THE DEVELOPMENT OF CLONING VECTORS FOR NOCARDIOFORM BACTERIA

Anne Lee Daffey

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DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Master of Science in Biotechnology in the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination in any other university.

Daffy

28 day of FEBRUARY, 1989.

ABSTRACT

Progress in the investigation of nocardioform bacteria, which are of considerable commercial value, has been hampered by the absence of resistance plasmids suitable for development into a cloning vector. A primarily genetic approach succeeded in generating a set of readily detectable plasmids which between them carried four resistance determinants, to sodium arsenate, sodium arsenite, cadmium chloride and chloramphenicol. These plasmids have since been reduced in size and only confer resistance to arsenate and arsenite.

The method of generating these plasmids was repeated in an attempt to obtain plasmids conferring resistance to chlorampenicol or cadmium chloride. Five transductants were obtained of varying phenotype. All transductants were resistant to arsenite. Only one transductant had resistance to chloramphenicol. Further analysis indicated that this resistance was not plasmid born.

Size determinations of the existing plasmids were made: pDA20 was found to be approximately 70 kb, pDA40 approximately 100 kb and, pDA 30 approximately 14 kb. An attempt was made to reduce the plasmids pDA20, pDA22 and pDA30 in size in order to create a set of small plasmids

suitable for cloning vectors, but no reduced plasmids were obtained.

pDA22 and pECOR251 were ligated together at their BamHI sites in order to develop a shuttle vector. Two orientations (A and B) of shuttle vector were expected. Both orientations proved to be viable. Orientation A subsequently proved to be more suitable for cloning purposes.

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ABBREVIATIONS

°C	degrees celcius
g	gram
kb	kilobase
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
M	molar
ng	nanogram
rpm	revolutions per minute
ug	microgram
ul	microlitre
UV	ultra violet
V	Volts

1. INTRODUCTION

1.1 Development of cloning vectors

Nocardioform bacteria of the genus Rhodococcus are a group of soil-dwelling gram-positive microorganisms with the ability to degrade toxic compounds such as acrylamide (Arai et al., 1981; cited in Dabbs, 1987), phenolic compounds (Haider et al., 1981; cited in Dabbs, 1987), and insecticides (Ferguson et al., 1981). Rhodococcus spp. also have the capability of synthesizing antibiotics such as hygromycin (Wakisuki et al., 1980) and rifamycin (Cross, 1982). In addition, they have the propensity for interconverting steroid compounds, for example, converting cholesterol to substances which are the precursors of steroid hormones or oral contraceptives (Ferreira et al., 1984). They are therefore of considerable commercial importance. Individual species are also the aetiological agents of tuberculosis, leprosy and nocardiosis.

Progress in the investigation of nocardioform has been hampered by the absence of resistance plasmids suitable for development into a cloning vector. Plasmids have been identified in several strains (Kasweck et al., 1982), but no phenotype has been ascribed to these. Another approach has been based on attempts to develop the lytic bacteriophage ØEC into a cloning vector (Brownell et al., 1982).

To obtain resistance plasmids for a well-characterised nocardioform, Rhodococcus erythropolis ATCC 12674, an alternative approach - primarily genetic - was adopted in this work (Dabbs and Sole, 1988). Among the streptomycetes as a whole there is evidence of low copy number plasmids which have been identified on the basis of their phenotype rather than by the presence of a plasmid band in a gel. Two examples are plasmid SCP1 of Streptomyces coelicolor (Chater and Hopwood, 1983) and a plasmid of S. fradiae (Stonesifer et al., 1986). No plasmid band was detected in R. erythropolis strain ATCC 12674 but it was hypothesized that a similar situation might prevail in this organism.

The following strategy was adopted to investigate and exploit this (Dabbs and Sole, 1988). The level of resistance of strain ATCC 12674 to a range of antibiotics and heavy metal compounds was determined. The organism was then grown in the presence of any of a number of plasmid curing agents, after which all resistance phenotypes were again determined. Any spontaneous reductions in resistance, as assayed by screening a large number of clones, were also monitored.

On the assumption that reductions in resistance might reflect loss of plasmid-coded genes, the parental strain was then used to select mutants with increased resistance to any of the compounds for which reduced resistance was identified

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On the assumption that reductions in resistance might reflect loss of plasmid-coded genes, the parental strain was then used to select mutants with increased resistance to any of the compounds for which reduced resistance was identified

in the "putative" cured strain. It was possible that in some cases increased resistance might arise as a result of increased copy number of the supposed plasmid, rendering it detectable on agarose gels. This strategy was successful in producing a set of related plasmids bearing several genes of resistance to heavy metal compounds (arsenate and arsenite, and cadmium chloride) or an antibiotic (chloramphenicol). The plasmids (designated pDA20 and pDA40) were large, migrating more slowly than chromosomal DNA in agarose gels, and were made up of resistance determinants (including Q4 immunity) from the host organism together with part of the genome (probably the replicon) of nocardiophage Q4. The plasmid pDA20 has since been reduced in size by a series of digestions and ligations, and its two derivatives, pDA22 and pDA30 confer only resistance to arsenate and arsenite. (Fig. 1.1).

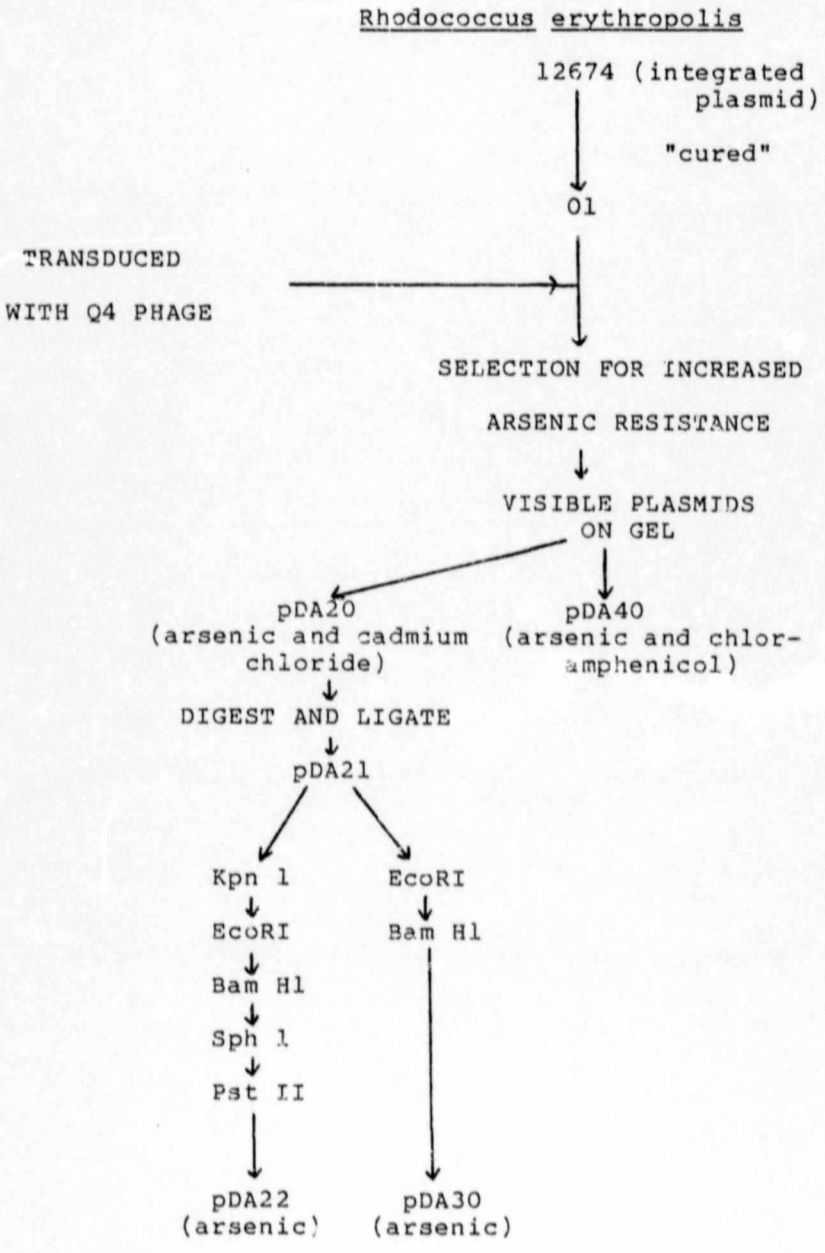

Figure 1.1 Schematic representation of the relationship between Q4 nocardiophage and the development of cloning vectors for nocardioform bacteria. The derivation of plasmids pDA22 and pDA30 from pDA21 by a series of digestions and ligations is shown. The resistance conferred by the various plasmids is indicated in brackets.

1.2 The Nocardiophage Q4

Q4 is a generalised transducing bacteriophage isolated from soil, and it has been shown to mediate the transduction of a number of unlinked chromosomal markers in *Rhodococcus erythropolis* (Dabbs, 1987).

1.2.1 The role of Q4

As mentioned, there is evidence in other actinomycetes of very low copy number plasmids undetectable or very hard to detect in plasmid screens but manifesting themselves by their phenotype (Chater and Hopwood, 1983; Stonesifer et al 1986). It has been hypothesised that these plasmids integrate into the chromosome and much of the time do not exist as autonomously replicating units. In *Bacillus stearothermophilus* a plasmid has been identified which goes from being an autonomous unit to being integrated into the chromosome when the organism is stressed by raising the temperature to near the maximum for growth (Koizumi et al., 1986).

On the assumption that an analogous situation exists in *R. erythropolis* strain ATCC 12674, attempts were made to disrupt the normal structure of a putative plasmid to prevent it from integrating into the chromosome. The agent of disruption was the generalised transducing phage Q4

(Dabbs and Sole, 1988). This virus would be expected to package host plasmid DNA as well as host chromosomal DNA.

A Q4 lysate of the wild type was used as a simultaneous donor of arsenate and arsenite resistance (100mM and 10mM, respectively) into strain CW22 (a spontaneously cured derivative of ATCC 12674). No colonies grew up on these selective plates when the cells of CW22 alone were plated. Four transductants, td 1 - td 4, were selected from plates where the cells had been exposed to a lysate of Q4. These transductants showed no evidence of a plasmid band. However, when strains td 1, 2, 3 or 4 were plated on higher concentrations (50mM) of arsenite many of the resulting putative copy number mutants showed an additional band in agarose gels.

By the nature of the selection all transductants had increased arsenate/arsenite resistance, compared with strain CW22. Three additionally had increased cadmium resistance but unchanged chloramphenicol resistance, while one had increased chloramphenicol resistance but unchanged cadmium resistance. Therefore, though the resistances are linked, arsenate/arsenite, cadmium, and chloramphenicol can segregate from one another. Furthermore, arsenate was constitutively expressed in ATCC 12674 and its derivatives whereas arsenite resistance was inducible, suggesting that more than one gene product was responsible for resistance to

these related compounds, as s been shown in other organisms (Mobley et al, 1984). ..ltogether therefore, four resistance determinants are pl.. ly located on the DNA segment which is spontaneously lost from strain ATCC 12674.

The occurrence of detectable plasmids only following a transduction step supported the interpretation that the plasmid of the wild - type strain may be integrated in the chromosome much of the time.

The relationship between Q4 phage and the development of the resistance plasmids is summarised in figure 1.1.

1.3 Requirements for cloning vectors

The requirements for a good cloning vector are

1) A replicon which is stably maintained in the organism.

2) A means of selection for the vector, that is, a resistance determinant.

3) A means of selecting cloned fragments into the vector.

4) A method of screening cloned DNA for the desired gene.

Meeting these requirements in nocardioform

1) A replicon derived from the generalised transducing phage Q4.

2) Arsenic resistance determinant obtained from an unstable genetic element in a nocardioform.

3) The Escherichia coli suicide vector pECOR251.

4) Bacterial mutants unable to utilise compounds as sole carbon, nitrogen sources.

By combining 1 and 2 (see 1.1.1.; fig. 1.1), nocardioform resistance plasmids have been obtained.

1.4 Aims

1. To repeat the transduction experiment described above with Q4 phage in order to obtain plasmids conferring resistance to chloramphenicol and cadmium chloride.

2. To combine the nocardioform resistance plasmid, pDA22, conferring arsenate and arsenite resistance, with the E. coli suicide vector pECOR251.

3. To determine the size of the nocardioform plasmids pDA20, pDA30 and pDA40.

4. To optimise nocardioform transformation conditions.

5. To digest and ligate the nocardioform resistance plasmids pDA20, pDA22 and pDA30 so as to reduce them in size, and to transform them into a suitable recipient.

2. METHODS AND MATERIALS

2.1 Bacterial strains

	Source
Rhodococcus erythropolis ATCC 12674 - parental strain	N. Ferreira
01 - "cured" parental strain	E. Dabbs
011 - 01 with streptomycin resistance	H. Golob
Escherichia coli MM294	
λMM294	

2.2 Plasmids

	Source
pECOR251 - *Escherichia coli* suicide vector	
pDA20 - Nocardioform plasmid with resistance to arsenate/arsenite and cadmium chloride	E. Dabbs
pDA22 - Nocardioform plasmid with resistance to arsenate/arsenite	E. Dabbs
pDA30 - Nocardioform plasmid with resistance to arsenate/arsenite	E. Dabbs
pDA40 - Nocardioform plasmid with resistance to arsenate/arsenite and chloramphenicol	E. Dabbs
pDA27 - Putative shuttle vector (Orientation A)	This work
pDA28 - Putative shuttle vector (Orientation B)	This work

2.3 Media

Luria Broth

1% Tryptone

0.5% Yeast Extract

0.5% NaCl

Luria Agar

1% Tryptone

0.5% Yeast Extract

0.5% NaCl

15 g/l Agar

Minimal Media Agar

Minimal media agar was prepared in two flasks as follows:

Flask 1 - 50 ml 10 x A - N stock

0.5 g NH_4Cl

200 ml H_2O

Flask 2 - 250 ml H_2O

7.5 g Agar

2.5 g Glucose

The flasks are autoclaved separately, and mixed. B1 and glutamate are each added to a final concentration of 0.1 ug/ml.

10 x A-N Stock

91.7 g $K_2HPO_4.3H_2O$

26.8 g KH_2PO_4

5 g sodium citrate

1 g $MgSO_4.H_2O$

The volume is made up to 1 litre with H O. The pH of the solution should be 7.9. Since the solution is not autoclaved, chloroform is added to keep it free of contaminants.

Protoplast buffer

Sucrose 10.3 g

K_2SO_4 0.025g

$MgCl_2.6H_2O$ 0.202

Distilled H_2O to 80 mls.

Autoclaved.

10 ml TES (N-tris (hydroxymethyl) methyl-2 amino ethanesulphonic acid) buffer (0.25M, adjusted to pH 7.2) is added. The protoplast buffer is then dispensed into 9.75 ml aliquots and frozen until required. Immediately before use, 250 ul of 1M $CaCl_2$ and 100 ul of 0.25M KH_2PO_4 are added.

Regeneration plates

0.9 g NaCl

3 g Tryptone

1.5 g Yeast Extract

35 g Sucrose

The sucrose is allowed to dissolve. The volume is then made up to 280 ml.

4 g of agar is added.

After autoclaving 6 ml 1M $CaCl_2$ and
10 ml TES buffer (0.25M, pH 7.2) are added.

TY

1% Tryptone

0.5% Yeast Extract

TY Agar

1% Tryptone

0.5% Yeast Extract

15 g/l Agar

TYG

1% Tryptone

0.5% Yeast Extract

1 - 1.2 % Glycine

TYM

1% Tryptone

0.5% Yeast Extract

$MgSO_4$ is added after autoclaving to a final concentration of 10mM.

TYMA

1% Tryptone

0.5% Yeast Extract

15 g/l Agar

After autoclaving, $MgSO_4$ is added to a final concentration of 10mM.

TYMCA

1% Tryptone

0.5% Yeast Extract

15 g/l Agar

After autoclaving, $MgCl_2$ and $CaCl_2$ are each added to a final concentration of 10mM.

TYMKA

1% Tryptone

0.5% Yeast Extract

15 g/l Agar

After autoclaving, $MgSO_4$ and kanamycin are added to final concentrations of 10mM and 15 ug/ml, respectively.

Top Agar

1% Tryptone

0.5% Yeast Extract

0.5% Agar

Stock solutions of antibiotics were generally 10 mg/ml. The stock solutions of other compounds were generally 1M.

2.4 Methods

2.4.1 Nocardiophage Q4

Single plaques of the original Q4 phage were isolated. 20 ul of the phage, stored in TYM, was taken and added to 2 ml TYM. Various dilutions were made. 20 ul of cells (*R. erythropolis* ATCC 12674) was added. 2 ml of top agar was added. The phage solution was then poured over chilled TYMCA plates. Once the top agar had solidified, the plates were incubated at 26°C for 24 hours.

2.4.1.1 Preparation of lysates (Dabbs, 1987)

Individual plaques were picked up with toothpicks from the above plates and transferred to a sterile tube containing 2 mls TYM. 20 ul of cells was added and vortexed gently. 2mls of top agar was added. After mixing, the suspension was poured onto chilled TYMKA plates. After 24 hours

incubation at 26°C, scoring of clear, turbid, or no lysis were made. The minimal conditions for clear lysis were used in subsequent plates to build up titres.

The plate lysate was heated to 40 °C for 1 hour to liquify the top agar. This was tipped of into a centrifuge tube, cell debris and agar were pelleted by centrifugation at 10 000 rpm for 10 minutes and the supernatant passed through a .22 um filter. To 2 mls of this lysate was added 200 ul cells and the procedure repeated as before. For a third round of build up 350 ul of cells were added to the 2 mls of filtered lysate from the second round. After this titres were determined.

2.4.1.2 Transductions (Dabbs, 1987)

Transductions were generally performed as follows: 0.5 ml of a stationary phase culture in a 1.5 ml Eppendorf tube was pelleted by spinning for 30 seconds in a microfuge. Phage lysate (routinely 0.5 ml) was added to the pellet, which was gently resuspended. The suspension was incubated without agitation for 2 hours at 26°C. Cells were then sedimented by 30 seconds centrifugation in a microfuge. Cells were then resuspended in the remaining volume after decanting and then spread on appropriate selective plates and incubated at 26°C. The selective media was spread under the agar a few hours before spreading to allow for diffusion. Colonies growing up were streaked on selective plates. These

colonies were then spotted onto appropriate media to determine the phenotype.

2.4.2 Plasmid Screens

for

2.4.2.1 Nocardioform (Dabbs and Sole, 1988, with modifications)

One ml of a stationary phase culture grown in TYG at 26 °C, to which arsenate had been added to a final concentration of 50mM, was taken and pelleted in an Eppendorf tube by microfuging for 30 seconds. 30 mg of lysozyme was dissolved in 7 ml 0.5M sucrose, 100mM Tris, pH 8.0 - 800 ul of which was added to each tube. The cells were resuspended and incubated for an hour at 37 °C with shaking. The cells were then pelleted and resuspended in 280 ul TE (10mM Tris (Tris-(hydroxymethyl)-amino methane), 10mM EDTA (ethylenediaminetetra-acetic acid), pH 8.0), after which 40 ul of 10% SDS (sodium dodecyl sulphate) in the same buffer was added. Mixing was by gentle inversion. After incubation at 60 °C for 10 minutes, 40 ul of 4.5M sodium acetate, pH 6.0 was added, and mixed in the same manner. Samples were placed on ice for 30 minutes then centrifuged in a microfuge for 20 minutes. The supernatant was extracted once with phenol, and once with 24:1 chloroform:isoamylalcohol. The nucleic acid was then ethanol precipitated. The pellet was dried by incubating at 60 °C for 20 minutes and then dissolved in 20 ul of TE buffer

containing 240 ug/ml pancreatic ribonuclease. Mixed with loading buffer (40% sucrose (w/v), 0.25% bromophenol blue) samples were analysed by electrophoresis at 80 V in agarose gels buffered with TBE (0.089M Tris, 0.089M Boric acid, 0.002M EDTA).

2.4.2.2 Escherichia Coli (Maniatis et al.,1982)

Bacterial cells were grown overnight at 37 °C in 2 ml LB containing ampicillin at a final concentration of 50 ug/ml at 37 C with aeration. One ml of the overnight culture was harvested by microfugation, the pellet was resuspended in 100 ul of 50mM glucose, 25mM Tris pH 8.0, 10mM EDTA, and allowed to stand at room temperature of 5 minutes. 200 ul of 0.2 N NaOH, 10% SDS was added and mixed in gently. This was then allowed to to stand for 5 minutes on ice. 150 ul of precooled 5M potassium acetate pH 4.8 was added to the solution and mixed in gently. This was allowed to stand on ice for 5 minutes, then centrifuged for 1 minute. The supernatant was transferred to a sterile Eppendorf tube and one volume of isopropanol was added. The solution was allowed to stand at room temperature for 5 minutes. The DNA was precipitated by centrifuging for 5 minutes in a microfuge. The resulting pellet was washed once with cold 70% ethanol, and centrifuged for 5 minutes in a microfuge. The pellet was dried and resuspended in 20 ul TE containing 240 ug/ml pancreatic ribonuclease. The samples, mixed with

loading buffer, were analysed by electrophoresis at 80 V in agarose gels buffered with TBE.

2.4.3 Plasmid Purification

for

2.4.3.1 Nocardioform (Dabbs and Sole, 1988, with modifications)

The organism was grown in 250 ml TYG at 26°C in the presence of selective pressure (50mM arsenate). Cells usually required two days in which to reach stationary phase. The cells were then harvested by centrifuging at 6000 rpm for 20 minutes. The pellet was resuspended in 20 ml of 10mM Tris pH 8.0, 10% sucrose in which 100 mg lysozyme had been dissolved. After incubation for 2 hours at 37°C, the cells were pelleted by centrifugation at 12 000 rpm for 15 minutes and then resuspended in 8 ml TE. A further 0.8 ml of 10% SDS in the same buffer was added. The cells were incubated at 37 C for 2 hours and then transferred to 50 Ti tubes and centrifuged for 30 minutes at 35 000 rpm. The DNA solution was decanted off and the volume measured. Caesium chloride (1 g/ml) was added and allowed to dissolve. This was then centrifuged for 15 minutes at 18 000 rpm. The clear solution was decanted out from under the tough scum which had aggregated on the top. Ethidium bromide (0.8 ml of a 10mg/ml stock) was added. The refractive index was measured and adjusted to 1.392. DNA was resolved on a CsCl (caesium

loading buffer, were analysed by electrophoresis at 80 V in agarose gels buffered with TBE.

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chloride) gradient, generated at 45 000 rpm for 12 hours. For any plasmid band detected, fractions containing the band from five or six tubes were pooled and rerun on a CsCl gradient.

2.4.3.2 Escherichia coli (Clewell and Helinski, 1969)

An overnight culture of 250 ml LB plus ampicillin (50 ug/ml) was grown at 37 °C. The cells were then pelleted by centrifugation at 6000 rpm for 10 minutes. The pellet was washed by resuspending the cells in 10 ml cold TE. Cells were again pelleted (6000 rpm for 10 minutes). The cells were then resuspended in 2 ml cold 25% sucrose, 50mM Tris pH 8.0. 0.25 ml of a fresh lysozyme solution (10 mg/ml) in the same buffer was added and the resuspended cells were swirled gently on ice for 15 minutes. After the addition of 0.25 ml 0.5M EDTA pH 8.0 the cells were swirled on ice for a further 5 minutes. 2.5 ml of cold detergent solution (20% Triton X - 100, 0.5M EDTA pH8.0, 1M Tris pH 8.0) was added to promote lysis. The cells were once again swirled on ice for 10 minutes until the solution was clear and highly viscous. The material was centrifuged at 18 000 rpm for 45 minutes. The supernatant was decanted off and the volume was determined. One gram of CsCl was added per ml of supernatant. A short clearing spin (10 000 rpm for 10 minutes) was then done to clear the liquid. Subsequent

procedures are the same as those described for nocardioform (Methods and Materials 2.4.3.1).

2.4.3.3 The removal of Ethidium Bromide

An equal volume of TE saturated butanol was added to the DNA solution. The solutions were gently mixed by inversion. The Eppendorf tubes were spun for 30 seconds in the microfuge. The solutions separated into two distinct layers. The top layer containing the ethidium bromide was discarded. The procedure was repeated until the DNA solution was colourless.

2.4.3.4 The removal of Caesium Chloride

The DNA solution was dialysed against TE buffer for 4 hours.

2.4.4 Determination of DNA concentration

The concentration of the DNA was determined spectrophotometrically. An absorbance reading of 1 at a wavelength of 260nm is equal to 50 ug/ml DNA.

2.4.5 Restriction endonuclease digestion of DNA

DNA was stored in TE. Therefore, prior to digestion with restriction endonucleases, the appropriate amount of DNA was ethanol precipitated, dried and resuspended in 18 ul of sterile H_2O per digestion. Two microlitres of the appropriate buffer (as specified by the manufacturers) was added. Finally, 1 ul of enzyme was added. The samples were then incubated at the appropriate temperature until digestion was complete.

2.4.6 Agarose gel electrophoresis

In most cases 4% agarose gels were prepared but when it was necessary to determine the size of fragments, a 1.2% agarose gel was also prepared.

The agarose was prepared by dissolving the appropriate amount of agarose in 200 ml TBE buffer. This was then autoclaved. When required, the agarose was melted by microwaving. A 25 ml aliquot of molten agarose, to which 1.5 ul of ethidium bromide (10 mg/ml) had been added, was then taken and poured into the gel former. The gels were allowed to polymerize for at least 2 hours at 4 °C. The gel running buffer was TBE. Ethidium bromide was added to the buffer to a final concentration of 0.5 ug/ml. Gels were run at 8 V/cm. When electrophoresis was complete the DNA was

visualised over UV light on a transilluminator and then photographed on polaroid 665 film.

2.4.6.1 Determination of size of fragments

The distance of migration of fragments through agarose gels is inversely proportional to the logarithm of the molecular weight. The distance of migration of molecular weight markers (lambda DNA digested with Hind III, and lambda DNA digested with Hind III and EcoRI), which were electrophoresed simultaneously with the samples, were measured. A graph of molecular weight versus distance migrated was drawn. The sizes of the fragments of the samples was then determined by reading the molecular weight off the graph from the relative distances migrated.

2.4.7 Transformation

2.4.7.1 Nocardioform (Dabbs and Sole, 1988; with modifications)

Cells were grown in TYG at 26 °C. One ml of the culture was taken, pelleted and resuspended in 1 ml protoplast buffer containing 5 mg/ml lysozyme. The cells were then incubated at 37°C for one hour. Thereafter the cells were pelleted, resuspended gently in 1 ml protoplast buffer, pelleted again and resuspended in a final volume of 500 ul protoplast

buffer. 100 ng DNA, usually in 5 ul TE, was added to 75 ul aliquots of cells. 80 ul of 50% polyethyleneglycol (PEG), dissolved in protoplast buffer, was added making a final concentration of 25% w/v and mixed in gently. The samples were left standing for 3 - 4 minutes after which they were spread over regeneration plates. The plates were incubated at 26 °C. After a 12 hour delay, in order to allow for phenotypic expression of introduced resistance genes, 500 ul of a 3M arsenate, 0.5M arsenite solution was added as an underlay. The plates were placed back in the 26 °C incubator and after 5 - 7 days the number of transformants was assessed.

The putative transformants were then replica patched onto TY agar and TY agar plates containing arsenate and arsenite to final concentrations of 50mM and 10mM, respectively. This patching discriminates between true transformants and spontaneous mutants (no spontaneous mutants are ever found which are simultaneously resistant to both arsenate and arsenite). Plasmid screens were also done to confirm the presence of a plasmid.

2.4.7.2 Escherichia coli (Maniatis, 1982; with modifications)

Conditions were optimised by S. Andersen and H. Golob.

A 100 ml culture of LB in a 500 ml side arm flask was inoculated with 1 ml of an overnight bacterial culture. The cells were grown with vigourous shaking at 37 °C until they reached a density of ~5 x 10 cells/ml. For λMM294 and MM294 1 OD_{550} = 0.2 (~5 X 10 cells/ml).

The cells were then chilled on ice for 10 minutes. The cell suspension was then centrifuged at 4000 g for 5 minutes at 4 °C. The supernatant was discarded and the cells were resuspended in half of the original culture volume of an ice-cold, sterile solution of 50mM $CaCl_2$ and 10mM Tris pH 8.0. The cell suspension was placed in an ice bath for 15 minutes and then centrifuged at 4000 g for 5 minutes at 4°C. The supernatant was discarded. The cells were resuspended in one fifteenth of the original culture volume of an ice-cold, sterile solution of 50mM $CaCl_2$ and 10mM Tris pH 8.0. 0.2 ml aliquots were dispensed into prechilled tubes and stored on ice for a maximum of one hour. The DNA in ligation buffer or TE was added. The samples were then transferred to a water bath, preheated to 42 °C, for 1 minute. One ml of LB was added to each tube and incubated at 37°C for 1 hour without shaking. An appropriate quantity

of cells was spread onto selective media (LA containing 50 ug/ml ampicillin). The plates were incubated at 37 °C. Colonies appeared in 12-16 hours.

2.4.8 Dephosphorylation of plasmid DNA (Maniatis, 1982, with modifications)

The plasmid DNA was digested to completion with the appropriate restriction enzyme. It was then extracted once with phenol and once with chloroform. It was then precipitated with ethanol. After allowing the DNA pellet to dry, it was dissolved in a minimum volume (5 ul) of 10mM Tris pH 8.0. Five microlitres of 10 X CIP buffer (0.5M Tris (pH (9.0), 10mM $MgCl_2$, 1mM $ZnCl_2$, 10mM spermidine), water to 48 ul and 1 ul of calf intestinal phosphatase (CIP) was added.

In order to dephosphorylate the protruding 5' termini, the sample was incubated at 37 C for 60 minutes. A second aliquot of CIP was added, and the incubation was continued for a further 60 minutes. After completion of incubation, 40 ul of water, 10 ul of 10 X STE (100mM Tris (pH 8.0), 1M NaCl, 10mM EDTA), and 5 ul of 10% SDS was added. The sample was then heated to 68 °C for 15 minutes. (This completely inactivates the calf intestinal phosphatase). After extracting the sample once with phenol and once with chloroform, the volume of the sample was increased to 500 ul with TE and dialysed against TE for 1 hour. The DNA was

ethanol precipitated, the pellet was allowed to dry and then dissolved in an appropriate volume of ligation buffer.

2.4.9 Ligation

DNA was ethanol precipitated, the pellet was allowed to dry and then dissolved in an appropriate volume of ligation buffer (20mM Tris (pH 7.6), 10mM $MgCl_2$, 10mM dithiothreitol, 0.6mM ATP). If the DNA had been digested with a restriction endonuclease, the DNA was first phenol extracted. After allowing the DNA to resuspend in the ligation buffer, 1 ul of T4 DNA ligase was added and the samples were incubated overnight at 14 °C.

3. RESULTS

3.1 Transductants

Q4 phage has been a means of introduction of resistance genes back into the cured strain 01/011. Arsenate/arsenite resistant plasmids were obtained in this manner (Dabbs and Sole, 1988). It was necessary to obtain plasmids conferring resistance to chloramphenicol or cadmium chloride, so the experiment, as reviewed in the Introduction (1.2.1), was repeated in order to achieve this. (Figure 3.1).

011 was grown in TYM until stationary phase was reached. One millilitre of the culture was taken and pelleted in a 1.5 ml Eppendorf. The pellet was resuspended in 0.2 mls TYM. One millilitre of filter sterilised phage lysate (4 phage lysates, namely 3A, 3B, 4A and 4B, had been prepared as described in Methods and Materials 2.4.1.1) was added and then incubated at 26°C without agitation. The cells were pelleted, the supernatant decanted and the cells resuspended in the remaining volume. The cells were then spread over a TYMK plate which 7 hours previously, had had an arsenate/arsenite underlay. The plates were then incubated at 26 °C. After 5-7 days the number of transductants was determined (Table 3.1).

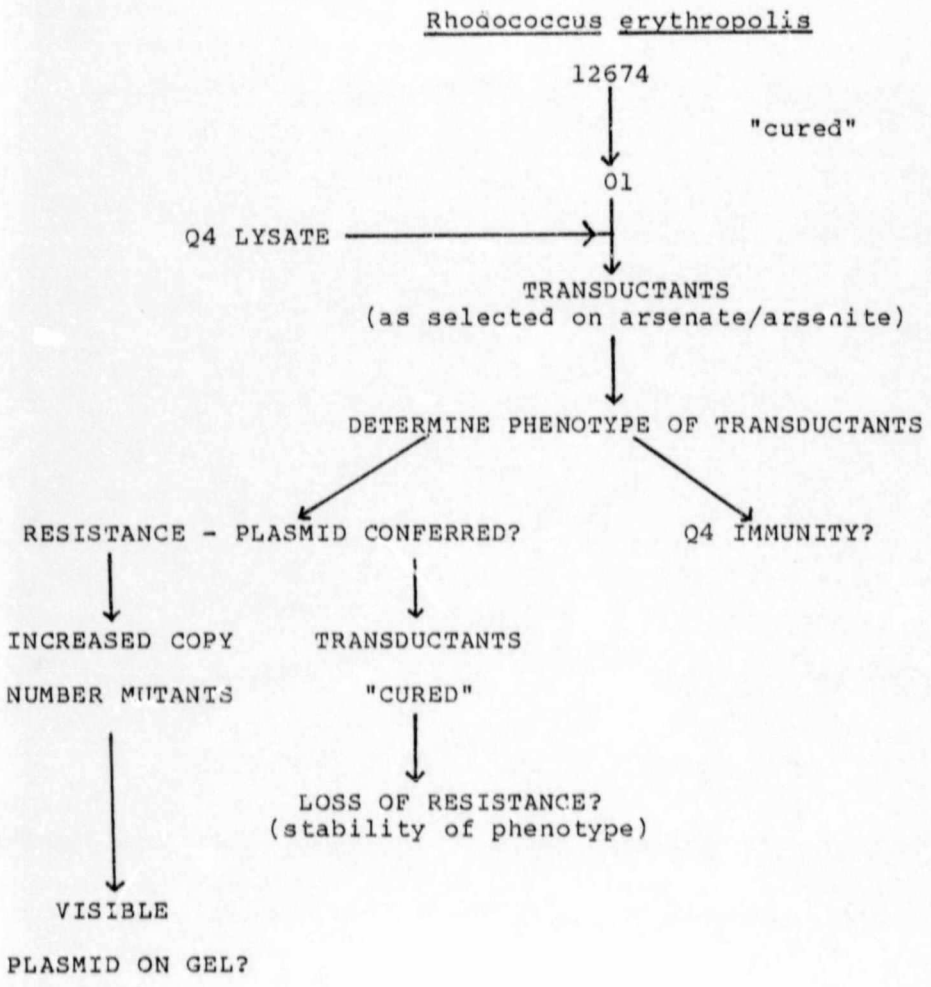

Fig. 3.1 Schematic representation of transduction experiments. The phenotype of the transductants is determined and they are analysed further for Q4 immunity, stability of phenotype and for the presence of a plasmid band on agarose gels.

Table 3.1 Table showing number of transductants obtained for the 4 Q4 phage lysates. Phage titres were determined and the number of transductants per plaque forming units (pfu) was calculated.

Phage lysate	Titre pfu/ml	Number of transductants	Number of transductants per pfu $X10^{10}$
3A	$6X10^{9}$	1	1.5
3B	$7X10^{9}$	2	2.5
4A	$12X10^{9}$	1	.75
4B	$6x10^{9}$	1	1.5

They were streaked on TYA plates containing 10mM arsenite, on which they grew. The results of replica plating the transductants on TYA plates with various antibiotic and heavy metal compound additions are summarised in table 3.2.

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4B	$6x10^{9}$	1	1.5

They were streaked on TYA plates containing 10mM arsenite, on which they grew. The results of replica plating the transductants on TYA plates with various antibiotic and heavy metal compound additions are summarised in table 3.2.

Table 3.2 Phenotype of the transductants. The level of resistance of the transductants to various antibiotic and heavy metal compounds was determined.

Transductant	Antibiotic or heavy metal compound additions			
	Arsenite 30mM	Arsenate 50mM	Chloramphenicol 20 ug/ml	CdCl 0.1mM
3A 1	+++	+++	+	-
3B 1	++	+	-	-
3B 2	+++	++	-	-
4A 1	+	+	-	-
4B 1	+	++	-	-

(+++ = good growth, ++ = moderate growth, + = poor growth, + = very poor growth)

3.1.1 Is the resistance plasmid born?

From a stationary phase culture of the transductant, 3A 1, (chosen because it demonstrated resistance to chloramphenicol as well as arsenate and arsenite) in TY containing 10mM arsenite, 20 ul was diluted in 4 ml TY (no selective pressure). Cultures were allowed to reach

stationary phase (10 generations) and streaked onto TYA plates. Twenty single colonies were then replica patched onto TYA and TYA containing 10mM arsenite plates. A further 20 ul was taken from the 10 generation culture and diluted into 4 ml TY. This procedure of patching and diluting was repeated again. The results are summarised in table 3.3.

Table 3.3 Table showing stability of the phenotype of transductant 3A 1. After growth in the absence of selective pressure, cultures were diluted onto TY plates. Single colonies grown up were patched onto TYA supplemented with 10mM arsenite and the number of colonies showing loss of resistance determined.

Generation	No. of colonies patched	No. of colonies with loss of resistance
11	20	1
21	20	1
31	20	2

The number of colonies with loss of resistance should increase with prolonged growth in media without selective pressure if the resistance is plasmid determined. From the

above results it can be seen that the number of colonies with loss of resistance did not increase. It would therefore, appear that the resistance is not plasmid born.

3.1.2 Increased copy number mutants

100 ul of a stationary phase culture in TY containing 10mM arsenite was spread over TYA plates containing various concentrations of arsenite (30mM, 40mM and 50mM) in order to obtain increased copy number mutants. The increased resistance could possibly be as a result of increased copy number of the "putative" plasmid which would then make it visible on an agarose gel. The plates were incubated at 26 °C for 5 days. The results are summarised in table 3.4.

Six colonies of transductant 3A 1 were taken from the 50mM and 40mM arsenite plates. Cultures were grown in 5 ml TYG containing 10mM arsenite and plasmid screens were done. No plasmids were observed. (Results not shown).

3.1.3 Q4 immunity

Q4 phage is not only the donor of resistance to various antibiotics and heavy metal compounds but also Q4 immunity. Cultures of the transductants were spread onto TYA plates. 50 ul of Q4 phage lysate was spotted in the centre of the plate. There was clearing on all the cultures therefore there was no Q4 immunity.

Table 3.4 Number of colonies obtained on TYA plates containing increasing concentrations of arsenite.

Transductant	Arsenite concentration		
	30mM	40mM	50mM
3A 1	confluent	14+	3
3B 1	confluent	4+	-
3B 2	confluent	6+	-
4A 1	confluent	1	-
4B 1	large no. (but not confluent)	-	-