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

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TABLE 15: COMPARISON BETWEEN NTG MUTAGENESIS OF AN EXPONENTIALLY GROWING CULTURE AND A STATIONARY PHASE CULTURE OF 14887-1

GROWTH PHASE OF CULTURE	PERCENTAGE AUXOTROPHS
Exponentially growing culture	1.58%
Stationary phase culture	0.32%

The mutagenized exponentially growing cultures yielded more auxotrophs than the stationary phase cultures. The former cultures yielded more colonies on TYA plates after mutagenesis. Exponentially growing cultures of 14887-1 were therefore preferably used in subsequent mutageneses.

3.6.2 ETHYL METHANE SULPHONATE (EMS)

The concentration of EMS and the time of exposure to this mutagen was varied in an attempt to obtain optimum numbers of mutants. The efficiency of mutagenesis was assayed by the production of auxotrophs.

TABLE 16: EFFECT OF VARYING CONCENTRATION OF EMS AND EXPOSURE TIME ON EXPONENTIALLY GROWING CULTURES OF 14887-1

CONC. OF EMS	EXPOSURE TIME HOURS	# COLONIES PATCHED	# OF AUXOTROPHS	PERCENT
2%	2	642	0	0
1%	2	566	0	0
1%	1	508	5	0.98%
1%	3	112	0	0

* # = Number Exposure of an exponentially growing culture of 14887 to 1% EMS for 1 hour gave the best frequency of auxotrophs.

3.6.3 ULTRAVIOLET LIGHT (U.V.)

One very successful round of mutagenesis using U.V light as a mutagen, yielded 0.50% auxotrophs. This mutagenesis, however was never successfully repeated and the reason for this failure was unexplained. It ap-

peared that the U.V was causing killing resulting in very few, if any colonies growing up on the plates. This means of mutagenesis was not pursued any further.

3.6.4 A[illegible]E ORANGE AND ETHIDIUM BROMIDE

Mutageneses using acridine orange and ethidium bromide as mutagens were not successful. The effect of various concentrations of the mutagens on the growth of 14887 were recorded. Acridine orange caused severe killing above a concentration of 1µg/ml. Growth was not observed above a concentration of 30µg/ml of ethidium bromide.

3.7 THE ENRICHMENT PROCEDURE

This alternative procedure of obtaining sodium taurocholate mutants, described in 2.2.8.5 yielded 5 auxotrophs (1.59%) and 19 possible sodium taurocholate mutants (2.56%). These 19 mutants had a substantially reduced ability to utilize sodium taurocholate and the other sterols as sole carbon sources, as compared to the growth of the wild type. See 3.8.2

An experiment was carried out in order to compare the enrichment procedure to NTG mutagenesis alone. The following results were obtained.

TABLE 17: COMPARISON OF NTG MUTAGENESIS + ENRICHMENT WITH NTG MUTAGENESIS ALONE

TYPE OF MUTAGENESIS	PERCENTAGE AUXOTROPHS		
	# PATCHED	# AUXOTROPH	PERCENT
NTG mutagenesis	112	1	0.89%
NTG mutagenesis + 1 round enrichment	314	5	1.59%

The enrichment procedure is a superior method of obtaining auxotrophs when compared to NTG mutagenesis alone.

3.8 MUTANTS OBTAINED

A total of 9938 colonies were patched after mutagenesis, including those obtained by the enrichment procedure. Of this total 65 were mutants with reduced or no ability to utilze the carbon sources. KD1 was the only mutant that was totally unable to utilize any of the carbon sources tested

with the exception of glucose. (See Table 19). This is referred to as a clean mutant.

3.8.1 AUXOTROPHS

Of the 65 mutants, 38 were auxotrophs. These auxotrophs may prove valuable for future work in mapping the 14887-1 chromosome.

Of these 38 auxotrophs, 15 were tested for growth requirements by spotting onto minimal media plates containing either adenine, B1 or one of the amino acids.

TABLE 18: REQUIREMENTS OF 15 OF THE 38 AUXOTROPHS

MUTANT TYPE	REQUIREMENT OF AUXOTROPH							
	ADE	ARG	HIS	LEU	PRO	TRYP	VAL ISO	
U.V			x		x	x		
U.V		x						
U.V				x				
U.V	x							
U.V			x					
NTG						x		
NTG						x		
NTG			x					
NTG			x					
NTG			x				x	
NTG			x					
NTG			x	x				
NTG		x						
NTG		x						
NTG						x		

KEY: ADE = Adenine PRO = Proline ARG = Argenine TRYP = Tryptophan

HIS = Histidine VAL ISO = Valine + Isoleucine LEU = Leucine

3.8.2 MUTANTS OBTAINED FROM THE ENRICHMENT PROCEDURE

Three rounds of the enrichment procedure described in 2.2.8.5 were carried out on a mutagenized culture of 14887-1. After the second round of enrichment, 19 colonies, patched onto minimal media, 0.02% sodium taurocholate plates, were identified as possible mutants. These candidates were streaked to single colonies. A spot test was done on a TYA plate and a series of minimal media plates containing the carbon sources of interest.

The majority were hypersensitive to 0.02% sodium taurocholate. Growth of all candidates was very poor on 0.01% cholesterol, stigmasterol and ß-sitosterol compared to growth of the wild type 14887. Only one colony appeared to be unable to utilize hyroxybenzoate.

All these candidates had a reduced ability to utilize the sterols and sodium taurocholate as compared to the growth of the wild type. However no nonutilizing mutant of the above carbon sources was obtained.

One round of enrichment in glucose of a NTG mutagenized culture of 14887 Rif&spr. yielded 5 auxotrophs out of 314 patched colonies. The requirements of these auxotrophs was not determined.

3.8.3 MUTANTS RESULTING FROM MUTAGENESIS, EXCLUDING THE ENRICHMENT PROCEDURE

Seven mutants, excluding auxotrophs resulted from the numerous rounds of mutagenesis. These had reduced ability or no ability to utilize the sterols, sodium taurocholate and other carbon sources, as shown by spot testing. The results of these spot tests are represented in table 19.

Several faster growing colonies resulted from a NTG mutagenesis. These colonies were larger than the rest and appeared to grow up before the other colonies. One of these faster growers was identified as a 14887 derivative using the K3 phage. This was designated KD 2. It was stored in glycerol at -80°C.

TABLE 19: MUTANTS OF 14887-1 WITH REDUCED ABILITY TO UTILIZE STEROLS, SODIUM TAUROCHOLATE AND OTHER CARBON SOURCES

MUTANTS	A	B	C	D	E	F	G	H	I	J	K
WILD TYPE	+++	++	+++	++	+	+	+/-	++	+	+++	+
U.V1 M 1	+++	+++	++	+	+/--	+/-	+/-	++	[illegible]	+	+
U.V M 2	+++	+++	++	+	+/--	+/-	+/-	[illegible]	-	+	+
NTG KD 1	+++	+++	++			-	-	-	-	-	-
EMS M 4	+++	+++	++	+	+/--	+/-	+/-	++	+	++	=
EMS M 5	+++	+++	+	+	+/--	+/-	+/-	++	+	++	+
NTG M 6	+++	+++	++	+	+/-	+/-	+	++	+	++	+
U.V M 7	+++	+++	[illegible]	[illegible]	+	+	-	++	-	++	+

A = TYA

B = MM + Glucose

C = MM + 0.02% Sodium taurocholate + glucose

D = MM + 0.02% Sodium taurocholate

E = MM + 0.01% Cholesterol

F = MM + 0.01% Stigmasterol

G = MM + 0.01% β-sitosterol

H = MM + 0.2% Hydroxybenzoate

I = MM + 0.2% Tween 80

J = MM + 2% Ethanol

K = MM Stock 3

KEY: +++ Very good growth ++ Good growth + Moderate growth +/- Slight growth +/-- Very slight growth ⁄ As appeared on plate - No growth

Mutant 3 was the only clean sodium taurocholate mutant that resulted. It was a nonutilizer of sodium taurocholate since it was able to grow on MM + 0.02% sodium taurocholate + glucose. It was also unable to utilize any of the other carbon sources shown in table 19 including agar. This mutant was designated KD 1.

Mutants 2 and 7 were unable to utilize Tween 80 as sole carbon source. Mutant 2 was also unable to utilize hydroxybenzoate.

All the mutants were poor utilizers of cholesterol. Stigmasterol and β-sitosterol seemed to be used better than cholesterol.

The mutants can be grouped into 4 different classes namely:

1. KD 1
2. Mutant 7
3. Mutants 4, 5 and 6
4. Mutants 1 and 2 -

The reversion frequency of KD 1 was 0.

The altered response of mutants KD 1 and 7 to sodium taurocholate was investigated. Liquid minimal media cultures were set up containing 1.2%

glucose. The following volumes of a 10% stock of sodium taurocholate were also added:

0.3µl 1µl 3µl 10µl 30µl 100µl

Each set of sterile tubes was inoculated with one of the following organisms:

14887-1, KD 1, Mutant 7

The sets of tubes were incubated at 26°C with aeration and optical density (OD) readings at a wavelength of 540nm were taken immediately one set showed turbidity.

The results of the experiment are shown in Figure 3.

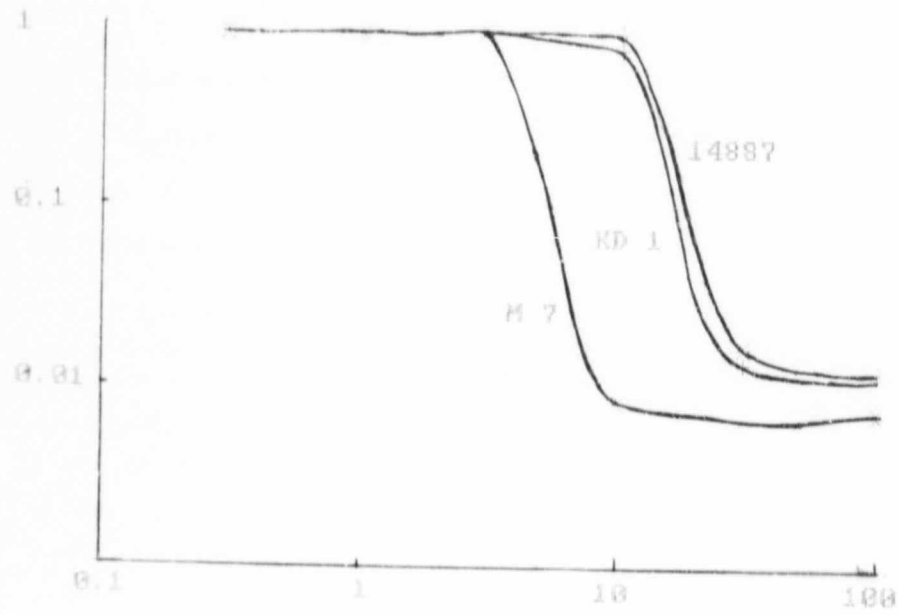
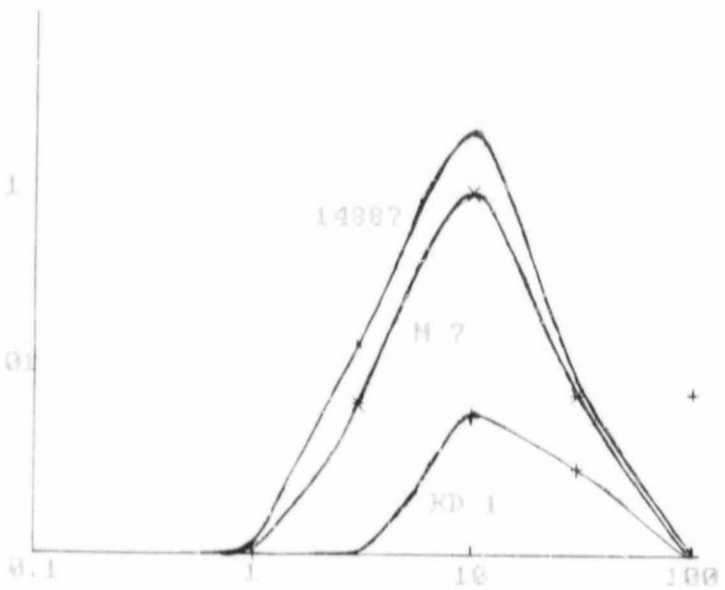


Figure 3. Graphical representation of the altered response of mutants KD 1 and 7 to sodium taurocholate

Mutant 7 is hypersensitive to sodium taurocholate at a concentration of 0.02% (or 10µl of a 10% stock in 5ml). There was no inhibition of growth of either 14887 or KD 1 at 0.02% sodium taurocholate but there was at 0.06%.

The optimum concentration of sodium taurocholate for, Mutant 7 and KD 1 was determined. A similiar experiment to the one above was set up without glucose.

The results are represented in figure 4



VOLUME OF SODIUM TAUROCHOLATE (ml)

Figure 4. Graphical representation of the optimum concentration of sodium taurocholate for mutants KD 1 and 7

The optimum concentration of sodium taurocholate for growth of both 14887 and mutant 7 was 0.02%. This supports the results obtained previously. KD 1 could not use sodium taurocholate to any significant extent.

3.9 CONSTRUCTION OF AN E.COLI LIBRARY CONTAINING 01 DNA

The ultimate aim of this work was to clone the genes for the utilization of sodium taurocholate and hopefully cholesterol into the mutant KD 1 by complementation.

The DNA of choice to clone into KD 1 would have been 14887. However, nocardioform bacteria do not possess a recombination minus system therefore the 14887 DNA would be integrated into that of the mutant.

Attempts were made to induce a rec^- mutant by exposing a mutagenized culture of 14887 patched onto TYA to ultraviolet light. The phenotype of rec^- mutants is U.V sensitivity. The mutants would be retrieved from TYA control plates.

The optimum time of exposure of 14887 to U.V light was 3 minutes. No rec^- mutants were obtained.

To determine which nocardioform DNA would be a suitable alternative to 14887 to clone into KD 1, a spot test was done using the organisms listed below. The object of the spot test was to determine which of these organisms possessed the ability to utilize sodium taurocholate.

TABLE 20: UTILIZATION OF SODIUM TAUROCHOLATE BY NOCARDIOFORM BACTERIA

NOCARDIOFORM BACTERIA	PLATES				
	A	B	C	D	E
ATCC 25593	+++	++	::	+	+
ATCC 14887	+++	++	+++	++	+
ATCC 4277	+++	++	+++	++	+
ATCC 12674	+++	+	++	++	+
A 448	+++	++	++	++	+
A 554	+++	++	+	+	+
M.smegmatis	+	++	+		+

A = TYA

B = MM + glucose

C = MM + 0.02% sodium taurocholate + glucose

D = MM + sodium taurocholate

E = MM - carbon source.

It appeared that 12674, 14887, 4277 and 448 were able to utilize sodium taurocholate better than the other three organisms. *M.smegmatis* and 25593 were not able to utilize the steroid compound as a carbon source.

The derivative of 12674, 01 was chosen as the chromosomal DNA to be cloned into the mutant.

An alternative cloning strategy using the shuttle vector pDA27 was used. This required the construction of an *E.coli* library containing clones of 01 chromosomal DNA. The library was constructed in *E.coli* MM294-1 which was a rifampicin resistant derivative of *E.coli* MM294.

The library had to be large enough to represent the entire 01 chromosome. The 01 chromosomal DNA, restricted with an endonuclease was ligated to the shuttle vector, restricted with the same restiction endonuclease. This was then used to transform *E.coli* MM294-1.

3.10 THE SHUTTLE VECTOR PDA27

The shuttle vector pDA27 was constructed by a colleague, A.Daffey. It consisted of the *E.coli* plasmid pEcoR251 (the suicide vector) and the nocardioform plasmid pDA22 which carried the genes conferring arsenic resistance. The *E.coli* plasmid carried the gene for ampicillin resistance and the gene for the Eco R1 endonuclease.

The suicide vector has a single BamH1 site outside the Eco R1 endonuclease gene and the ampicillin resistance gene. It also has a unique Bgl II site. The pDA22 plasmid has a single Bam H1 site. These two were digested with Bam H1 and ligated to form the shuttle vector.

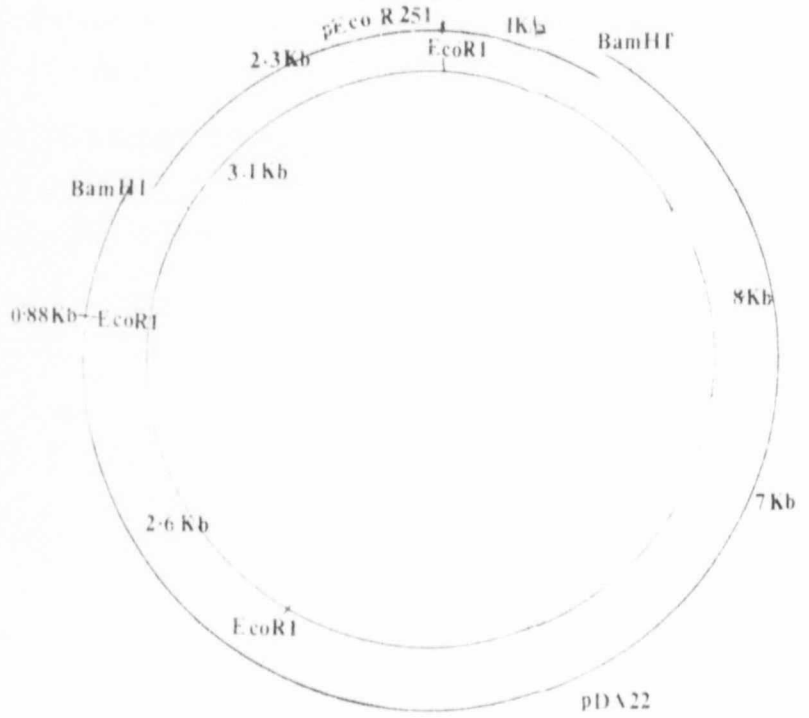


Figure 5. Diagram of the shuttle vector, orientation A constructed by A.Daffey.

The suicide vector and pDA22 were combined in two different orientations, known as shuttle vector 1 and 2, by A.Daffey. Both orientations of the shuttle vector possessed a replicon, an arsenic resistance determinant as a means of selection and a means of selecting cloned fragments in the

vector. Nocardioform DNA, digested with Bgl II was cloned into these two orientations to determine which of these was suitable for cloning.

A plasmid screen was carried out on the transformants obtained using the shuttle vectors 1 and 2.

Figure 6 shows that the plasmid screen of transformants obtained from DNA cloned into shuttle vector 1 resulted in heterogeneity of inserts whereas shuttle vector 2 yielded very little heterogeneity of inserts (not shown). Shuttle vector 1 was therefore used for all subsequent cloning of DNA for the construction of the *E.coli* library.

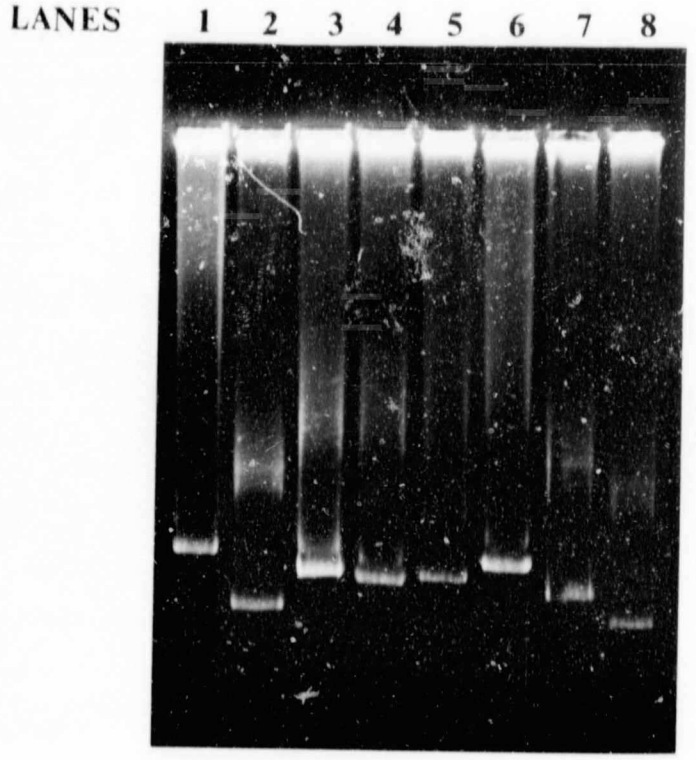


Figure 6. Plasmid screen of transformants resulting from ligation of nocardioform DNA and the shuttle vector

3.11 01 CHROMOSOMAL DNA

The shuttle vector had a single Bgl II site. Other restriction endonucleases share compatible cohesive ends with Bgl II. Sau 3A, Bcl I and Bam H1 were available.The recognition sites of each enzyme are shown in Table 21.

TABLE 21: RECOGNITION SEQUENCES OF Bam H1, Bcl I and Bgl II

e. Sau 3A

RESTRICTION ENDONUCLEASE	RECOGNITION SEQUENCE
Bam H1	G↓GATCC
Bcl I	T↓GATCA
Bgl II	A↓GATCT
Sau 3A	↓GATC

3.11.1 PARTIAL DIGESTION OF 01 CHROMOSOMAL DNA WITH SAU 3A

01 chromosomal DNA was prepared as described in 2.2.10.1. Aliquots of this DNA were digested with 0.35, 0.035, 0.0035 and 0.00035 units of SAU 3A for 30 minutes at 37°C. The samples were then run on a 0.4% agarose gel. Figure 7 shows that 0.0035 units of SAU 3A partially digested 01 chromosomal DNA. The DNA was totally digested by 0.35 and 0.035 units of enzyme whereas 0.00035 units of SAU 3A did not cut it significantly.



Figure 7. A 0.4% agarose gel illustrating the digestion of 01 chromosomal DNA: 0.55, 0.055, 0.0055 and 0.00055 units (lanes 3-6) of Sau 3A at 37°C for 30 minutes. An aliquot of the λ Hind III molecular weight marker was run in lane 1 and an undigested sample of 01 chromosomal DNA in lane 2.

The concentration of 01 chromosomal DNA was 268µg/ml. This was calculated from an absorbance reading of an aliquot of 01 chromosomal DNA in TE at 260nm.

The calculation was as follows:

OD_{260} x 50 x dilution factor

= 0.067 x 50 x 800/10

= 268µg/ml.

The amount of DNA used for each digestion was 0.241µg.

3.11.2 PARTIAL DIGESTION OF 01 CHROMOSOMAL DNA WITH BGL II

Bgl II was used in preference to Sau 3A to cleave the 01 chromosomal DNA and the snuttle vector, pDA27. Since both were restricted with Bgl II it would be easier to remove the cloned chromosomal insert from the shuttle vector .

Partial Bgl II digestion would result in larger fragments of chromosomal DNA. The enzyme would not cut as often as it would if a total digest occured. Hence the likelihood of cleaving a gene of interest would therefore be decreased.

01 chromosomal DNA was prepared as described in 2.2.10.2. Each digestion had 1µg of DNA .

Bgl II was diluted as follows:

0.9, 0.09, 0.009 and 0.[illegible] units of enzyme/µl DNA.

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