was aliquoted into an eppendorf tube and the final volume was made up to 200µl with TE pH 8.0. (Appendix C.1). Then 80 µl of TE- saturated phenol (Appendix C.3) was added and centrifuged for 5 minutes. The upper layer was drawn off and placed in a fresh tube, to which 80 µl of chloroform : iso amyl alcohol (Appendix C.4) was added, and centrifuged for 1 minute. The upper layer was drawn off into a fresh tube, and the DNA was ethanol precipitated. (Appendix D.2).

2. ETHANOL PRECIPITATION

One tenth volume of 1M NaCl was added to the volume of DNA solution and two and a half volumes of ice-cold absolute alcohol were added. The DNA was centrifuged for 20 minutes in the cold. The supernatant was gently poured off, and the pellet was dried for 20 minutes at 60°C. The pellet was resuspended in sterile water, or a buffer of choice, at 42°C for a minimum of 1 hour. The more ions present in the buffer, the longer would be the time of resuspension.

3. PREPARATION OF DIALYSIS TUBING

In 300 ml of sterile water, a 2% bicarbonate of soda (SAARchem) solution was prepared. The dialysis tubing was added, and boiled for 5 minutes. The tubing was then washed three times in sterile water, and finally stored in fresh sterile water at 4°C.

E. AGAROSE GEL ELECTROPHORESIS.

1. AGAROSE AND BUFFERS.

i) 5xTBE (Tris, Boric Acid, EDTA)

54g Tris

27.5g Boric Acid (Sigma)

20ml 0.5M EDTA pH8.0

The EDTA was made up to 900ml with distilled water, the pH was adjusted and then the other components were added. The final volume was made up to a volume of 1 litre.

ii) 0.4% Agarose.

0.8g High Gelling Temperature Agarose (Sea Kem) (HGT)

200ml 1 x TBE (Appendix E.1)

The agarose was first autoclaved, and then stored at room temperature.

iii) 1.2% Agarose

2.4g HGT Agarose

200ml 1 x TBE(Appendix E.1)

The agarose was first autoclaved, and then stored at room temperature.

iv) Gel loading Buffer.

Buffer type II (Maniatis et al., 1982.)

0.25% Bromophenol Blue (SIGMA)

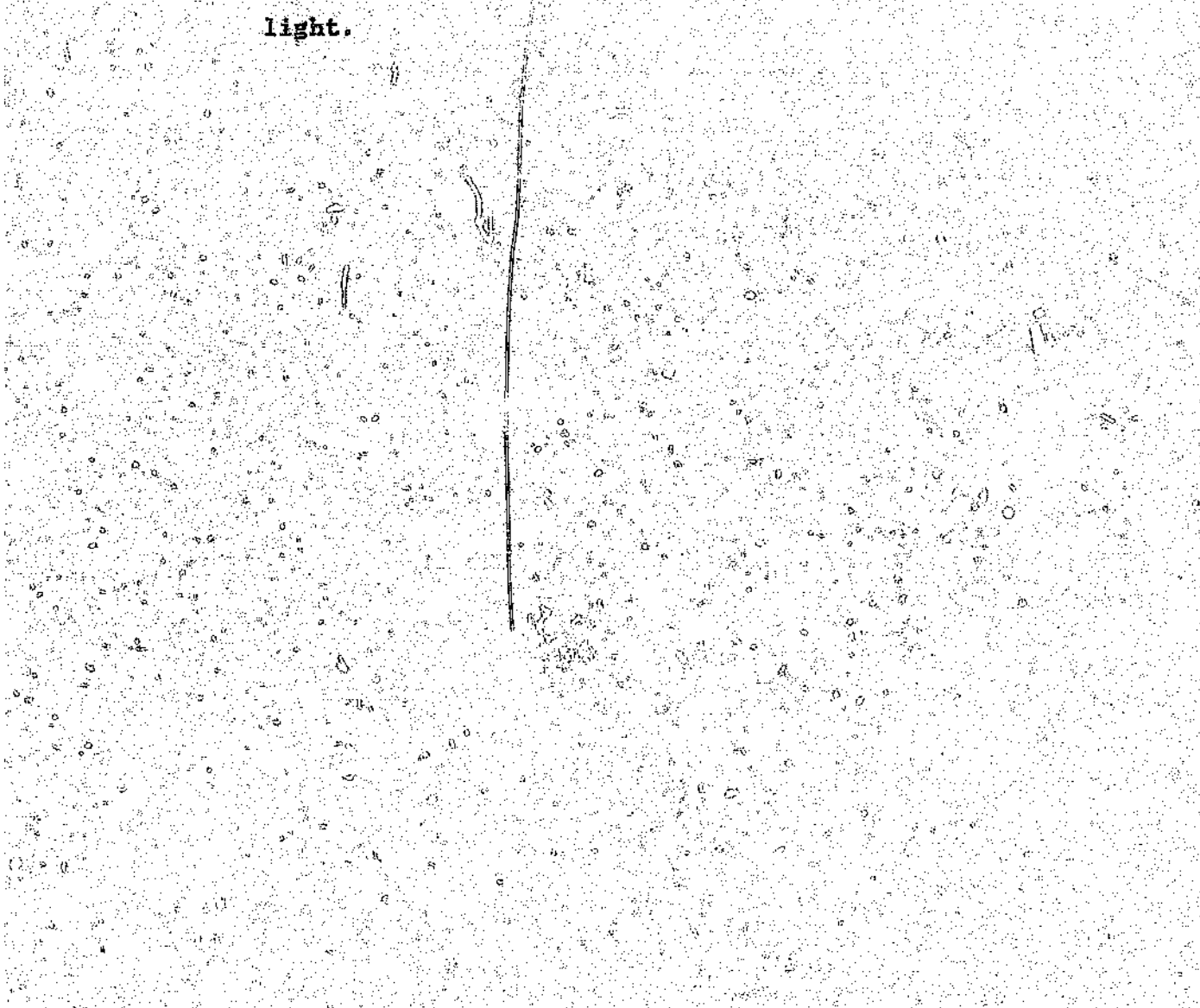
40% (w/v) Sucrose in distilled water.

The buffer was stored at 4°C.

2. THE POURING AND ELECTROLYSIS OF GELS.

The gel tray (minigel) was first sealed with tape and the comb was positioned and levelled. Then 25ml of melted agarose was mixed with 1.5µl of EtBr (Appendix C.5), and poured into the tray. The gel was allowed to set, for a minimum of 2 hours, or over night if covered, at 4°C. The running buffer was prepared by using 200ml of 1 x TBE, mixed with 15µl of EtBr. The gel comb and sealing tape were removed, and the gel tray was placed into the electrophoresis chamber. The running buffer was poured over the gel, ensuring that the gel was amply covered. The samples were then all mixed with 2ml of gel loading buffer, and loaded into the gel wells by means of a

Gilson pipette. The electrical connections were made, and the set voltage was chosen, ensuring that the amperage was only half that of the voltage. The gels were run for the desired time, or until all the loading buffer had run off the gel. The gels were then viewed by ultra-violet light.



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