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

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erythropolis and *Rhodococcus equi* were capable of utilizing 0.1% w/v citric acid as sole carbon source at a frequency of 100%. *Rhodococcus rubropertinctus* could utilize it at a frequency of 92% whereas *R. australis* was unable to utilize citrate as sole carbon source.
It was therefore necessary to use A - N stock without citrate for making up minimal media. This is in contrast to *E.coli* for which A - N stock was developed.

Cholesterol and the other sterols, stigmasterol and β-sitosterol were very poorly soluble in water. Cholesterol has a maximum solubility in aqueous solutions of 1.8 µg/ml and undergoes thermodynamically reversible monomer micelle equilibrium. (Haberland and Reynolds 1973). Several approaches to solubilizing these sterols have been suggested in the literature.

4.2.3 TWEEN

Tween is a non ionic detergent and a surface active agent. It has been used by several researchers in an attempt to solubilize sterols by trying to avoid problems of aggregation. Wovcha *et al* (1978) included 0.1% Tween 80 in a culture of a *Mycobacterium fortuitum* containing β-sitosterol. Nakamatsu *et al* (1983) added 0.01% Tween 80 to a culture of a mutant *Nocardia corallina* strain in order to solubilize cholesterol (below 7%). These authors found that Tween 80 had a marked stimulative effect on the

conversion rate of the sterol to the intermediate HIL. They believed that Tween stimulated sterol degradation not by improving the substrate suspension but by providing a fatty acid component for biosynthesis of surface active cell wall lipids. (Nakamatsu *et al* 1983). Ferreira *et al* (1984) prepared sterol suspensions in a 2% Tween 80 solution and the sterol and Tween were added to a final concentration of 2g/l respectively. This was used for the production of HIL from cholesterol by a mutant strain of *Rhodococcus australis*. Ferreira and Tracey (1984) showed that *Rhodococcus australis* was able to hydrolyse both Tween 20 and Tween 80 very efficiently. *Rhodococcus coprophilus* used these less efficiently. Tarnok (1976) reported that Tweens 20, 40, 60, and 80 were hydrolyzed by certain *Nocardia* and *Rhodococcus* species.

Three mutants I isolated in this work were unable to utilize Tween 80 as sole carbon source.

4.2.4 ETHANOL

Cholesterol was reported to be slightly soluble in alcohol but more soluble in hot alcohol. (Merck Index Tenth edition). It is also soluble in benzene. Solubilizing cholesterol in cold and hot alcohol was not successful and when tested as a carbon source, proved to be very suitable for 14887 and 4277.

According to Ferreira and Tracey (1984) *Rhodococcus erythropolis*, *rubropertinctus*, *australis* and *equi* can all use butanol, octanol, propanol and amylalcohol very efficiently as carbon sources.
The results obtained with the growth of 14887 and 4277 in the presence of ethanol as sole carbon source are in agreement with this report.

4.2.5 DMSO

DMSO has been used to dissolve sterols in order to obtain finely distributed substrate suspensions. (Martin 1984). DMSO at a concentration of 1% was not used as a carbon source by 14887 which was a very promising result. However growth in the presence of cholesterol and DMSO was very poor.

4.2.6 SONICATION OF STEROLS

This means of solubilizing sterols gave the best results. Nakamatsu *et al* 1983 and Watanabe *et al* 1987 both used homogenization of cholesterol by sonication for the microbial degradation of the sterol to the intermediates cholesten-3-one, ADD and HIL.

4.2.7 SODIUM BENZOATE AND HYDROXYBENZOATE

Ferreira and Tracey (1984) have shown that *Rhodococcus equi* can utilize 2,5 - dihydroxybenzoic acid very efficiently as a carbon source whereas benzoic acid is not utilized as well. The results obtained in this work showed that 14887 was able to utilize hydroxybenzoate at a concentration of 0.01% efficiently whereas very little growth occurred in the presence of sodium benzoate at the same concentration.

4.3 PRODUCTION OF MUTANTS

Mutants were produced by NTG at a much lower frequency in nocardioform bacteria than in *Escherichia coli* where 60 -80% of colonies, after heavy NTG mutagenesis, can be auxotrophs. (E. Dabbs personal communication). Conditions of NTG mutagenesis for the *Rhodococcus* species were optimized. Optimization of conditions specifically for NTG and EMS mutagenesis of 14887 was also carried out. Auxotrophs were produced in this organism at a frequency of 0.37%. This was lower than the frequency obtained for 4277

which was 1.8 - 2.5%. (B.Gowan). The frequency of auxotrophs was 1 - 2% for 12674. (S. Andersen).

Ultraviolet light was more effective as a mutagen than NTG and EMS, producing the highest percentage of auxotrophs. However the most suitable mutant arising from my work, KD 1 was produced by NTG mutagenesis. This mutant was unable to utilize several of the carbon sources mentioned above, notably sodium taurocholate and agar.

The enrichment procedure for production of mutants was a modification of the penicillin selection technique of *E.coli*. This procedure resulted in approximately double the number of auxotrophs produced by NTG mutagenesis alone in 14887. It was therefore a superior method of obtaining 14887 auxotrophs. A greater number of mutants with reduced ability to utilize sterols, sodium taurocholate and other carbon sources also resulted from this procedure compared with the non enrichment mutageneses.

4.4 ACTION OF MUTAGENS

NTG acts by alkylation meaning an alkyl group is added to N-7 of the purine ring (either guanine or adenine). This alkylation weakens the N-glycosylic bond which is hydrolyzed resulting in depurination. (Freifelder 1983). Bacteria carrying an NTG induced mutation often have additional mutations in the same gene and in other genes. This is possibly true for KD 1 which was unable to utilize several carbon sources. The

mutations are clustered in genes that are adjacent in the genetic map and the explanation for this phenomenon is that NTG exerts its effect in a replication fork. Therefore mutations are clustered in the region of the DNA that was traversed by the replication fork during exposure to the mutagen. (Freifelder 1983).

EMS is also an alkylating agent which causes transversions. This is a substitution of a purine for a pyrimidine or vice versa.

Ultraviolet irradiation causes thymine dimers. These cause inhibition of the advance of the replication fork.

Acridine orange and ethidium bromide form stacked arrays in aqueous solution and are able to stack with a base pair. This is done by insertion between two base pairs, known as intercalation. The intercalation causes adjacent base pairs to move apart resulting in frameshift mutations.

4.5 MICROBIAL TRANSFORMATION OF STEROLS AND BILE ACIDS

The pathway of microbial degradation of cholesterol is as follows: Cholesterol→cholest-4-en-3-one→cholesta-1,4-dien-3-one→ 3 oxobisnorchola-1,4-dien-22-oic acid→androsta-1,4-diene-3,17-dione (ADD)→3-hydroxy-9,10-secoandrosta-1,3,5(10)-tricne-9,17-dione→3aα-H-4α -[3'-propionic acid]-7aβ-methylhexahydro-1,5,indanedione (HIL) →5-methyl-4-oxooctane-1,8-dioic acid→succinic acid→CO_2 H_2O. (Nakamatsu *et al* 1980).

The pathway of microbial degradation of bile acids to CO_2 and water is not known although studies on the metabolism of the bile acids by microorganisms has been carried out.

A variety of metabolites of these compounds have been isolated and identified, which would enable researchers to predict possible pathways for bile acid degradation.

Research on microbial degradation of steroids has demonstrated that several species of microorganisms are able to degrade some steroidal compounds to a common intermediate, HIL from ADD (see above). It is possible that bile acids such as cholic acid are degraded to this intermediate via ADD. (Hayakawa 1973). A hypothesis as to why the mutant KD 1 was unable to utilize cholesterol, stigmasterol, β-sitosterol and sodium taurocholate based on this information could be made. It is possible that the gene/s coding for the enzyme necessary for the microbial reaction responsible for the degradation of ADD to HIL was altered during mutagenesis.

It does not however, explain why KD 1 was unable to utilize the other carbon sources excluding glucose. It is possible that the gene sequence/s for some enzyme/s common to the degradation pathways of all these carbon sources was altered due to the action of NTG.

4.6 OPTIMIZATION OF POLYETHYLENEGLYCOL TRANSFORMATION

Optimization of the efficiency of nocardioform transformation was based in part on work done by Bibb *et al* 1978 on streptomycetes.

The growth phase which gave the optimum number of transformants for KD 1 was log phase. This differed from 01 and 4277 whose optimum phases were post exponential / pre stationary and post stationary phase respectively.

The DNA concentration of pDA30 was apparently not a limiting factor on the frequency of transformants of KD 1.

The length of incubation before arsenic challenge was 12 hours for 01 and 4277 whereas 18 - 24 hours was the optimum for KD 1.

The optimum PEG 6000 concentration appeared to be 25% for KD 1 and 01 and 30% for 4277. This is probably not a significant difference. Biphasic growth of transformants was observed with 01 when PEG concentrations were varied. Initial growth of 01 exposed to 10% PEG on regeneration plates gave the best number of transformants/µg DNA. However, with continued incubation at 26°C, smaller colonies grew up on plates of cultures exposed to higher concentrations of PEG. (A.Daffey).

The optimum time of exposure to lysozyme was 60 minutes for all three organisms.

These results from nocardioform transformations of 01 and 4277 were obtained from A.Daffey and B.Gowan respectively. It is evident that each of these nocardioform bacteria and their derivatives had specific optimal conditions for transformation.

4.7 CONSTRUCTION OF THE E.COLI LIBRARY

The estimated genome size of mycobacteria is 3 x 10^9 daltons. (Pao *et al* 1988). This is equivalent to 6,3 x 10^6 bp. The average insert size in the library was 5,46 Kb. Therefore in order to cover the entire chromosome of 01, a total of 3433 clones would be necessary. The number of clones presently in the library is 1811 of which 92% have inserts. Once the library was complete, plasmid DNA would be extracted from this bulk pool and used to transform into KD 1. Complementation of the mutation would be sought. Before this approach to cloning could be attempted, a restriction minus mutant would have to be obtained to prevent the restriction of the *E.coli* DNA by the nocardioform bacterium.

5.0 CONCLUSION

Although the cloning of the gene/s for the interconversion of sterols to compounds of pharmacological interest was unsuccessful, several essential steps towards this aim were achieved. Mutagenesis resulted in the production of a number of mutants, notably KD 1, which had reduced ability to utilize several carbon resources. Conditions of nocardioform transformation for this mutant were optimized, which was essential for the continuation of this work. The phage ϕ 3 specific for 14887 , was isolated and successfully used in the identification of 14887 derivatives, specifically the faster growing derivative KD 2.

An alternative approach to cloning was considered. To this aim, an *E.coli* library containing 1811 clones was constructed. These clones contained 01 chromosomal DNA. A future objective would be to obtain the remaining clones to complete the library covering the 01 chromosome. Successful cloning into the nocardioform KD 1 would require a restriction minus mutant. The generation of a recombination minus mutant would also be advantageous. These must therefore be obtained in order to pursue this work. Once the gene/s for either sterol or sodium taurocholate interconversion have been cloned, the degradation products of the recombinants will be analyzed. This will be achieved via thin layer chromatography. It is hoped that these intermediates will be of pharmacological importance.

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