3.2 Development of putative shuttle vectors

pECOR251 (fig. 3.2) and pDA22 (fig. 3.3) were digested with BamH1 and ligated together. The pDA22 was prepared in excess of the pECOR251. (The ligation mix contained pDA22 and pECOR251 DNA in a raio of 3:1.) Prior to ligation, the pECOR251 DNA was dephosphorylated to minimise self-ligation. The ligation product was transformed into λMM294 and transformants were selected on LA plates containing ampicillin (50 ug/ml). Nineteen transformants were observed and these were patched onto another LA plate containing ampicillin (50 ug/ml). Of the nineteen transformants only 17 grew. E. coli plasmid screens were done on these transformants (Plate 3.1). Eleven transformants contained the product of the ligation between pECOR251 and pDA22 (putative shuttle vectors). The other six only contained pECOR251. Two of the putative shuttle vectors were of intermediate size. BamH1 digests were done to confirm the presence of the two plasmids.

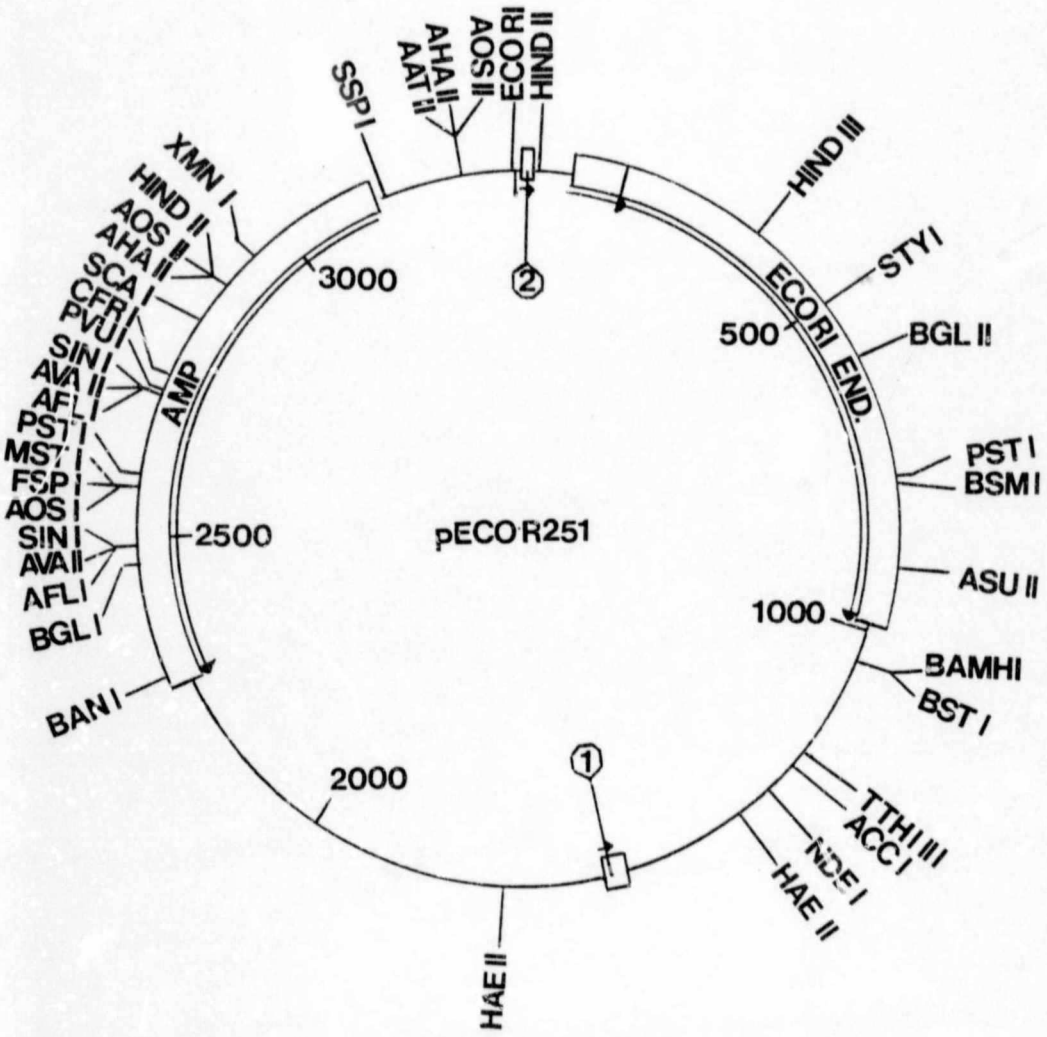


Fig. 3.2 The Escherichia coli suicide vector pECOR251. pECOR251 has a single BamHI site, outside either the ampicillin or EcoRI endonuclease gene, suitable for ligation with pDA22. The single recognition sites for Bgl II and Hind III in the EcoRI endonuclease gene are suitable for cloning chromosomal fragments.

1 - Origin

2 - Promotor

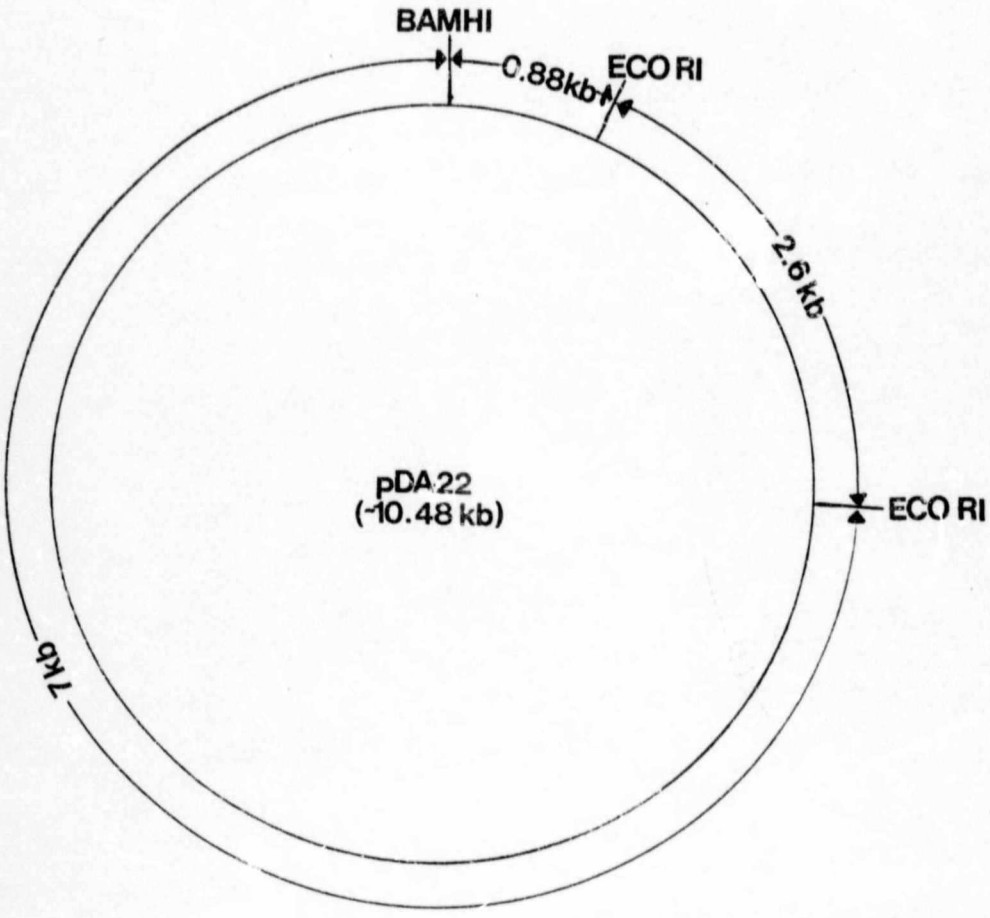


Fig. 3.3 Restriction map of pDA22. pDA22 is a nocardioform plasmid conferring resistance to arsenate and arsenite. It has a single BamHI site outside any critical part of the pDA22 genome. It has no Bgl II recognition site. (A. Daffey and K. Downing; M.Sc Biotechnology course, February 1988).

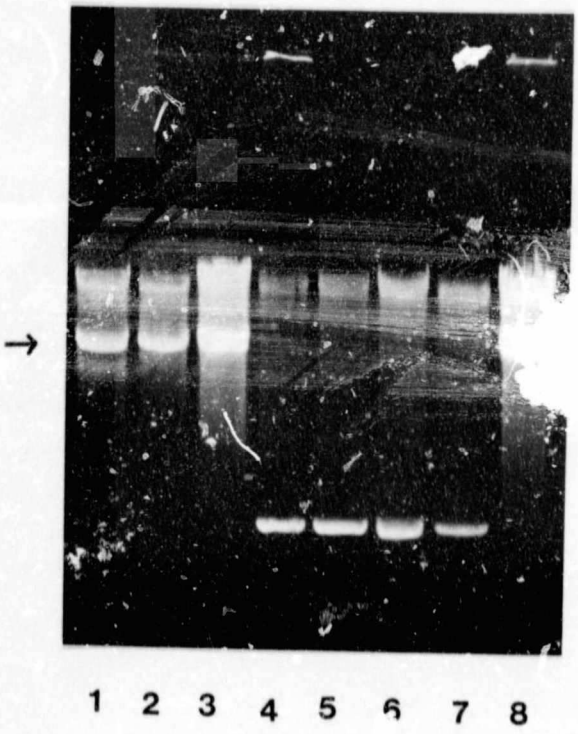
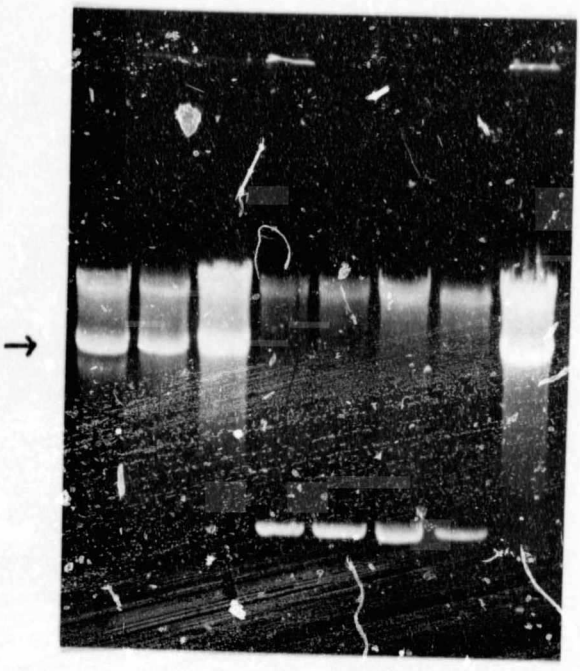


Plate 3.1 0.4% agarose gel, run at 80 V for 2 hours, of DNA from 1 ml of culture of 8 transformants of the ligation of pECOR251 and pDA22. Lanes 1, 2, 3, and 8 have a band (arrowed) running slightly faster than the chromosomal DNA. This band is the putative shuttle vector. The band in lanes 4 - 7 is pECOR251.



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3.2.1 Establishing the orientation of the putative shuttle vectors

Two orientations (designated A and B) are expected to arise as a result of the ligation between pECOR251 and pDA22 (figs 3.4a and b). In order to establish whether both orientations where present, EcoRI digestions were done. Two of the putative shuttle vectors were of orientation A, while the remainder, 7, were orientation B.

Examples of both orientations and the two shuttle vectors of intermediate size were prepared for a bulk plasmid purification. Further studies were confined to these putative shuttle vectors. The two full size plasmids were designated pDA27 (orientation A) and pDA28 (orientation B).

The BamHl and EcoRl digests were repeated (plates 3.2 and 3.3, respectively). Size determinations were made (tables 3.5 and 3.6) from the graphs of molecular weight versus distance migrated (figs 3.5 and 3.6). The sizes determined are those that are expected (figs 3.4a and b). The results also suggest that putative shuttle vector 19 has a deletion of DNA which includes one of the EcoRl sites.

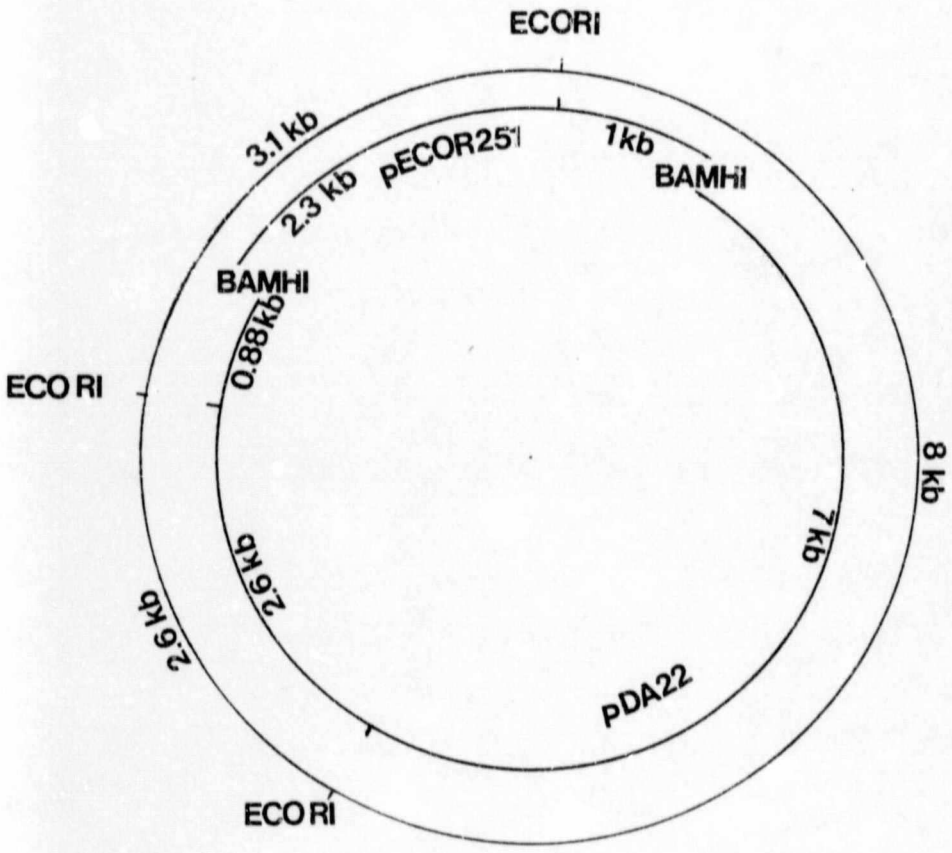

Fig. 3.4a Diagrammatic representation of orientation A of the shuttle vector (outer circle). pDA22 and pECOR251 (inner circles) were digested with BamHI and ligated together. EcoRI digestions distinguish between the two orientations. The expected size of the fragments obtained after an EcoRI digestion are indicated on the outer circle.

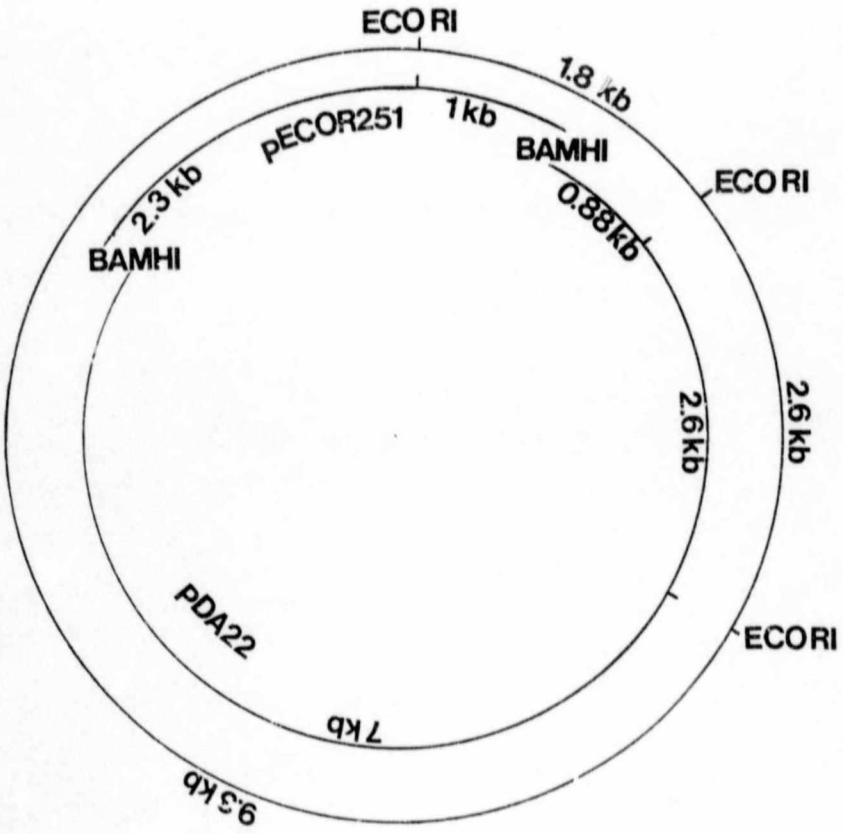

Fig. 3.4b Diagrammatic representation of orientation B of the shuttle vector (outer circle). pDA22 and pECOR251 (inner circles) were digested with BamHI and ligated together. EcoRI digestions distinquish between the two orientations. The expected size of the fragments obtained after an EcoRI digestion are indicated on the outer circle.

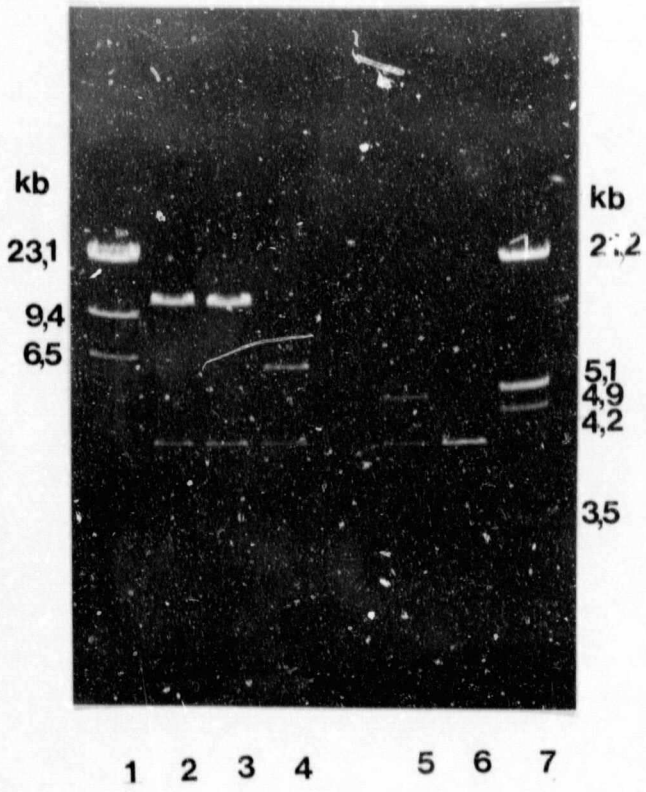

Plate 3.2 0.4% agarose gel of BamHI digestions of purified DNA of the putative shuttle vectors, pDA27 (lane 2), pDA28 (lane 3), 15 (lane 4) and 19 (lane 5), and pECOR251 (lane 6). Lanes 1 and 7 are the DNA molecular weight markers*, λII and III, respectively.

* λ II - DNA digested with Hind III

λIII - DNA digested with Hind III and EcoRI

(for sizes of λ DNA markers see Appendix)

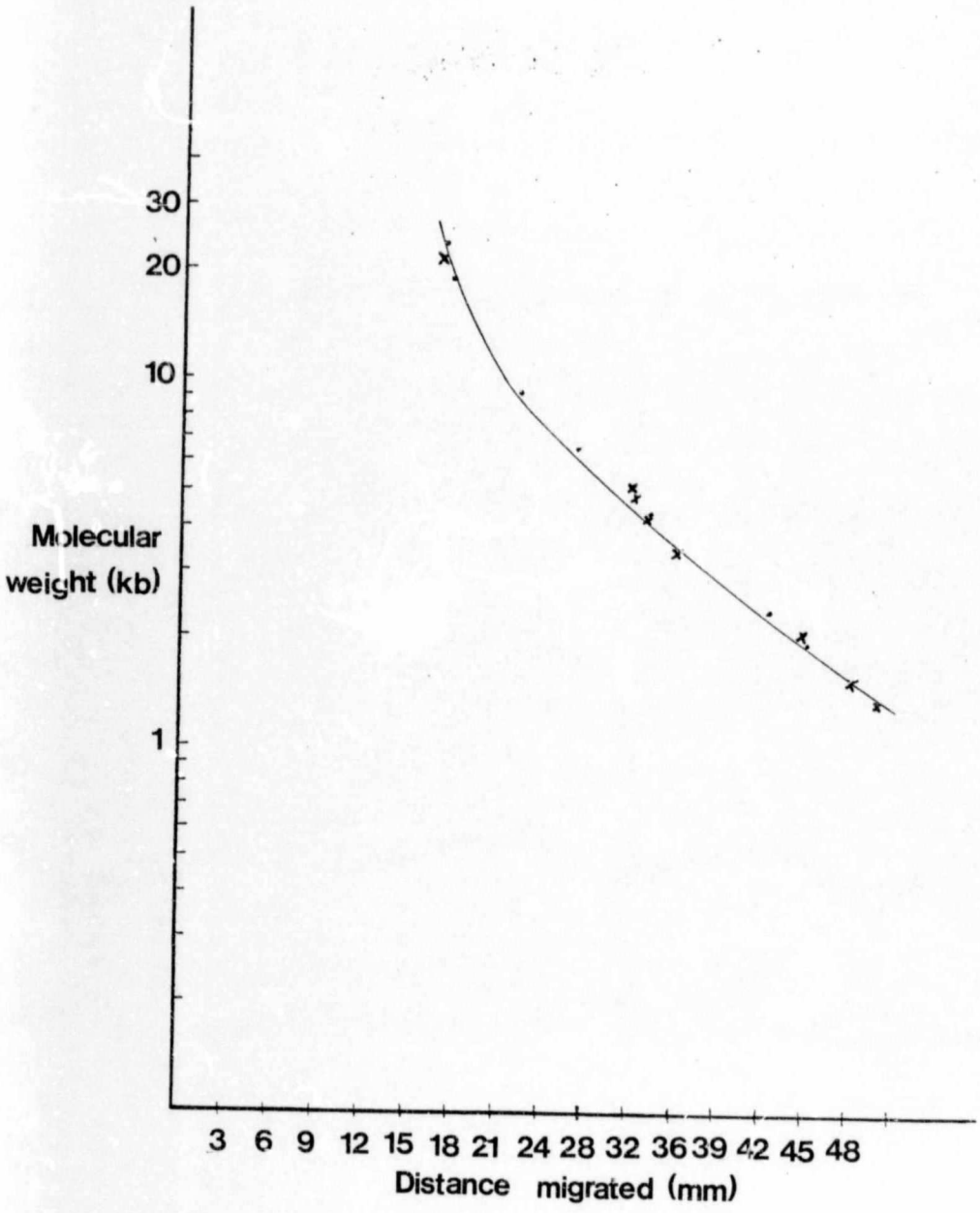

Fig. 3.5 Graph of molecular weight versus distance migrated: Determination of fragment size for BamHI digestions of the putative shuttle vectors.

· - λ II DNA molecular weight marker

x - λ III DNA molecular weight marker

Table 3.5 Size determination of fragments obtained with BamHl digestions of the putative shuttle vectors.

	Putative shuttle vector				pECOR251
	pDA27	pDA28	15	19	
Size of fragments (kb)	10.3 3.3	10.3 3.3	5.8 3.3	4.8 3.3	3.3
Size of vector (kb)	13.3	13.3	9.1	8.1	

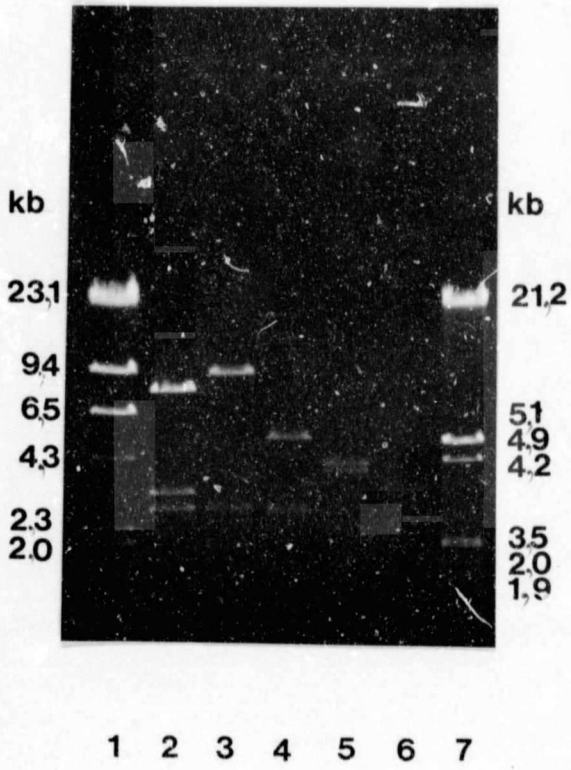

Plate 3.3 0.4% agarose gel of EcoRI digestions of purified DNA of the putative shuttle vectors, pDA27 (lane 2), pDA28 (lane 3), 15 (lane 4) and 19 (lane 5), and pECOR251 (lane 6). Lanes 1 and 7 are the DNA molecular weight markers, λII and III, respectively.

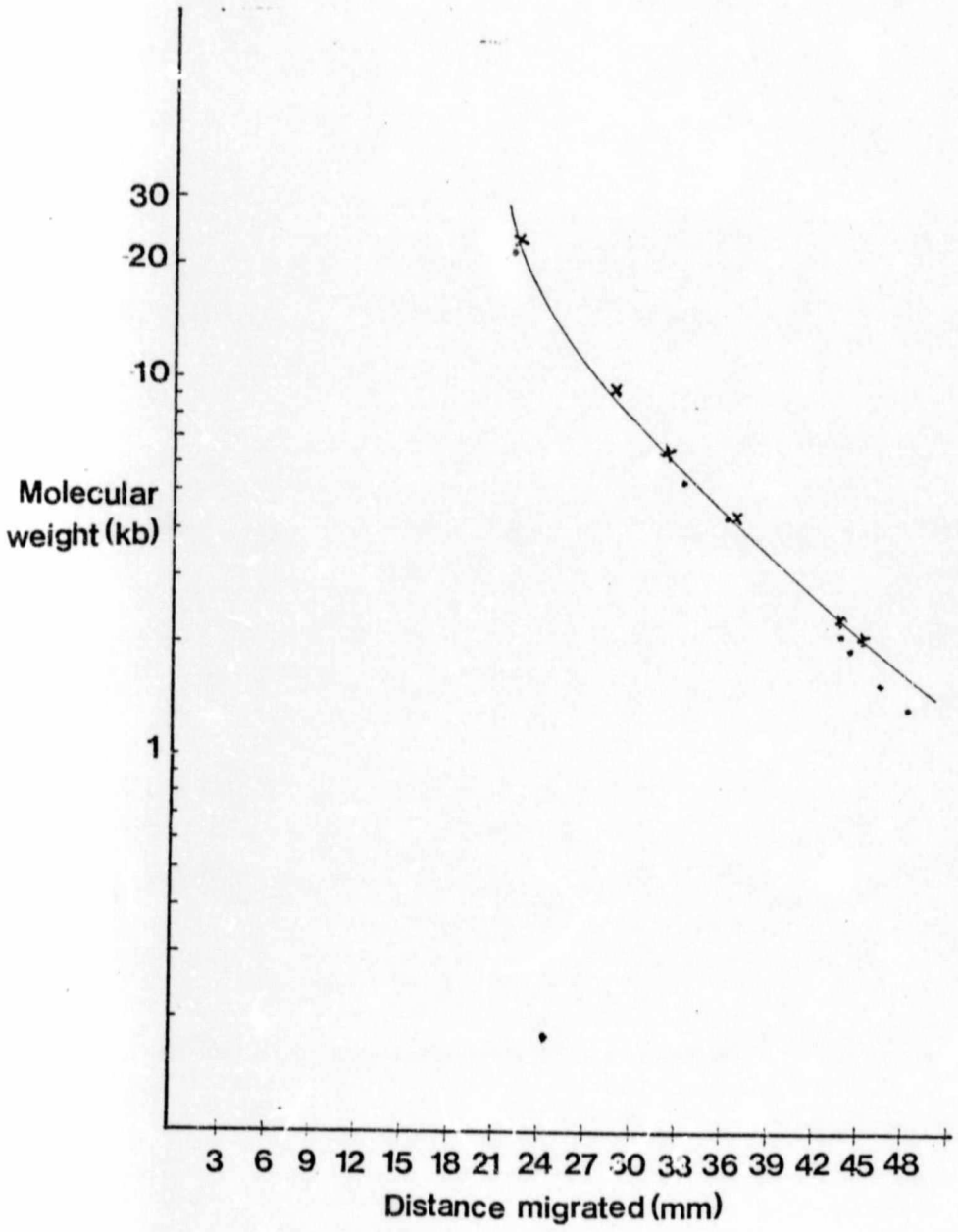

Fig. 3.6 Graph of molecular weight versus distance migrated: Determination of fragment size for EcoRI digestions of the putative shuttle vectors.

x - λII DNA molecular weight marker

· - λIII DNA molecular weight marker

Table 3.6 Size determinations of fragments obtained with EcoR1 digestions of the putative shuttle vectors.

	Putative shuttle vector				pECOR251
	pDA27	pDA28	15	19	
Size of fragments (kb)	7.4 3.0 2.8	8.3 2.8 2.1	4.9 2.8 1.5	4.2 3.8	3.3
Size of vector (kb)	13.2	13.2	9.2	8.0	

3.2.2 Shuttle vectors - expression of arsenic resistance in E. coli

In order to obtain information on the locality of the arsenate/arsenite resistance genes on the nocardioform DNA it was necessary to determine whether nocardioform genes (in particular the arsenic genes) were expressed in E. coli. The shuttle vector, which would express resistance to both ampicillin and arsenic, could then be selected for its ampicillin resistance while the pDA22 region of the shuttle vector could undergo a series of deletions. The arsenic resistance of the shuttle vector would then be determined; if the arsenic resistance has been lost, the restriction map of the reduced plasmid could then be compared with that of the complete pDA22 and the fragment conferring arsenic resistance, determined.

The putative shuttle vectors, pDA27 and pDA28, were replica plated onto LA plates containing ampicillin (50 ug/ml) and various concentrations of arsenate (5mM, 10mM and 15mM) and arsenite (1mM, 3mM and 5mM) to see whether there was any difference in the expression of the arsenic genes in comparison with the pECOR251.

No initial difference was observed between the growth of the putative shuttle vectors and pECOR251 on the 5mM and 10mM arsenate, and 1mM and 3mM arsenite plates. There was a minimal growth on the 15mM arsenate and 5mM arsenite plates. These plates were incubated for longer and individual colonies

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The putative shuttle vectors, pDA27 and pDA28, were replica plated onto LA plates containing ampicillin (50 ug/ml) and various concentrations of arsenate (5mM, 10mM and 15mM) and arsenite (1mM, 3mM and 5mM) to see whether there was any difference in the expression of the arsenic genes in comparison with the pECOR251.

No initial difference was observed between the growth of the putative shuttle vectors and pECOR251 on the 5mM and 10mM arsenate, and 1mM and 3mM arsenite plates. There was a minimal growth on the 15mM arsenate and 5mM arsenite plates. These plates were incubated for longer and individual colonies

that grew up in the areas where the putative shuttle vectors had been spotted were then taken for further analysis in the hope that they were plasmid mutants with increased resistance to arsenate.

In order to determine whether the increased resistance was plasmid conferred, the strains were grown in LB in the absence of any selective pressure (that is, in the absence of ampicillin and arsenate). The "cured" strains were then compared to the parental strains by replica plating on LA plates containing ampicillin (50 ug/ml), and arsenate and arsenite at varying concentrations.

The arsenite resistance appeared to be chromosomally determined - there was no difference in the level of resistance between the parental and the "cured" strains. The arsenate resistance, however, appeared to be plasmid born - the "cured" strain showed decreased resistance as compared to the parental strain.

3.3 Nocardioform transformation: Optimisation of conditions

Preliminary transformation experiments of stationary phase cultures only gave a transformation frequency of 21 transformants per microgram of DNA. This frequency is far from efficient and various conditions were varied in order to optimise conditions for transformation. 100 ng of plasmid pDA30 DNA was used per transformation in each of the experiments described below, unless otherwise stated.

3.3.1 Agitation

Incubation at 37 °C without agitation was shown to increase the transformation efficiency. (Table 3.7).

Table 3.7 Number of transformants obtained with cultures of different optical density with and without agitation.

OD_{540}	Number of transformants	
	with agitation	without agitation
1.110	5	7
2.760	6	13
5.160	4	6
3.370	2	

3.3.2 Growth stage of the culture

This was done in collaboration with S. Andersen and H. Golob.

One millilitre of a stationary phase culture of 01 was diluted into 100 ml TYG. The culture was incubated at 26 °C, and at various intervals, 20 x 1 ml samples were taken and placed in 1.5 ml Eppendorf tubes. The samples were then frozen at -70 °C. One tube from each sampling was then thawed slowly, and transformed with 100 ng of plasmid pDA30. The results are summarised in table 3.8.

Table 3.8 Number of transformants obtained at different stag f growth of the 01 culture.

Time after dilution	OD_{540}	Number of transformants	No. of transformants per ug DNA
14 hours	0.820	0	0
22	1.940	5	30
30	3.330	0	0
38	3.890	20	120
46	4.150	9	90

From the above results it appears that the optimum growth stage for transformation is post-exponential and pre-stationary.

All following optimisation experiments were done with the 1 ml samples taken 38 hours after dilution of the stationary phase culture, that is, with an optical density of 3.890. Only one parameter was changed at a time, otherwise the same procedure was followed as described in Methods and Material 2.4.7.1.

3.3.3 Polyethyleneglycol (PEG) concentration

The amount of PEG added to the samples was adjusted as to vary the concentration. The results of the experiment are shown in table 3.9.

Table 3.9 The number of transformants obtained with various concentrations of PEG.

% PEG (w/v)	Number of transformants	Number of transformants per ug of DNA
0	22	220
10	62	620
20	25	250
25	35	350
30	37	370

From the above results it would appear that 10% PEG (w/v) is the best concentration of PEG. Prolonged incubation of the regeneration plates, however, resulted in the appearance of a large number of smaller colonies. These colonies, when patched onto TYA plates containing 60mM arsenate and 10mM arsenite, grew and are therefore real transformants (no spontaneous mutants are ever found which are simultaneously resistant to both arsenate and arsenite). This biphasic growth of transformants would appear to a be a function of the PEG concentration since it was found that with increasing concentrations of PEG, the number of smaller colonies also increased (table 3.10).

Table 3.10 The number of smaller colonies obtained with different concentrations of PEG.

% PEG	Number of smaller colonies
0	-
10	100
20	240
25	350
30	1000

3.3.4 Length of incubation with lysozmye

The length of time of incubation with lysozyme was varied. The results are shown in table 3.11.

Table 3.11 Table showing number of transformants obtained with varying lengths of lysozyme incubation.

Length of lysozyme incubation	Number of transformants	Number of transformants per ug DNA
0 minutes	0	0
15	25	250
30	29	290
60	51	510
120	38	380

3.3.5 Length of time before underlay

The delay before adding the arsenate and arsenite underlay allows for phenotypic expression of the introduced resistance genes. The length of time before doing the underlay was varied and the results are summarised in table 3.12.

Table 3.12 The number of transformants obtained with increased length of time before arsenate/arsenite underlay.

Length of time before underlay	Number of transformants	Number of transformants per ug of DNA
0 hours	0	0
4	20	200
8	20	200
12	28	280
16*	19	190

* With a 16 hour underlay there was increased background growth.

3.3.6 Amount of DNA

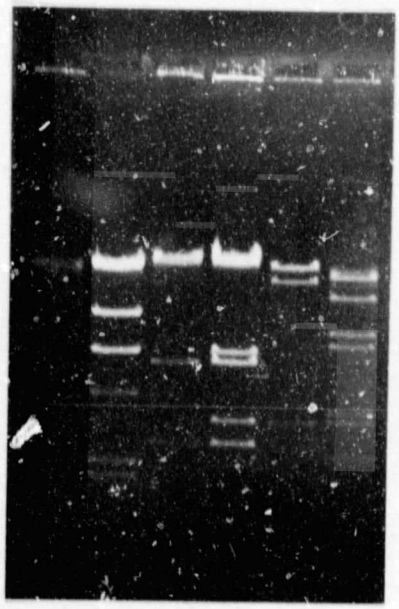
The amount of DNA was trebled, that is, 300ng of DNA was added to the cells. The number of transformants was 101.

3.3.7 Number of cells

One third of the number of cells was used for the transformation. The number of transformants was 10.

3.4 Size determination of Plasmids

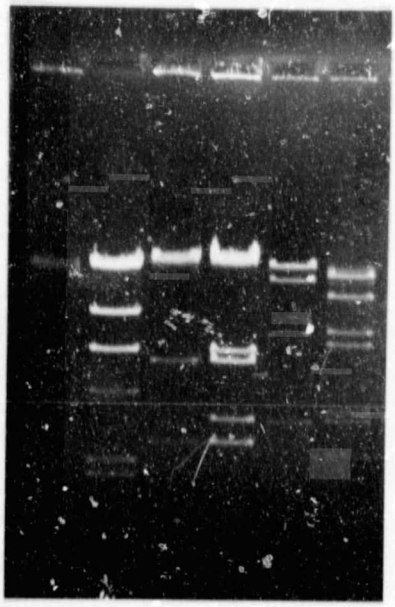
Size determinations were made of the plasmids pDA20, pDA30 and pDA40. pDA20 and pDA40 are large plasmids, size estimated to be approximately 100kb, migrating slower than chromosomal DNA on agarose gels. pDA20 confers resistance to arsenate/aresenite and cadmium chloride. pDA40 confers resistance to arsenate/arsenite and chloramphenicol. The plasmids were digested with various enzymes, the samples were run on agarose gels (plates 3.4 and 3.5) and size determinations were made from the graphs of molecular weight versus distance migrated (figs. 3.7 and 3.8). The results of the size determinations are shown in tables 3.13 and 3.14.



1 2 3 4 5 6

Plate 3.4 0.4% agarose gel of pDA20 digested with various restriction enzymes : Bgl II (lane 3), Bgl II and Not I (lane 4), Not I (lane 5), EcoRI (lane 6). Lanes 1 and 2 are uncut λDNA and λII, respectively.

(The large fragment migrating in lane 4 was taken to be the same size as the large fragment in lane 5 (information obtained from another gel - not shown).



1 2 3 4 5 6

Plate 3.4 0.4% agarose gel of pDA20 digested with various restriction enzymes : Bgl II (lane 3), Bgl II and Not I (lane 4), Not I (lane 5), EcoRI (lane 6). Lanes 1 and 2 are uncut λDNA and λII, respectively.

(The large fragment migrating in lane 4 was taken to be the same size as the large fragment in lane 5 (information obtained from another gel - not shown).

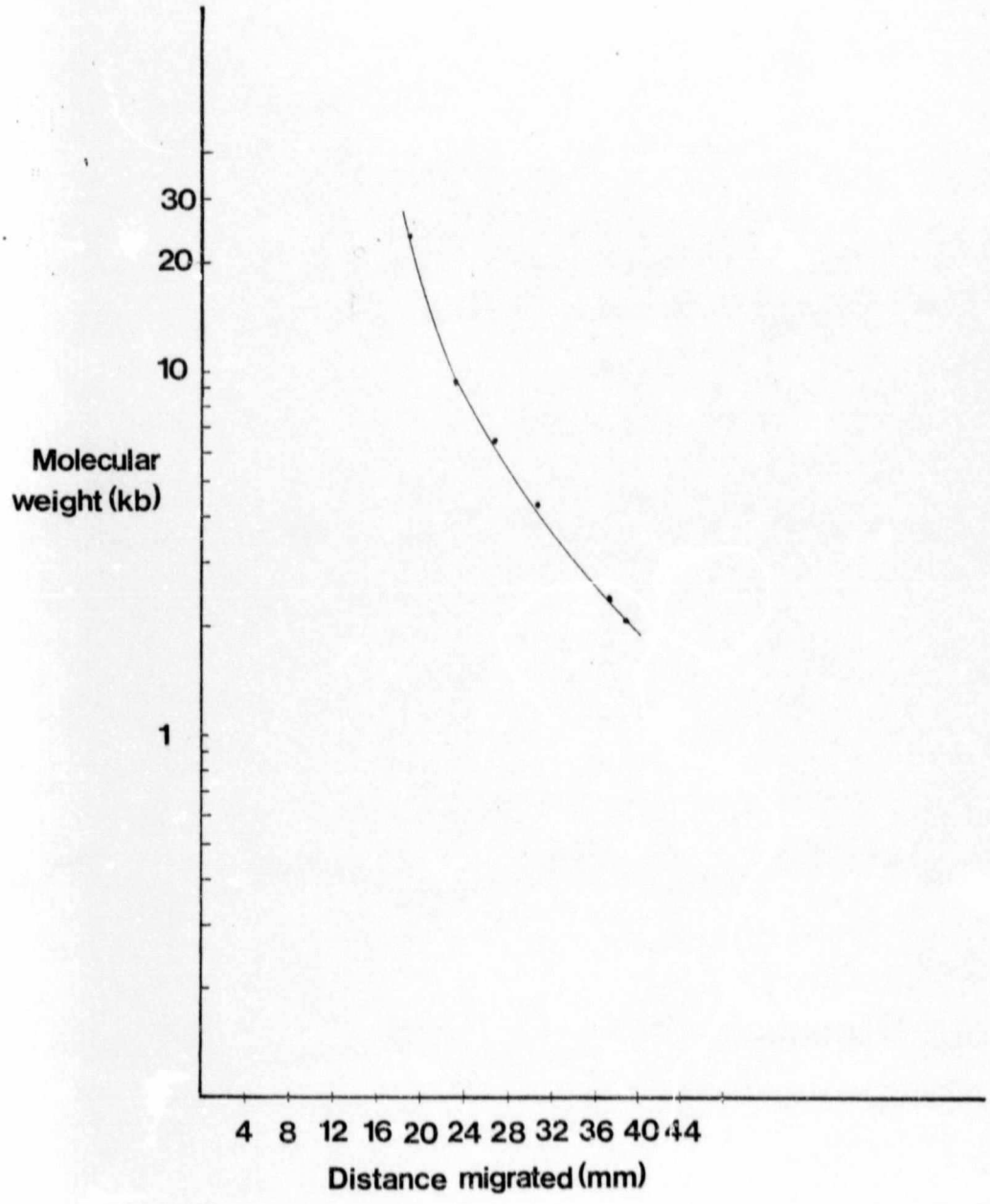


Fig. 3.7 Graph of molecular weight versus distance migrated: Size determination of pDA20.

• - λII DNA molecular weight marker

Table 3.13 pDA20: Size determination of fragments obtained when the DNA was digested with various enzymes

	Restriction enzyme			
	Bgl II	BglI + Not I	Not I	EcoRI
Size of fragments (kb)	? 5.9 2.8	?* 6.6 5.9 3.7 2.8 1.05	?* 22.0 3.7	22.5 18.0 9.1 8.0 4.1 3.8 2.9 1.8
Total (kb)				70.2

? Even though lambda DNA was run on the gel as a size marker, size determination of the large fragments is inaccurate.

* If the two fragments marked with an asterisk are given the value of the size of lambda (+ 48 kb), the sum of the fragments will equal 68kb for the Bgl II + Not I digestion and 73.7 for the Not I digestion.

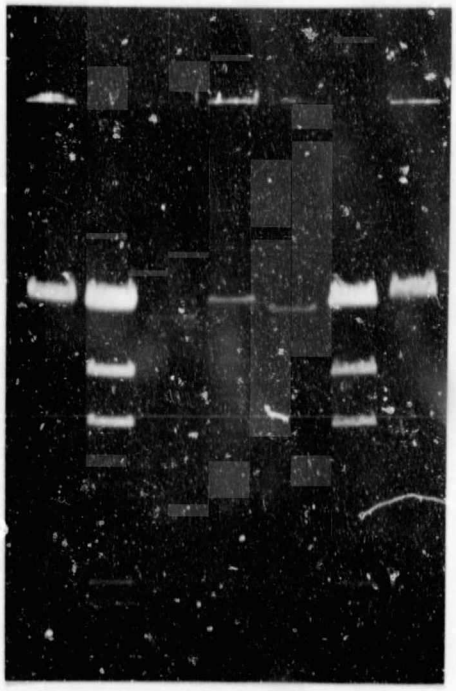


Plate 3.5 0.4% agarose gel of pDA40 digested with various restriction enzymes : EcoRI (lane 3), Bgl II (lane 4), Not I (lane 5).
Lanes 1 and 7 are uncut λDNA, and lanes 2 and 6 are λII.

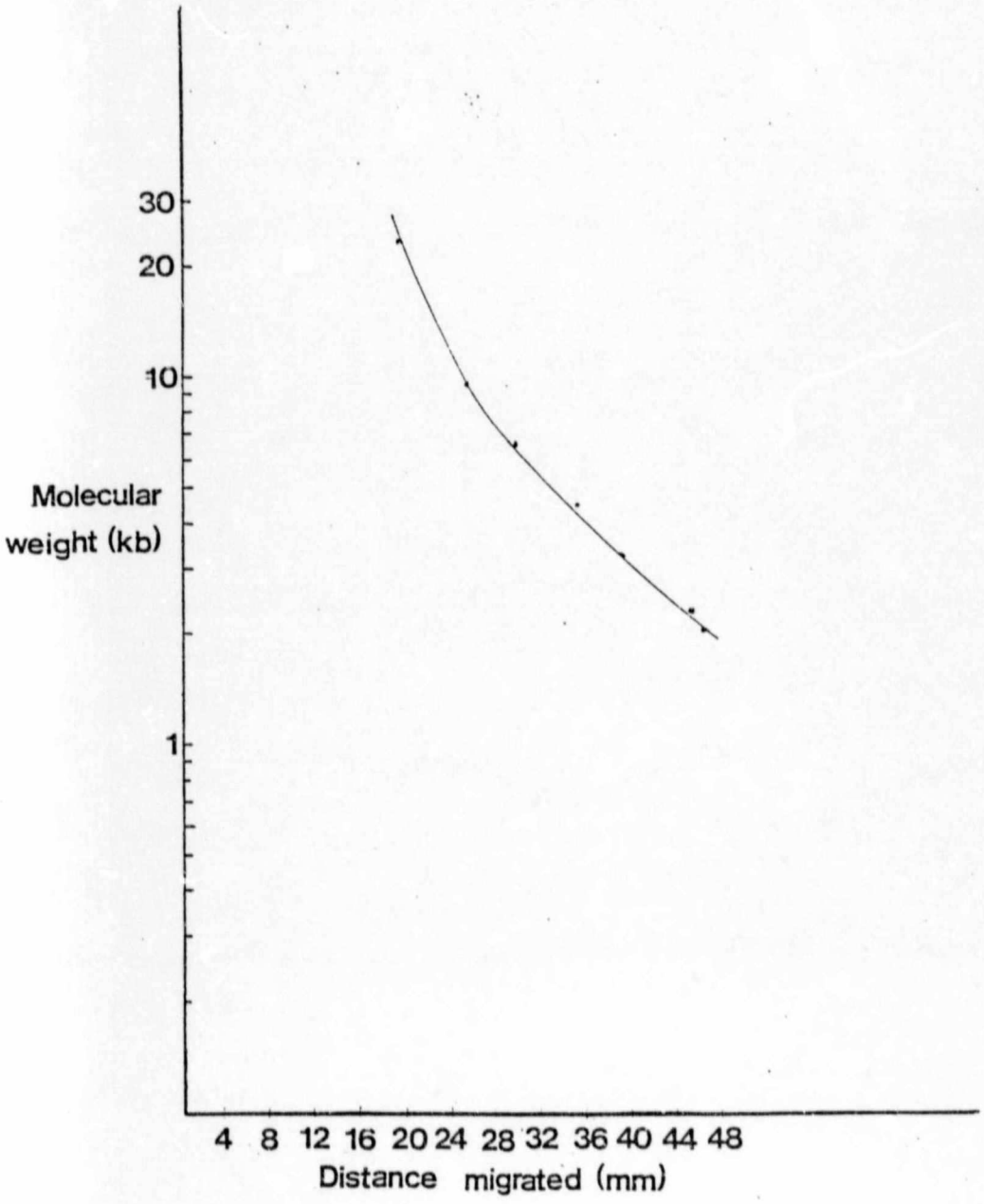


Fig. 3.8 Graph of molecular weight versus distance migrated: Size determination of pDA40.

• - λII DNA molecular weight marker

Table 3.14 pDA4 ize determination of fragments obtained when ' DNA was digested with various enzymes

	Restriction enzyme		
	EcoRI	Bgl II	Not I
Size of fragments (kb)	22.0(?) 18.5 8.0 6.2 5.2 4.5 3.3 2.8	?* 9.8 9.4 6.1 6.1 5.7 5.2 4.2 3.3	23.0(?) 23.0(?) 20.0 4.8 4.3
Total (kb)	70.5		75.1

? Even though lambda DNA was run on the gel as a size marker, size determination of the large fragments is inaccurate.

* If the fragment marked with an asterisk is given the value of the size of lambda (+ 48 kb), the sum of the fragments will equal 102 kb for the Bgl II digestion.

No absolute size determination of pDA30 has been made. Various digestions of pDA30 DNA were done and sizes determined ranged between 14.8 and 11.4 kb.

The size determination of pDA20 suggests that the plasmid is approximately 70 kb. The size of pDA40 is estimated to be approximately 100 kb (Bgl II digestion).

3.5 Reduction in size of Plasmids

Preliminary digestions of pDA20, pDA22 and pDA30 showed that various enzymes were probably suitable for digesting, ligating and then transforming the plasmids into an appropriate recipient, namely, 01 with the aim in mind of reducing the size of the plasmids.

Two micrograms of pDA22 and pDA30 DNA was used per digestion. For pDA20, 5 ug of DNA was used per digestion. Uncut plasmid (100 ng of DNA) was transformed into 01 as a positive control. The results of the digest and ligate experiments are summarised in table 3.15.

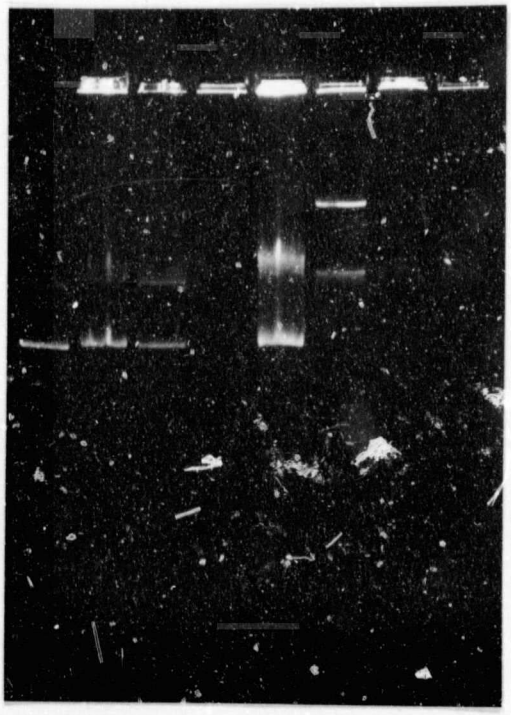
Plasmid screens were carried out on a number of the transformants, listed in table 3.16, to check for the presence of a reduced plasmid (Plate 3.6)

Table 3.15 The number of transformants obtained with the plasmids pDA20, pDA22 and pDA30 digested with various restriction enzymes. Two experiments, 1 and 2, were done.

Plasmid	Restriction enzyme	Number of transformants	
		1	2
pDA20	Uncut	55	120
	Bgl II	21	4
	Not I	2	1
pDA22	Uncut	40	120
	Bcl I	1	-
	Cla I	16	-
	Pst II	3	-
	Pvu II	20	-
pDA30	Uncut	58	38
	BamHl	-	17
	Bcl I	11	-
	Cla I	5	2
	Pvu II	1	4

Table 3.16 Number of transformants checked by plasmid screen for the presence of a reduced plasmid.

	Plasmid		
	pDA20	pDA22	pDA30
Number of transformants checked	3 x Bgl II 1 x Not I	2 x Cla I 2 x Pvu II	5 x BamHl 3 x Bcl I 1 x Cla I 5 x Pvu II



1 2 3 4 5 6 7 8

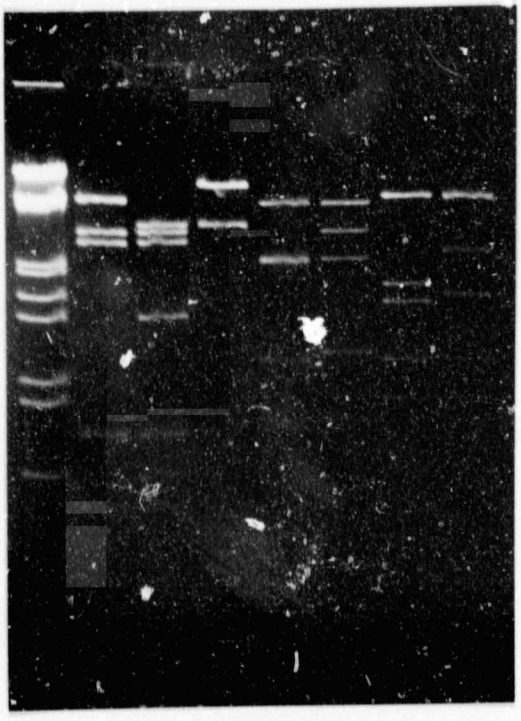
Plate 3.6 0.4% agarose gel of DNA from 1 ml of culture of pDA30 and pDA20 transformants. pDA30 DNA was digested with Bcl I, Cla I, Pst II and Pvu II. pDA20 was digested with Bgl II and Not I. The DNA was religated and transformed into 01. Random transformants were grown in TYG supplemented with arsenate and screened for plasmids that would possibly be reduced in size. Lane 1 is undigested pDA30 DNA. Lanes 2 - 5 were transformants of pDA30 digested with Bcl I, Bcl I, Cla I and Pvu II, respectively. Lane 6 is undigested pDA20 DNA. Lanes 7 and 8 were transformants of pDA20 digested with Bgl II.

The plasmid screens showed either the absence of plasmid or the presence of a plasmid of similar size to that of the undigested plasmid.

For cutting and ligating, it is best to use enzymes which cut the DNA into a maximum of 3 fragments. Pst II and Pvu II cut pDA30 into at least 6 fragments (plate 3.7). The number of combinations of reassociation are large and the chances of obtaining a reduced reassociated plasmid that is viable are greatly minimised, as proved to be the case.

pDA22 may be reduced as much as is possible - any further reduction in size may remove a critical portion of the plasmid.

Additional digestions of pDA22 and pDA30 were done with Bst EII, Mlu I, Nde I, Sph I and Sal I. It appears that Bst EII and Sal I give 3 and 2, and 4 and 3 fragments, respectively. This makes these enzymes very suitable for further attempts at reducing plasmids pDA22 and pDA30 in size.



λIII A B C D E F G

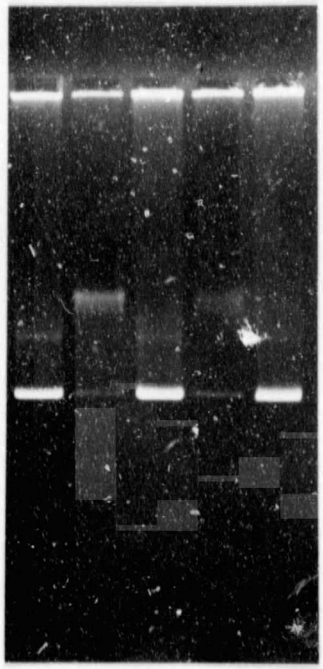
Plate 3.7 1.2% agarose gel of pDA30 DNA digested with A, Pst II; B, Pst II and BamHI; C, BamHI; D, Pvu II and BamHI; E, Pvu II; F, Bcl I and BamHI; G, Bcl I.

3.5 Plasmid Screens

3.5.1 Increasing plasmid copy number

An attempt was made to see whether the plasmid copy number of the nocardioform plasmids could be increased by the addition of antibiotics. A control culture and those with additions were started at the same time under as identical conditions as possible. Chloramphenicol, kanamycin and tetracycline were added to 5 ml cultures in TYG to a final concentration of 100 ug/ml, whereas spectinomycin was added to a final concentration of 1000 ug/ml.

The results of the plasmid screen as seen in plate 3.8 show that no increase in plasmid copy number was obtained, and that, in fact, chloramphenicol and spectinomycin actually tended to decrease plasmid copy number. This is interesting since chloramphenicol is used to increase the plasmid copy number in *E. coli* (Maniatis et al, 1982).



A B C D E

Plate 3.3 0.4% agarose gel of DNA from 1 ml of culture supplemented with A, no addition; B, chloramphenicol; C, kanamycin; D, spectinomycin; E, tetracycline. The plasmid copy number has not increased, and it appears that chloramphenicol and spectinomycin rather decrease the plasmid copy number.

4. DISCUSSION

4.1 Transductants

The transduction experiment of Dabbs and Sole (1988) was repeated in an attempt to obtain plasmids which confer resistance to cadmium chloride or chloramphenicol. The transductants obtained during these experiments had either arsenate or arsenite resistance except for transductant 3A 1, which appeared to have resistance to both. Transductant 3A 1 was also the only transductant with resistance to another compound, namely chloramphenicol. This is unlike the results obtained by Dabbs and Sole (1988) who found that all the transductants obtained had resistance to both arsenate and arsenite as well as to either cadmium chloride or chloramphenicol. It seemed therefore that in my experiments the generalised transducing phage Q4 was packaging smaller pieces of DNA. (The head volume of Q4 phage has been shown to be at least twice that of lambda, therefore it should be able to package at least 100 kb of DNA (Dabbs and Sole, 1988)).

It was necessary to determine whether the resistance conferred by transductant 3A 1 was consistent with the presence of an unstable genetic element. Selection was made for transductants with increased resistance to arsenite. These transductants may be increased plasmid copy number mutants and this would possibly result in the appearance of

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a visible plasmid band on agarose gels. No plasmid band was visible, again in contrast to previous findings.

Dabbs and Sole (1988) found that the arsenate, arsenite, cadmium, and chloramphenicol resistance phenotype of the parental strain ATCC 12674 was very stable. In the absence of positive selective pressure, the phenotype of the transductants was found to be less stable. Under the same conditions, the phenotype of increased copy number mutants was even more unstable. The instability occurred only for the set of resistances identified as plasmid coded. The stability of phenotype of transductant 3A 1 was determined. The phenotype appeared to be stable.

The stability of phenotype and the non-appearance of a plasmid band of the "transductants" with increased resistance seemed to indicate that the resistance was chromosomally determined.

Q4 phage functions not only as the means of introduction of resistance genes back into the cured strain, but also confers Q4 resistance (Dabbs and Sole, 1988). The plasmids obtained by Dabbs and Sole (1988) were chimaeras, with part of their DNA (including resistance determinants) being derived from strain ATCC 12674 and part (including Q4 immunity) from the phage. The ability of the phage to plaque on the transductant strains was determined. If there

was no plaquing it could be assumed that part of the phage (namely, Q4 immunity) was present in the DNA of the transductant. The transductants had no Q4 immunity.

4.2 Development of putative shuttle vectors

One of the requirements for a suitable cloning vector for nocardioform bacteria is a means of selecting fragments cloned into the vector. This requirement can be met by the formation of a shuttle vector using the Escherichia coli suicide vector pECOR251.

pECOR251 is a suitable candidate for joining with pDA22 since it has a single BamHl site outside either the ampicillin gene or the EcoRl endonuclease gene. pDA22 has a single BamHl site which seems to be outside any critical region of the pDA22 genome. The pECOR251 has single Hind III and Bgl II sites within the EcoRI endonuclease gene suitable for cloning chromosomal fragments, as well as providing a means of selection, while pDA22 has no Hind III or Bgl II site.

Eleven putative shuttle vectors were obtained from the ligation of pECOR251 and pDA22. Two were of intermediate size, that is, were between the size of pECOR251 and the ligation product of pDA22 and pECOR251. Of the remaining 9, two were of orientation A and seven were of orientation B. Both orientations were therefore viable. Further studies by

B. Gowan however, showed that pDA27 (orientation A) was more suitable for cloning.

An attempt was made to transform a heterogenous mix of putative shuttle vector pDA27, containing chromosomal inserts in the endonuclease gene, into nocardioform strain 01. No transformants were obtained. The nocardioform DNA probably restricts DNA with the E. coli pattern of modification. The experiment was repeated with an increased amount of DNA in the hope of obtaining at least one transformant which might have arisen because the recipient nocardioform cell was a restriction minus mutant. This transformant could then be cured of its plasmid and used for subsequent transformation experiments.

4.2.1 Expression of arsenic resistance in E. coli

In order to obtain information on the locality of the arsenate/arsenite resistance genes on the nocardioform DNA it was necessary to determine whether nocardioform genes (in particular the arsenic genes) were expressed in E. coli. It was determined that the arsenate resistance was plasmid conferred. The arsenite resistance was, however, chromosomal conferred.

4.3 Nocardioform transformation

The transformation frequency of nocardioform bacteria was greatly improved by optimising various conditions during the transformation procedure.

The optimal growth stage for transformation of 01 appeared to be post - exponential and pre - stationary. The optimal length of incubation of the cells at 37°C in the presence of lysozyme was found to be one hour. The transformation of the protoplasts was found to be more efficient if the incubation was without agitation.

The delay before the arsenate/arsenite underlay allows for the phenotypic expression of the introduced resistance genes. The optimal length of time of delay is between 8 and 12 hours. After 12 hours, the background growth is increased.

The amount of DNA as well as the number of cells seems to influence the number of transformants. With a trebling of the amount of DNA there was almost a three times increase in the number of transformants (100 transformants versus 35). One third of the number of cells was used and resulted in approximately one third of the number of transformants (10 transformants versus 35).

Initially it was indicated that 10% PEG (w/v) was probably the optimal PEG concentration, but with prolonged incubation, a large number of smaller colonies started to appear. With this biphasic growth of transformants 25% PEG was decided upon as the optimal concentration. Transformants were obtained in the absence of PEG and at 30% the PEG did not appear to be toxic. The mechanism by which PEG induces transformation is not known. It may interact with the cell membrane to make it more permeable to DNA. Alternatively, because nucleic acid molecules have been shown to adopt compact forms in solutions containing high concentrations of PEG (Jordan et al., 1972; cited in Bibb et al., 1978), it is possible that the stimulation results from a conformational change in the DNA molecules facilitating penetration into the protoplasts. How the biphasic growth of transformants is influenced by these interactions is not known but it appears that with increased concentrations of PEG, this process is enhanced.

4.3.1 Transformation of ATCC strains 14887 and 4277

Conditions for transformation for ATCC strains 14887 and 4277 were optimised by K. Downing and B. Gowan, respectively. These strains are closely related to ATCC strain 12674 but for some parameters very different conditions were optimal for transformation.

The growth stage of ATCC 14887 that is optimal for transformation is early logarithmic phase, whereas for ATCC 4277 the growth stage best suited for transformation is post stationary phase.

The PEG concentration and length of time of incubation with lysozyme seemed to be similar for all three strains. The length of delay before underlay may be a function of the rate of growth of the different organisms. ATCC 14887, a slower growing organism, was shown to produce more transformants when the underlay was delayed until 18 - 24 hours after spreading onto the plates.

The number of transformants was greater when incubation was done with agi ation for ATCC strains 14887 and 4277, but the opposite was the case for strain 12674 derivatives. The successful production of protoplasts may require more vigorous conditions in strains 14887 and 4277.

The biphasic growth of transformants was not found in ATCC strains 14887 and 4277.

pDA30 DNA from a 01 background was transformed into an agarase mutant of ATCC strain 14887 (this was done in collaboration with K. Downing) and the number of transformants, as compared to the number of transformants obtained by transforming the same DNA into 01, was determined. There was no significant difference in the

number of transformants obtained. This suggests that the restriction of ATCC 12674 DNA by ATCC 14887 is minimal. The two organisms probably have similar modication and restriction systems.

4.4 Size determination of plasmids

The size of the plasmids pDA20, pDA30 and pDA40 was determined. Early estimates of the plasmids pDA20 and pDA40 suggested that they were approximately 100 kb. The sizes determined in this study suggest that pDA20 is approximately 70 kb, pDA30 appears to be about 14 kb and pDA40 is approximately 100 kb.

4.5 Reduction in size of the plasmids

Various enzymes were used to digest the DNA of plasmids pDA20, pDA22 and pDA30 in an attempt to reduce the size of the plasmids with the view of obtaining small plasmids suitable for cloning vectors. Plasmid screens showed no plasmids of reduced size.

The possibility exists that pDA22 and pDA30 have been reduced in size as much as is possible, and that any further reduction in size may remove a critical part of the genome.

The enzymes Bst EII and Sal I appear to be suitable for further attempts at reducing the plasmids pDA22 and pDA30 in size.

4.6 Conclusion

The plasmid pDA22 which confers resistance to arsenate/arsenite was ligated with the E. coli suicide vector pECOR251. The shuttle vector pDA27 obtained from this ligation has successfully been used to clone heterogeneous fragments of chromosomal DNA from Rhodococcus erthropolis ATCC strains 12674, 14887 and 4277 (S. Andersen, H. Golob, K. Downing, B. Gowan). It still has not been successfully transformed into nocardioform bacteria but the possibility exists of obtaining a restriction minus mutant that can be used for further transformation experiments.

APPENDIX

Fragment sizes of DNA molecular weight markers

Fragment length (base pairs)	II	III
23 130	+	
21 226		+
9 416	+	
6 557	+	
5 148		+
4 973		+
4 361	+	
4 227		+
3 530		+
2 322	+	
2 027	+	+
1 904		+
1 584		+
1 330		+
983		+
831		+
564	+	+
125	(+)	(+)

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