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

Downing, Katrina Jo

2015-01-19
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and samples of the DNA were as digested with each dilution for 3 hours at 37°C. The aliquots were run on a 0.4% agarose gel, the results of which can be seen in figu e 8. 0.0009 units Bbl II/µl.DNA. dilution did not significantly restrict the DNA. The other three digestions partially digested the DNA.

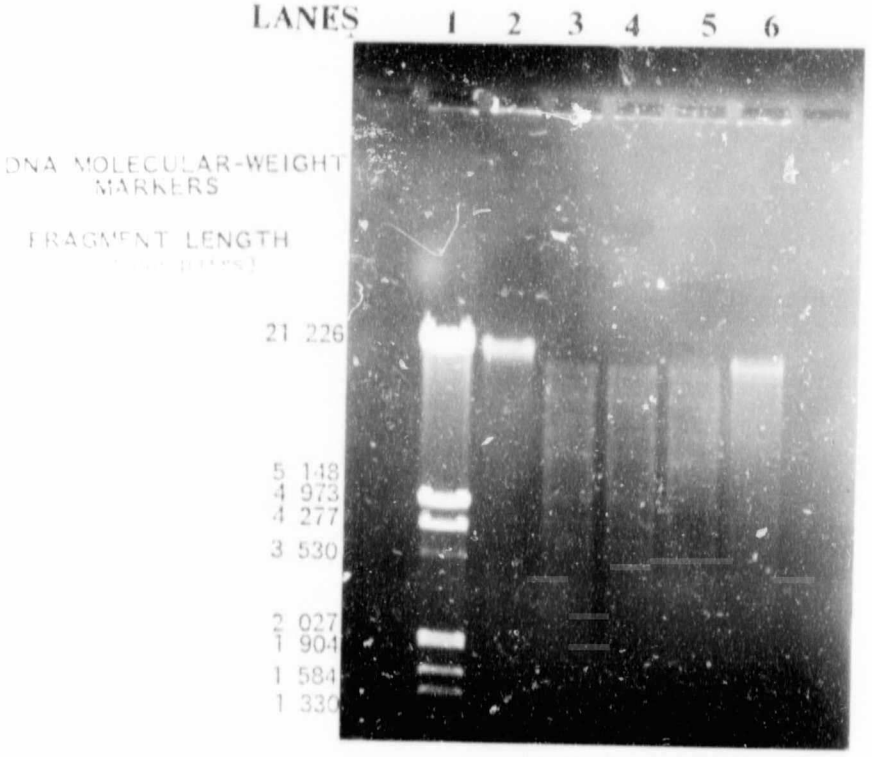


Figure 8. A 0.4% agarose gel illustrating digestion of Φ1 chromosomal DNA. Digestions were with 0.9, 0.09, 0.009 and 0.0009 units Bgl II µl DNA. A VIII molecular weight marker and an uncut aliquot of Φ1 DNA are evident in lanes 1 and 2 respectively.

3.12 CALCIUM CHLORIDE TRANSFORMATION

For the initial *E.coli* transformations the concentration of 01 chromosomal DNA and shuttle vector 1 DNA was 0.314µg/µl and 0.371µg/µl respectively. However for the majority of ligations the concentrations of the DNA were 0.2µg/µl and 0.108µg/µl.

Ligations using these latter concentrations were set up as in 2.2.11.3.

3.12.1 OPTIMIZATION OF TRANSFORMATION

Two colleagues, S.Andersen and H.Golob optimized conditions for calcium chloride transformation. The length of time that cells were left on ice in the absence of the DNA and the length of time of heat shock were varied. Samples were left on ice for 0,1,2 and 24 hours. The greatest number of transformants were obtained when samples were left on ice for 1 hour.. Samples of the cells and ligation mixture were subjected to heat for 0, 1, 2 and 3 minutes, with 1 minute exposure giving the best results.

3.12.2 DURATION OF 01 CHROMOSOMAL AND SHUTTLE VECTOR DNA IN LIGATION BUFFER

Four sets of ligation mixtures and controls were prepared as in 2.2.11.3. The amount of chromosomal DNA was 8x that of the vector. These sets were treated as follows:

TABLE 22: DURATION OF DNA IN LIGATION BUFFER WITH AND WITHOUT LIGASE AT 14°C

LIGATION MIXTURE	T4 LIGASE	INCUBATION AT 14°C	NUMBER TRANSFORMANTS
1	-	+/- 18 hours	128
2	-	+/- 42 hours	6
3	+	+/- 18 hours	82
4	+	+/- 42 hours	16

Sets 1 and 2 (above) were prepared in order to allow hydrogen bonding between DNA molecules to occur, prior to the addition of ligase. The number of transformants obtained from prolonged incubation with and without T4 ligase was compared. There was a 22% increase in the number of transformants when the DNA was left in ligation buffer overnight (approximately 18 hours) prior to the addition of T4 ligase and a 45% increase if T4 ligase was added for approximately 42 hours. Obviously longer

periods in ligation buffer without T4 ligase decreases the efficiency of transformation but prolonged time in the presence of T4 ligase increses the efficiency of transformation.

3.12.3 OPTIMUM AMOUNTS OF 01 CHROMOSOMAL DNA PER LIGATION

The optimum amount of 01 chromosomal DNA per ligation was determined. Ligation mixtures were prepared as shown in 2.2.11.3. The optimum quantity of 01 DNA was 16x the amount of shuttle vector DNA.

3.12.4 DETERMINATION OF HETEROGENEITY OF INSERTS OF 01 CHROMOSOMAL DNA IN THE E.COLI LIBRARY

A plasmid screen (see 2.2.9.4) was carried out on 12 *E.coli* transformants. The DNA pellet was dried and resuspended in 12µl of boiled TE + RNase. The RNAse was boiled to remove any DNase present. The samples were loaded on a 0.4% agarose gel.

Figure 9 shows the plasmid screen of 6 transformants, all of which appear to have inserts.

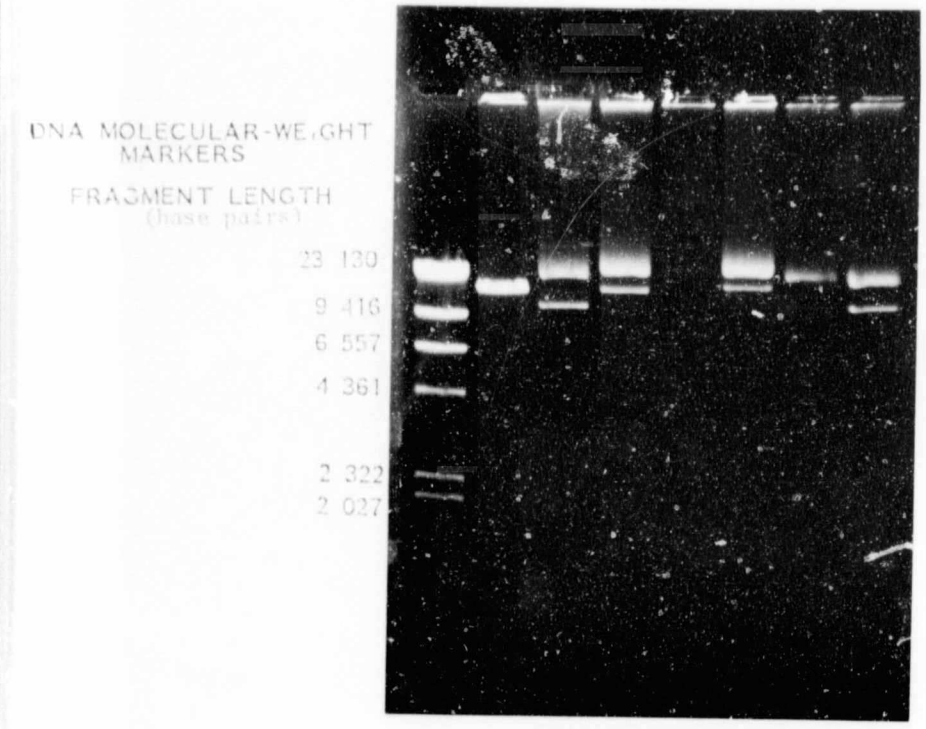


Figure 9. Plasmid screen of 6 *E.coli* transformants, (lanes 3-8).: Lanes 1 and 2 represent the λII molecular weight marker and uncut shuttle vector 1 respectively. (0.4% agarose gel).

The plasmid screen was repeated on each of these 12 transformants. The pellet was resuspended in 15µl of medium buffer + ribonuclease + the appropriate concentration of Bgl II.

Figures 10 and 11 show heterogeneity in the size of the inserts. Lane 1 of both figures represents a λII molecular weight marker. Lane 2 in Figure 10 represents Bgl II digested shuttle vector only.

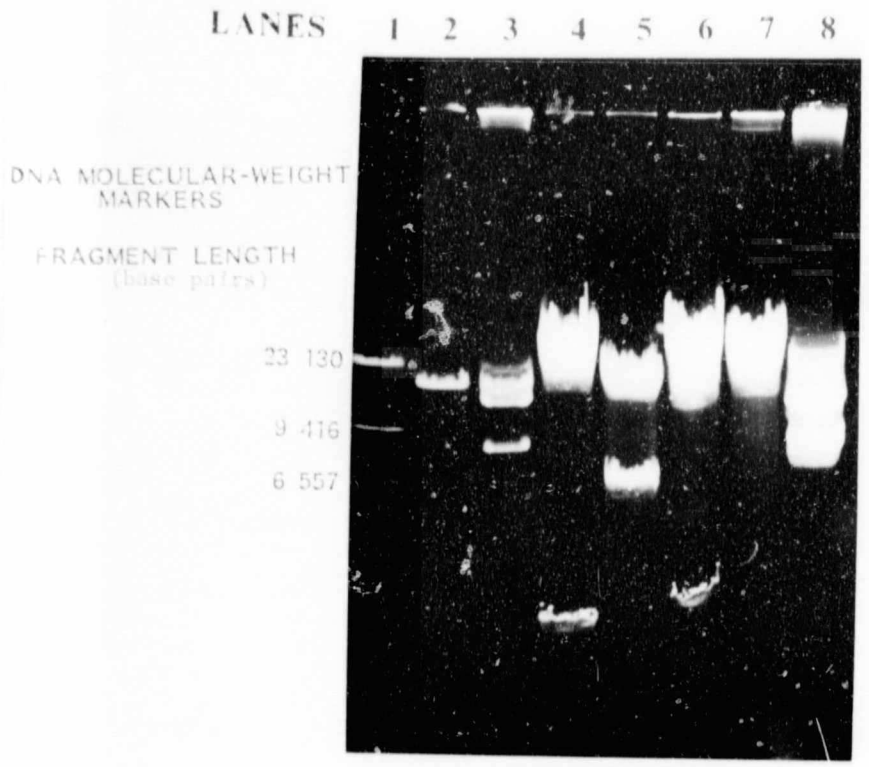


Figure 10. Plasmid screen of 6 Bgl II digested transformants showing heterogeneity in the size of the inserts. (0.4% agarose gel)

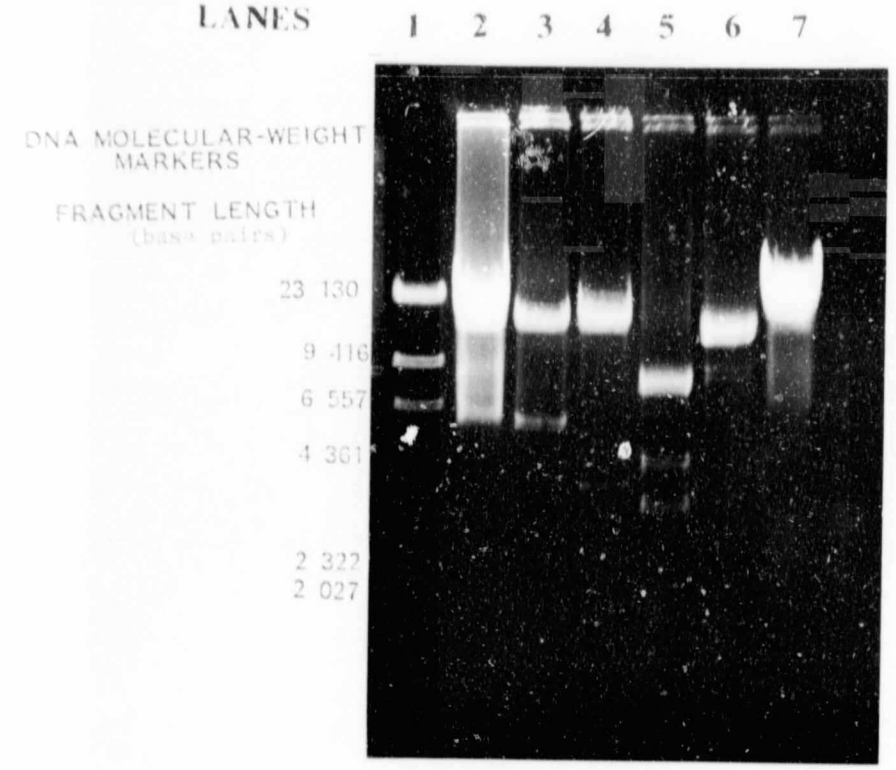


Figure 11. Plasmid screen of 6 additional Bgl II transformants showing heterogeneity in the size of inserts. (0.4% agarose gel).

The number of clones presently in the *E.coli* library is 1811. The average size of inserts was calculated to be 5.46 Kb.

From the plasmid screens performed on the 12 randomly chosen transformants, 11 had inserts.

3.13 NOCARDIOFORM TRANSFORMATION

3.13.1 RESTRICTION MINUS MUTANTS

The plasmid pDA[illegible] (construct of the shuttle vector + a pool of inserts) was used for nocardioform transformation in an attempt to find a restriction minus mutant.

A nocardioform transformation (2.2.13) using both pDA29 and pDA30 (a plasmid from 12674 or 01 with arsenic resistance)transformed into KD 1 gave the following results.

TABLE 23: NOCARDIOFORM TRANSFORMATION OF KD 1

KD 1	# TRANSFORMANTS	# TRANSFORMANTS PER µg DNA
0.64µg pDA30	9	14
4.4µg pDA29	0	0

This result suggested that *E.coli* DNA was restricted in the nocardioform whereas plasmid DNA from a 12674 or 01 background was restricted much less by KD 1. Although this latter result was favourable, it was decided to extract the pDA30 plasmid from the KD 1 background to be used in all subsequent nocardioform transformations using KD 1.

It was still necessary to find a restriction minus mutant before cloning into nocardioform bacteria could be attempted.

3.14 EXTRACTION OF PDA30 FROM KD 1 BACKGROUND

Three of the 9 nocardioform transformants were selected for a plasmid screen (see 2.2.9.5). All three of the nocardioform transformants pos-

sessed the pDA30 plasmid.(see figure 12). One of these three was chosen for the extraction of the pDA30 plasmid from a KD 1 background.

LANES 1 2 3 4

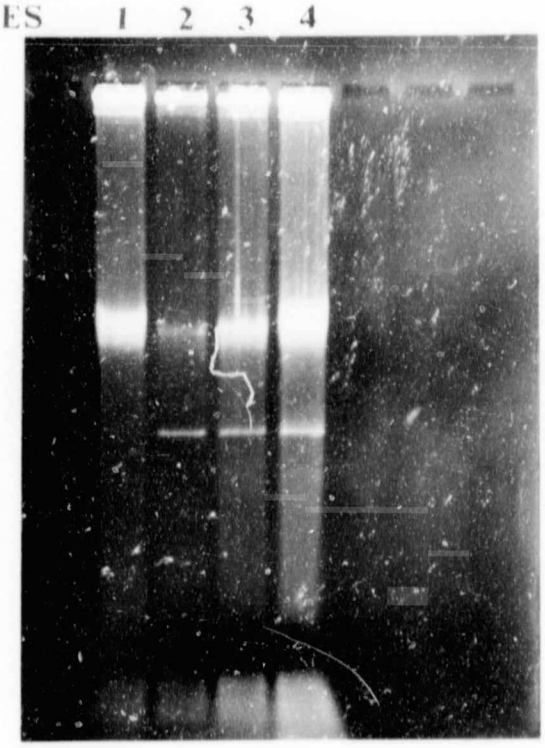


Figure 12. Plasmid screen of 3 of the 9 nocardioform transformants of pDA30 in KD 1.Lane 1 is a control with no plasmid.

3.15 OPTIMIZING CONDITIONS FOR NOCARDIOFORM TRANSFORMATIONS.

Transformation frequency in nocardioform bacteria was very low. It was essential to optimize conditions of the polyethyleneglycol transformation for each organism and/or mutant in order to obtain the maximum number of transformants.

Several variables in the nocardioform transformation were changed to optimize efficiency of transformation.

3.15.1 ARSENIC CONCENTRATIONS FOR SELECTION OF TRANSFORMANTS

The concentrations of 100mM sodium arsenate and 10mM sodium arsenite were used for selection of strain ATCC 12674 transformants (Dabbs and Sole 1987). However, 14887-1 and KD 1 were sensitive to sodium arsenate and arsenite at concentrations of 100mM and 40mM respectively. Hence a stock solution of 3M arsenate, 1M arsenite was used in all subsequent work with 14887 and derivatives.

3.15.2 GROWTH PHASE.

An experiment was carried out in order to determine what phase of growth of the culture yielded the best number of transformants. A culture of KD 1 was initiated in T2 as a preculture. Once it had reached stationary phase it was diluted 10^{-2} times and incubated with aeration at 26°C. After every 12 hours an aliquot was removed and a transformation carried out. In each transformation, 100ng of DNA was used. The results are shown in Table 24 and Figure 13.

TABLE 24 : OPTIMUM GROWTH PHASE OF KD 1 CULTURE GIVING HIGHEST NUMBERS OF TRANSFORMANTS.

ALIQUOTS HOURS	OD READINGS 540nm	NUMBER OF TRANSFORMANTS PER 100ng DNA	NUMBEROF TRANSFORMANTS PER µg DNA
18	0.44	71	710
26	1.34	518	5180
38	2.52	30	300
50	3.84	176	1760
62	4.10	44	440
74	4.0	19	190
86	3.87	0	0
98	3.02	0	0

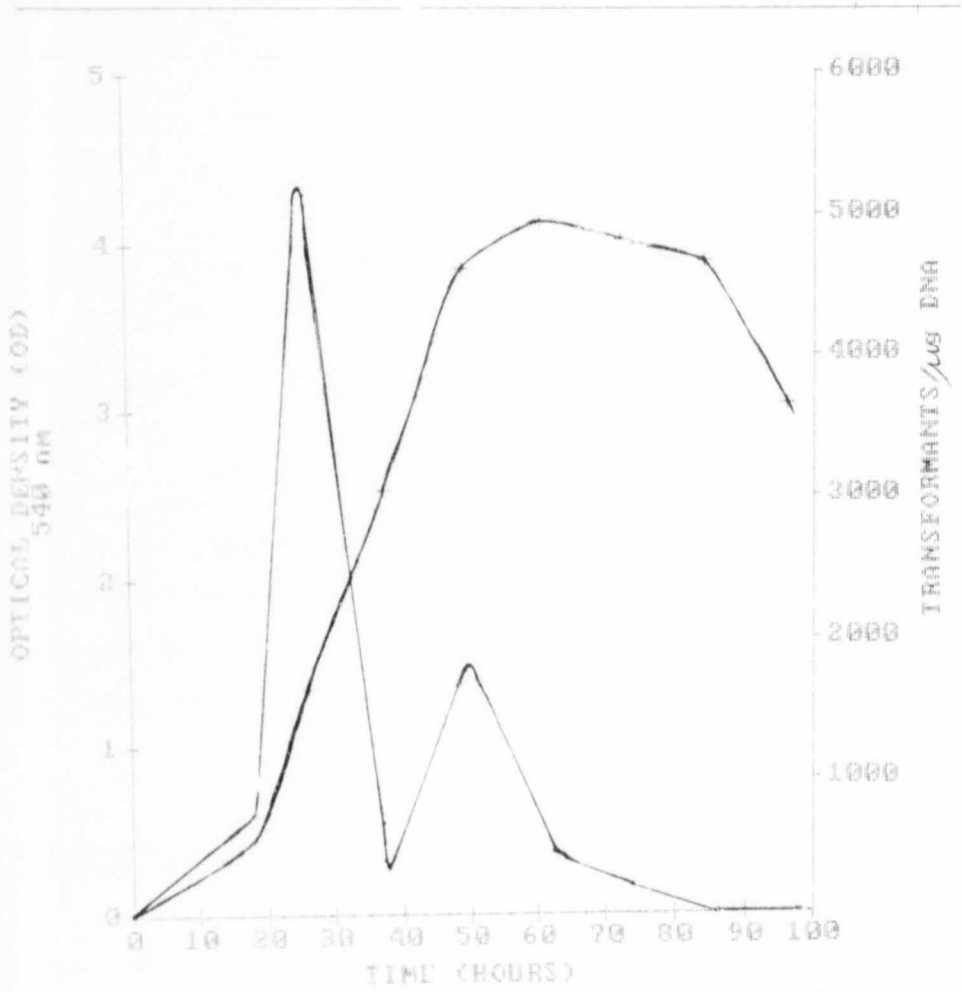


Figure 13. Graphical representation of the growth phase of KD 1 vs time and the number of transformants vs time.

Samples 2 and 4 in Table 24, with OD's of 1.34 and 3.84 respectively were combined and a series of transformations carried out. One of the following was varied with each transformation, the other conditions remained constant as in 2.2.13.1.

3.15.3 LENGTH OF INCUBATION BEFORE ARSENIC CHALLENGE.

This time of incubation was to allow phenotypic expression of the transformants before selection with arsenic solution.

The length of incubation was varied as follows:

0hr 6hr 12hr 18hr 24hr

3.15.4 LENGTH OF LYSOZYME INCUBATION.

The length of incubation of the cells in the prescence of lysozyme was varied as follows:

Lysozyme time: 30min 60min 120min

3.15.5 CONCENTRATION OF POLYETHYLENE GLYCOL (PEG).

The final concentration of PEG 6000 was varied as follows:

0% 10% 20% 25% 30%

3.15.6 THE AMOUNT OF CELLS.

The amount of cells was varied as follows:

25µl 50µl 100µl

3.15.7 THE CONCENTRATION OF DNA.

Keeping all other variables constant, the concentration of DNA was changed as follows:

1µl(20ng) 3µl(60ng) 7µl(140ng) 9µl(180ng)

TABLE 25 : OPTIMIZATION OF NOCARDIOFORM TRANSFORMATION.

VARIABLES	TRANSFORMANTS	
DNA		
1µl(20ng)	09	
3µl(60ng)	41	
5µl(100ng)	37	
7µl(140ng)	24	
9µl(180ng)	23	
	NUMBER OF TRANSFORMANTS per 100ng DNA	NUMBER OF TRANSFORMANTS per µg DNA
TIME OF UNDERLAY		
0	0	0
6	6	60
12	37	370
18	96	960
24	96	960
CELLS		
25µl	54	540

50µl	167	1670
75µl	37	370
100µl	208	2080
PPEG		
0%	0	0
10%	64	640
20%	86	860
25%	259	2590
30%	140	1400
LYSOZYME TIME		
0 min	0	0
30 min	0	0
60 min	204	2040
120 min	29	290

The transformants from each plate were patched onto TYA and TYA + 50mM arsenate , 100mM arsenite plates in order to check if they were true transformants. All the transformants tested were genuine as they were all able to grow on the TYA plates containing arsenate and arsenite.

From the results represented in Table 24:

i) The optimal time of exposure to lysozyme was 60 minutes.

ii) The DNA concentration was not a rate limiting step since the number of transformants did not vary widely.

iii) The optimum amount of cells appeared to be 100µl.

iv) The optimum length of incubation before arsenate/arsenite challenge had an effect on the number of transformants obtained. Incubation for less than 12 hours resulted in fewer transformants, and the optimal length of incubation appeared to be 18 or 24 hours.

v) The PPEG final concentration optimum is 25%.

It was necessary to repeat this experiment to obtain reproducible results.

3.16 SUMMARY OF RESULTS.

The *Rhodococcus* strains used in this work were capable of utilizing a variety of compounds, notably citrate, agar and sodium taurocholate as sole carbon source. Several mutants with reduced or no ability to utilize these carbon sources were obtained. One of the mutants, KD 1, was unable to use all carbon sources tested, including agar. This was therefore ideal for subsequent cloning of nocardioform DNA. Optimum conditions for both the nocardioform transformations and the *E coli* transformations were obtained.

The availability of a suitable cloning vector, pDA27 enabled the construction of an *E.coli* library containing inserts of 01 chromosomal DNA. This will be used in an alternative approach to cloning. The number of clones in the library was 1811. A further 1622 clones must be obtained in order to have a complete representation of the entire 01 chromosome.

Cloning of the nocardioform DNA into the mutant KD 1 was hindered by the lack of a restriction minus mutant. Obtaining this mutant is therefore a prerequisite for future work.

When the gene/s for interconversion of sodium taurocholate or cholesterol to pharmacologically important compounds have been cloned into KD 1, it will be of interest to analyze the resulting degradation products. This can be achieved by thin layer chromatography.

4.0 DISCUSSION

4.1 PHAGE TYPING

There are several characteristics to be considered when characterizing and identifying the nocardioform bacteria.

The cell wall composition has been discussed in the Introduction. Lipid analyses have been used in classification and identification. Nocardiae produce a family of lipid-soluble, iron-binding compounds called the nocobactins which can be divided into three distinguishable types that correspond to three *Nocardia* species. Small amounts of analogous compounds have been detected in certain species of *Rhodococcus* but not in the other species, thereby providing a means of identification between *Rhodococcus* species.

Pigmentation is not a stable character for identification of actinomycetes and pigments have to be chemically analyzed. There is some evidence that analyses of the chemical composition of chromosomal DNA could be of value in the classification of *Rhodococcus* species whereas *Nocardia* strains have a narrow G + C range.

One useful means of identifying nocardioform bacteria and specifically identifying species within a genus is phage typing. Nocardioform bacteria are sensitive to phage and although phages only attack bacteria of a given wall chemotype, a particular phage does not necessarily attack every strain with the same wall type. (Goodfellow and Minnikin 1977). For the

majority of the nocardioform species, phage have not yet been isolated. (Prauser 1976).

The phage K3 isolated from soil was specific for *Rhodococcus equi* ATCC 14887. Two of the remaining four phages isolated from a lawn of 14887 were able to infect 12674 and 4277 as well as 14887 but not A 448. This confirms that 12674 and 4277 are closely related to one another and that 14887 is also related to these strains whereas 448 is more distantly related.

Similiar work done by a colleague, B.Gowan, showed that 5 phages isolated on 4277 were able to infect 14887 as well.

A nocardioform phage R1 has been reported in the literature to infect a strain of *Rhodococcus equi* (Prauser 1976).

4.1.1 NUTRITION AND GROWTH OF NOCARDIOFORM BACTERIA

Members of the genus *Nocardia* and *Rhodococcus* are heteromorphic, aerobic organisms. However some *Nocardia* species are autotrophic and can grow in an atmosphere consisting of hydrogen, carbon dioxide and oxygen and in media which contain a nitrogen source with the necessary salts. However, none of the *Rhodococcus* species used in this work were autotrophic.

A great number of organic substances can be used as carbon and carbon + nitrogen sources.

The nocardioform bacteria are able to utilize alkanes which serve as good carbon sources. These include paraffin and pesticides such as DDT. They are also able to use phenolic compounds and acrylamide. (cited in Dabbs 1987). The degradation of these toxic compounds is of industrial and agricultural importance.

Glucose can be used as sole carbon source by *Nocardia* and *Rhodococcus* strains. Ferreira and Tracey (1984) found that *R .erythropolis*, *R. erythropertinctus*, *R.australis* and *R. equi* were able to use glucose as sole carbon source with a frequency of 100%. A similiar result was obtained with fructose. However glucose was the carbohydrate of choice for this work.

Other carbohydrates such as sucrose, galactose, maltose and L-arabinose can be used in different degrees by the *Rhodococcus* species. (Ferreira and Tracey 1984).

Inorganic compounds such as NH_4^+, NO_2^- and NO_3^- can be utilized as sole nitrogen source. (Tarnok 1976). The minimal media used in this work contained NH_4Cl as a nitrogen source.

Some nocardioform bacteria require vitamins and additional growth factors. *Rhodococcus erythropolis* ATCC 12674 required vitamin B1 (Thiamin) and glutamate for growth. The organism 14887 on the other hand did not require these and was therefore the organism of choice for carbon source work.

The generation time for the nocardioform bacteria could be between 3 and 7 hours under optimum growth conditions for the majority of the

Nocardia. *Rhodococcus equi* 14887 was a much slower grower in both minimal media and TY or T2 than 12674 and 4277. However 25593 was an even slower grower than 14887. This may again be an indication of relatedness between these species.

Some *Nocardia* are able to grow at extreme temperatures such as 10°C or 50°C. The best temperature range is reported to be between 27° and 37°C. The *Rhodococcus* species were grown at a temperature of 26°C. The pH of the minimal media was pH 7.9 which is in the pH range suggested in the literature. (cited in Tarnok 1976).

4.2 CARBON SOURCES

As mentioned previously the nocardioform bacteria can use numerous organic substances. Table 8 (see results) lists the carbon sources utilized by 14887.

4.2.1 AGAR

It was shown that both 14887 and 4277 could utilize agar as a carbon source, although not as efficiently as they could use glucose. A close relative of the nocardioform bacteria, *Streptomyces coelicolor* A3(2) can also utilize agar as a carbon source. (cited in Buttner *et al* 1987). An extracellular agarase is produced in this organism which hydrolyses the glycosidic bonds linking the alternating units of (1→3)β-D-galactopyranoside and (1→4)3,6,anhydro-α-L-galactoside which constitute the agar polymer. (cited in Kendall and Cullum 1984). The gene coding for this enzyme is known as the agarase (dag A) gene and is expressed subject to catabolite repression by glucose and several other sugars. (Buttner *et al* 1987). The coding and regulatory regions of this gene have been sequenced. (Buttner *et al*)

It is therefore highly probable that the *Rhodococcus* species possess an agarase gene similiar to that of *S.coelicolor* enabling them to utilize agar as a carbon source.

4.2.2 CITRATE

Both the organisms 4277 and 14887 were able to utilize citrate, present in minimal media, as a carbon source. This result is in agreement with those obtained by Ferreira and Tracey, 1984 who showed that *Rhodococcus*

Author Downing Katrina Jo
Name of thesis Towards Molecular Biological Characterisation Of The Genes For Sterol And Bile Acid Metabolism In Nocardioform Bacteria. 1989

PUBLISHER:
University of the Witwatersrand, Johannesburg
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