The nocardiform bacteria can be classified, on this basis, into 7 families and 20 genera. The family of interest in this work was the Nocardiaceae consisting of 3 genera, *Micropolyspora*, *Nocardia* and *Rhodococcus* formerly the "rhodochrous" complex, *Mycobacterium rhodochrous* or *Proactinomyces* (cited in Goodfellow and Minnikin 1977).

The nocardiform bacteria are actinomycetes. They are branching bacteria that reproduce by fragmentation of their hyphae, into bacilli and coccoid elements.

It has been suggested that the term nocardiform is merely one of convenience and that the bacteria in this group should be regarded as a collection of individual genera and species. Therefore one can define the two genera of interest in the family Nocardiaceae as follows.

The genus *Rhodococcus* are aerobic, nonsporing actinomycetes that are pleomorphic but often form a primary mycelium that soon fragments into rod and coccoid elements. The strains contain mycolic acids and have a wall chemotype IV. The complex structure of the wall, consisting of a peptidoglycan, lipid constituents and other polysaccharide or polypeptide compounds, can be divided into chemotypes on the basis of the major sugars and amino acids found in the wall. Chemotype IV strains contain major amounts of meso-diaminopimelic acid, arabinose and galactose. The G + C content of the *Rhodococcus* DNA ranges from 59% - 69%.

The genus *Nocardia* is now a relatively homogeneous taxon and are aerobic, gram-positive actinomycetes that produce a primary mycelium that fragments into rod- and coccoid-like elements. Aerial hyphae are usually formed, strains contain mycolic acids and have a wall chemotype.
In addition to their ability to interconvert steroid compounds such as cholesterol to precursors of steroid hormones and oral contraceptives, the nocardioform bacteria of the genus *Rhodococcus* are able to degrade toxic compounds such as acrylamide, insecticides and phenolic compounds. They are also able to degrade lignin and capable of synthesizing antibiotics (cited in Dabbs 1987).

Some individual species are the aetiological agents of tuberculosis, leprosy and nocardiosis.

Four strains of nocardioform bacteria were of particular interest for the work in this thesis. They are, in order of relatedness, *Rhodococcus erythropolis* ATCC 12674 and 4277, *Rhodococcus equi* ATCC 14887, *Rhodococcus rubropertinctus* ATCC 23793 and *Rhodococcus australis* 4448 and 534.

The introduction of new steroids into commerce is limited and so new developments in microbial biotechnology of steroids is restricted. New developments in the older manufacturing protocols for the important steroid hormones and analogs are not receiving much attention. Some present interests include the preparation of isotopically labelled steroids for metabolic studies and production of enzymes that transform steroids. The microbial enzyme can be used in assay procedures for clinical estimations such as that of plasma cholesterol.

The literature dealing with the microbial transformation of steroids is considerable. This includes individual journal articles and specialized reviews. Additionally, a substantial patent literature exists. However
there is no report in the literature of cloning the genes for interconverting steroids to pharmacologically important substances in the nocardioform bacteria. This was the major aim of the work for this thesis. Cloning the genes would be by complementation of the appropriate mutation. The first priority of this work therefore was to make mutants incapable of utilizing the steroid of choice, such as cholesterol, as the sole carbon source. Mutagenesis using mutagens such as N-ethyl-N-nitro-N-nitrosoguanidine (NTG) and ultraviolet light would be carried out in order to make these mutants. Mutagenesis would be assayed by auxotroph production. Once mutants had been obtained, nocardioform chromosomal DNA would be cloned into a suitable vector and transformed into these mutants. Complementation of mutations would be observed on minimal media plates containing the steroid of choice such as cholesterol.

A suitable cloning vector for nocardioform bacteria had to be developed. The requirements for a good cloning vector were the following:

1. A replicon that was stably maintained in the organism. This requirement was met by the replicon derived from the generalized transducing bacteriophage for Rhodococcus erythropolis, Q4.
2. A means of selecting for the vector. This was achieved by the arsenic (arsenate and arsenite) resistance determinant obtained from an unstable genetic element in a nocardioform bacteria.
3. A means of selecting cloned fragments in the vector. The Escherichia coli suicide vector pEcoR251 is digested if DNA is not cloned in to the E. coli endonuclease gene at the unique Bgl II site. The presence of a λ repressor also turns off the transcription of the endonuclease hence
preventing the suicide of the plasmid. However in the absence of a λ repres sor and a cloned insert, the vector will not survive.

4. A method of screening the cloned DNA for the desired gene involves the bacterial mutants unable to utilize the compound of interest as the sole carbon/nitrogen source.

Nocardioform resistance plasmids were obtained by combining the resistance determinants from Rhodococcus erythropolis ATCC 12674 and part of the genome of nocardioophage 14. (Dabbs and Sale 1987). (See 1 & 2). These were then joined to the suicide vector pBest251 to generate a shuttle vector, pDB7 constructed by A. Saffey.

Heterologous chromosomal DNA as opposed to homologous DNA would be preferable for cloning in the absence of a recombination minus strain to prevent the cloned DNA from recombining with that of the host. The phenotype of Ren− strain is ultraviolet light hypersensitivity. A minor aim of the work described in this thesis was to obtain a Ren− strain of Rhodococcus equi. The availability of this Ren− strain would therefore facilitate the cloning of homologous nocardioform DNA into a nocardioform mutant.

The approach to cloning the genes of interest into the nocardioform mutant involved the construction of a library of nocardioform chromosomal DNA in E.coli. The object was to extract the plasmid from the bulk pool and use it to transform into the nocardioform mutant and observe for complementation. However before this can be achieved, a restriction minus mutant must be obtained since nocardioform bacteria restrict Escherichia coli DNA.
Two types of transformations will be necessary, namely transformation into the E.coli strain and the nocardioform mutant. It is essential to optimize the conditions of transformation in order to obtain the maximum number of transformants.

Once the gene/s had been cloned into the mutant the product resulting from the degradation of the steroid of interest would have to be characterized. This would be done by biochemical studies such as thin layer chromatography.

The auxotrophs obtained by mutagenesis could be used to carry out genetic mapping of the organism.
2.1 MATERIALS

2.1.1 ORGANISMS

2.1.1.1 NOCARDIOFORM BACTERIA

<table>
<thead>
<tr>
<th>ORGANISMS</th>
<th>STRAINS</th>
<th>ORIGIN</th>
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</thead>
<tbody>
<tr>
<td><em>Rhodococcus erythropolis</em></td>
<td>ATCC 12674</td>
<td>N. Ferreira</td>
</tr>
<tr>
<td></td>
<td>01</td>
<td>E. Dabbs</td>
</tr>
<tr>
<td><em>Rhodococcus equi</em></td>
<td>ATCC 14887</td>
<td>N. Ferreira</td>
</tr>
<tr>
<td></td>
<td>14887-1</td>
<td>E. Dabbs</td>
</tr>
<tr>
<td></td>
<td>KD 1</td>
<td>K. Downing</td>
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</table>

2.1.1.2 *Escherichia coli*

<table>
<thead>
<tr>
<th>STRAIN</th>
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</thead>
<tbody>
<tr>
<td>N1 294</td>
<td>E. Dabbs</td>
</tr>
</tbody>
</table>
2.1.1.3 PLASMIDS

PLASMID

p8A22
p8A30

ORIGIN

E. Babbs
E. Babbs

2.1.1.4 VECTORS

VECTOR

p8A27
p8A29

ORIGIN

E. Babbs
A. Daffey
B. Gowan
### 2.1.1.5 PHAGES

<table>
<thead>
<tr>
<th>PHAGE</th>
<th>ORIGIN</th>
</tr>
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<tbody>
<tr>
<td>K3</td>
<td>K.Downing</td>
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</tbody>
</table>

#### DERIVATIVES OF *Rhodococcus*

<table>
<thead>
<tr>
<th>PHENOTYPE</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin resistant</td>
<td>S.Andersen</td>
</tr>
<tr>
<td>Rifampicin resistant</td>
<td>E.Dabbs</td>
</tr>
<tr>
<td>Sodium taurocholate mutant</td>
<td>K.Downing</td>
</tr>
<tr>
<td>Agarase&quot; mutant</td>
<td>K.Downing</td>
</tr>
<tr>
<td>Faster grower</td>
<td>K.Downing</td>
</tr>
</tbody>
</table>

#### DERIVATIVE OF *E.coli*

<table>
<thead>
<tr>
<th>PHENOTYPE</th>
<th>ORIGIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin resistant</td>
<td>E.Dabbs</td>
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</table>

#### PLASMIDS AND VECTORS

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>ORIGIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>nocardioform plasm. conferring arsenic resistance</td>
<td>E.Dabbs</td>
</tr>
</tbody>
</table>
pDA27  

pEcoK251 + pDA22  

A.Daffey

pDA29  

pDA27 + chromosomal inserts  

K.Downing

pDA30  

Plasmid from 12674 with E.Dabbs  

with arsenic resistance

2.1.2 MEDIA.

2.1.2.1 A - N STOCK.

458.5g K2HPO4 . 3H2O

134.0g KH2PO4

25.0g tri sodium citrate . 2H2O

5g MgSO4 . 7H2O

This was made up to 5l with distilled water. This stock was not autoclaved but 10ml/l chloroform was added to prevent contaminant growth.

A - N STOCK WITHOUT CITRATE
The A-N stock without citrate was used most frequently for making minimal media to which various carbon sources were to be added. It was made up as in 2.1.2.1 eliminating tri sodium citrate. This stock was known as A-N-citrate or ST3. The media used were the following:

2.1.2.2 MINIMAL MEDIA A (MM) (Hopwood et al. 1985)

Minimal media A was prepared in two separate Erlenmeyer flasks, autoclaved separately, then the contents of one flask transferred into the other flask.

Flask 1 contained:
- 100ml A-N stock (see 2.1.1)
- 1g NH4Cl
- 400ml Distilled H2O

Flask 2 contained:
- 12-13g Agar Noble (Difco)
- 5g glucose
- 500ml H2O

This makes up 11 of media.

The glucose was usually left out of all minimal media when used in conjunction with other carbon sources and, if necessary, the required concentration was added from a 20% stock of glucose.
2.1.2.3 TY

- Tryptone: OXOID ENGLAND
- Yeast extract: OXOID ENGLAND

2.1.2.4 TY AND AGAR (TYA).

- 1% of Agar bacteriological grade: OXOID ENGLAND

2.1.2.5 T2 (Dabbs personal communication).

- 10 g/l NaCl
- 2 M MgCl₂
- 2 M MgSO₄
2.1.2.6 TYG

TY plus
1% glycine

2.1.2.7 TYMC

TYA plus
10mM CaCl₂
10mM MgCl₂

2.1.2.8 TYM

TY plus
10mM MgSO₄
2.1.2.9 TYC

TY plus
10mM CaCl₂

2.1.2.10 LURIA BROTH (LB)

0.5% Yeast extract
0.5% NaCl

2.1.2.11 LURIA AGAR (LA)

LB plus
13g/l Agar bacteriological grade OXOID ENGLAND
2.1.2.12 REGENERATION MEDIA

0.9g NaCl
3g Tryptone
1.5g Yeast extract
35g sucrose

A small volume of distilled water was added to dissolve the sucrose. The volume was made up to 280ml with distilled water to which 4g of agar was added. After autoclaving 6ml 1M CaCl₂ and 10ml TES buffer (2.1.4.13) were added. Each regeneration plate contained 25ml of Regeneration Media.

2.1.3 ADDITIONAL GROWTH REQUIREMENTS FOR NOCARDIOFORM BACTERIA

2.1.3.1 GLUTAMATE

0.2μg/ml OF A 10% STOCK.
2.1.3.2 VITAMIN B1

0.1 μg/ml of a 100 μg/ml stock.

2.1.4 BUFFERS

2.1.4.1 BUFFERS FOR MUTAGENESIS

TRIS HCl pH 8.0

10 mM Tris to pH 8.0 with HCl

TRIS HCL pH 7.0

10 mM Tris to pH 7.0 with HCl

SODIUM ACETATE BUFFER pH 4.8

23 ml 1 M acetic acid (filter sterilized)
10 ml 1 M NaOH
2.1.4.2 TRIS EDTA BUFFER (TE) (Maniatis et al. 1982)

10mM Tris
10mM EDTA

2.1.4.3 TRIS EDTA BUFFER PLUS 10% SDS

2.1.4.4 TRIS HCl WITH 10% SUCROSE

10mM Tris HCl pH 8.0 with 10% SDS

2.1.4.5 DETERGENT SOLUTION FOR DNA PREPARATION

0.25ml 20% Triton X100
3.125ml 0.5M EDTA pH 8
1.25ml 1M Tris HCl pH 8.0
20.375ml sterile H₂O
2.1.4.6 ELECTROPHORESIS BUFFER TRIS BORATE (TBA)

**Working solution**

- 0.089M Tris borate
- 0.089M Boric acid
- 0.002M EDTA

**Concentrated stock solution (per liter)**

5X:
- 54g Tris borate
- 27.5g Boric acid
- 20mL 0.5M EDTA pH 8.0

2.1.4.7 RUNNING BUFFER FOR ELECTROPHORESIS

40 mL 5X TBE

140mL sterile H₂O 15μL of a 10mg/mL stock of ethidium bromide.
2.1.4.8 GEL LOADING BUFFER (Maniatis et al 1982)

Buffer type III (10ml)

0.25% bromophenol blue
30% glycerol in water

Storage temperature was 4°C

2.1.4.9 BUFFERS FOR E.coli PLASMID SCREEN (Maniatis et al)

SOLUTION 1

50mM glucose autoclaved separately
25mM Tris pH 8.0
10mM EDTA

SOLUTION 2

0.2 N NaOH
10% SDS

SOLUTION 3
SM KAC pH 4.8

2.1.4.10 TRIS CaCl₂

50mM CaCl₂
10mM Tris Cl (pH 8.0)

2.1.4.11 LIGATION BUFFER

200mM Tris HCl
100mM MgCl₂
100mM DTT (dithiothreitol)
0.6mM ATP pH 7.6

The volume was made up to 10ml with sterile H₂O.
2.1.4.12 PROTOPLAST (P) BUFFER

The following basal solution was made up:

Sucrose 10.3g  
\( \text{K}_2\text{SO}_4 \) 0.025g  
\( \text{MgCl}_2 \cdot 6\text{H}_2\text{O} \) 0.202g  
Distilled water to 87.5ml

This was autoclaved and 10ml 0.25 M pH 7 TES buffer was added. This was then dispensed into 10 aliquots of 9.75ml each and stored at -80°C. Just before use, 250μl 1M CaCl\(_2\)·2H\(_2\)O and 100μl KH\(_2\)PO\(_4\) were added to each aliquot.

2.1.4.13 TES (N-tris(hydroxymethyl)methyl-2amino ethanesulfonic)

0.25M pH 7.2
2.1.5 ANTIBIOTICS

2.1.5.1 STREPTOMYCIN BOEHRINGER M.W. 1457.4

Concentration of the stock solution was a 100mg/ml of H₂O.

2.1.5.2 RIFAMPICIN BOEHRINGER

Concentration of stock solution was 30mg/ml made up with methanol.
Concentration added to cultures and plates: 20mg/ml.

2.1.5.3 AMPICILLIN SODIUM SALT SIGMA

Concentration of stock solution was 30mg/ml made up with 0.5ml H₂O and 0.5ml ethanol.
Concentration added to cultures and plates: 60μg/ml.
2.1.6 AGAROSE GELS

0.4% agarose gels were used and made up as follows:

0.8g Agarose (Seakem FMC)
160ml H₂O
40ml 3 x TBE

Ethidium bromide from a 10mg/ml stock (1.5 µl) was added.

2.1.7 TOP AGAR

1% Tryptone
0.5% Yeast extract
0.5% Agar
2.1.8 CARBON SOURCES

POLYOXYETHYLENE JITANMONOLEAT (TWEEN 60) MERCK.
DIMETHYLSULFOXIDE (DMSO)
CHOLESTEROL UNILAB M.W. 386.66
B-SITOSTEROL FLUKA M.W. 414.72
STIGMASTEROL FLUKA M.W. 412.70.
SODIUM BENZOATE MERCK M.W. 144.11.
SODIUM TAUROCHOLATE BDH CHEMICALS Ltd.

2.1.9 MUTAGENS

2.1.9.1 N-METHYL-N-NITRO-N-NITROSOGUANIDINE (NTG)

1 mg in 2.5 ml buffer either pH 8 (4.4.1) or pH 4.8 (4.4.3)

Gentle heat was necessary to dissolve the NTG.
2.1.9.2 METHANESULFONIC ACID ETHYL ESTER (EMS) SIGMA

1% or 2% made up with pH 7 buffer (4.4%).
The volume made up was usually 2mls.

2.1.9.3 ACRIDINE ORANGE

Stock concentration of 10mg/ml made up with sterile H2O.

2.1.9.4 ETHIDlUM BROMIDE

Stock concentration of 10mg/ml made up with sterile H2O.

2.1.9.5 ULTRAVIOLET LIGHT
2.1.10 ARSENICAL SOLUTIONS

Arsenate stock solution : 3M
Arsenite stock solution : 1M

The solutions were made up with water, microwaved to dissolve, then filter sterilized.
2.2 METHODS

2.2.1 CHOICE OF ORGANISMS

Two sets of minimal media plates were prepared, one set with and the other without glutamate and B1. Each plate contained approximately 22ml of media. The nocardioform organisms referred to by their ATCC (American Type Culture Collection) classification numbers as 25593, 4277, 14887 and 12674 were grown on both sets of plates at 26°C.

2.2.2 PREPARATION OF HIGH LEVEL RESISTANT STREPTOMYCIN MUTANTS

In order to spontaneously induce mutants with a high level of resistance to the antibiotic streptomycin in the two organisms 14887 and 4277, a loopful of each was added to 5ml of T2 and placed on a shaker at 26°C for 24-48 hours. When each organism had grown up, 0.1ml of each of the cultures was plated on a TYA plate containing streptomycin to a final concentration of 200µg/ml. The plates were incubated at 26°C.
2.2.3 PREPARATION OF HIGH LEVEL RESISTANT RIFAMPICIN MUTANTS

The procedure described in 2.2.2 was used to obtain mutants with a high level of resistance to the antibiotic rifampicin. The TYA plate contained rifampicin to a final concentration of 100μg/ml.

2.2.4 CARBON SOURCES

2.2.4.1 METHODS USED TO SOLUBILIZE CHOLESTEROL

Cholesterol is insoluble in water, therefore attempts were made to solubilize it in the following:

TWEEN 80

The concentrations 0.1%, 0.2% and 1% cholesterol were added to 1% Tween 80 solutions prepared with sterile water.