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Towards molecular biological characterisation of the genes for sterol and bile acid metabolism in nocardioform bacteria

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The nocardioform 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 *Proactinomycetes* (cited in Goodfellow and Minnikin 1977).

The nocardioform 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 nocardioform 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 Nocardiacea as follows.

The genus *Rhodococcus* are aerobic, nonsporing actinomycetes that are pleiomorphic 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

IV. The G + C content of their DNA ranges from 64% - 69%. (definitions from Goodfellow and Minnikin 1977).

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 25593 and *Rhodococcus australis* A448 and 554.

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 recciving much attention. Some present interests include the preparation of isotopically labelled steroids for metabolic studies and production of enzymes that transform steroids. The microbial enzymes 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 of 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-methyl-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 generalised transducing bacteriophage for *Rhodococcus erythropolis*, Q4.
2. A means of select g 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 repressor 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 nocardiophage Q4. (Dabbs and Sole 1987). (See 1 & 2). These were then joined to the suicide vector pEcoR251 to generate a shuttle vector, pDA27 constructed by A. Daffey.

Heterologous chromosomal DNA as opposed to homologous DNA would be preferential 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 Rec^- strain is ultraviolet light hypersensitivity. A minor aim of the work described in this thesis was to obtain a Rec^- strain of *Rhodococcus equi*. The availability of this Rec^- 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

ORGANISMS	STRAINS	ORIGIN
Rhodococcus erythropolis	ATCC 12674	N.Ferriera
	01	E.Dabbs
Rhodococcus equi	ATCC 14887	N.Ferreira
	14887-1	E.Dabbs
	KD 1	K.Downing

2.1.1.2 Escherichia coli

STRAIN	ORIGIN
MM 294	E.Dabbs

MM 294-1	E.Dabbs

2.1.1.3 PLASMIDS

PLASMID	ORIGIN
pDA22	E.Dabbs
pDA30	E.Dabbs

2.1.1.4 VECTORS

VECTOR	ORIGIN
pDA27	E.Dabbs A.Daffey
pDA29	B.Gowan

2.1.1.5 PHAGES

PHAGE		ORIGIN
K3		K.Downing

DERIVATIVES OF *Rhodococcus*	PHENOTYPE	ORIGIN
01	Rifampicin resistant	S.Andersen
14887-1	Rifampicin resistant	E.Dabbs
KD 1	Sodium taurocholate mutant Agarase$^-$ mutant.	K.Downing
KD 2	Faster grower	K.Downing

DERIVATIVE OF *E.coli*		
MM 294-1	Rifampicin resistant	E.Dabbs

PLASMIDS AND VECTORS		
pDA22	nocardioform plasm.[3] conferring arsenic resistance	E.Dabbs

pDA27	pEcoR251 + pDA22	A.Daffey
pDA29	pDA27 + chromosomal inserts	K.Downing
pDA30	Plasmid from 12674 with with arsenic resistance	E.Dabbs

2.1.2 MEDIA.

2.1.2.1 A - N STOCK.

458,5g $K_2HPO_4.3H_2O$

134,0g KH_2PO_4

25,0g tri sodium citrate - $2H_2O$

5g $MgSO_4 - 7H_2O$

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 Erlenmyer 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	NH_4Cl
400ml	Distilled H_2O

Flask 2 contained :

12-13g	Agar Noble (Difco)
5g	glucose
500ml	H_2O

This makes up 1l 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).

Agar bacteriological grade OXOID ENGLAND

2.1.2.5 T2 (Dabbs personal communication).

K_2HPO_4

NaCl

$CaCl_2$

$MgSO_4$

2.1.2.6 TYG

TY plus

1% glycine

2.1.2.7 TYMC

TYA plus

10mM $CaCl_2$

10mM $MgCl_2$

2.1.2.8 TYM

TY plus

10mM $MgSO_4$

2.1.2.9 TYC

TY plus

10mM $CaCl_2$

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 bacteriolegical 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 $CaCl_2$ 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

0.1µg/ml OF A 100mg/ml STOCK.

2.1.4 BUFFERS

2.1.4.1 BUFFERS FOR MUTAGENESIS

TRIS HCl pH 8.0

10mM Tris to pH 8.0 with HCl

TRIS HCL pH 7.0

10mM Tris to pH 7.0 with HCL

SODIUM ACETATE BUFFER pH 4.8

23ml 1M acetic acid (filter sterlized)

10ml 1M 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 H_2O

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

160ml sterile H_2O 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

5M KAC pH 4.8

2.1.4.10 TRIS $CaCl_2$

50mM $CaCl_2$

10mM Tris Cl(pH 8.0)

2.1.4.11 LIGATION BUFFER

200mM Tris HCl

100mM $MgCl_2$

100mM DTT (Dithiothreitol)

0.6mM ATP pH 7.6

The volume was made up to 10ml with sterile H_2O.

2.1.4.12 PROTOPLAST (P) BUFFER

The following basal solution was made up:

Sucrose	10.3g
K_2SO_4	0.025g
$MgCl_2.6H_2O$	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 $CaCl_2.2H_2O$ and 100µl KH_2PO_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 H_2O

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 H_2O and 0.5ml ethanol.

Concentration added to cultures and plates : 60 µg/ml.

2.1.6 AGAROSE GELS

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

0.8g Agarose (Seakem FMC)

160ml H_2O

40ml 5 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

POLYOXYETHYLENSORBITANMONOOLEAT (TWEEN 80) MERCK.

DIMETHYESULFOXIDE (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.

HYDROXYBENZOIC ACID E.C.C M.W. 138.12.

SODIUM TAUROCHOLATE BDH CHEMICALS Ltd.

2.1.9 MUTAGENS

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

1.5mg in 2.5ml 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 H_2O

2.1.9.4 ETHIDIUM BROMIDE

Stock concentration of 10mg/ml made up with sterile H_2O.

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.

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