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

Downing, Katrina Jo

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HOT ETHANOL

A 20% suspension of cholesterol in ethanol was made and heated gently over a flame.

DMSO

A 1% solution of DMSO was prepared in sterile water to which 0.1% cholesterol was added.

SOLUBILIZING CHOLESTEROL BY SONICATION

An aqueous suspension of cholesterol was prepared and homogenized by sonication for approximately 1 minute.

DISPERSAL OF β -SITOSTEROL AND STIGMASTEROL

Aqueous solutions of 0.1% of each of these phytosterols were homogenized by sonication for approximately 1 minute.

2.2.4.2 PREPARATION OF SODIUM BENZOATE AND HYDROXYBENZOATE

Both of these were prepared as 10% stocks in sterile water. They were both autoclaved before use.

2.2.4.3 SODIUM TAUROCHOLATE

This was soluble in water. A 10% stock was prepared with sterile water and was autoclaved before use. The final concentration of sodium taurocholate used in this work was 0.02%.

2.2.5 LIQUID CULTURES

All glass flasks used for liquid cultures of nocardioform bacteria were filled with distilled water and autoclaved prior to use, in order to remove any detergent remaining from the washing procedure. Liquid cultures were set up for testing the suitability of each of the carbon sources mentioned in 2.1.8. These cultures consisted of 5ml of minimal media A

without agar and glucose. The desired concentration of the appropriate carbon source was added to the cultures together with a loopful of the organism of interest. Rifampicin was added to a final concentration of 20µg/ml in order to eliminate contaminant growth. These cultures were incubated at 26°C on a shaker until growth was observed.

2.2.6 PREPARATION OF MINIMAL MEDIA PLATES

Minimal media was prepared as described in 2.1.2.2 with the exclusion of glucose. The desired concentration of the appropriate carbon source was added to a sterile glass test tube into which freshly autoclaved minimal media was poured. The contents of the tube were mixed and transferred into a sterile plastic petri dish. Plates were allowed to harden and were dried overnight at 37°C. Each petri dish contained approximately 22ml of media.

2.2.7 ISOLATION OF PHAGE TO BE USED FOR TYPING

A quantity of soil was collected from the Johannesburg area. From this, 1g was added to 20ml of T2 and was incubated at 26°C on a shaker for 6

hours. After this time the soil was centrifuged at 15 000 rpm for 15 minutes. The supernatant was removed and filter sterilized.

The organism 14887 was incubated overnight in 5ml of T2 at 26°C. When this had grown up, 1ml of the overnight culture was spun down in an eppendorf tube and resuspended in 1ml of T2.

The following were added to sterile glass tubes:

+ Tube	- Tube
1ml T2	2ml T2.
20µl 14887	20µl 14887
1ml soil supernatant	

To each tube 2ml of top agar was added, the contents mixed and then poured onto TYMC plates (2.1.2.7). After growth at 26°C, the plates were checked to determine the number of plaques. Each plaque was removed from the TYMC plate with a sterile flat edged toothpick and placed in 100µl of T2, vortexed briefly then shaken at 26°C for 1 hour in order to release the phage. After shaking, the eppendorf tubes were centrifuged for 1 minute and the supernatant transferred to a new sterile eppendorf tube.

A lawn of cells of each of 4 organisms, 14887, 4277, 12674 and 448 were grown up on TYMC plates. They were prepared as follows:

1ml of T2 + 20µl organism + 2ml top agar poured onto TYMC plates and incubated at 26°C.

Once the lawn had grown up, each of the plaques prepared above were spotted onto the top agar in 10µl aliquots. The plates were incubated at 26°C and observed for clearing in the lawn.

In order to determine the divalent ion requirement of each of the resulting phages, an aliquot of each was spotted onto a lawn of 14887, prepared as above, on TYA, TYMC, TYC and TYM plates (2.1.2.3 -2.1.2.8).

The titre of a phage specific for 14887, namely K3 (See Results Table 10) was increased. The K3 phage was removed from the TYC plate with a sterile flat edged toothpick and placed into 0.5ml TYC. This was vortexed briefly and shak at 26°C for 1 hour to release the phage from the agar. The phage was centrifuged for 1 minute and the lysate placed in 2ml TYC together with 20µl of 14887 in a glass tube. Top agar was added and the contents of the tube poured as a top layer onto a TYC plate. After 1 days growth at 26°C the top agar was allowed to melt at 42°C, collected, centrifuged at 12 000 rpm for 10 minutes and the phage lysate recovered. The procedure was repeated and the resulting phage lysate filter sterilized and kept at 4°C for the identification of 14887.

Controls without phage lysates were also prepared.

2.2.8 MUTAGENESIS

2.2.8.1 MUTAGENESIS USING NTG AND EMS AS MUTAGENS

The rifampicin derivative of 14887 (14887-1) was grown up in 5ml of T2 until it reached log phase, which was determined by observation only. The mutagens NTG and EMS and the respective buffers were made up as described in 2.1.4.5 and 2.1.9.

A 1ml aliquot of the culture was centrifuged for a few seconds. The cells were washed in the respective buffers, namely 1ml of the Tris HCl pH 8 buffer for cells to be mutagenized with NTG and 1ml of the Tris HCl pH 7 buffer for the cells to be mutagenized with EMS.

The washed cells were centrifuged again. If NTG was used as a mutagen, the pelleted cells were resuspended in 0.9ml buffer + 0.1ml NTG, hence a final concentration of 100µg/ml.

If EMS was used as a mutagen, the pelleted cells were resuspended in 1ml of 1% EMS (or 2% EMS). The resuspended cells were placed at 37°C for 2 hours, after which the cells were centrifuged briefly for 10 seconds, washed with sterile water and recentrifuged. The supernatant was discarded and the pellet resuspended in 100µl of T2. A volume of 5ml of T2 was added to a sterile flask treated as described in 2.2.5, to which the 100µl of T2 was added. Rifampicin to a final concentration of 20µg/ml was also added to the flask to prevent contaminant growth. The cultures were allowed to "outgrow" at 26°C with agitation. Once the culture was turbid an aliquot was sonicated for 5 seconds to reduce clumping. This sonicated

culture was diluted in T2 to 10^{-6} and 100µl of this dilution was plated onto TYA plates. The plates were incubated at 26°C.

PATCHING

When single colonies had grown up on the TYA plates, these were "patched" onto a series of plates using sterile flat edged toothpicks. A total of 112 colonies per plate were patched. The plates used to assay for mutants were, in order of patching:

1) A minimal media plate with no additional carbon source, in order to detect agarose$^-$ mutants;

2) A minimal media plate with the addition of the appropriate carbon source to detect mutants unable to utilize the carbon source;

3) A minimal media plate with glucose to detect auxotroph production which was a means of assaying the mutagenesis

4) A TYA plate as a control.

SPOT TESTS OR REPLICA PLATING

Possible mutants which exhibited poor growth on the plates described in 2.2.8.1.1 were streaked to single colonies on TYA. A single colony representing the candidate mutant was mixed in a drop of sterile water in the female part of a replicator and was spotted onto the appropriate plates.

A TYA, minimal media + glucose and a minimal media with no additional carbon source were always included in each spot test.

Plates were incubated at 26°C and observed for spots with little or no growth.

2.2.8.2 ULTRAVIOLET LIGHT MUTAGENESIS

An overnight culture of 14887-1 prepared as in 2.2.8.1 was sonicated for 5 seconds to reduce clumping. This sonicated culture was diluted in sterile H_2O to 10^{-4}. Drops of 150µl of the 10^{-4} dilution were placed in a sterile petri dish and exposed ultraviolet light for 10 minutes, after which 100µl of each drop was plated onto TYA and rifampicin (15µg/ml) plates. A control TYA Rifampicin plate of 100µl of the unexposed 10^{-4} was set up.

The plates were placed in a light tight container and incubated at 26°C. When single colonies grew up they were patched as described previously and any candidate mutants were checked via the spot test described above.

2.2.8.3 MUTAGENESIS USING ACRIDINE ORANGE AS A MUTAGEN

A stock solution of acridine orange was prepared as described in 2.1.9.3. A liquid culture of 5ml of T2 containing 5µl of a stationary phase culture

of 14887-1 was set up to which acridine orange was added. Several different concentrations of acridine orange were used namely 1, 2, 3, 5, 10 and 20µg/ml. A control culture containing no mutagen was also included. The cultures were incubated at 26°C with agitation.

2.2.8.4 MUTAGENESIS USING ETHIDIUM BROMIDE AS A MUTAGEN

A liquid culture was set up as in 2.2.8.3 except ethidium bromide was added using a stock solution described in 2.1.9.4. The concentrations of ethidium bromide used were 10, 20 and 30µg/ml. A control culture without ethidium bromide was included. The cultures were incubated at 26°C with shaking.

2.2.8.5 THE ENRICHMENT PROCEDURE FOR OBTAINING MUTANTS

This procedure was based on that used for obtaining auxotrophs in *Escherichia coli* which is known as the penicillin selection or auxotroph enrichment procedure. A modification of this procedure was used to select for auxotrophs and sodium taurocholate mutants.

OBTAINING AUXOTROPHS BY ENRICHMENT

A culture of 14887-1 grown up in T2 was mutagenized using NTG as described (2.2.8.1).

The culture, after mutagenesis was outgrown in T2. In order to obtain auxotrophs, 1ml of the culture was washed twice with sterile distilled water and 0.1ml of this added to 5ml of minimal media containing glucose and rifampicin to a final concentration of 20µg/ml. Rifampicin was added to prevent contaminant growth. This culture was allowed to grow at 25°C with agitation for 24 hours. Ampicillin to a final concentration of 60µg/ml was added to the culture which was incubated at the same temperature for a further 24 hours. An aliquot of the culture was centrifuged, washed with sterile water, diluted in water and 100µl of the appropriate dilution plated on TYA plates. The single colonies which grew up were patched onto TYA and minimal media + glucose plates. This was the equivalent of one round of enrichment which was carried out in order to determine the number of auxotrophs generated by this procedure compared to the numbers generated by the NTG mutagenesis alone described in 2.2.8.1.

NTG mutagenesis was carried out simultaneously with the enrichment procedure so that a valid comparison could be made.

SELECTING SODIUM TAUROCHOLATE MUTANTS BY ENRICHMENT

A culture of 14887-1 grown up in T2 was mutagenized as described in 2.2.8.5.1. The culture was outgrown and 1ml of it was washed twice in sterile distilled H_2O. A liquid culture of 5ml of minimal media without

glucose but with the addition of the optimal concentration of sodium taurocholate was set up to which 100µl of the washed culture was added. Rifampicin to a final concentration of (20µg/ml) was also added for the same reason as in 2.2.8.5. The culture was incubated at 26°C on a shaker overnight. The following day, the culture was centrifuged and the pellet washed with sterile water. The cells were added to a liquid culture of minimal media containing glucose and rifampicin to a final concentration of (20µg/ml). The culture was incubated at 26°C and once it was turbid, the procedure was repeated twice more. After the third round of enrichment the culture was washed, resuspended, diluted to single colonies, and plated onto TYA plates. The resulting single colonies were patched onto the series of plates described previously.

2.2.9 EXTRACTION OF DNA

2.2.9.1 BULK E.coli PLASMID PREPARATION (Clewell and Helinski, 1969)

A 5ml LB + Amp preculture of *E.coli* λMM294 was grown overnight at 37°C and used to inoculate a sterile flask of 250ml LB + ampicillin to a final concentration of (50µg/ml). The flask was incubated in a 37°C waterbath with agitation. When the culture had grown up the cells were pelleted at

6000rpm for 10 minutes. The supernatant was poured off and the pellet resuspended and washed in 10ml of cold TE. The cells were pelleted again by centrifuging at 6000rpm for 10 minutes. They were resuspended in 2ml cold 25% sucrose, 50mM Tris HCl pH8.0. A lysozyme solution was made up by dissolving lysozyme (10mg/ml) in 10mM Tris Hcl pH8.0. of which 0.25 ml was added to the cells. This was swirled gently on ice for 15 minutes after which 0.25ml of 0.5M EDTA pH8.0 was added. The material was swirled gently for a further 5 minutes on ice. To promote lysis, 2.5ml of cold detergent solution (2.1.4.5) was added and the material swirled gently on ice for 10 minutes until partial clearing occurred, at which stage the material was expected to be highly viscous. A 45 minute centrifugation was performed at 18 000rpm. The supernatant was decanted off, the volume measured and 0.95g of CsCl per ml of supernatant added. A short clearing spin was done at 10 000rpm for 10 minutes to clear lipid after the CsCl addition. The cleared lysate was recovered and 0.1ml of ethidium bromide (10mg/ml) per ml was added. The refractive index was measured and adjusted to 1.392.

The material was loaded into Quickseal ultracentrifuge tubes, balanced in pairs and sealed. The tubes were centrifuged for approximately 16 hours at 40 000rpm in a VTi 65.2 vertical rotor. The plasmid band was extracted from the tube. This was reloaded with cesium chloride (1g/ml) and a second centrifugation was performed in order to remove residual chromosomal DNA. The plasmid band was extracted and decolourized by the addition of approximately the same volume of TE saturated butanol. This was mixed and centrifuged for 2-3 minutes. The top layer containing the ethidium bromide was removed. The procedure was repeated until all the colour had been removed. The DNA was dialyzed against TE for approximately 4 hours and

then transferred to eppendorf tubes. The concentration of the DNA was determined by spectrophotometric readings at 260n..

2.2.9.2 BULK NOCARDIOFORM CHROMOSOMAL DNA PREPARTION

The nocardioform organism 01, a derivative of 12674, was grown up in TY 1% glycine at 26°C on a shaker. In order to ensure the culture was not contaminated, a loopful of the culture was plated on a TYA plate and incubated at 26°C.

Cells were harvested by centrifugation at 6 000rpm for 15 minutes. The supernatant was poured off and the pellet resuspended in 10ml Tris HCl with 10% sucrose. A lysozyme solution of 50 - 100 mg lysozyme in 5ml Tris HCl + 10% sucrose was added and the suspension incubated at 37°C for 2 hours in order to weaken cell walls. The cells were centrifuged for 15 minutes at 12 000 rpm in a low speed rotor. The supernatant was poured off and the pellet resuspended in 8ml TE containing approximately 1-2 mg proteinase K to which 0.8ml TE + 10% SDS was added. The material was incubated at 37°C for 2 hours. It was then transferred to 50 Ti tubes and centrifuged at 35 000rpm for 30 minutes to pellet the cell debris. The DNA solution was decanted off and the volume measured. An equal weight of CsCl was added and dissolved. The DNA solution was centrifuged at 18 000rpm for 15 minutes and was decanted off from under the scum which formed during centrifugation. The refractive index was measured and adjusted to 1.395 prior to addition of ethidium bromide. Approximately 0.6ml

of a 10mg/ml stock solution of ethidium bromide was added and the refractive index remeasured and adjusted to 1.390. The material was loaded into Quickseal ultracentrifuge tubes, balanced in pairs and sealed. The tubes were centrifuged at 40 000rpm for approximately 12 hours. The chromosomal DNA band was removed and a second CsCl run was not necessary. The DNA was decolourized with TE saturated butanol as described in 2.2.9.1 and dialyzed against TE. The concentration of DNA was measured using a spectrophotometer.

2.2.9.3 CALCULATING THE DNA CONCENTRATION

A Spectronic 601 spectrophotometer (Milton Roy Company) was used to obtain absorbance readings for the calculation of DNA concentration. A dilution of the dialyzed DNA was made in TE, usually a 1/100 dilution. All absorbance readings were taken at 260nm using quartz cuvettes and a TE blank.

The concentration of the DNA in µg/ml was calculated as follows:

A260 x dilution factor x 50

2.2.9.4 PLASMID DNA ISOLATION OF E.coli (Maniatis et.al 1982)

The bacterial cells were grown overnight in 5ml of L B at 37°C with aeration of which 1ml was harvested. The pellet was resuspended in 100µl of solution 1 (2.1.4.9.1) and allowed to stand at room temperature for 5 minutes. To this 200µl of solution 2 (2.1.4.9.2) was added and mixed gently. The material was placed on ice for 5 minutes after which 150µl precooled solution 3 (2.1.4.9.3) was added and mixed gently. The material was placed on ice for a further 5 minutes, centrifuged for 1 minute and the supernatant removed to a sterile eppendorf tube. One volume of isopropanol was added to the supernatant which was allowed to stand at room temperature for 5 minutes. DNA was precipitated by centrifuging for 5 minutes. The pellet was washed with cold 95% ethanol and centrifuged a second time for 5 minutes. The pellet was dried at 60°C for 15-20 minutes and resuspended in 12µl boiled TE buffer with ribonuclease to a final concentration of (1mg/ml). If the plasmid was to be enzymatically digested then the dried pellet was resuspended in 15µl of medium buffer and ribonuclease (final concentration of 1mg/ml) and the appropriate concentration of the restriction endonuclease Bgl II.

2.2.9.5 NOCARDIOFORM PLASMID DNA ISOLATION

The organism was grown up overnight in TY medium supplemented with 1% glycine and 1ml of this overnight culture was centrifuged for 30 seconds. The supernatant was discarded and the pellet resuspended in 800µl lysozyme and Tris HCl with 10% sucrose (3.75mg/ml lysozyme). The material was incubated for 1 hour at 37°C with agitation and was then centrifuged for 30 seconds. The pellet was resuspended in 280µl TE to which 40µl TE+10% SDS was added and mixed by gentle inversion. The material was incubated at 60°C for 10 minutes after which 40µl 4.5m sodium acetate pH 6.0 was mixed with the material. This was left in an ice water slurry for 30 minutes and then centrifuged for 20 minutes. The upper layer, containing the plasmid, was decanted and the plasmid extracted with 80µl phenol saturated with TE which was centrifuged for 5 minutes. The top layer was collected and 80µl chloroform/isoamylalcohol (24:1) was added. The top layer was collected after a second 5 minutes centrifugation, to which 2.5 volumes of cold ethanol were added. This was centrifuged for 20 minutes and resuspended in 20µl boiled TE and ribonuclease (1mg/ml). The plasmid band was observed by running the sample on a 0.4% agarose gel.

2.2.9.6 POURING AGAROSE GELS

Agarose was made up as described (2.1.6). Only 0.4% agarose gels were used. Each gel had a volume of 25ml to which 1.5µl of a 10mg/ml stock solution of ethidium bromide was added. The gel was poured into the gel former of a Hoefer Minnie submarine agarose gel unit and allowed to polymerise at 4°C for 2 hours.

2.2.9.7 LOADING GELS

Each gel had 8 wells. Usually a DNA molecular weight marker, either λII or λIII were used in addition to a control. The amount of material loaded varied between 12 and 17µl.

To each sample 2µl of gel loading buffer was added prior to loading into the wells. (2.1.4.8).

2.2.9.8 ELECTROPHORESIS

Once the agarose gel had polymerised, the gel was placed in the Hoefer Minnie submarine agarose gel unit, model HE 33. The gel apparatus was filled with 200ml of running buffer containing 15µl of a 10mg/ml stock solution of ethidium bromide (2.1.4.7) and the samples loaded into the wells. The gels were run at 80 volts for approximately 2 hours or until the dye front had reached the end of the gel.

The bands on the gel were observed using a Transilluminator. Photographs of relevant gels were taken.

2.2.9.9 DIGESTING DNA

The 01 chromosomal DNA was prepared according to the procedures described in 2.2.9.2 and 2.2 The DNA was stored in TE which might have interfered with the action of some restriction endonucleases. Therefore it was necessary to remove the TE and resuspend the DNA in H_2O. This was done by taking an aliquot of the DNA in TE representing the desired amount of DNA, adding a volume of sterile distilled water and 10% of the total volume of 1M NaCl.

This was mixed, then 2.5 volumes of cold 95% ethanol was added. The mixture was centrifuged for 20 minutes. This is known as ethanol precipitation. The pellet was dried at 60°C for 15-20 minutes and resuspended

in an appropriate volume of sterile distilled water, usually 90µl, for 1 hour or more at 42°C. The restriction endonucleases used were Sau 3A and Bgl II. Partial digestions of the chromosomal DNA using these enzymes were required, hence it was necessary to dilute the enzymes and determine which dilution partially digested the 01 chromosomal DNA. The enzymes were diluted in their correct buffers. The shuttle vector (pDA27) DNA was totally digested. An aliquot of the DNA was digested with the desired quantity of enzyme and the appropriate ionic buffer at 37°C which was the temperature at which both Bgl II and Sau 3A digested DNA. The DNA was digested for varying amounts of time. Aliquots of the digested samples were run on 0.4% agarose gels (2.2.9.6 - 2.2.9.8).

2.2.9.10 PARTIAL DIGESTION OF 01 CHROMOSOMAL DNA WITH SAU 3A

The 01 chromosomal DNA was ethanol precipitated by aliquoting 9µl of the DNA in TE, adding 36µl of sterile distilled water and 5µl 1M NaCl. This was mixed and 125µl ice'd 95% ethanol was added. The material was centrifuged for 20 minutes and the pellet dried at 60°C for 15-20 minutes then resuspended in 90µ sterile distilled water.

To determine the correct quantity of SAU 3A which would partially digest the 01 chromosomal DNA, 0.35, 0.035, 0.0035 and 0.00035 units of enzyme were prepared with medium ionic buffer.

A 9µl aliquot of the resuspended 01 chromosomal DNA was digested with 1µl of each of these dilutions along with 1µl of 10X medium buffer for 30 minutes at 37°C. Aliquots were run on a 0.4% agarose gel for 2 hours.

2.2.9.11 PARTIAL DIGESTION OF 01 CHROMOSOMAL DNA WITH Bgl II

To determine the correct dilution of Bgl II which would partially digest the 01 chromosomal DNA, several dilutions of the enzyme were made. Each digestion had to have 1µl of DNA therefore the appropriate volume of 01 chromosomal DNA was taken. This worked out to be 20µl of 01 DNA in TE (5.3oµl of DNA to which 70µl sterile distilled water and 10µl 1M NaCl was added and mixed. Two and a half volumes of cold 95% ethanol (250µl) was added and the material centrifuged for 20 minutes. The dried pellet was resuspended in 53.6µl sterile water and was aliquoted 5 ways resulting in 1µl of DNA per aliquot. To each aliquot, 1.1µl of medium ionic buffer was added.

The Bgl II enzyme was diluted as follows: 0.9, 0.09, 0.009 and 0.0009 units enzyme/µl DNA with 1 x medium buffer. To four of the aliquots, 1µl of each dilution was added. The samples were digested for 3 hours at 37°C and were run on a 0.4% agarose gel along with a λIII DNA molecular weight marker.

2.2.9.12 DIGESTION OF THE SHUTTLE VECTOR WITH Bgl II

The DNA of the shuttle vector (pDA27) was prepared using the procedure described in 2.2.9.1 and the concentration determined as in 2.2.9.3. An aliquot of the DNA was ethanol precipitated as described in 2.2.9.9 and resuspended in an appropriate volume of sterile distilled H_2O.
Originally the DNA was digested with undiluted Bgl II for 3 hours at 37°C but when new shuttle vector DNA was prepared by a colleague, H.Golob, it was digested for 5 hours at 37°C with 1 unit of Bgl II.

2.2.10 LIGATION OF 01 CHROMOSOMAL DNA AND SHUTTLE VECTOR DNA

2.2.10.1 PREPARATION OF 01 CHROMOSOMAL DNA FOR LIGATION

The concentration of the 01 chromosomal DNA had been worked out according to the procedure described in 2.2.9.3 for each preparation of DNA, an appropriate volume of 01 DNA in TE was aliquoted in order to give 100µl of DNA. The DNA was ethanol precipitated and resuspended in 90µl sterile distilled water. When DNA was resuspended, 10µl of 10 x medium ionic buffer was added giving a concentration of 100µl DNA in 100µl. The ap-

propriate volume of the desired dilution of Bgl II was added and the DNA digested at 37°C for 3 hours.

In order to remove the enzyme, a phenol extraction was done by adding an equal volume of phenol to the sample which was centrifuged for 10 minutes. The top layer was retained to which an equal volume of chloroform : isoamylalcohol (24 : 1) was added. This was centrifuged for 5 minutes and the top layer retained which was then ethanol precipitated, the pellet dried and resuspended in an appropriate volume of ligation buffer. (2.1.4.11).

2.2.10.2 PREPARATION OF THE SHUTTLE VECTOR DNA FOR LIGATION

The concentration of the shuttle vector DNA was determined. This DNA was ethanol precipitated resuspended in an appropriate volume and digested with undiluted Bgl II for 5 hours. The procedure to remove the enzyme was carried out as described in 2.2.11.1 and the DNA resuspended in an appropriate volume of ligation buffer.

2.2.10.3 LIGATIONS

For each ligation, 0.3µl of shuttle vector was used. In order to determine the optimal concentration of 01 chromosomal DNA to give the most transformants a series of ligations were set up as in table 1. The volume of the ligation was 50µl and each ligation was made up to this volume with ligation buffer.

TABLE 1 : PREPARATION OF LIGATION MIXTURES OF 01 CHROMOSOMAL DNA AND pDA 27 DNA

AMOUNT OF 01 CHROMOSOMAL DNA (PER AMT PLASMID)	AMOUNT OF SHUTTLE VECTOR	AMOUNT OF LIGATION BUFFER
1x = 0.3µg = 1.5µl	0.3µg = 2.7µl	47.3µl
2x = 0.6µg = 3µl	0.3µg = 2.7µl	45.8µl
4x = 1.2µg = 6µl	0.3µg = 2.7µl	44.3µl
6x = 1.8µg = 9µl	0.3µg = 2.7µl	41.3µl
8x = 2.4µg = 12µl	0.3µg = 2.7µl	38.3µl
16x = 4.8µg = 24µl	0.3µg = 2.7µl	23.3µl
24x = 7.2µg = 36µl	0.3µg = 2.7µl	11.3µl
32x = 9.6µg = 48µl	0.3µg = 2.7µl	0

To each ligation, 1µl of T4 ligase (Boehringer) was added and the ligations were placed in a 14°C waterbath overnight.

2.2.11 CALCIUM CHLORIDE TRANSFORMATION (MANIATIS ET AL 1982)

This procedure was used to transform *E.coli* MM294 - 1 with 01 chromosomal DNA ligated to shuttle vector pDA27. A sidearm flask containing 100ml of sterile LB was inoculated with 1ml of an overnight culture of *E.coli* MM294-1 grown in 5ml LB at 37°C with aeration. The cells were grown with vigorous shaking at 37°C until an optical density reading at 550nm of 0.2 was obtained. This corresponded to approximately 5 x 10^7 cells/ml. This took approximately 2 hours. The culture was chilled on ice for 10 minutes then centrifuged at 6 000rpm for 10 minutes at 4°C. The supernatant was discarded and the cells resuspended in 50ml (half the original culture volume) of an ice cold, sterile solution of 50mM $CaCl_2$ and 10mM Tris HCl pH 8.0. The cell suspension was centrifuged at 6 000rpm for 10 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 6.66 ml (1/15 of the original volume) of an ice cold, sterile solution of 50mM $CaCl_2$ and10mM Tris HCl pH 8.0. The cells were dispensed in a volume of 0.2ml into prechilled eppendorf tubes and stored on ice for 1 hour. The plasmid containing the 01 chromosomal inserts in ligation buffer was added to the cells and mixed. The tubes were transferred to a water bath preheated to 42°C for 1 minute to heat shock. To each tube 1 ml of LB was added and incubated at 37°C for 1 hour without shaking. This period allowed the bacteria to recover and to enable phenotypic expression to occur. The cells were centrifuged for a short time and the supernatant poured off. The pellet was resuspended in the remaining LB and the cells were spread very gently onto LA and 60µg/ml ampicillin plates. The plates were incubated at 37°C overnight.

2.2.12 POLYETHYLENEGYLCOL TRANSFORMATION

This transformation procedure was used to transform the plasmid pDA30 extracted from the 14887 derived mutant KD, background into the mutant KD 1.

KD 1 was grown up in 5ml of T2 and was diluted 1/100 times. This diluted culture was grown up at 26°C and 1ml of this centrifuged and resuspended in 1ml of P buffer (2.1.4.12) containing lysozyme (5mg/ml). This was incubated at 37°C for 1 hour without agitation. The cells were centrifuged and resuspended in 1ml P buffer very gently. They were centrifuged briefly and resuspended in 0.5ml P buffer. The cells were aliquoted in volumes of 75µl into sterile eppendorf tubes one of which was to serve as a control. The following were added to each eppendorf.

Control : 5µl TE and 80µl PEG 6 000 (25% w/v)

Experiment : 5µl of a 1/10 dilution of pDA30 and 80µl PEG (25% w/v) The PEG was made up under as sterile conditions as possible. A 50% w/v solution was made up with P buffer.

The 50% PEG in P buffer was mixed thoroughly with the protoplasts and left at room temperature for 4 minutes.

The mixture was then spread onto chilled regeneration plates (2.1.2.11) and incubated at 26°C. After 12 hours, 0.5ml of a 3M arsenate, 1M arsenite solution was added as an underlay. The plates were incubated at 26°C until transformants appeared.

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