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

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2.2.12.1 OPTIMIZATION OF THE PEG TRANSFORMATION

The procedure described in 2.2.13 was the general procedure used but it was necessary to determine the optimal conditons for the transformation. Firstly, the culture of KD 1 diluted 1/100 times was grown up at 26°C with agitation and every 12 hours a 1ml aliquot was removed. An absorbance reading at 540nm was taken and the transformation carried out in order to determine the growth stage which gave the best transformation efficiency. Other aliquots or the culture were removed and frozen at 80°C for subsequent work. The following variables were also changed:

Lysozyme time

Final concentration of P PEG

The amount of KD 1 cells

The amount of pDA30 DNA

The time of arsenate/arsenite underlay

Tranformation using these variables were carried out changing only one parameter at a time, keeping the others constant as described in 2.2.13. These variables will be discussed in the results section.

3.0 RESULTS

3.1 CHOICE OF ORGANISMS

The nocardioform bacteria are noted for the diversity in their catabolic activities especially for interconverting steroids. They were therefore attractive candidates for this work.

Four species of nocardioform bacteria of the genus *Rhodococcus* were chosen and will be referred to as strains ATCC 12674, 4277, 14887 and 25593. The correct taxonomic names of these bacteria are given in 2.1.

It was of necessity to determine the quality of growth of these organisms on minimal media and any additional growth requirements.

Two sets of minimal media plates were prepared, one set containing glucose only, as the carbon source and the other set containing glucose and supplementso of sodium glutamate and thiamin (vitamin B1).

The results are shown in Table 2.

TABLE 2: DETERMINATION OF GROWTH AND GROWTH REQUIREMENTS OF *Rhodococcus* SPECIES ON MINIMAL MEDIA

	GROWTH	
ORGANISM	MINIMAL MEDIA + glucose	MINIMAL MEDIA + GLUCOSE, GLUTAMATE, B1
12674	++	+++
4277	+++	+++
14887	+++	+++
25593	+	+

KEY: +++ Very good growth ++ Good growth + moderate growth +/- Slight growth +/-- Very slight growth

Sodium glutamate can be utilized as a carbon source by the bacteria, therefore ATCC 12674 which requires glutamate and B1 for growth would not be suitable to use in this work. The strains 14887 and 4277 were chosen for this work as they grew well on minimal media and did not have additional growth requirements. Of these two, 14887 was chosen specifically for the steroid work.

3.2 PREPARATION OF HIGH LEVEL RESISTANT STREPTOMYCIN AND RIFAMPICIN MUTANTS

To eliminate the growth of contaminants during the long periods of incubation and manipulation of the bacteria, it was desirable to have a mutant of the strain, which had a high level of resistance to a particular antibiotic. This antibiotic could then be included in plates or liquid cultures of the resistant strains, at an appropriate concentration, thus minimizing the risk of contamination.

Approximately 300 high level resistant mutants of both 4277 and 12674 to a concentration of 200µg/ml streptomycin resulted. However, only 2 14887 streptomycin resistant mutants resulted, one of which was a low level resistant mutant the other a slow growing high level resistant mutant. Neither of these were suitable.

Rifampicin was used as an alternative antibiotic. One high level resistant rifampicin mutant was selected and was designated 14887-1.

3.3 CARBON SOURCES

The sterols, cholesterol, ß-sitosterol and stigmasterol and the bile acid, sodium taurocholate were used as carbon sources in an attempt to obtain mutants. These were to be used for cloning of the gene/s respon-

sible for interconverting the above steroid compounds into substances of pharmacological interest

A major problem was the insolubility of the sterols in water. Four different approaches to preparing cholesterol were attempted, namely

1. Solubilizing cholesterol in the presence of Tween 80.
2. Solubilizing cholesterol in hot ethanol.
3. Solubilizing cholesterol in DMSO.
4. Making an aqueous suspension and homogenizing by sonication. These four approaches are discussed in sections 3.3.5 - 3.3.8.

Suitable concentrations of both the cholesterol and the solvent had to be determined. It was also essential to determine whether these solvents, namely Tween 80, ethanol and DMSO could themselves be used by ATCC 14887-1 as sole carbon source.

3.3.1 AGAR AS A CARBON SOURCE

Some microorganisms possess an agarase gene which enables them to utilize agar as a carbon source. It was therefore necessary to determine whether the above was true for the organisms 14887, 4277 and 12674. These organisms were streaked on minimal media lacking glucose. All were able

to grow on this media therefore it was assumed that they possessed an agarase gene. Thus agarase$^-$ mutants had to be made, using agar noble. Three different types of agar were available in the laboratory, each differing in purity. A colleague, B.Gowan showed that growth of 14887 and 4277 on the purest agar, Difco agar noble was the poorest and therefore this agar was suitable for incorporation into minimal media plates.

3.3.2 CITRATE AS A CARBON SOURCE

Citrate was present in the A - N stock described in 2.1.2.1. The ability of 14887-1 to utilize this compound as sole carbon source was investigated.

Liquid minimal media cultures were prepared containing A - N stock with citrate (called stock 1) and without citrate (known as stock 3). The nitrogen source was NH_4Cl. Glucose was added to one set of liquid cultures.

The results were as follows.

TABLE 3: INVESTIGATION OF THE UTILIZATION OF CITRATE AS SOLE CARBON SOURCE BY 14887-1 IN LIQUID MINIMAL MEDIA

MINIMAL MEDIA CULTURES	GROWTH OF 14887-1
Stock 1 + glucose	+++
Stock 3 + glucose	++
Stock 1 - glucose	+
Stock 3 - glucose	+/-

These results were scored after 5 days.

Similiar results were obtained for 4277 by B.Gowan. A spot test on minimal media plates made with A - N stocks 1 and 3 confirmed that 14887 ,4277 and 12674 were able to utilize citrate. From these results it was evident that citrate can be used as sole carbon source by these *Rhodococcus* species, although less efficiently than glucose.

3.3.3 OPTIMAL CONCENTRATIONS OF TWEEN 80, ETHANOL AND DMSO TO BE USED AS SOLVENTS

The optimal concentrations of Tween 80, Ethanol and DMSO that would either enhance or inhibit growth of 14887 and 4277 were ascertained.

Separate experiments were performed for each solvent. The organisms were spotted onto minimal media plates containing glucose and the various concentrations of solvent.

The optimal concentrations of each solvent at which growth was not inhibited were the following.

TABLE 4: OPTIMUM CONCENTRATIONS OF THE SOLVENTS TWEEN 80, AND DMSO FOR 14887 AND 4277

SOLVENTS	OPTIMUM CONCENTRATION FOR 14887 AND 4277
Tween 80	1%
Ethanol	2-4%
DMSO	2%

3.3.4 UTILIZATION OF SOLVENTS AS SOLE CARBON SOURCES

The utilization of Tween 80, ethanol and DMSO as carbon sources by 14887 -1 was investigated. Liquid minimal media cultures were necessary to perform these experiments due to the lack of a 14887 agarase mutant. Glucose was eliminated from the cultures and replaced with either Tween 80, ethanol or DMSO.

Control cultures including glucose as sole carbon source were set up. Results were recorded after 4 days incubation at 26°C. The results of three separate experiments are summarized in table 5.

TABLE 5: UTILISATION OF TWEEN 80, ETHANOL AND DMSO BY 14887-1

IN LIQUID MINIMAL MEDIA AT 26°C

CARBON SOURCE	GROWTH OF 14887
0.5% glucose (control)	+++
1% Tween 80	++
0.2% Tween 80	++
0.02% Tween 80	+
0.002% Tween 80	+/-
2.5% Ethanol	++
1% DMSO	-

The growth of 4277 on 1% Tween 80 and 2.5% ethanol was also investigated. It appeared that 14887 could utilize 1% Tween 80 better than 4277 but there was no difference in the growth between the two organisms on 2.5% ethanol plates.

The solvents DMSO and Tween 80 at concentrations of 1% and 0.002% respectively were not used by 14887 as sole carbon source.

3.3.5 SOLUBILIZING CHOLESTEROL WITH TWEEN 80

Tween 80 was used in an attempt to solubilize cholesterol by trying to avoid the problems of aggregation.

Several concentrations of cholesterol in combination with a number of concentrations of Tween 80 were attempted.

TABLE 6: COMBINATIONS OF CHOLESTEROL AND TWEEN 80

CONCENTRATION	
0.1% cholesterol in 1% Tween 80	Well dissolved
0.2% cholesterol in 1% Tween 80	Partially dissolved
1% cholesterol in 1% Tween 80	Suspension
1% cholesterol in 10% Tween 80	Suspension

The ideal combination of 0.1% cholesterol in 1% Tween 80 was unsatisfactory because Tween 80 above a concentration of 0.002% could be utilized as sole carbon source by 14887. (see table 5).

A liquid minimal media culture of 14887 with the addition of 0.1% cholesterol and 0.002% Tween 80 was prepared. A control culture lacking the 14887 was also set up in order to determine whether the cholesterol precipitated out of solution. This was the case, causing both the control and the culture to be turbid. This turbidity could therefore not be attributed to the growth of 14887.

3.3.6 SOLUBILIZING CHOLESTEROL IN HOT ETHANOL

A 20% suspension of cholesterol in ethanol was prepared and heated gently over a flame in order to dissolve the cholesterol. As soon as the solution cooled, it solidified.

If this method had been successful, attempts would have been made to ob-
a ethanol mutants.

3.3.7 SOLUBILIZING CHOLESTEROL IN DMSO

A liquid culture of 14887 was set up as described in 3.3.6 to which 0.1% cholesterol and 1% DMSO was added. A control culture lacking 14887 was also prepared, which served the same purpose as in 3.3.6. After incubation at 26°C both the control and culture were turbid indicating that cholesterol had precipitated out of solution. Growth of 14887 could not therefore be distinguished.

3.3.8 SONICATION OF CHOLESTEROL

A 1% and 0.1% suspension of cholesterol in sterile water was made and sonicated for several seconds. The cholesterol was dispersed in both cases, but the 0.1% cholesterol was dispersed better.
Sonication was therefore the best approach to solubilizing cholesterol.

Minimal media plates were made with the incorporation of the sonicated cholesterol suspensions to a final concentration of 0.1% and 0.01%. Plates containing 0.01% cholesterol were better dispersed than those containing 0.1%.

It was very difficult to observe differences in the quality of growth of organisms on plates containing either 0.1% or 0.01% cholesterol and therefore alternative sterols were sought.

3.3.9 β-SITOSTEROL AND STIGMASTEROL AS ALTERNATIVE STEROLS

Liquid minimal media cultures were set up to determine whether 14887-1 could utilize two alternative sterols, namely β-sitosterol and stigmasterol. A culture containing cholesterol was also incubated for comparison. All sterols were sonicated and the concentration of all was 0.1%.

The results are shown in table 7.

TABLE 7: UTILIZATION OF STEROLS BY 14887-1 IN LIQUID MINIMAL MEDIA

STEROL	TURBIDITY OF CULTURE
No C control	+++
Glucose control	+++
0.1% Stigmasterol + cells	+++
0.1% Stigmasterol - cells	++
0.1% β-sitosterol + cells	+++
0.1% β-sitosterol - cells	++
0.1% cholesterol + cells	+++
0.1% cholesterol - cells	++

KEY: +++ Very turbid ++ Turbid

The sterols precipitated out of solution resulting in the turbidity observed in the no cells control. However there was a slight difference between the cells controls and no cells controls. There was no difference in turbidity between the cultures of the different sterols.

This experiment was repeated with lower concentrations of sterols, namely 0.01% but no growth was observed.

3.3.10 SODIUM TAUROCHOLATE AS AN ALTERNATIVE STEROID

Sodium taurocholate, a bile acid and steroid compound has a similiar structure to cholesterol and was very freely soluble in water. This was therefore a suitable alternative to the sterols mentioned previously.

The optimum concentration of sodium taurocholate for 14887, 4277 and 12674, which did not inhibit growth, was 0.02%. The bile acid was also utilized as sole carbon source by 14887 at this concentration.

3.3.11 ALTERNATIVE CARBON SOURCES, SODIUM BENZOATE AND HYDROXYBENZOATE

Sodium benzoate and hydroxybenzoate were tested as possible carbon sources in liquid minimal media cultures of 14887-1

TABLE 8: UTILIZATION OF SODIUM BENZOATE AND HYDROXYBENZOATE AS CARBON SOURCES FOR 14887-1

CARBON SOURCES	GROWTH OF 14887
No C control	+/-
0.1% Sodium benzoate	+/-
0.1% Hydroxybenzoate	++

These results were recorded after three days incubation at 26°C.

TABLE 9: COMPARISON OF UTILIZATION OF THE CARBON SOURCES TESTED BY 14887-1 LIQUID CULTURES AND PLATES

SOLE CARBON SOURCE	LIQUID CULTURE	MINIMAL MEDIA PLATES
Glucose	+++	+++
Citrate	+/- → +	Not tested
Agar noble	Not tested	+/- → +
0.2% Tween 80	++	++
2% Ethanol	+	+++
1%/2% DMSO	-	+/-
0.01% Cholesterol	-	+
0.01% Stigmasterol	-	+
0.01% β-sitosterol	-	+/-
0.1% Sodium benzoate	+/-	+/-
0.1% Hydroxybenzoate	+++	+++
0.02% Sodium taurocholate	++	+++

Ethanol, hydroxybenzoate and sodium taurocholate were utilized by 14887-1 as well as glucose. These three were better carbon sources than Tween 80. Cholesterol, β-sitosterol and stigmasterol were not good carbon sources. Citrate and agar noble were poor carbon sources, whereas DMSO and sodium benzoate can not be utilized by 14887-1 as sole carbon source.

3.4 ISOLATION OF PHAGE TO BE USED FOR TYPING

The nocardioform bacteria 12o74, 4277 and 14887 are closely related and there are no specific markers that distinguish between the strains.

A useful means of distinguishing and identifying 14887 from the other strains and from contaminants is by phage typing.

Phages were prepared from a soil sample as described in 2.2.7. The filter sterilised soil supernatant was mixed with 14887 which was allowed to grow on TYMC plates. After 2 days incubation at 26°C, 3 definite plagues and 2 possible plagues were observed in the lawn of 14887.

Each plague was removed and treated as in 2.2.7. A lawn of cells of each of the four organisms 14887, 4277, 12674 and 448 were grown up on TYMC plates. Once the lawn had grown up, each of the plagues prepared above were spotted onto it.

The results were as follows:

TABLE 10: LYSIS OF ORGANISMS BY PHAGES ISOLATED FROM SOIL.

ORGANISM	PHAGES				
	K1	K2	K3	K4	K5
12674	+/-	+	+/-	+/-	+
14887	+	+	+	+	+
4277	+/-	+	-	-	+
448	-	-	-	+/-	-

KEY: + Lysis - No Lysis +/- Turbid plaques

All five phages isolated from the soil lysed 14887-1.One of the phages, known as K3 was more specific than the other four phages in lysing 14887. It appeared that there were four kinds of phages namely:

1. K2 and K5
2. K4
3. K3
4. K1

These results also showed that 14887, 12674 and 4277 are closely related whereas 448 is not.

The five phages were tested on a series of plates (see 2.2.7) to determine their divalent ion requirements.

TABLE 11: DETERMINING THE DIVALENT ION REQUIREMENT OF THE FIVE PHAGES SPECIFIC FOR 14887-1

PHAGES	PLATES			
	TYA	TYMC	TYM	TYC
PHAGE K1	-	+	+/-	+/-
PHAGE K2	+	+	+/-	+
PHAGE K3	-	+	+	+/-
PHAGE K4	-	+	+/-	+/-
PHAGE K5	+	+	+/-	+

KEY: + Lysis - No Lysis +/- Turbid plaques/slight lysis

TYA = TY and Agar TYMC = TYA + $CaCl_2$ and $MgCl_2$ TYM = TYA $MgSO_4$ TYC = TYA + $CaCl_2$

These results showed that phage K3 was a calcium dependent phage, therefore calcium was added to media in all subsequent work with the phage .

K3 proved to be very useful in identifying several mutants of 14887 which had quite different growth properties than the parent.

3.5 MUTAGENESIS

The ultimate aim of this work was to clone the genes for converting sterols into substances of pharmacological importance by complementation of the appropriate mutation. It was therefore essential to obtain mutants which had lost the ability to utilize the carbon source of interest, namely sodium taurocholate.

3.5.1 OPTIMUM CONDITIONS FOR NTG MUTAGENESIS

Optimization of NTG mutagenesis was carried out by a colleague, B.Gowan, with 01, a derivative of 12674. She reported that the following conditions were optimum for NTG mutagenesis of 0

TABLE 12: OPTIMUM CONDITIONS FOR NTG MUTAGENESIS

CONDITIONS	OPTIMUM
pH of buffers	pH 8
Concentration of NTG	100µg/ml
Media	T2
Vigorous vortexing	80 seconds
Sonication	3-6 seconds (with medium tip)

3.6 MUTAGENS

The different mutagens used for mutagenesis are listed in 2.1.9.

Most mutagens act by virtue of an ability either to act on a particular base of DNA or to become incorporated into the nucleic acid.

N-methyl-N'-nitro-N-nitrosoguanidine or NTG is a powerful mutagen that acts by alkylation. It alters a base that is already incorporated in DNA and thereby changes its hydrogen-bonding specificity.

Ethyl methane sulfonate (EMS) is also an alkylating agent that reacts primarily with guanine.

Ultraviolet light is a fairly potent mutagen. It causes thymine dimers. Acridine orange and ethidium bromide are intercalating substances. They insert between bases and cause frameshift mutations.

The generation of mutants was assayed by the production of auxotrophs. From a series of experiments a total of 8869 colonies growing up after mutagenesis were patched (2.2.8.1.1). Of these, a total of 33 auxotrophs resulted from the different mutageneses. (0.37% auxotrophs).

TABLE 13: COMPARISON OF THE DIFFERENT MUTAGENS BASED ON THE NUMBER OF AUXOTROPHS PRODUCED

MUTAGEN	PERCENTAGE AUXOTROPHS		
	# COLONIES PATCHED	AUXOTROPHS FROM NTG	PERCENT
NTG	5853	22	0.37
EMS	1828	5	0.27
U.V.	1182	6	0.50
ACRIDINE ORANGE	6	0	0

* # = number of

From the table it can be seen that ultraviolet light was the best mutagen, producing the highest percentage of auxotrophs. However subsequent rounds of mutagenesis using ultraviolet light as a mutagen did not yield very many colonies and the reason for this problem was not found.

Nitrosoguanidine (NTG) produced the second highest number of auxotrophs, with EMS a close third. However the NTG mutagen was used in subsequent rounds of mutagenesis.

No comment can be made on acridine orange as a mutagen. See 3.6.4

3.6.1 N-METHYL-N'-NITRO-N-NITROSOGUANIDINE (NTG)

In order to determine the optimum time of exposure of the cells to NTG, a culture of 14887-1 was grown up in T2 and was subjected to NTG for 30 minutes, 1 hour, 2 hours and 3 hours.

The number of auxotrophs per mutagenesis was recorded.

TABLE 14: OPTIMUM TIME OF EXPOSURE OF 14887-1 TO NTG

TIME OF EXPOSURE	PERCENTAGE AUXOTROPHS		
	NUMBER COLONIES PATCHED	NUMBER OF AUXOTROPHS	PERCENT
#30 minutes	164	0	0
1 hour	406	3	0.73
2 hour	305	3	0.98
3 hour	267	0	0

The optimum time of exposure of 14887-1 to NTG was between 1 and 2 hours. Exposure for 30 minutes and 3 hours was unsuitable.

A comparison between mutagenesis of exponentially growing cells and stationary phase cultures was made. The growth phase was determined by observation only, and the efficiency of mutagenesis assayed by the production of auxotrophs.

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