Development of an autonomously replicating vector for filamentous fungi.

A thesis submitted for the degree of Doctor of Philosophy at the University of Glasgow

by

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Dedicated to my family and friends for their support and understanding over the last three years.

The research reported in this thesis is my own original work except where otherwise stated and has not been submitted for any other degree.

James Scott Robertson

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Abbreviations.

1. Chemicals

AC - acetate

AMPPD - 3-(2′-Spiroadamantane)-4-methoxy-4-(3′′-phosphoryloxy)-phenyl-1,2-dioxetane

ATP - adenosine triphosphate

BSA - bovine serum albumin

DIG - digoxigenin

DMSO - dimethyl sulphoxide

DNA - deoxyribonucleic acid

DNase - deoxyribonuclease

DTT - dithiothreitol

dATP - deoxyadenosine triphosphate

dCTP - deoxycytidine triphosphate

dGTP - deoxyguanosine triphosphate

dTTP - deoxythymidine triphosphate

dUTP - deoxyuridine triphosphate

ddATP - dideoxyadenosine triphosphate

ddCTP - dideoxycytidine triphosphate

ddGTP - dideoxyguanosine triphosphate

ddTTP - dideoxythymidine triphosphate

EDTA - ethylenediamine tetra-acetate (diNa salt)

EtBr - ethidium bromide

EtOH - ethanol

HCl - hydrochloric acid

ITPG - isopropylthiogalactoside

LiCl - lithium chloride

Na - sodium

NaAc - sodium acetate

NaCl - sodium chloride

NaOH - sodium hydroxide

PEG - polyethylene gylcol

rDNA - ribosomal DNA

RNA - ribonucleic acid

RNase - ribonuclease

SDS - sodium dodecylsulphate

TE - Tris/EDTA (DNA storage buffer)

Tris - tris(hydroxymethyl)aminoethane

X-Gal - 5-bromo-4-chloro-3-indoyl-beta-D-galactopyranoside.

2. Measurements

Ci - Curie(s)

mA - milliamps (10^{-3}A)

bp - base pair

kb - kilobase (1000bp)

kda - kilodalton

^{o}C - degrees Centrigrade

g - centrifugal force equal to gravitional acceleration

g - gramme

mg - milligramme (10^{-3}g)

µg - microgramme (10^{-6}g)

ng - nanogramme (10^{-9}g)

nm - nanometre

l - litre

ml - millilitre (10^{-3}l)

µl - microlitre (10^{-6}l)

M - molar (moles per litre)

mM - millimolar (10^{-3}M)

uM - micromolar (10^{-6}M)

pH - acidity [negative $\log_{10}$(molar concentration H^+ ions)]

V - volts

W - watts

3. Miscellaneous

log - logarithim

dH_2O - distilled water

ss - single stranded (DNA)

ds - double stranded (DNA)

UV - ultra violet light

% - percentage

ARV - automomously replicating vector

TSB - transformation and storage buffer

SCGB - single colony gel buffer

TEM - transmission electron microscope

MW - molecular weight

(w/v) - weight for volume

CCC - closed covalent circle (plasmid DNA)

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Summary.

The plasmid ARp1 contains a sequence called amal; amal appears to confer the ability to replicate autonomously. The amal sequence is an inverted repeat of about 6kb in size and consists of two 3kb arms separated by a 345bp unique region.

The unique central region and one complete arm of the amal insert has been sequenced, (Chapter 3). The amal sequence appears to consist of Aspergillus genomic DNA and rearranged pUC-like DNA. No recognisable ARS sequences were found in amal, but amal does contain A/T rich regions. Northern blots suggest that amal is transcribed not at all frequently.

The involvement of the amal sequence in plasmid transformation frequency, instability and plasmid rearrangements was studied using subclones of ARp1, (Chapter 4). The results suggest that no single identifiable amal region is responsible for either enhancing transformation frequency or plasmid maintenance. The Chapter 4 results indicate that some component of pUC8 plays a role in the autonomous replication of Aspergillus plasmids.

ARp1 plasmid DNA isolated directly from Aspergillus transformants has been photographed using Electron Microscopy techniques, (Chapter 5). The results indicate that ARp1 is present in Aspergillus in monomeric, dimeric, trimeric and

tetrameric forms.

A number of *amal*-like sequences have been identified in an *Aspergillus* cosmid library, constructed by Brody. These sequences have been investigated, (Chapter 6). Cotransformation experiments with some of these *amal*-like sequences suggest that these sequences are capable of promoting autonomous replication.

The ARp1-derived *amal* sequence was used as a probe to isolate similar sequences from *Penicillium chrysogenum* and *Cephalosporium acremonium*, (Chapter 7). Three such sequences were isolated from *Penicillium*. These sequences were called *pam1a*, *pam1b* and *pam2*. Transformation experiments with these sequences suggest that these sequences are capable of promoting autonomous replication in both *Penicillium* and *Aspergillus*. Similarly, ARp1-derived *amal* subclones give rise to autonomously replicating plasmids in *Penicillium*.

ARp1-derived *amal* subclones were used to clone the *argB*, *niaD* and *nirA* genes from wild type *Penicillium* via cotransformation with fragmented genomic DNA, (Chapter 8).

This technique has been called The Instant Gene Bank, (Gems, 1990). Fungal transformants grown on selective medium were obtained, but in all cases it was not possible to isolate plasmid DNA containing the genes of interest.

Chapter 1.

Introduction.

INTRODUCTION.

1.1 General fungal transformation.

Transformation can be defined as the process by which naked DNA is introduced into a recipient cell. Much of the work on transformation in fungi is based on the yeast Saccharomyces cerevisiae. The transformation systems developed for Saccharomyces cerevisiae and the behaviour of plasmids within this yeast are the models on which much of the work on Aspergillus and Neurospora are based. Transformation systems have been developed for: Aspergillus nidulans, Ballance and Turner (1985), Beri et al (1988), Gems et al (1991), Tilburn et al (1983) and Wernars et al (1985); Neurospora crassa, Paietta and Marzluf (1985), Dhawale and Marzluf (1985); Penicillium chrysogenum, Cantoral et al (1987), Beri and Turner (1987) and Bull et al (1988); Cephalosporium acremonium, Whitehead et al (1990) and Skatrud et al (1987). These transformation systems have opened up new avenues of research e.g. it is possible to study gene structure, function and regulation and also clone genes by transformation e.g. Birse and Clutterbuck (1991).

1.2 Approaches to transformation.

1.2.1. Cell preparation.

The first step in most protocols for transforming fungi is the removal of the complex cell wall because the cell wall acts as a barrier to entry of DNA into the recipient cell. Digestion of the cell wall is accomplished enzymatically by using a complex mixture of glucanases and chitinase to produce protoplasts/sphaeroplasts. After digestion, the protoplasts have a semi-permeable membrane which in the presence of PEG, allows entry of the transforming DNA under certain conditions. During both protoplasting and subsequent transformation it is essential to have an osmotic stabiliser present. Commonly used osmotic stabilisers include inorganic salts e.g. $MgSO_4$, sugars e.g. sucrose and sugar alcohols e.g. sorbitol, Peberdy (1979).

There are at least four recognisably different ways of introducing transforming DNA into fungal cells. The most common method involves incubating protoplasts with the transforming DNA in the presence of $CaCl_2$ or LiCl; PEG is added resulting in protoplast fusion and DNA uptake.

Ward et al (1989), have used electroporation to transform A.awamori. Electroporation involves applying an electrical pulse to a protoplast suspension. This pulse appears to produce transient

pores in the protoplast wall allowing entry of the transforming DNA.

Armalo et al (1990), have transformed both yeast and N.crassa using biolistic transformation. This procedure entails coating tungsten microprojectiles with the transforming DNA and then "firing" these projectiles into either prepared conidia or stationary phase yeast cells.

Zucchi et al (1989), have developed an RNA mediated transformation system termed Retrotransformation.

Two additional transforming protocols which do not rely on protoplasting have been developed for yeast. Costanzo and Fox (1988), have transformed yeast cells by agitating such cells with glass beads, in the presence of plasmid DNA, using a vortex mixer. This method is not as efficient as the protoplasting methods. Ito et al (1983), have obtained good transformation yields using Lithium acetate.

It is possible to transform fungal cells using both integrative plasmids and autonomously replicating plasmids.

1.2.2. ARS containing plasmids.

DNA can be introduced into a fungal cell by an autonomously replicating vector (ARV). Such plasmids are capable of replicating independently of the genome and so be present in a high copy number e.g there may be up to 100 copies of the 2u plasmid in a haploid yeast cell.

Autonomously replicating vectors contain elements called ARS or autonomously replicating sequences. Chan and Tye (1980), estimated that in yeast, ARS sequences are present in the genome every 30-40kb. Various ARS elements have been characterised in yeast. Each yeast ARS consists of an AT rich core sequence which is essential for ARS function, and flanking regions which are required for efficient ARS function. The core regions so far described have the consenus sequence: 5'A/TTTTATPuTTT(A/T)3', Kearsey (1984). The flanking regions do not appear to fit into a recognisable consensus sequence. Bouton (1986), suggested that it is the secondary structure of the ARS flanking regions which are important for autonomous replication, rather than specific DNA sequences.

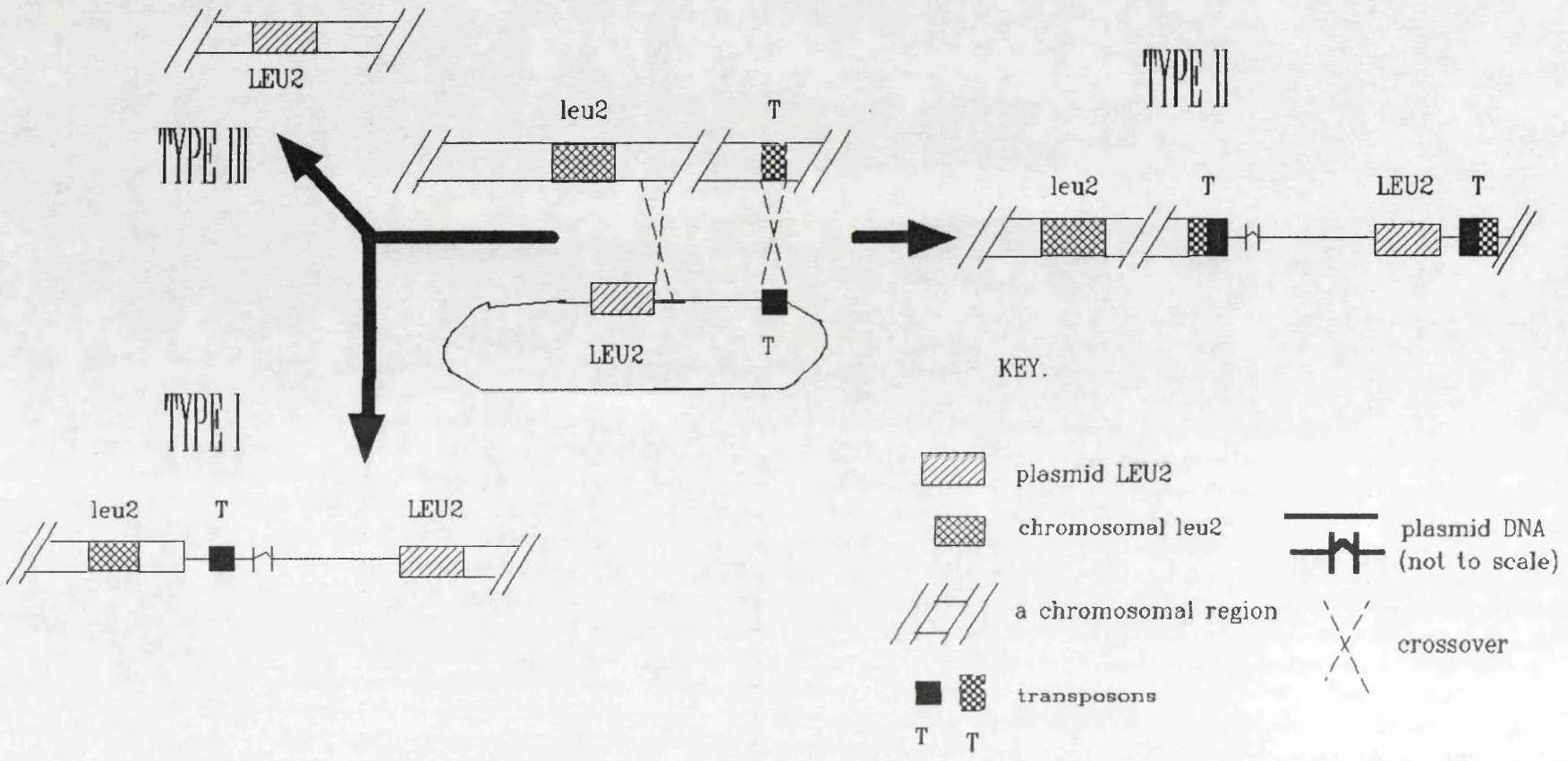

Figure 1.1: Three distinct integration events. Type I events result in the integration of the complete plasmid so two copies of the leu2 gene are present. Type II events also result in a second integrated copy of the leu2 gene in a non-homologous site, leaving the genomic leu2 locus undisturbed. Type III events lead to a simple replacement of the genomic leu2 with the plasmid-borne LEU2.

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1.2.3. Integrative plasmids.

In comparison, integrative plasmids are not capable of autonomous replication but such plasmids are much more stable than ARVs. This stability is a consequence of the vector integrating into the genome. Integrative transformation is the most common outcome of transformation in the filamentous fungi.

Hinnen (1978), characterised three distinct types of integration event (figure 1.1) in a leu2 Saccharomyces cerevisiae mutant, using a plasmid carrying the LEU2 gene which would complement the mutant gene, so that transformed cells would be LEU^{+}.

Type I events involve a crossover between the homologous genomic leu gene and the plasmid-borne LEU gene. This crossover event results in the plasmid being integrated into the chromosome in such a way that the plasmid DNA is flanked by both copies of the leu gene. Type I events are also known as homologous additive integration events.

Type II or ectopic integration events were thought to involve crossovers between non-homologous regions since the plasmid was found to be integrated at sites other than the resident leu gene. However, this may no longer be true. Andreadis et al (1982) have demonstrated that homologous crossovers between transposon elements, present in both the plasmid and Saccharomyces cerevisiae genome, are responsible for

the apparent ectopic integration events. No transposons have yet been identified in A.nidulans so Type II integration events are probably due to interactions between genuinely non-homologous DNA.

Type III events involve gene replacement i.e leu to LEU but there is no permanent integration of the plasmid. Type III transformants may result from either a double crossover event, or gene conversion, or integration followed by excision of the plasmid from the chromosome, leaving the transforming copy of the selectable marker behind.

In the filamentous fungi, all three of the integration patterns mentioned above have been identified. Selker (1987), demonstrated type I integration in Neurospora crassa. Nonhomologous integration (type II) appears to be the most common in Neurospora; Paietta (1985), demonstrated that 90% of Neurospora transformants in one experiment were due to type II integration. In Aspergillus, type I integration is the most prevalent. Upshall (1986), showed that 17 out of 33 Aspergillus transformants were the result of type I integration. Type II integration in Aspergillus was observed by Tilburn (1983), using the amdS (acetamidase) gene. Other workers have found evidence for Type II events: Ballance and Turner (1985), have shown that although A.nidulans DNA has low homology with the N.crassa pyr-4 gene it is possible to obtain integrative transformants with these DNAs. Both Upshall (1986)

and Johnstone (1985), showed that some Arg^+ transformants resulted from gene conversion/Type III events.

1.3 Transforming plasmids in yeast.

Various integrative and replicative plasmids have been constructed in yeast, Gunge (1983) and Volkert *et al* (1989). The yeast integrative plasmids (YIp) are based on the bacterial ColE1 plasmid. The transformation frequencies of YIp plasmids were initially low but were increased by the inclusion of rDNA in the vector and by transforming with linearised plasmid.

Yeast episomal plasmids (YEp) are based on the endogenous, autonomously replicating 2μ plasmid and give a high frequency of transformation; about 10,000 colonies/μg DNA. The yeast 2μ plasmid is discussed in detail in Chapter 4.

Yeast plasmids were also constructed which contain yeast genomic ARS sequences, such plasmids were named Yeast replicating plasmids (YRp). ARS sequences have been isolated from various genes e.g. *ura1*, Sakaguchi and Yamamoto (1982) and *trp1*, Struhl *et al* (1979) and from yeast mitochondrial regions e.g. *ori6*, Delouya and Nobrega (1991).

Plasmids containing ARS sequences give up to a thousand-fold increase in the frequency of transformation in yeast when compared to integrative

vectors. Both YEp and YRp plasmids are unstable i.e. the plasmids are inherited at low frequency by daughter cells. In the case of YRp plasmids this instability is due partly to preferential segregation of the plasmid into the mother cell at budding; probably due to the ars sequence temporarily binding to the nuclear scaffold, Conrad and Zakian (1989) and Amati and Gasser (1988). The mitotic stability of ARVs has been increased by cloning centromeric (cen) sequences such as CEN3 and CEN11 into the YCp plasmids (yeast centromeric plasmids) so that the plasmid acts as a minichromosome i.e. it is mitotically stable and segregates properly at mitosis and meiosis, Fitzgerald-Hayes et al (1982).

The inclusion of ARS sequences, centromeres and telomeres in yeast plasmids has led to the development of Yeast Artificial Chromosomes (YAC) which have been used to make libraries of complex genomes, Schlessinger (1990).

1.4 Transforming plasmids in the filamentous fungi.

Attempts have been made to construct YRp like plasmids i.e. autonomously replicating plasmids in N.crassa. Stohl and Lambowitz (1983), successfully inserted a mitochondrial ARS (from strain p405-labelle) into a plasmid (pBR322) containing the Neurospora ga2 gene. Subsequent transformation of a

Neurospora intermedia species resulted in a five- to ten-fold increase in the frequency of transformation. It is not entirely clear that the mitochondrial ARS is responsible for the increase in transformation frequency because of the difficulties involved in recovering the inserted DNA from Neurospora. In contrast, Paietta and Marzluf (1985), have identified BamH1 restriction fragments from chromosomal material which appear to have ARS function, although there is no substantial increase in the frequency of transformation with these chromosomal ARS fragments.

To date, no endogenous autonomously replicating plasmids have been isolated from Aspergillus nidulans that could be used as a basis for genetically engineered ARVs. Reddy et al (1991), have isolated an endogenous, autonomously replicating plasmid, designated pME, from the lignin-degrading Basidiomycete Phanerochaete chryosporium.

Initially, attempts to generate ARVs for use in Aspergillus nidulans met with little success. Ballance and Turner (1985), isolated a sequence designated ans1 which increased the transformation frequency of a pyr^{+} (pyrimidine) transforming plasmid by fifty to one hundred fold. Examination of DNA isolated from transformants showed that the transforming DNA containing the ans1 sequence had integrated into the genome. It was therefore concluded that the ans1 sequence was enhancing the frequency of integration and was not acting as a

typical ARS sequence. Cullen et al (1987), used ans1 in cotransformations selecting for argB, trpC and pyr-4 and found that ans1 increased the frequency of transformation. Sequencing of part of ans1 showed that it was A-T rich and contained an 11bp sequence identical to the yeast ARS sequence. The data presented in this paper suggests that ans1 either promotes integration at non-related sequences between the genome and the plasmid or else it acts as an ARS for a short time, thereby increasing the transformation frequency, after which integration takes place.

At the time of writing, ARS sequences have been isolated from various fungi: Mucor cincinelloides, Roncero et al (1989), Ustilago mydis, Tsukuda et al (1988) and Phanerochaete chryosporium, Randall et al (1991).

1.5 Project aims and background.

The development of an autonomously replicating vector for use in the filamentous fungi is of some interest to Biotechnologists. Such vectors result in an increase in transformation frequency and may be present within the host cells in high copy number (a definite advantage over integrative plasmids). High copy number results in the amplification of the cloned DNA sequence and so potentially increased yield of the gene product which could be an antibiotic,

fungicide, human interleukins etc. One potential drawback with using an ARV is that autonomously replicating plasmids are significantly less stable than integrative vectors. Instability is characteristic of autonomously replicating plasmids; transformant colonies containing such a plasmid grow slower under selective conditions and can display a different morphology during the initial stages of growth when compared to colonies containing an integrative plasmid.

The research described in this thesis details the behaviour and characterisation of an autonomously replicating plasmid called Aspergillus Replicating plasmid 1 or ARp1. The plasmid ARp1 contains an Aspergillus genomic-derived DNA sequence called ama1 (Automomous Maintenance in Aspergillus), the A.nidulans argB gene and pUC8. ARp1 was originally isolated from a slow-growing Aspergillus nidulans transformant by Johnstone (1985). Johnstone constructed an Aspergillus gene bank in the integrative vector pILJ16 which contains only the A.nidulans argB and pUC8. Johnstone reasoned that if Aspergillus contained ARS sequences then such sequences would be represented in the gene bank. Therefore, transformation of Aspergillus with the gene bank, followed by selection for argB, identifying and then testing unstable transformant colonies allowed him to isolate an ARS-containing plasmid.

Once ARp1 was isolated, Johnstone (1985) and subsequently Gems (1990) demonstrated that ARp1 transformed an argB- strain to argB+ at a frequency of up to 100-fold greater than pILJ16. Approximately 65% of asexual progeny were ARG$^-$, so ARp1 is unstable. Southern blots showed clearly that ARp1 had not integrated into the genome. Further work by Gems (1990), showed that ARp1 was present as monomer, dimer and possibly higher forms. The copy number for ARp1 was estimated at 10 per transformed nucleus. Gems (1990), also showed that ARp1 could transform both Aspergillus oryzae and Aspergillus niger at a higher frequency than pILJ16.

The only conclusion that explains these observations is that ARp1 is an autonomously replicating vector.

However, ARp1 does not seem to be unique: Wright and Philipson (1991), have developed a plasmid called pAG-1 which appears to replicate autonomously in the filamentous ascomycete fungus Ashbya gossypii. The plasmid contains the S.cerevisiae ARS1 and the 2uARS autonomously replicating sequences. Initial analysis shows that pAG-1 transforms at a higher frequency than the original, non-ARS containing integrative vector. The plasmid is also unstable but no figures for plasmid instability are quoted in the paper. Southern analysis has also shown that pAG-1 does not integrate into the genome and that it is present in monomer, dimer and possibly higher forms.

There are clearly many similarities between ARp1 and pAG-1: increased frequency of transformation, plasmid instability and structural forms. The most apparent difference is that pAG-1 contains yeast ARS sequences; ARp1 contains a piece of Aspergillus genomic DNA with ARS-like properties.

Both ARp1 and pAG-1 underline both the importance and the potential of ARVs for the filamentous fungi. The results presented in this thesis provide the necessary groundwork for the development and possible applications of ARp1 and ARp1-derived autonomously replicating vectors.

Chapter 2.

Materials and methods.

2.1 List of materials.

Material	Source
General chemicals and organic solvents	B.D.H., Hopkins & Williams Koch-Light Laboratories, May and Baker.
Acrylamide/Bisacrylamide	Sigma.
Media	Davies, Oxoid.
Agar	Davies, Difco.
Biochemicals	Sigma.
Antibiotics	Sigma.
Agarose	Sigma.
Radiochemicals	New England Nuclear.
ECL kit	Amersham.
Hybond-N Nylon Membrane	Amersham.
Random priming kit	Boehringer Mannheim.
DIG Luminescent Detection kit	Boehringer Mannheim.
Prepagene kit	Bio-Rad.
Sequenase kit	United States Biochemical.
Frozen competent E.coli cells	Stratagene.
Gigapack II Gold Packaging extract	Stratagene.

All enzymes were obtained from Gibco BRL and Promega except the following:

NovoZym 234. Novo Biolabs.

2.2 Bacterial Strains.

All strains used were derivatives of E.coli K-12.

Name	Genotype	Source.
DS941	recF143, proA7, Str31, thr1, leu6, tsx33, mtL1, his4, argE3, lacY1, galK2, ara14, lambda$^-$, lacI^q, lacZ M15, lacY$^+$.	Hori and Clarke 1973.
SURE	(hsdRMS), mcrA, mcrB, mrr, endA1, supE44, thi-1, -, gyrA96, relA1, lac-, recB, sbcC, umuC, uvrC, [F', proAB, lacIqz M15, Tn10, (tetr)].	Stratagene catalogue.
XL-Blue	recA-, (recA1, lac-, endA1, gyrA96, thi, hsoR17, supE44, relA1, {F', proAB, lac19, lacZ m15, Tn10})	Bullock et al 1987

2.3 Plasmids and Bacteriophage.

The list includes those plasmids and bacteriophages whose construction is not described in this thesis. All selectable markers denoted with a * are fungal markers:

Plasmid	Description	Selectable marker	Reference
pUC8	Derived from pBR322	$\underline{amp}^R$	Vieira & Messing, 1982.
pBLUESCRIPT II KS^+	M13-based sequencing vector	$\underline{amp}^R$	Stratagene catalogue.
pILJ16	integrative vector	<u>argB</u>*	Johnstone <u>et al</u> 1985.

Plasmid	Description	Selectable marker	Reference
ARp1	ARV	<u>argB</u>*	Johnstone 1985a.
pY184	pBLUESCRIPT trimer	<u>ampR</u>	Johnstone 1992.

pILJ20, 23 and 25	subclones of ARp1	argB*	Johnstone 1985a.
pDHG24, 25	subclones of ARp1	argB*	Gems 1990.
0 pHELP1	subclone of ARp1	none	Gems 1990.
pMW14	integrative vector	oli^R*	Ward 1984.
pIH4	integrative vector	hyg^R*	GlaxoChem.

Bacteriophage	**Description**	**Reference.**
EMBL3	Lambda replacement vector	Frischauf et al 1983.
M13mp18, M13mp19	sequencing vectors	Yanisch-Perron et al 1985.

2.4 Fungal strains.

All Aspergillus strains used in this work were either from the Glasgow stocks (Clutterbuck, 1974) or from A.J.Clutterbuck's personal collection. Both the Penicillium and Cephalosporium strains were gifts from GlaxoChem.

Strain	Genotype.
A.nidulans	
G34	yA2; methH2; argB2.
G833	yA2; pyroA4; nirA1.
G0125	biA1; niaD17.
G051	biA1.
P.chrysogenum	
V992 CMI40233	niaD19
NRRL1951	wild type.
C.acremonium	
M8650	wild type.

2.5 E.coli Culture Media.

LB-Broth: 10g tryptone, 5g yeast extract, 5g NaCl, 1g glucose, 20mg thiamine, made up to 1 litre in distilled water and adjusted to pH7.0 with NaOH. For phage work 10mM $MgSO_4$ was added to LB-broth.
Solid L-agar: as LB-Broth with the addition of 12g N^{O}.3 Oxoid agar.

BBL agar: 10g trypticase peptone, 5g NaCl, made up to 1 litre in distilled water, adjusted to pH7.2 with NaOH, then add 10g Taiyo agar.

BBL agarose overlay: as for BBL agar but with the addition of 2.5g $MgSO_4.6H_2O$ and 6.5g of type 1 low EEO A6013 agarose.

TSB broth: as for LB-broth but with the addition of 10% PEG (MW=3,350), 10mM $MgCl_2$, 10mM $MgSO_4$, adjusted to pH6.1. Filter sterilised 5% DMSO is added after autoclaving.

2.6 Fungal Culture Media.

The following media were used to grow Aspergillus, Penicillium and Cephalosporium.

Liquid Minimal Media (LMM): 10g glucose, 2g $NaNO_3$, 10mls -CN solution, 1ml Trace Elements Solution, made up to 1 litre with distilled water.

Solid MM: as LMM but with the addition of 12g Tayio agar.

-C and -N versions of both LMM and solid MM were made up with the exclusion of glucose and $NaNO_3$ respectively.

Complete Media (CM): as for MM plus 2g peptone, 1g Yeastrel, 1.5g Casein Hydrolysate, 1ml Vitamin Solution.

Nitrogen free Sucrose osmotically stabilised media (SOS-N): 342g sucrose, 10mls -CN solution, 1ml Trace Element Solution, 20g Difco agar, made up to 1 litre with distilled water. 0.7% top agar made up as for SOS-N but with 7g Difco agar.

-CN Solution: 140g KH_2PO_4, 90g $K_2HPO_4.3H_2O$, 10g KCl, 10g $MgSO_4$, made up to 1 litre with distilled water.

Trace Element Solution: 40mg $Na_2B_4O_7.10H_2O$, 400mg $CuSO_4$, 800 mg $FeSO_4$, 800 mg $MnSO_4.4H_2O$, 800mg $NaMoO_4.2H_2O$, 8g $ZnSO_4$, made up to 1 litre with distilled water.

Vitamin Solution: 1g riboflavin, 1g nicotinamide, 0.1g p-amino benzoic acid, 0.5g pyridoxine HCl, 0.5g aneurine HCl, 10mg biotin, made up to 1 litre with distilled water.

Media Supplememts.

Only those supplements required by fungal strains used in this project are listed.

Supplement	Final Concentration.
ammonium	5.0mM
biotin	0.04µg/ml
glucose	1%
L-arginine	1.0mM
L-methionine	1.0mM
nitrate	10.0mM
pyridoxine HCl	0.05µg/ml

2.7 Sterilisation.

All growth media were sterilised by autoclaving at 120°C for 15 minutes. Some supplements and buffers were autoclaved at 108°C for 10 minutes. Both Oligomycin and Hygromicin were filter sterilised using a 0.22µm filter, taking the necessary safety precautions when handling poisons.

2.8 Buffer Solutions.

Electrophoresis.

10x TBE buffer: 109g Tris, 55g H_3BO_3, 9.3g $Na_2EDTA.2H_2O$, made up to 1 litre with distilled water, pH to 8.3.

10x TAE buffer: 48.8g Tris, 3.6g NaAc, 3.6g $Na_2EDTA.H_2O$, made up to 1 litre with distilled water, pH to 8.2 with acetic acid.

Single Colony Gel Buffer: 2% Ficoll, 1% SDS, 0.01% bromophenol blue, 0.01% orange G, in 1x TAE buffer.

Final Sample Buffer: 10% Ficoll, 0.5% SDS, 0.06% bromophenol blue, 0.06% orange G, in 1x TAE buffer.

DNA Manipulation.

All restriction and ligation buffers were obtained from Gibco BRL and Promega.

ATP stock solution: dissolve 60mg ATP in 0.8ml distilled water, pH to 7.0 with 0.1M NaOH, make volume up to 1ml with distilled water; store at $-20^{o}C$.

TE Buffer: 10mM Tris, 1mM EDTA, pH to 7.0.

DNA Hybridisation.

Random prime reaction buffer: Boehringer Mannheim.

Hybridisation solution: 11.4 mls distilled water, 0.3mls 10% SDS, 3mls SSPE.

20x SSC: 3M NaCl, 300mM Na_3Cit, pH to 7.0.

20x SSPE: 3.6 NaCl, 200mM NaH_2PO_4, 20mM EDTA, pH to 7.4.

Denaturing solution: 1.5M NaCl, 0.5M NaOH.

Neutralising solution: 1.5M Tris, 1mM EDTA, pH to 7.2.

Pall Blot-wash: 0.2% SDS, 1mM EDTA, 5mM NaH_2PO_4.

DIG hybridisation solutions.

Buffer 1: 11.6g maleic acid, 8.7g NaCl, pH to 7.5 with concentrated NaOH, distilled water to 1 litre.

Blocking stock solution: blocking agent, 10% (w/v) in buffer 1. Store at $4^{o}C$.

DIG Hybridisation buffer: 10mls formamide, 5mls 5x SSC, 400µls of blocking stock solution, 20uls 10% N-lauroylsarkosine, 4µls 10% SDS, distilled water to 20mls.

Washing buffer: 0.3% (w/v) Tween-20 in buffer 1.

Buffer 2: blocking stock solution diluted 1:10 in buffer 1.

Buffer 3: 0.1M Tris-HCl, 0.1M NaCl, 0.05M $MgCl_2$, pH to 9.5.

AMPPD stock solution: 10mg/ml; 23.5mM.

AMPPD substrate solution: stock solution freshly diluted 1:100 in buffer 3. Stored in the dark at $4^{o}C$, can be reused upto 5-6 times.

DNA extraction, purification and general purpose.

Phenol: all phenol used in the purification of DNA contained 0.1% 8-hydroquinoline and was buffered against 0.25M Tris, pH 8.0.

Chloroform: a mixture of chloroform and isoamyl alcohol (24:1) was used to reduce foaming during extraction and improve phase separation of the aqueous and organic phases.

SM Buffer: used for bacteriophage storage and dilutions, 5.8g NaCl, 2g $MgSO_4$, 2% gelatin, 1mM Tris, pH to 7.5 in total volume of 1 litre of distilled water.

Birnboim Doly Buffer I: 50mM glucose, 25mM Tris, 10mM EDTA, pH to 8.0.

Birnboim Doly Buffer II: 0.2M NaOH, 1% SDS, make up fresh before use.

Birnboim Doly Buffer III: 3M KAc pH4.8, mix equal volumes of 3M CH_3COOK and CH_3COOH

Transformation.

(i) Fungal

ATB: 1.2M sorbitol, 10mM $CaCl_2$, 10mM Tris pH to 7.5.

APB: 1.2M $MgSO_4$, 5mM B-mercaptoethanol, 2mg/ml BSA, 10mM phosphate buffer, pH to 5.6.

(ii) <u>E.coli.</u>

TfbI: filter sterilised, 30mM potassium acetate, 100mM $RbCl_2$, 10mM $CaCl_2$, 50mM $MnCl_2$, 15% (v/v) glycerol, pH to 5.8 with acetic acid.

TfbII: filter sterilised, 10mM MOPS, 75mM $CaCl_2$, 10mM $RbCl_2$, 15% (v/v) glycerol, pH to 6.5 with KOH.

TSB: as described in section 2.5.

DNA nested deletion series buffers.

10x S1 buffer: 1.1mls 3M KOAc, pH4.6, 5mls 5M NaCl, 5mls glycerol, 30mg $ZnSO_4$.

Exonuclease III buffer: 66mM Tris/HCl, pH 8.0, 0.66mM $MgCl_2$.

S1 mix: 172µls distilled water, 27µls 10x S1 buffer, 60 units S1 nuclease.

S1 stop solution: 0.3M Trizma base (no HCl), 0.05M EDTA, pH 8.0.

Klenow mix: 3µls 0.1M Tris/HCl, pH 8.0, 6µls 1M $MgCl_2$, 20µls distilled water, 3 units Klenow fragment.

dNTP's: 0.125mM of each of the four deoxynucleoside triphosphates.

Antibiotics.

Antibiotic	stock soln.	final conc.
Ampicillin	100mg/ml in water	50µg/ml
Chloramphenicol	34mg/ml in 100% EtOH	10µg/ml
Tetracycline	12mg/ml in 80% EtOH	100µg/ml
Oligomycin	5mg/ml in DMSO	100µg/ml
Hygromycin	10mg/ml in EtOH	100µg/ml

In all cases the antibiotics were filter sterilised and added to media cooled to <55^{o}C.

2.9 *E.coli* growth conditions.

Liquid cultures for transformation, plasmid or phage DNA preparations were grown in L-broth at 37°C with vigorous shaking.

Plate cultures were grown on solid L-agar with any required antibiotic.

For phage DNA preparations, phage particles were mixed and incubated with the plating cells at 37°C for 20 minutes to allow phage adsorption. This phage/cell suspension was added to precooled (47°C) BBL Agarose overlay, then poured onto a preset BBL agar plate. Once the agar has solidified, the plates were incubated at 37°C for 12-16 hours.

Bacterial strains were stored on both L-agar slopes at room temperature and in 50% LB-broth/40% glycerol at -20°C.

2.10 *Aspergillus* growth conditions.

All strains were maintained on solid complete media, transformants were maintained on selective solid minimal media. All plate cultures were grown at 37°C, healthy strains conidiate after 2-3 days.

Liquid media plus supplements were used to produce mycelia for both transformation and DNA preparation experiments.

2.11 *Penicillium* growth conditions.

Strains and transformants were maintained in similar fashion to the *Aspergillus* strains. *Penicillium* liquid cultures were incubated at 28°C for 2 days before harvesting. Transformants were incubated at 28°C, the time depending on the supplement e.g. on nitrate, transformants appear after 2-3 days; on oligomycin, transformants appear after 6-9 days.

2.12 *Cephalosporium* growth conditions.

Strains and transformants were maintained in similar fashion to the *Aspergillus* strains. *Cephalosporium* liquid cultures were incubated at 25°C for 2 days before harvesting. Transformants were incubated for 5-8 days at 25°C.

2.13 *In vivo* techniques: *E.coli*.

Competent cells: $CaCl_2$ method.

A single colony was picked and grown up in 5mls of LB-broth at 37°C until $O.D._{550}$=0.3. This culture was used to innoculate 100mls of prewarmed LB-broth. The culture was grown at 37°C until O.D.=0.5. The cells were chilled on ice for 10 minutes, transfered to 40ml Falcon tubes and spun down at 1,000g for 5

minutes at 0°C. The resulting pellet was resuspended in 30mls of ice-cold TfbI and then immediately spun down again at 1,000g for 5 minutes at 0°C. The cells were gently resuspended in 4mls of TfbII using a cut-tip pipette. The cell suspension was incubated for 15 minutes at 0°C, 200µl aliquots were transferred to pre-cooled 1.5ml Eppendorf tubes and snap frozen with liquid nitrogen. The competent cells were stored at -70°C.

Transformation of $CaCl_2$ competent <u>E.coli</u> cells.

Frozen aliquots of cells were thawed at room temperature then quickly stored on ice for 10 minutes. Transforming DNA at approximately 100ng/200µl cells was added and mixed by gentle stirring. The cells were left on ice for 30 minutes and heat-shocked at 42°C for 45-90 seconds. The cells were placed on ice to cool for 1-2 minutes, after which time 800µl of fresh LB-broth was added to each tube. The cells were incubated at 37°C for 1 hour to allow expression of the antibiotic resistance gene(s). The transformed cells were spread on selective L-agar plates and incubated at 37°C for at least 8 hours.

Competent cells: TSB method.

E.coli cells were grown up in LB-broth at 37°C until $O.D._{600}$=0.3-0.6. The cells were harvested by centrifugation at 1,000g for 5 minutes at 4°C. The cells were resuspended in 1/10th original volume in ice-cold TSB and left on ice for 10 minutes. 100µl aliquots of competent cells were transferred to pre-cooled 1.5ml Eppendorf tubes, frozen in a dry ice/ethanol bath and stored at -70°C.

Transformation of TSB competent <u>E.coli</u> cells.

For transformation, cells were thawed at room temperature and then placed on ice. Approximately 100ng of transforming DNA was gently mixed with the cells which were then left on ice for 5-30 minutes. There is no heat-shock step. The cells were mixed with 900µl of 20mM glucose supplemented TSB and incubated at 37°C for 1 hour to allow expression of the antibiotic resistance gene(s). The transformed cells were spread on selective L-agar plates and incubated at 37°C for at least 8 hours.

2.14 *In vivo* techniques: *Aspergillus.*

Transformation of *Aspergillus*: the transformation method is that described by Tilburn *et al*, 1984 and modified by Johnstone, 1985a.

Liquid culture: 200mls of liquid MM, supplemented as appropriate, was innoculated with approximately 10^8 conidia and incubated with vigorous shaking for 12-16 hours at 37°C. The mycelium was harvested under sterile conditions.

Protoplast production: the mycelium was resuspended with 5mls of ATB per 1g of mycelium in a 250ml conical flask. B-mercaptoethanol and BSA were added to final concentrations of 5mM and 2mg/ml respectively, the mixture was left at room temperature for 10 minutes. NovoZym 234 was then added to a final concentration of 4mg/ml. This protoplasting mixture was incubated at 30°C for 90-180 minutes in an orbital shaker. After this time, the mixture was transfered to 15ml corex tubes and carefully overlayed with 1ml of 0.5x ATB. The tubes were centrifuged at 5,000g for 10 minutes at room temperature in a swing out rotor. The protoplasts are found at the interface between the APB and the 0.5x ATB layers. The protoplasts were removed with a cut-tip pipette, resuspended in 30mls of ATB and pelleted by centrifugation at 4,000g for 5 minutes at room temperature. This wash step is repeated twice more and the protoplasts were

resuspended in ATB to give a final concentration of $2x10^8$ protoplasts/ml.

Transformation: 1-3µg of transforming DNA in 10µl of TE was added to 100µl (10^6 protoplasts) protoplast suspension, mixed gently and incubated at room temperature for 20 minutes. 1ml of 60% PEG MW 8,000 in ATB was added and the mixture was incubated at room temperature for a further 15 minutes; 5mls of ATB were added and the solution mixed gently.

Regeneration: 1ml aliquots of the protoplast suspension were pipetted (cut-tip pipette) onto appropriately supplemented pre-set SOS-N agar plates. The plates were overlayed with appropriately supplemented 0.7% SOS-N top agar. After setting, the plates were incubated at 37°C for 2-3 days.

2.15 In vivo techniques: Penicillium.

Transformation of Penicillium: basically the same as for Aspergillus but with the following differences.

Liquid culture: 200mls of liquid MM, supplemented as appropriate, was innoculated with approximately 10^8 conidia and incubated with vigorous shaking for 48-60 hours at 28°C. The mycelium was harvested under sterile conditions.

Protoplast production: exactly as described for Aspergillus.

Transformation: 20-50µg of transforming DNA in 30µl of TE was added to 200µl of protoplast solution, mixed

gently and incubated on ice for 20 minutes. 800µls of 60% PEG MW 8,000 in ATB were added and the mixture was incubated at room temperature for a further 20 minutes; 1ml of ATB was added and the solution mixed gently.

Regeneration: 0.1ml aliquots of the protoplast suspension were pipetted (cut-tip pipette) onto appropriately supplemented pre-set SOS-N agar plate, spread and allowed to dry. The plates were incubated overnight at 28°C and then overlayed with appropriately supplemented 0.7% SOS-N top agar. After setting, the plates were incubated at 28°C for 2-3 days if selecting on Nitrate and 6-9 days if selecting for oligomycin resistance.

2.16 *In vivo* techniques: *Cephalosporium*.

Transformation of *Cephalosporium*: basically the same as for *Aspergillus* but with the following differences.

Liquid culture: 200mls of liquid MM, supplemented as appropriate, was innoculated with approximately 10^8 conidia and incubated with vigarous shaking for 40-48 hours at 25°C. The mycelium was harvested under sterile conditions.

Protoplast production: exactly as described for *Aspergillus.*

Transformation: 40-60µg of transforming DNA in 30µl of TE was added to 250µl of protoplast solution,

mixed gently and incubated at room temperature for 20 minutes. 250µls of 60% PEG MW 8,000 in ATB were added and the mixture was incubated at room temperature for a further 10 minutes; 500µls of ATB were added and the solution mixed gently.

Regeneration: 0.1ml aliquots of the protoplast suspension were pipetted (cut-tip pipette) onto appropriately supplemented pre-set SOS-N agar plates. The plates were incubated overnight at 25°C and then overlayed with appropriately supplemented 0.7% SOS-N top agar. After setting, the plates were incubated at 25°C for 5-8 days.

2.17 *In vitro* techniques.

The standard methods were as described in Maniatis *et al*, 1989.

Plasmid preparation.

The plasmids from *E.coli* transformants were routinely examined by both Single Colony Gel Electrophoresis and restriction mapping following plasmid isolation.

Single Colony Gel Electrophoresis: A single colony is patched out on a selective plate and incubated overnight. A "blob" of cells is collected using a toothpick and resuspended in 100µls of Single Colony

Gel Buffer and left at room temperature for 15 minutes. The suspension is then centrifuged in a microfuge for 15 minutes and 25µls are loaded onto an agarose gel. It is not possible to restriction digest any DNA prepared in this way.

Birnboim-Doly large scale preparation of plamid DNA.

This method is a modification of the Birnboim-Doly, 1979 method. A 200ml overnight culture of transformant E.coli, grown under selective conditions was centrifuged at 5,000g for ten minutes at $4^{o}C$.

The pellet was resuspended in 5mls of Birnboim/Doly I solution and left for 10 minutes at room temperature. The cells were lysed by the addition of 10mls of freshly prepared Birnboim/Doly II solution, the solution was mixed by gentle inversion and placed on ice for 10 minutes. 7mls of ice cold Birnboim/Doly III solution were added, mixed by gentle inversion and stored on ice for 10 minutes. The solution was centrifuged at 35,000g for 20 minutes at $4^{o}C$. The supernatent was decanted carefully into a fresh tube containing 5mls of isopropanol. After mixing the tube was left at room temperature for 15 minutes and centrifuged at 35,000g for 10 minutes at room temperature. The resulting pellet was washed with 70% EtOH, dried and resuspended in TE. The plasmid DNA can now be

purified free of RNA, genomic DNA and protein in many ways. The two protocols outlined below are the two most commonly used in this lab.

(i) CsCl/EtBr Ultracentrifugation: the DNA was resuspended in 6mls of TE. 6g of CsCl were added and once the CsCl had dissolved, 240uls of a 15mg/ml solution of EtBr were added. The solution was transfered to a polyprop ylene ultracentrifugation tube and spun at 49,000rpm at 18^{o}C for 18 hours. The CCC plasmid DNA band was removed with a hyperdermic syringe.

The EtBr was removed by repeated extractions with water saturated Butanol. An equal volume of EtOH was added to the plasmid DNA, mixed and spun at 35,000g for 15 minutes at 4^{o}C. The plasmid DNA pellet was washed with 70% EtOH, dried and resuspended in TE to give the desired concentration.

(ii) PEG precipitation of plasmid DNA: the DNA was resuspended in 3mls of TE and stored on ice. 3mls of ice cold 5M LiCl were added, mixed gently and spun at 10,000rpm for 10 minutes at 4^{o}C; this step removes high MW RNA. The supernatent was decanted to a fresh tube containing 3 mls of isopropanol and mixed well. The DNA was pelleted by centrifugation at 10,000rpm for 10 minutes at room temperature. The supernatent was discarded and the pellet resuspended in 500uls of TE containing DNase free RNase (20ug/ml). The solution

was transfered to a 1.5ml Eppendorf tube and left at room temperature for 30 minutes. 500µls of a 1.6M NaCl solution containing 13% (w/v) PEG (MW 8,000) was added and mixed well. The DNA was centrifuged for 5 minutes at 4°C in a microfuge. The supernatent was discarded and the pellet resuspended in 400µls of TE. The DNA solution was extracted once with phenol, once with phenol/chloroform and once with chloroform. The aqueous phase from the final extraction was transfered to a fresh tube and 1ml of EtOH was added and mixed. The plasmid DNA was pelleted by spinning in a microfuge for 10 minutes at 4°C. The plasmid DNA pellet was washed with 70% EtOH, dried and resuspended in TE.

Bacteriophage DNA preparation.

Preparation of plating bacteria: 100mls of LB-broth supplemented with 0.2% sterile maltose and 10mM $MgSO_4$ was innoculated with a single colony of either E.coli SURE or XL-1BLUE strains. The culture was grown at 37°C for 5 hours and the bacteria were pelleted by centrifugation at 4,000g for 10 minutes at 4°C. The cells were resuspended in 50mls of sterile 10mM $MgSO_4$ and diluted to an $O.D._{600}$=0.5 with 10mM $MgSO_4$.

Packaging Lambda library DNA: this protocol was followed exactly as described in the Stratagene instructions. The sonic extract (yellow tube) was

place on ice to slowly thaw. Meanwhile the Freeze/Thaw extract (red tube) was thawed between fingers until just begining to thaw. 4µg of the Lambda library DNA, in 4ul of TE, was immediately added to the Freeze/Thaw tube which was then placed on ice. 15µls of the Sonic extract were added to the Freeze/Thaw extract containing DNA. After mixing, the tube was incubated at room temperature for 2 hours. 500µls of SM buffer and 20 µls of chloroform were added and the tube was inverted gently to mix. The tube was briefly spun in a microfuge and the supernatent titred.

Plating out of bacteriophage: consecutive serial dilutions of 10^{-4}, 10^{-6} and 10^{-8} were made from the packaged phage supernatent. 200µl aliquots of plating bacteria were innoculated with 10µls of each of the phage dilutions and incubated for 15 minutes at 37°C. The cell/phage suspensions were mixed with 3mls of BBL top agar cooled to 48°C and poured onto pre-warmed BBL agar plates. These plates were incubated at 37°C for 8 hours and the number of plaques counted.

Library amplification: 600µl aliquots of plating cells were innoculated with 100,000 phage particles and incubated for 15 minutes at 37°C. The cell/phage suspensions were mixed with 6.5mls of BBL top agar cooled to 48°C and poured onto pre-warmed BBL agar

150mm plates. These plates were incubated at 37°C for 8 hours. The library plates were then overlayed with 10mls of SM buffer and stored overnight at 4°C. The SM buffer containing the phage particles was transferred to a polypropylene flask containing 3mls of chloroform. After gentle mixing the cell debris was removed by centrifugation at 2,000g for 5 minutes at 4°C. The phage supernatent was decanted into a fresh container, titred as before and stored at 4°C.

Purification of Bacteriophage: a single phage plaque was picked from a BBL agar plate and added to 1ml of SM buffer and vortexed. 100µls of the resulting phage suspension was mixed with 600µls of plating bacteria, plated out and incubated overnight at 37°C. The phage were then washed from the plate with SM buffer and the phage supernatent was collected and treated as described in the previous section. After centrifugation, DNase and RNase were added to the phage supernatent to a final concentration of 2µg/ml and incubated for 20 minutes at room temperature. NaCl was added to a final concentration of 2% w/v and left on ice for 1 hour. This solution was centrifuged at 15,000g for 5 minutes at 4°C to remove unwanted proteins. The supernatent was decanted into a fresh flask and 8% (w/v) PEG (MW 6,000) was added to precipitate the phage. After overnight storage at 4°C, the phage were pelleted by spinning at 15,000g for 15 minutes at 4°C. The phage were resuspended in

12mls of SM buffer and 8.5g of CsCl were added. The addition of CsCl produces a phosphate precipitate which was removed by spinning at 12,000g for 10 minutes at 20°C. The phage/CsCl solution was transfered to a polypropylene tube; the phage were purified by density gradient ultracentrifugation at 270,000g for 4 hours at 20°C. The resulting phage band appears a pale blue against a black background, this band was removed using a hyperdermic syringe; the CsCl was removed by serial dialyses against SM.

DNA extraction: the purified phage were incubated with 20mM EDTA, 50µg/ml of proteinase K and 0.5% SDS for 1 hour at 37°C. The phage DNA was extracted twice with phenol/chloroform and twice with chloroform. The DNA was mixed with 2x volume EtOH and pelleted by spinning at 20,000g for 30 minutes at 4°C. The DNA pellet was dried and resuspended in TE.

Preparation of high MW DNA from <u>Aspergillus:</u> this protocol is adapted from the method of Raeder and Broda (1985) and was used as described to prepare genomic DNA from all the fungal species previously described.

Mycelium were harvested after the required growth period depending on species. The harvested mycelium was immersed in liquid nitrogen and ground into a fine powder using a mortar and pestle. 10mls of extraction buffer was quickly added for every 1g

of ground mycelium and then mixed well. 7mls of buffered phenol were added and mixed. 3mls of chloroform were added and mixed gently. The suspension was centrifuged at 13,000g for 30 minutes at room temperature. The aqueous top layer was carefully removed using a cut-tip pipette and placed in a fresh tube. RNase was added to this tube at a final concentration of 250µg/ml and left at 37°C for 15 minutes. 5mls of chloroform were added and mixed. The suspension was centrifuged at 13,00g for 10 minutes and the upper aqueous layer was removed and placed in a fresh tube. 6mls of isopropanol were added to this tube, mixed and left at room temperature for 1 hour. The DNA was pelleted by spinning at 13,000g for 15 minutes at room temperature. The pellet was washed with 70% EtOH, dried and resuspended in TE.

Restriction of DNA.

DNA was routinely digested in 0.5ml Eppendorf tubes. Reactions were carried out in a total volume of 20µls containing 0.1-1µg of DNA, 2µl of 10x restriction buffer, 10-20 units of restriction enzyme and finally distilled water making up the final volume. The reactions were incubated at the temperature recommended by the suppliers for 1-4 hours. Restriction was terminated by either addition of final sample loading buffer or phenol extraction.

Phosphatase treatment of restricted DNA.

Self-ligation of vector molecules severely reduces the DNA cloning efficiency. Removal of the 5′ terminal phosphate groups of linearised vector DNA by Calf Intestinal Phosphatase (CIP) minimises self-ligation and dramatically increased cloning efficiency. To phosphatase overhanging "sticky ends" 0.1 units of CIP were routinely added to a restriction digest reaction and incubated at 37°C for 30 minutes, after which time another 0.1 units of CIP were added and left for a further 30 minutes. To dephosphorylate blunt ended or recessed "sticky ends" 0.1 units of CIP were added, incubated at 37°C for 15 minutes and then at 56°C for 15 minutes.
CIP was removed by adding 2% SDS to the reaction mix and heating to 68°C for 15 minutes, extracting twice with phenol/chloroform and the DNA recovered by either precipitating with EtOH or by using the Prepagene system.

Ligation of DNA.

T4 ligase catalyses the formation of covalently joined hybrid DNA molecules from both "sticky" and "blunt ended" molecules. A typical 20µl ligation mix

comprised of 200ng of DNA (vector and fragment in a ratio of 1:2), 4µl of 5x ligation buffer, 0.1 unit of ligase for "sticky ends" or 1 unit of ligase for "blunt ends" and distilled water to make up the volume. "Sticky end" reactions were left at room temperature for four hours; "blunt end" reactions were incubated at 15°C overnight. Plasmid library ligation reactions were carried out in a final volume of 2mls to reduce the possibility of concatemerisation. All components of the ligation were scaled up from the standard 20µl reaction volume.

Prep-a-gene protocol.

This protocol was carried out according to the manufacturer's instructions. This method was used to purify DNA bands from agarose gels for a variety of purposes e.g. cloning, transformation of Aspergillus and for hybridisation probes.

After restriction digestion, the DNA bands were separated on an agarose gel, the desired band was excised from the gel and the resulting gel slice was placed in a 1.5ml Eppendorf tube. The volume of this gel slice was estimated and 3 volumes of Binding buffer were added to the tube which was then incubated at 50°C until the agarose gel had dissolved. 10ul of Glass milk (a suspension of powdered glass in distilled water) was added and

mixed by inversion for 5-10 minutes. The tube was spun in a microfuge for 30 seconds and the supernatent discarded. The glass milk pellet was resuspended in 500µls of Binding buffer and spun down as before. The pellet was resuspended in fresh Binding buffer and pelleted as before. The glass milk pellet was then resuspended in Wash buffer and pelleted a total of three times. The pellet was carefully dried and resuspended in 10µls of Elution buffer, incubated at 50°C for five minutes then spun for 2 minutes. The supernatent containing the DNA fragment was transfered to a fresh tube for use.

2.18 DNA electrophoresis through gels.

Agarose: agarose gels of 1%, 0.8% and 0.3% were used to separate DNA molecules and fragments. Agarose powder was dissolved in 1x TBE buffer at 100°C. The agarose was cooled to 55°C and 5µls of a 15mg/ml EtBr solution was added and mixed. The molten agarose was poured into a horizontal gel former fitted with a Teflon comb. After setting, the gel was transfered to a running tank and submerged in 1x TBE buffer. Once the DNA samples were loaded the gel was run for 2-12 hours at 20-100 volts.

Gels were photographed with UV transillumination (wavelength=240nm) using a Polaroid camera loaded with Polaroid 4x5 Land film (no. 57), fitted with a red Kodak Wratten Filter No.9.

2.19 Nucleic acid hybridisation.

Random priming: the random prime protocols were carried out as recommended by the manufacturer. Random sequence hexanucleotides can be used to prime DNA synthesis on a single stranded DNA template by the DNA polymerase I Klenow fragment; up to 80% incorporation of label can be achieved. The actual experimental protocol depends on whether a radioactive or non-radioactive label is used.

(i) Radioactive labelling: the probe DNA, 25ng in 10µls, was linearised, denatured by heating to 95°C for 10 minutes and then immediately cooled on ice for 2 minutes. 1µl of each of 0·125mM dCTP, dGTP and dTTP were added to the tube and carefully mixed; 2µls of Reaction buffer were added followed by 5µls of [alpha^{32}P] dATP, 3000Ci/mmol. 1µl of Klenow enzyme was added and the tube incubated at 37°C for 30 minutes in a pre-warmed lead pig. The tube was then heated to 65°C to denature the probe and added to the hybridisation solution containing the filter(s).

(ii) DIG labelling: the hapten digoxigenin (DIG) is bound via a spacer arm to uridine-nucleotides and can be incorporated into DNA or RNA probes by a variety of techniques including random prime labelling. The probe DNA, 25ng in 10µls, was linearised, denatured by heating to 95°C for 10 minutes and then immediately cooled on ice for 2 minutes. 1µl of each

of dATP, dCTP and dGTP were added to the tube and carefully mixed. 1.6µls of an 1:1 mixture of dTTP and 0.3mmol Digoxigenin-11-dUTP was added, followed by 2µls of Reaction buffer. The volume was made up to 19µls with distilled water. 1µl of Klenow enzyme was added and the tube incubated at 37°C for 60 minutes. The tube was then heated to 65°C to denature the probe and added to the hybridisation solution containing the filter(s).

Southern blotting.

DNA was transfered from agarose gels to Hybond-N (Amersham) nylon membrane using the basic protocol described by Southern (1975) and such modifications as recommended by the membrane manufacturers.

The agarose gel was first immersed in 200mls of 0.25M HCl if genomic DNA (>10kb) was present for 30 minutes. The gel was washed in denaturing solution for 30 minutes, rinsed in distilled water and washed twice in neutralising solution for 15 minutes. A glass plate spanning two reservoirs of 20x SSC was covered with a sheet of Whatman 3MM filter paper, taking care that the filter paper was submerged in the 20x SSC on all sides. The gel was placed on the paper and covered with a pre-sized piece of nylon membrane; all trapped air bubbles were carefully removed. The membrane was covered with two pieces of filter paper and then by a thick stack of paper

towels, a glass plate was placed on top, followed by a 1kg weight to ensure that all the various layers are compressed together. The DNA transfer takes 12-18 hours. The membrane was then removed, washed briefly in 2x SSC to remove any adhering agarose and exposed to U.V. radiation in a Stratalinker U.V. oven to crosslink the DNA to the filter.

Nucleic acid hybridisation conditions.

Radioactive probe (i) high stringency: the U.V. fixed filters were placed in hybridisation tubes and 11.4mls of hybridisation solution were added and incubated in a hybridisation oven for 4 hours at 65°C. The radioactively labelled probe was added to the tube and incubated at 65°C for upto 12 hours. The radioactive solution was discarded in a designated area. The membrane was washed two to three times in Pall blot wash for upto 20 minutes each time at room temperature. The number and length of the washes depended on how "hot" the membrane was. After washing, the membrane was wrapped in Saran Wrap and placed in an autoradiography cassette with a sheet of Kodak Xomat S1 and an intensifying screen. The cassette was placed in a -70°C freezer for 1 hour to 1 week, depending again on how "hot" the membrane was.

Radioactive probe (ii) low stringency: this was as high stringency except that the hybridisation buffer contained 40% deionised formamide. After the addition of the labelled probe, hybridisation was carried out at 42^{o}C for 12 hours. The membranes were washed serially in conditions of decreasing stringency: 2x SSC, 0.1% SDS, then 1x SSC, 0.1%SDS and then 0.5x SSC, 0.1% SDS. All washes were carried out at room temperature. All washes were repeated but at 42^{o}C.

DIG hybridisation and luminescent detection: after hybridisation and blocking, DIG-labelled probes are detected by high affinity anti-digoxigenin-antibody Fab fragments which are conjugated to alkaline phosphatase. The alkaline phosphatase dephosphorylates a substance called AMPPD which is the chemiluminescent substrate. The breakdown of AMPPD results in the formation of a light-emitting unstable intermediate.

(i) hybridisation: after Southern blotting, the membrane was prehybridised at 42^{o}C in 20mls of DIG hybridisation buffer per 100cm^{2} of membrane for at least 1 hour. This solution was replaced with 2.5mls/100cm^{2} filter of DIG hybridisation buffer containing 25ng of denatured DIG-labelled probe. The filter was incubated at 42^{o}C for at least 6 hours in a hybridisation oven. The hybridisation solution

containing the DIG probe was decanted off and stored at -70°C for reuse. The filter was washed twice in 2x SSC, 0.1% SDS for 5 minutes at room temperature. The filter was then washed twice in 0.1x SSC, 0.1% SDS at 68°C. Unless otherwise stated all washes were carried out at room temperature with gentle agitation.

(ii) chemiluminescent detection: the membrane was washed in 100mls of wash buffer for 5 minutes and incubated in 100mls of buffer 2 for 30 minutes. 2µls of anti-digoxigenin-AP Fab fragments were diluted 1:10000 in 20mls of buffer 2 and the filter incubated in the diluted antibody solution for 30 minutes. The filter was washed twice in 100mls of wash buffer for 15 minutes to remove any unbound conjugate. The filter was drained and incubated in 20mls of buffer 3 and incubated in 20mls of AMPDD substrate solution for 5 minutes. The filter was sealed in a hybridisation bag and incubated at 37°C for 15 minutes. The filter was placed in an autoradiography cassette and the film exposed for 15-25 minutes at room temperature and then developed.

2.20 DNA sequencing.

The M13 derived cloning vectors M13mp18, M13mp19 and pBLUESCRIPT II KS$^+$ were used to construct ama1 sequencing subclones. Insertions into the polylinker of these vectors disrupts the lac operon. This

disruption results in the failure to hydrolyse a colourless substrate commonly called XGal to a blue coloured derivative. The lac enzymatic pathway can be induced by IPTG. Therefore, wild type M13 derived vectors produce blue coloured colonies and plaques, M13 derived vectors containing cloned fragments produce white colonies and plaques. The Sanger chain terminator method was used to sequence amal.

Making the deletion series.

For each deletion series 10µg of purified CCC plasmid were completely digested with two different restriction digests; the first enzyme produces a 3'overhang which protects the vector from degradation by Exonuclease III, the second enzyme produces either a 5'overhang or blunt ends, both of which allow degradation by Exonuclease III. 10µg of plasmid are sufficient for 25 individual aliquots. The doubly digested DNA was extracted twice with phenol/chloroform and precipitated with EtOH, spun in a microfuge and resuspended in 60uls of Exo III buffer. 7.5µls of S1 mix were added to 25 0.5ml Eppendorf tubes and stored on ice. The resuspended DNA was pre-warmed to 37°C and 500 units of Exonuclease III were added, mixed as quickly as possible and incubated at 37°C; 2.5µl aliquots were removed every 30 seconds to the appropriate tube containing the S1 mix and S1 nuclease. This process

produces a series of deletions that are about 175-250 bp apart. Once the desired number of deletions were collected, all the S1/DNA containing tubes were incubated at room temperature for 30 minutes. 1µl of S1 stop solution was added to each tube which were incubated at 70°C for 10 minutes. After cooling, 2µl aliquots were run an a 0.8% agarose gel to check the extent of digestion, the tubes were meanwhile stored on ice. All tubes were incubated at 37°C and 1µl of Klenow mix was added to each tube and incubated at 37°C for 5 minutes. 1µl of dNTPs were added and incubated at 37°C for 5 minutes. All tubes were placed at room temperature and both ligation reactions and subsequent transformations were set up as described previously.

Isolation of template DNA.

(i) single stranded M13 phage DNA: transformations were carried out as described previously but the transformations were plated in 3mls of 0.6% top agar containing 200µls of TG1 cells, 20µls of 40µg/ml XGal/DMF solution, 20µls of 20µg/ml IPTG solution on minimal medium B1, D+M salts, glucose plates. After overnight incubation at 37°C cells from white plaques were transfered into 4mls of LB-broth containing XL-Blue cells in early log phase. These cultures were grown overnight at 37°C with vigorous shaking. 1.5ml aliqots were taken from these overnight

cultures and spun twice in a microfuge to remove host cells. The clear supernatent was transferred to fresh tubes and the phage precipitated by addition of 200uls of 40% PEG, 2.5M NaCl and a 30 minute incubation on ice. The phage were pelleted by a 15 minute spin in a microfuge. The supernatent was decanted and the pellet carefully dried and resuspended in 200µls of TE. The DNA was extracted twice with phenol/chloroform and precipitated with EtOH. The DNA pellet was dried and resuspended in 20µls of TE.

(ii) double stranded plasmid DNA: the plasmid DNA was prepared by the Birnboim/Doly method and CsCl/EtBr ultracentrifugation as described previously.

Sequenase labelling reactions.

The DNA was labelled using the USB Sequenase kit and protocol.

(i) plasmid DNA: the double stranded plasmid DNA must first be denatured before it is labelled. 4µg of plasmid DNA in 30µls of TE was denatured by addition of 3µls of 2M NaOH and incubated at 37^{o}C for 15 minutes. 120µls of ice-cold EtOH was added, followed by 15µls of 1M NaAc and incubated at room temperature for 5 minutes. The single stranded DNA was pelleted by spinning in a microfuge for 10 minutes, dried and resupended in 1µl of primer

solution. 2µls of Sequenase buffer and 7µls of distilled water were added and carefully mixed. The DNA was reannealed by incubating at 37°C for 45 minutes.

(ii) phage DNA: 2µls of the phage DNA was mixed with 1µl of primer solution. 2µls of Sequenase buffer and 7µls of distilled water were added and carefully mixed. The DNA was reannealed by incubating at 37°C for 45 minutes.

The reannealed template/primer DNA was then labelled as described here. The labelling mix was diluted 1:5 with distilled water. 2.5µls of each ddNTP termination mix was placed in appropriately marked 0.5ml Eppendorf tubes and pre-warmed to 42°C. The 10µls of template/primer mix was placed at room temperature and 1µl DDT, 2µls diluted labelling mix, 0.5ul ^{35}S dATP and 2µls of diluted Sequenase were added, mixed and incubated at room temperature for 3 minutes. 3.5µl aliquots were removed and added to the relevant termination tubes, mixed and incubated at 42°C for 5 minutes. 4µls of Stop solution were added and all tubes stored at -20°C until required.

Gel apparatus: the BioRad-Sequi-Gen 38cm x 50cm setup was used with 0.25mm-0.75mm wedge spacers and 48 well Sharks tooth combs.

Sequencing gels: 8% wedge gels were used and made as follows and:

40% acrylamide stock	20mls
5x TBE	20mls
distilled water	20mls
Urea	50g
10% ammonium persulphate	1ml
TEMED	20µls

The gels were run at 1800V for 4-8 hours.

Autoradiography: after electrophoresis the gels were fixed in 10% Acetic acid, 10% Methanol, 2% Glycerol solution for 30 minutes. After drying, the gels were exposed using Kodak XAR-5 film for 36-72 hours at room temperature.

2.21 Electron microscopy.

All protocols are as described in Sommerville and Scheer, 1988. All materials, except the ARp1 DNA were gifts from Dr Lesley W. Coggins.

As suggested in Chapter 1, ARp1 might replicate via a rolling circle model leading to the production of large molecules. If such large molecules could be visualised by Electron microscopy this would support the model. Such an experiment would require DNA prepared directly from Aspergillus transformants and not E.coli transformants.

Preparation of ARp1 DNA: Conidia from an Aspergillus ARp1 transformant were grown up under selective conditions in 1 litre of minimal media. The mycelia were collected and total genomic DNA was made as previously described. The genomic DNA was resuspended in 6mls of TE. 6g of CsCl was added and once the CsCl had dissolved, 240µls of a 15mg/ml solution of EtBr were added. The solution was transfered to a polypropylene ultracentrifugation tube and spun at 49,000rpm at 18°C for 18 hours. It was not possible to see a CCC plasmid band so the tube was pierced and the contents collected in 250µl aliquots. Each aliquot was treated with Butanol to remove the EtBr. A 20µl sample from each collected fraction was run on a 0.8% agarose gel. This gel was Southern blotted and probed with radiolabelled pUC DNA. This procedure identified the fractions containing only purified CCC ARp1. These fractions were then pooled, ethanol precipitated and resuspended to a final concentration of 10ng of ARp1/µl TE.

The ARp1 DNA was then prepared in two different ways before spreading onto the electron microscope grids. The DNA spreading solutions were prepared as follows:

1. **self annealed ARp1** : 50ng of ARp1 were resuspended in 8µls of water. 2µls of TE were added followed by 10µls of redistilled formamide. This solution was heated to 85°C for 2 minutes and

allowed to cool at room temperature for 20 minutes. After cooling 12µl water, 3µl TE, 10µl Formamide, 5µl of 0.4mg/ml Cytochrome C were added in this order and carefully mixed. The DNA was then spread onto the grids.

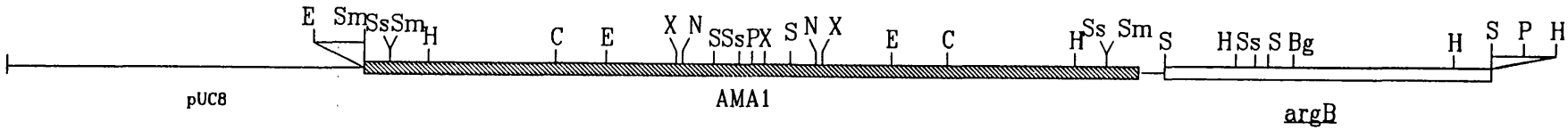
2. **untreated ARp1:** 20ng of ARp1 were resuspended in 20µl of water. 5ul ofTE, 20µl of Formamide and 5µl of 0.4mg/ml Cytochrome C were added in this order and carefully mixed. The DNA was then spread onto the grids.

DNA spreading.

The prepared ARp1 DNA was spread as follows: an acid washed glass slide was placed at an angle in a 30ml hypophase solution containing 10% Formamide, 1mM EDTA and 10mM Tris-HCl pH8.5. Talcum powder was sprinkled carefully onto the surface. The prepared DNA was placed on the glass slide and allowed to run down the slide, as it did so the DNA entered the hypophase and pushed the talc out of the way, leaving a clear patch which showed where the DNA was. The grids were touched gently to the surface of the clear patch and DNA was transfered to the grid. The grids were stained for 30 seconds in uranyl acetate, washed in 95% ethanol for 15 seconds and air dried. The dried grids were shadowed using Platinum and examined in the electron microscope.

Chapter 3.

Sequencing of *amal*.



KEY

C=ClaI Bg=BglII E=EcoRI H=HindIII

N=NruI P=PstI S=SalI Sm=SmaI

Ss=SstI X=XhoI

Figure 3.1: structure of ama1 as determined by restriction mapping, (Johnstone 1985, Gems 1990).

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3.1 Introduction.

The overall structure of ARp1 is shown in figure 3.1. The plasmid is 11.5kb in size and contains a 6kb region called *ama1*. Restriction digest mapping has shown that *ama1* contains two identical 3kb inverted repeats which are separated by a unique 345bp central region. Gems (1990), showed that the *ama1* arms are capable of "flipping", i.e. the arms can exchange positions, probably by homologous somatic recombination. I decided to sequence a 3kb SalI fragment which is essentially the right hand arm of the *ama1* insert. It was hoped that sequencing this *ama1* region would identify internal repeated regions, ORF's, protein binding sites etc. Database searches using this *ama1* sequence might identify origins of replication and any *ama1* related sequences and might give clues as to the normal function of the sequence from which *ama1* originated. The central 345bp unique region had already been sequenced by Johnstone (1985).

There are two techniques that can be used to generate sequence data; Gilbert and Maxαm's chemical degradation method and the Sanger *et al* dideoxy-mediated chain termination method.

For most DNA analysis/sequencing the Gilbert and Maxαm technique has been superceded by the Sanger *et al* method which is both faster and easier; most sequencing strategies are geared to this method.

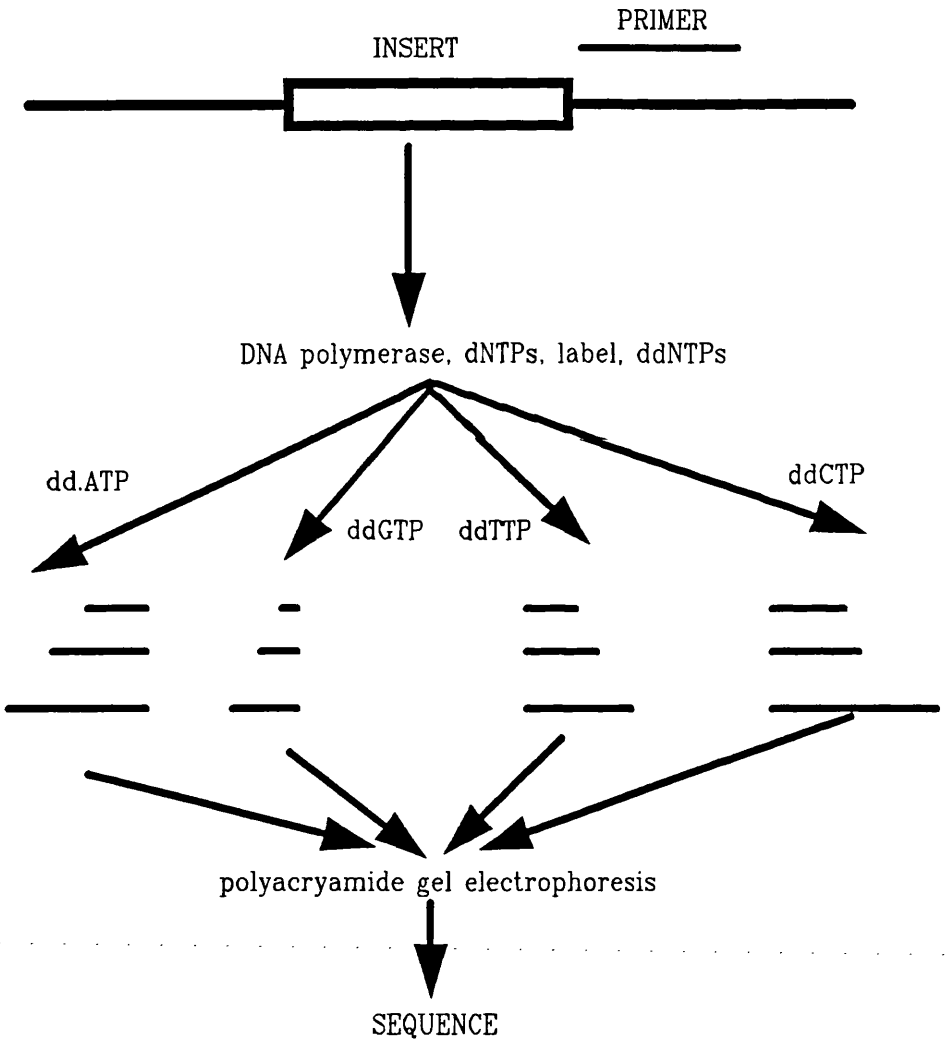

Figure 3.2: Sanger's sequencing protocol.

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The Sanger method is based on the properties of a DNA polymerase and dideoxy NTPs which lack a 3' hydroxy residue and therefore prevent further elongation of a DNA molecule. In practice, a typical reaction mix contains a small primer (13-29 bases) which binds to a single stranded template DNA molecule, dNTPs i.e. C, G, T, 35^{S}dATP and one specific ddNTP i.e one of either ddA or ddC or ddG or ddT; the ddNTP is present in low concentration so is more rarely incorporated into the growing DNA molecule than its dNTP counterpart. This DNA template molecule can be either single stranded phage or alkali denatured plasmid DNA or heat denatured plasmid DNA. A DNA polymerase then adds the dNTPs and more rarely the ddNTP to the template/primer. When the ddNTP is incorporated DNA elongation stops and the overall result is to produce a series of oligonucleotides which differ in size, depending on where the termination of DNA synthesis took place. It is therefore possible, by using the different ddNTPs, to identify the position of every A, C, G, and T on the template DNA by denaturing the oligonucleotides and then separating by gel elecrophoresis followed by autoradiography. This protocol is outlined in figure 3.2.

Many sequencing kits are available from a variety of manufacturers. The most commonly used sequencing primers include M13 primer, T3 primer, T7 primer and reverse primer. Various DNA polymerases

with different properties can be used; Klenow fragment of E.coli DNA polymerase I, Reverse transcriptase (AMV), Taq DNA polymerase and Sequenase (modified T7 polymerase).

In addition, there are three types of sequencing vector available: phage, plasmid and phagemid. The phage vector most often used is derived from the single stranded filamentous phage M13 e.g. M13mp18 . The pUC series of plasmids can be used for sequencing and contain the M13mp7 cloning site. Finally, phagemids are plasmids containing an origin of replication from a filamentous phage, which means that the template DNA can be prepared and sequenced from single stranded phage or denatured double stranded DNA, making phagemids a very flexible vector for sequencing.

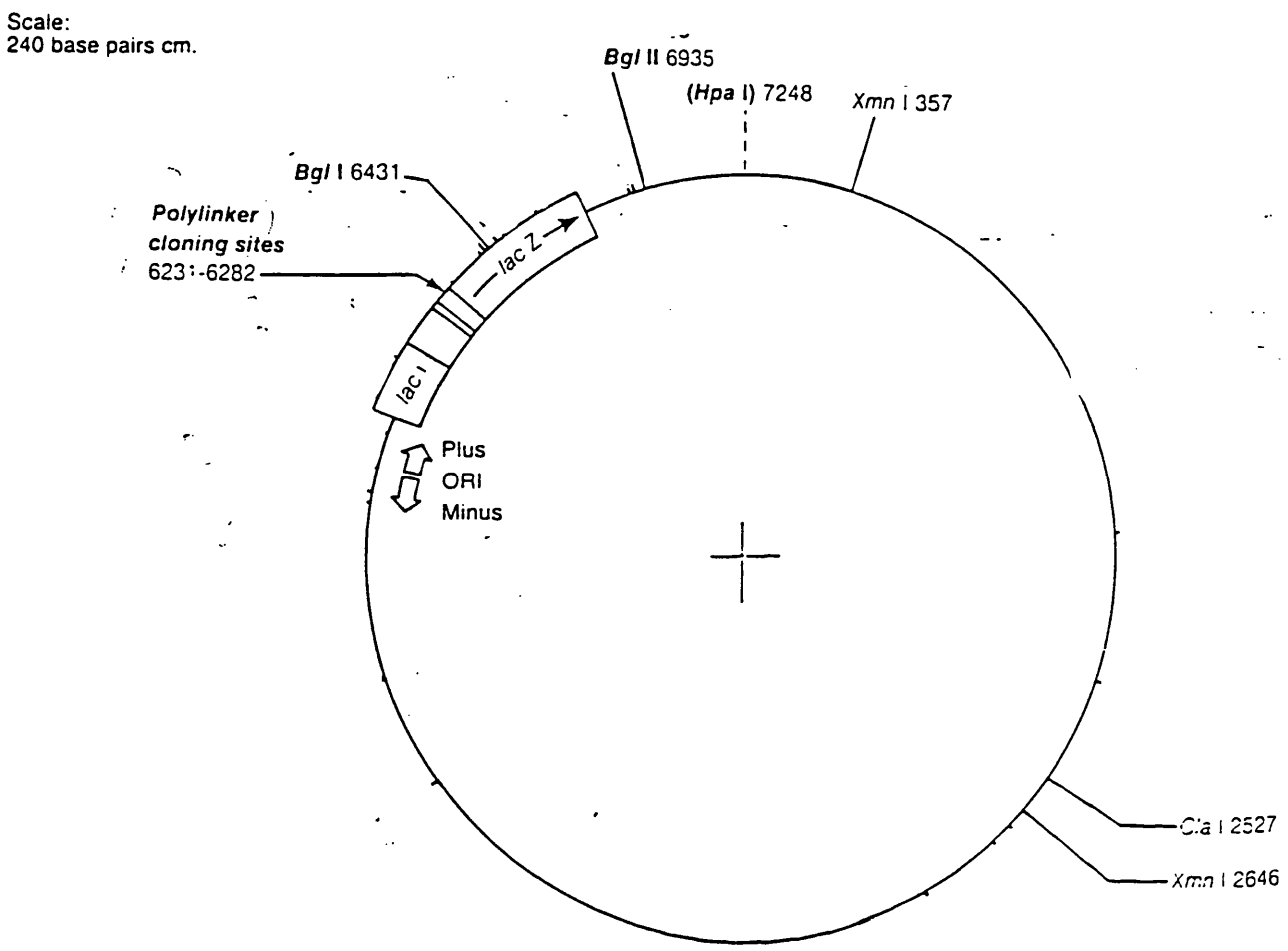
The bewildering choice of what vector to use etc is further complicated by consideration of what sequencing strategy to use.

Initially I considered three different approaches. The first method involves simply sequencing the first 200 or so bases of the template, collating the sequence data, designing a primer from the end of this sequence and then using this newly designed primer to sequence more of the template DNA. There are two major drawbacks with this method; it is very slow and it is expensive to sequence large pieces of DNA in this way.

The second approach is shotgun sequencing. This

approach involves taking the DNA to be sequenced and producing random DNA fragments by either sonication or partial digestion. The random fragments are then cloned into the sequencing vector and sequenced. The problem with this approach is that until all the data is collated and assembled on a computer, it is very difficult to determine (i) where the sequence data maps to the original DNA (ii) which strand is being sequenced (iii) whether or not gaps are present in the sequence data.

The procedure that I finally decided on was to clone the ama1 fragments into a phagemid called pBLUESCRIPT II and then generate a nested, overlapping set of systematic deletions using Exonuclease III (see sections 3.3 and 3.4). This approach makes it much easier to assemble the data but generating good deletions can be difficult. As a backup I also cloned ama1 fragments into M13mp18 and M13mp19 phage (see section 3.2). By preparing a set of deletions in the phagemid I could also use the whole series of plasmids in cotransformations with pILJ16 to test, functionally, for origins etc (see later) and I could prepare DNA in either single or double stranded form for sequencing.



polylinker cloning sites

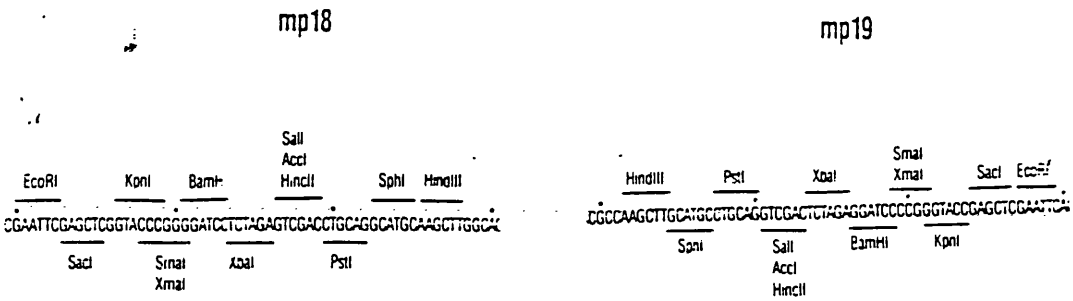


Figure 3.3: structure of M13mp18 and M13mp19 sequencing vectors.

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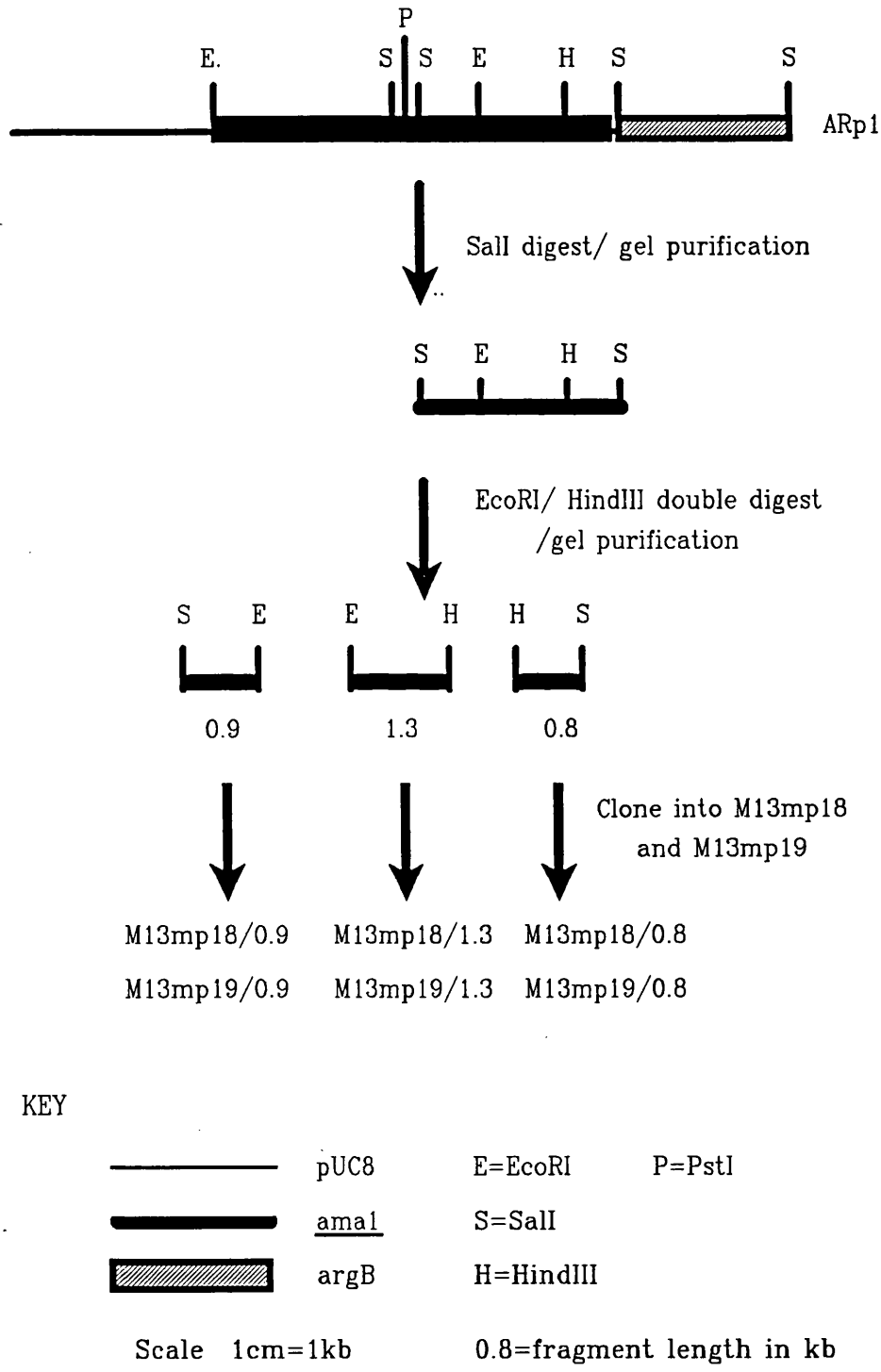
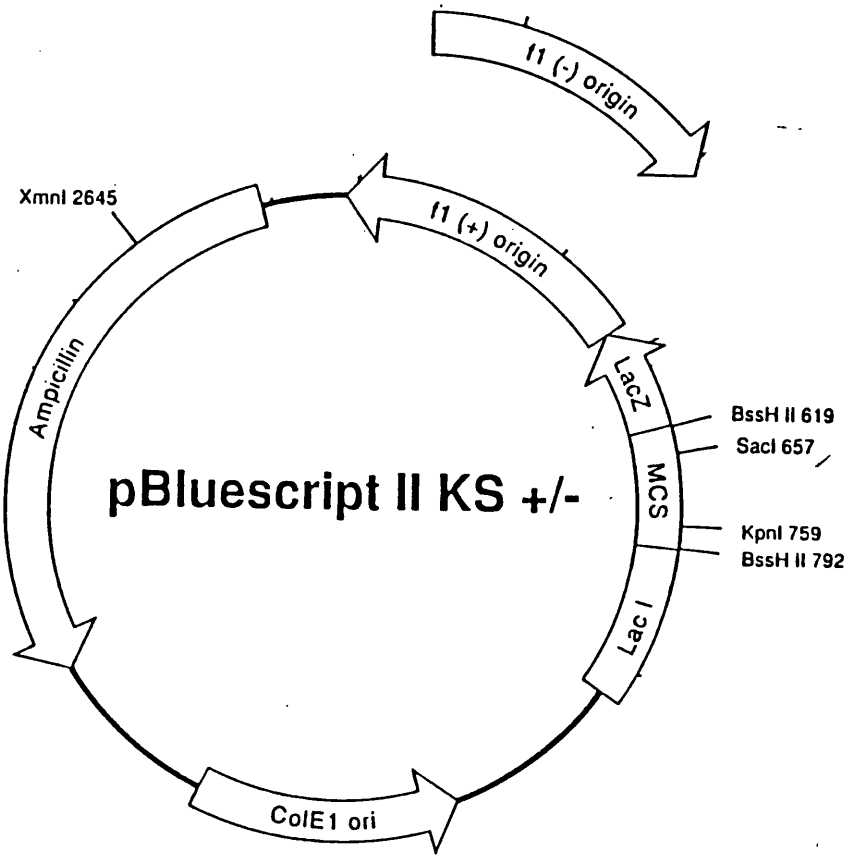


Figure 3.4: construction of M13 sequencing subclones.



Multiple Cloning Site (MCS) sequence

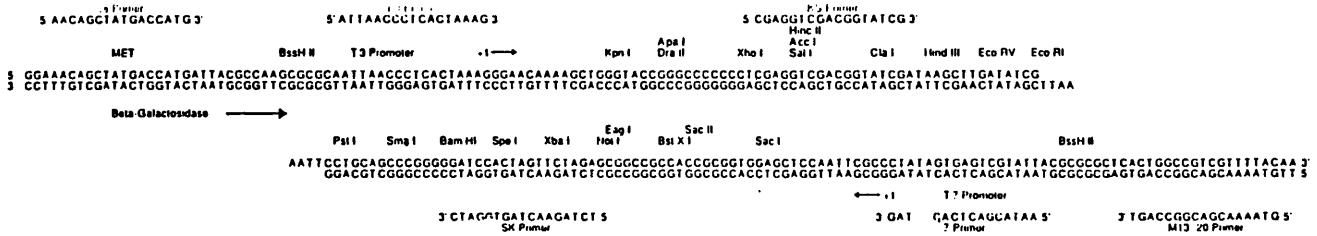
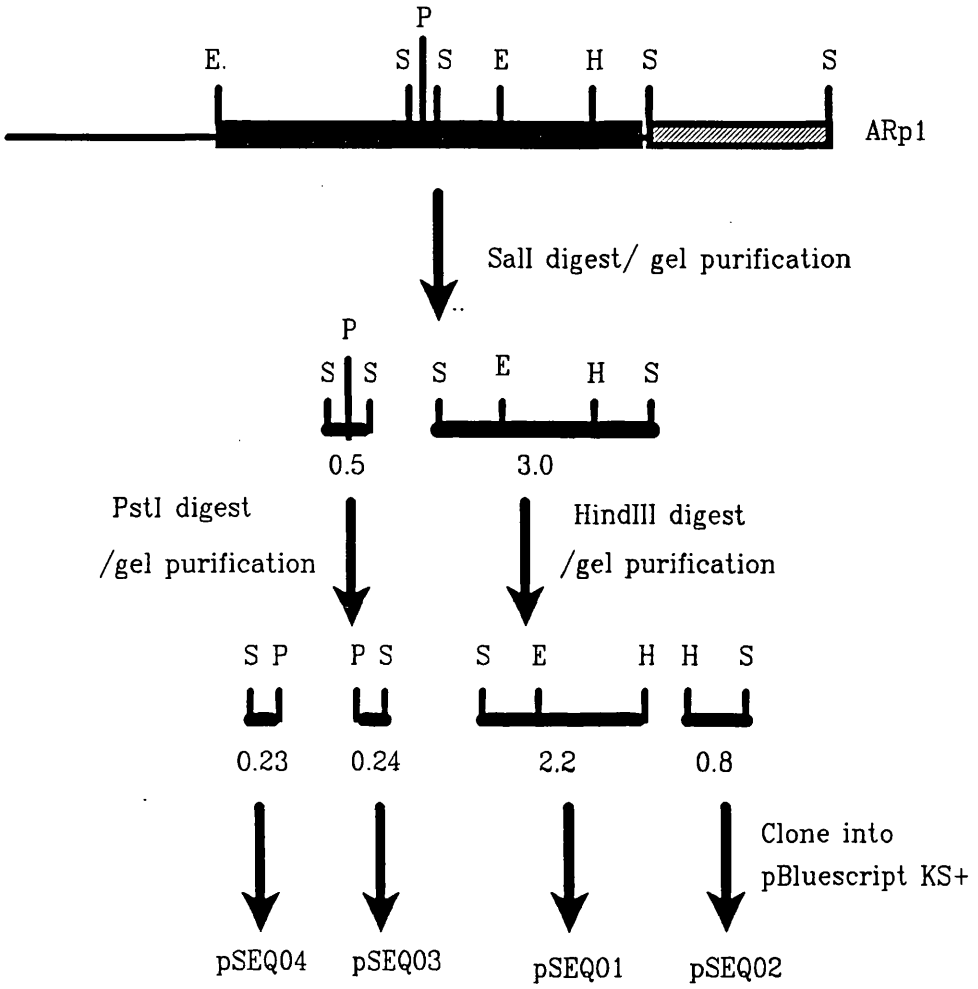


Figure 3.5: structure of pBLUESCRIPT II KS+/- sequencing vector, (Stratagene 1992). The upper DNA strand is designated the + strand and the lower DNA strand is designated the - strand.



KEY

pUC8 E=EcoRI P=PstI

ama1 S=SalI

argB H=HindIII

Scale 1cm=1kb 0.8=fragment length in kb

Fig 3.6: Construction of pSEQ series of sequencing subclones.

3.2 Construction of M13 sequencing subclones.

The structures of M13mp18 and M13mp19 are shown in figure 3.3. As can be seen the only difference between these two phages is the orientation of the multiple cloning site. The sequencing subclones were prepared as outlined in figure 3.4. The 3.0kb SalI fragment from ARp1 was gel purified on a 0.8% agarose gel then double digested with Hind III and EcoRI to produce three fragments of approximately 0.9kb, 1.3kb and 0.8kb in size. These fragments were ligated to M13mp18 and M13mp19 replicative forms which had been previously double digested with SalI/EcoRI, EcoRI/HindIII and HindIII/SalI. The ligation mixtures were transfected into E.coli strain XL-1 Blue. White (insert containing) plaques were selected for sequencing and single stranded DNA was prepared from these.

3.3 Construction of pBLUESCRIPT sequencing plasmids.

The structure of pBLUESCRIPT KS+/- is shown in figure 3.5. The +/- refers to orientation of the F1 phage origin of replication. The four sequencing plasmids were constructed as shown in figure 3.6. The 3.0kb and the 0.5kb SalI fragments were gel purified on a 0.8% agarose gel. The 3.0kb SalI fragment was digested with HindIII to produce two fragments of approximately 2.2kb and 0.8 kb in size.

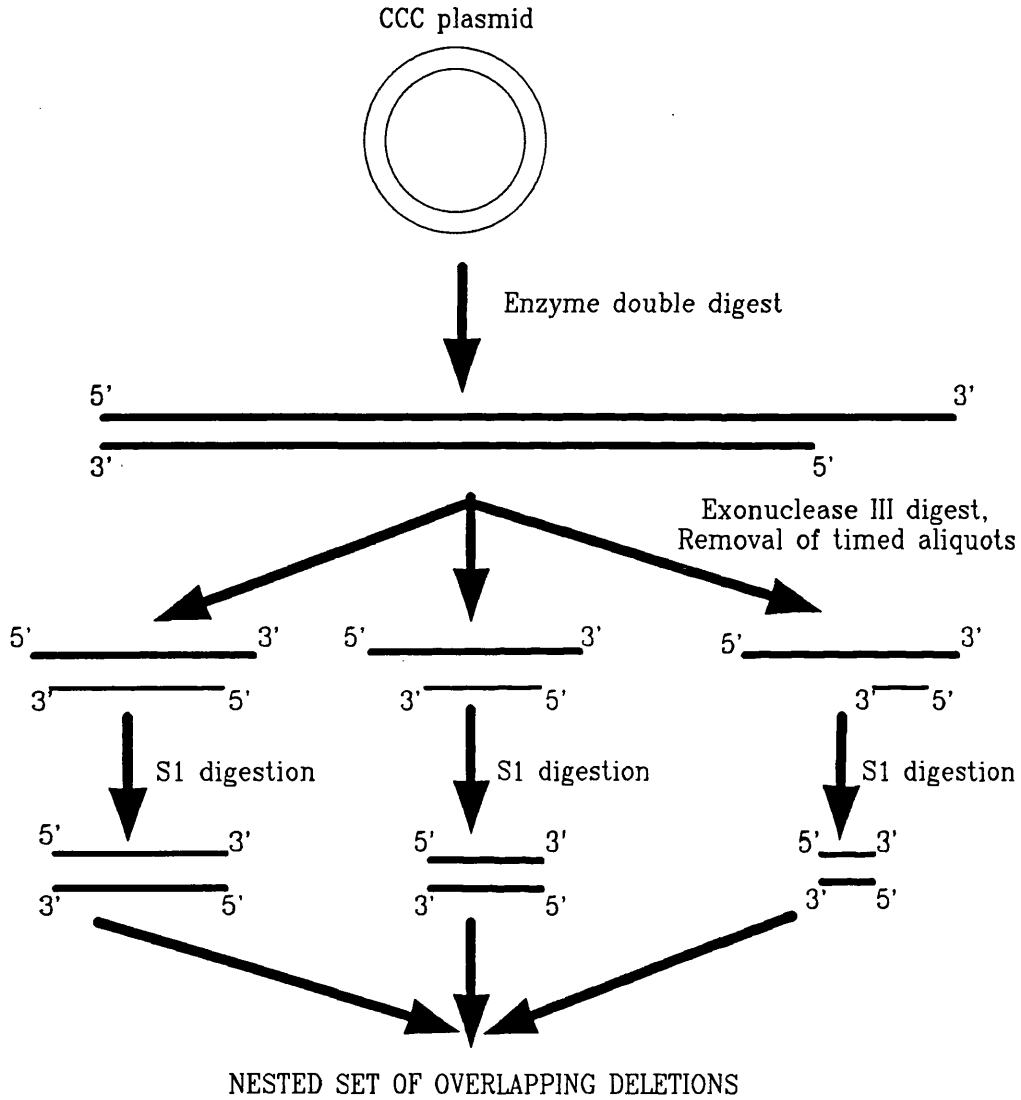
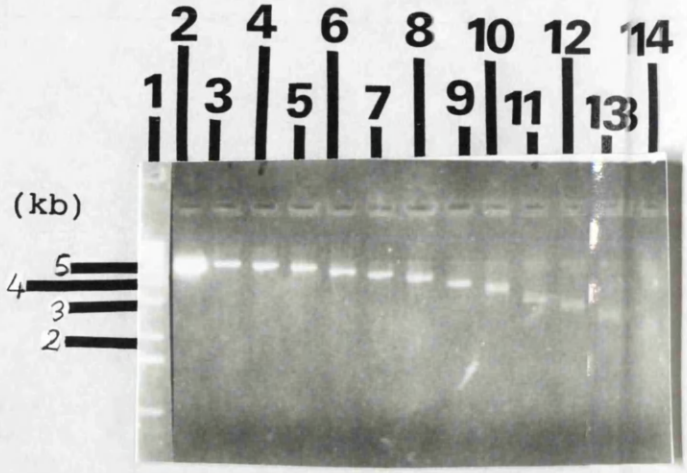


Figure 3.7: Exonuclease III/ SI nuclease nested deletion series.

The 0.5kb SalI fragment was digested with PstI to produce two fragments of 230bp and 240bp; the sizes of these two fragments were calculated from Johnstone's known DNA sequence. These four fragments were ligated into pBLUESCRIPT KS+ previously double digested with SalI/HindIII and SalI/PstI to produce four plasmids designated pSEQ01, pSEQ02, pSEQ03 and pSEQ04. The plasmid pSEQ01 contains the 2.2kb fragment; pSEQ02 contains the 0.8kb fragment; pSEQ03 contains the 240bp fragment with a XhoI site and pSEQ04 contains the 230bp fragment with the SstI site. All plasmid structures were confirmed by plasmid mini-preps and subsequent digestion (results not shown).

3.4 Construction of deletion series.

Nested sets of deletions were made for pSEQ01 and pSEQ02, while pSEQ03 and pSEQ04 were small enough to sequence without further modification. The deletion series were made as described in chapter 2, (Heinkoff 1987). The strategy is shown in figure 3.7. The first step is to cut the target DNA with a "protective enzyme" that produces a 3′ overhang (see list below). Exonuclease III does not attack 4-base 3′ protrusions but it does digest DNA from both 5′ protrusions and blunt ends. The DNA is then cut with a "digestion enzyme" that produces 5′ overhangs or blunt ends. The exonuclease then digests the DNA



LANE		LANE	
1	1kb ladder	8	+150 seconds
2	uncut pSEQ01 DNA	9	+180 seconds
3	timepoint 0	10	+210 seconds
4	+ 30 seconds	11	+240 seconds
5	+ 60 seconds	12	+270 seconds
6	+ 90 seconds	13	+300 seconds
7	+120 seconds	14	+330 seconds

Figure 3.8: exonuclease III/SI digestion of pSEQ01 NotI/SmaI deletion series. Samples run on a 0.8% agarose gel.

from the accessible end, base by base in a uniform manner at 37°C. The rate of digestion depends on temperature. Timed aliquots are removed from the exonuclease III digest mixture every 30 seconds. After buffer changes, the remaining undigested DNA strand is removed by addition of S1 nuclease in conditions that inhibit further digestion by the exonuclease. Klenow polymerase and dNTP's are then used to fill in the ends which are ligated and the ligation mixtures are used to transform bacteria. Figure 3.8 shows a sample of Exonuclease III digests from the pSEQ01 NotI/KpnI deletion series. For the subsequent sequencing reactions I chose plasmids that differed in size by approximately 200 bases.

As I wanted to sequence both strands of pSEQ01 and pSEQ02 it was necessary to make four separate deletion sets in total as described in the table below, see also figure 3.6:

PLASMID	PROTECTIVE ENZYME	DIGESTION ENZYME
pSEQ01	NotI	SmaI
pSEQ01	KpnI	XhoI
pSEQ02	NotI	PstI
pSEQ02	KpnI	XhoI

After ligation and transformation each deletion series was checked by enzyme restriction analysis

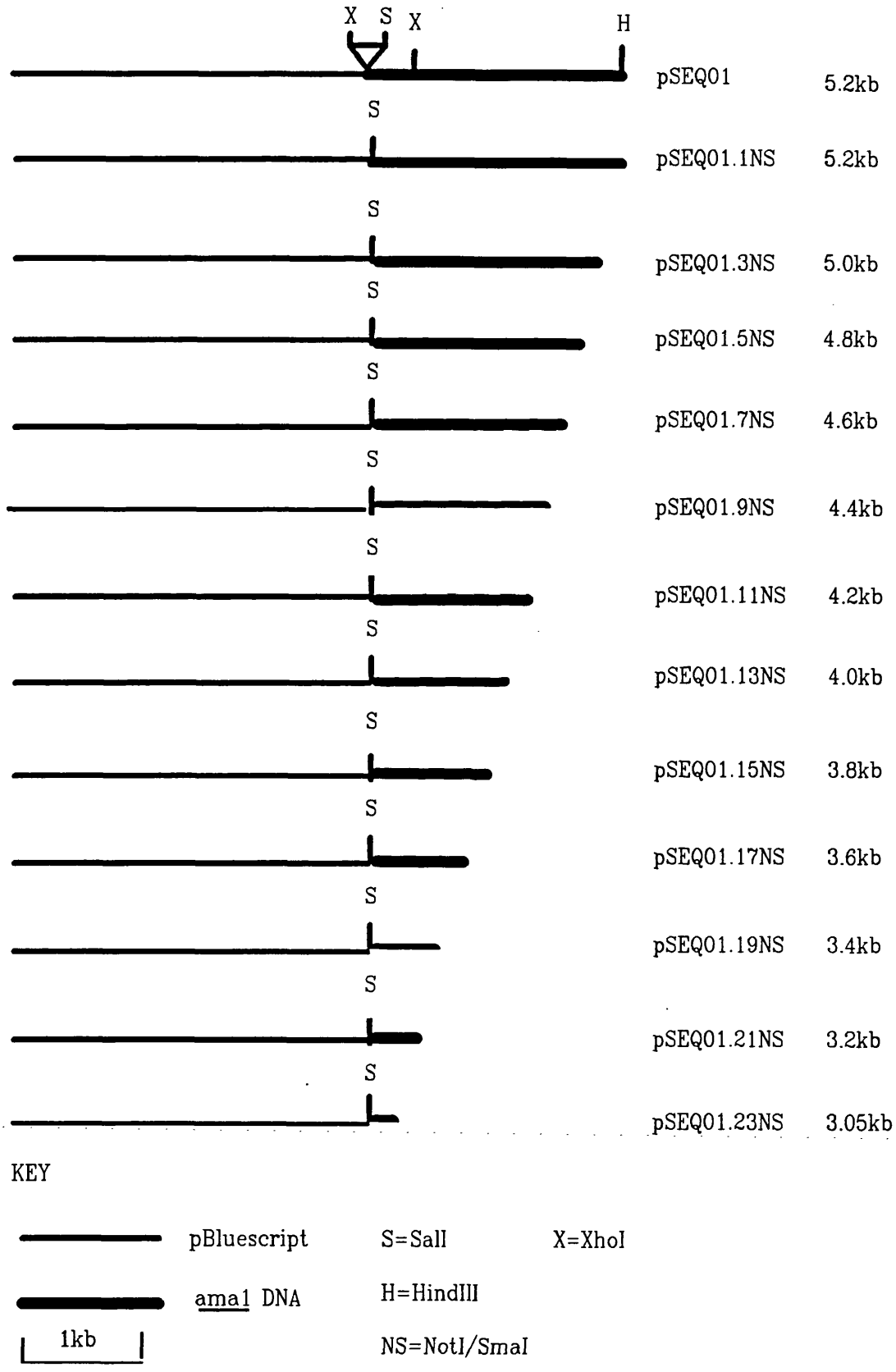
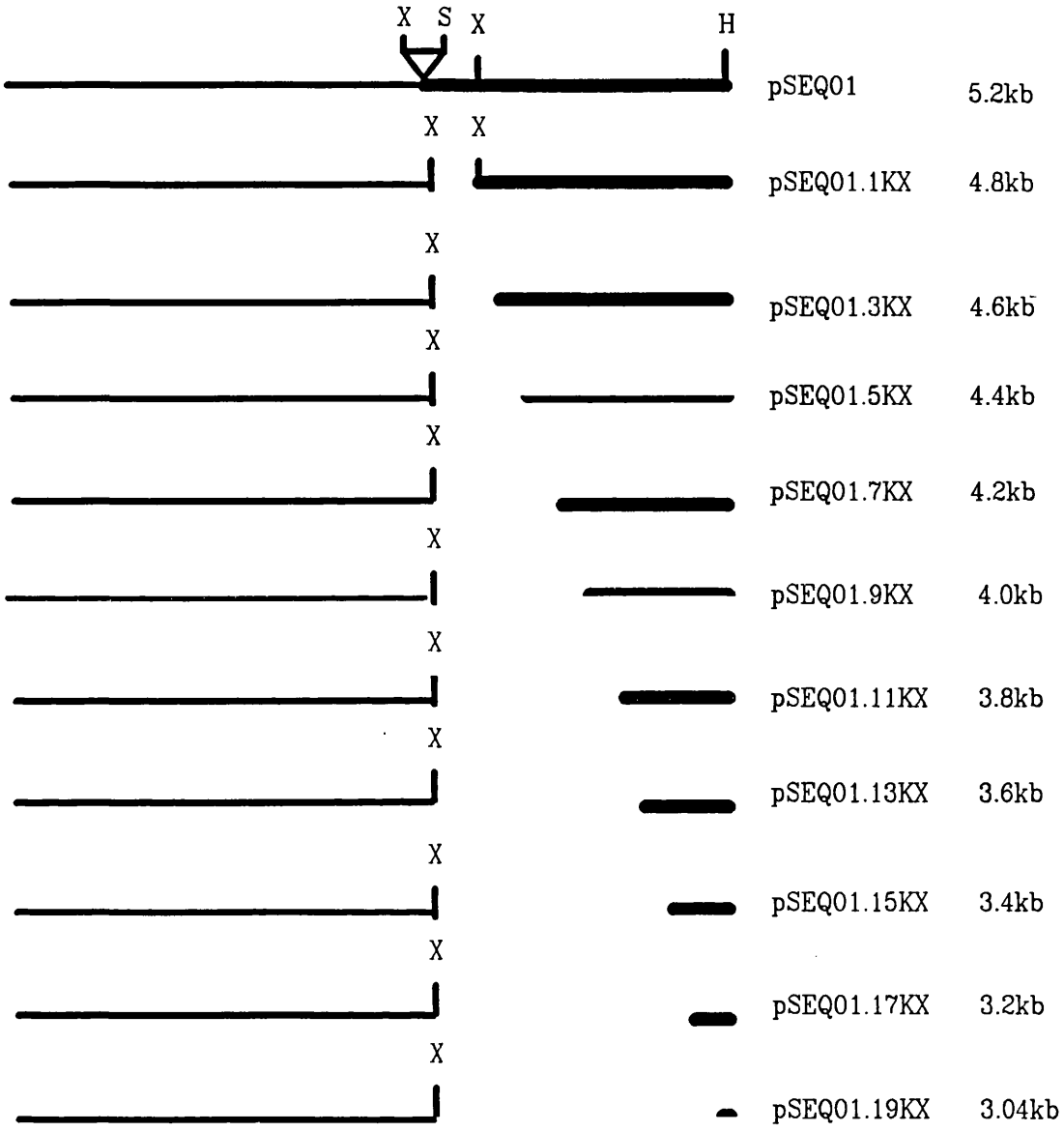


Figure 3.9a: Structure of pSEQ01 NotI/SalI deletion series plasmids.

facing page 68a



KEY

pBluescript S=SalI X=XhoI

ama1 DNA H=HindIII

1kb KX=KpnI/XhoI

Figure 3.9b: Structure of pSEQ01 KpnI/XhoI deletion series plasmids.

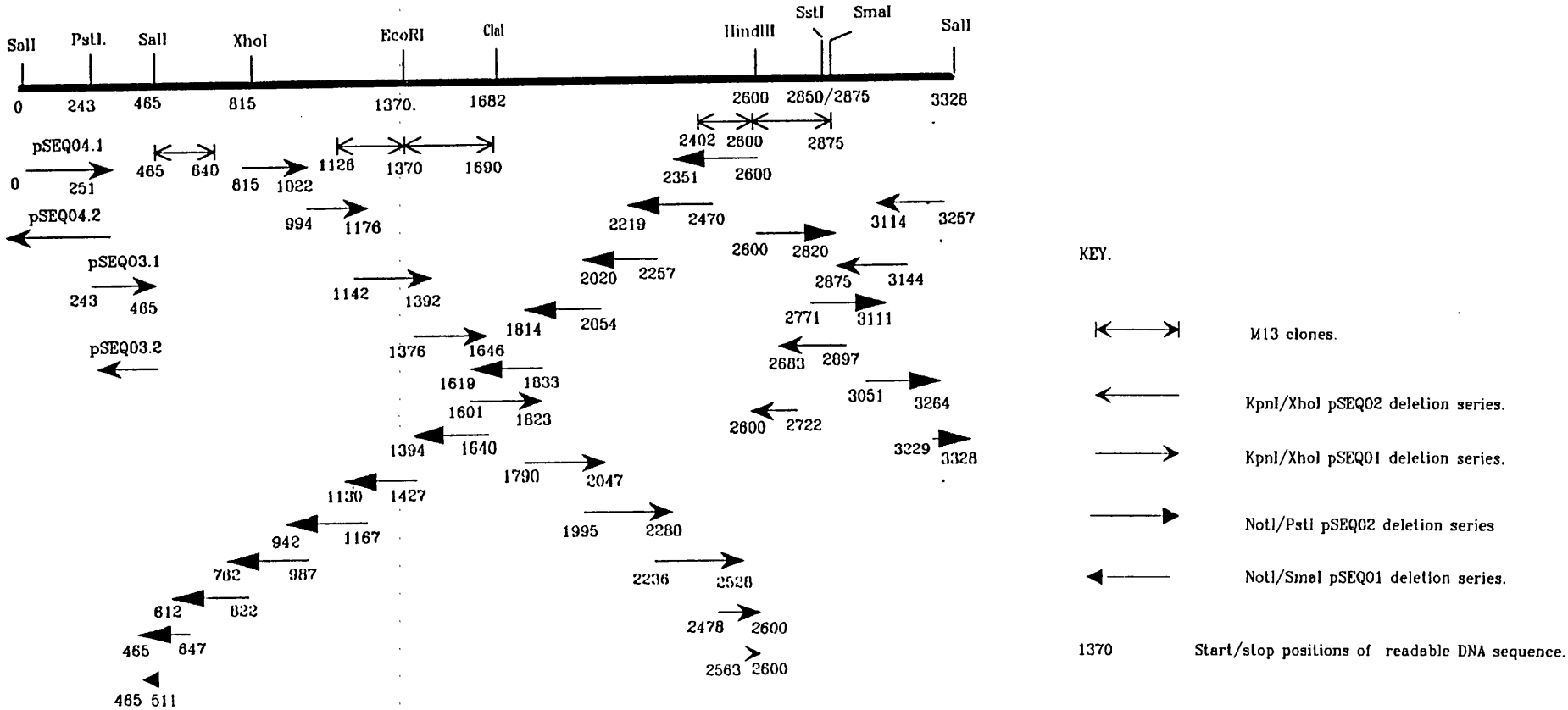


Figure 3.10: ama 1 sequencing strategy.

facing page 68

followed by gel electrophoresis (gels not shown). Plasmid DNAs were prepared from 200ml cultures and then by CsCl/EtBr ultracentrifugation. It should be noted that in making the KpnI/XhoI deletion series a 470bp fragment is deleted from pSEQ01 when it is digested with XhoI, see figure 3.9b.

3.5 Sequenase reactions and electrophoresis.

The sequencing reactions were carried out using the USB Sequenase kit as described in Materials and Methods. Electrophoresis and autoradiography were carried out as previously described. The sequence data was then entered into the IBI Sequenanalysis program and assembled. Database searches and DNA homology comparisons were carried out on the University mainframe using the GCG package.

The structure of each individual deletion plasmid is shown in figures 3.9a and 3.9b. The 2.2kb ama1 SalI fragment is drawn at the top of the page, below which is each numbered pSEQ01 deletion plasmid and it's sequence data. The structure of the M13mp18 and M13mp19 clones are shown in figure 3.4.

The overall sequencing strategy and the start/stop positions of the sequence data from all the deletion series plasmids and M13 clones are shown in figure 3.10.

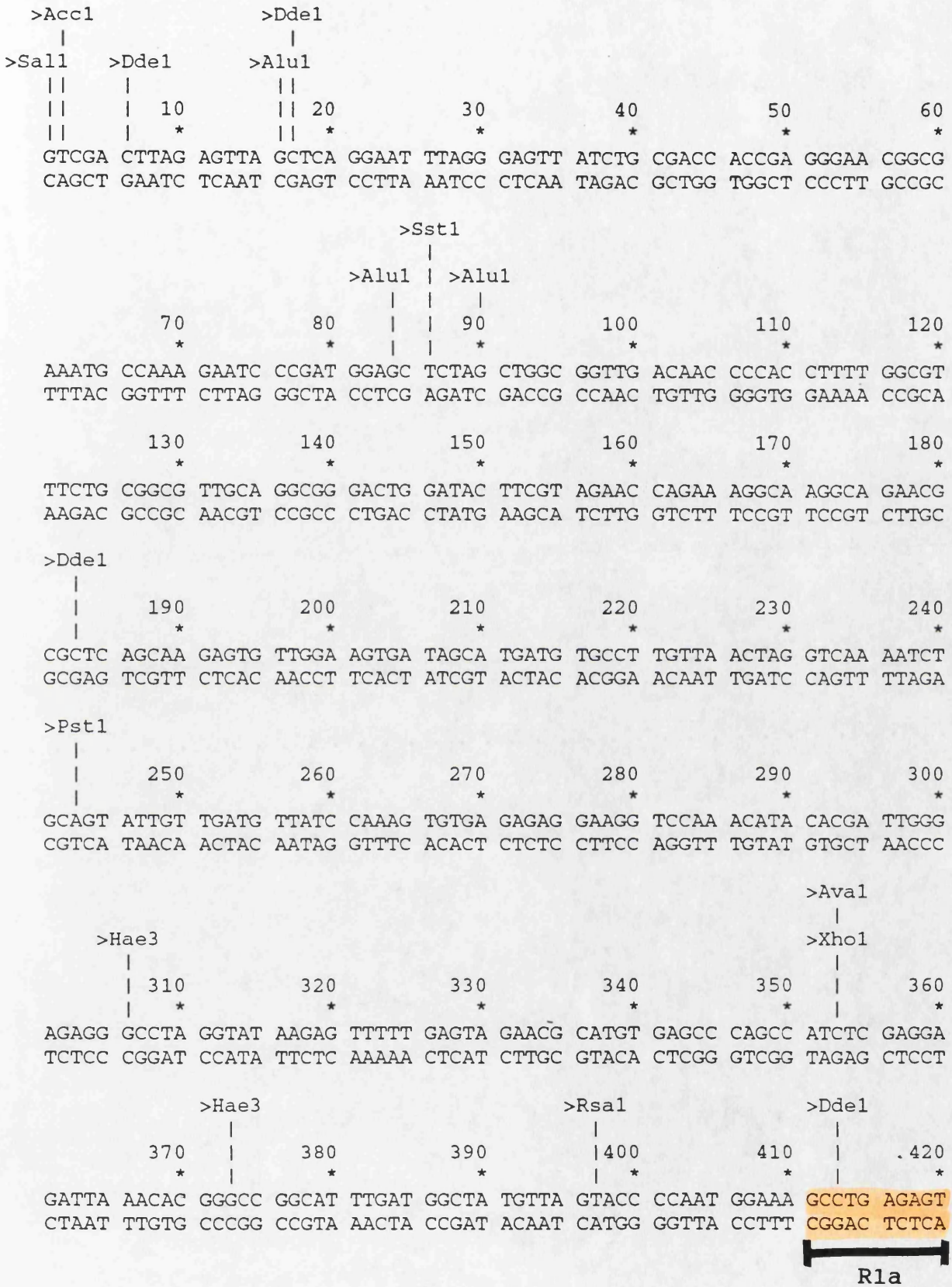
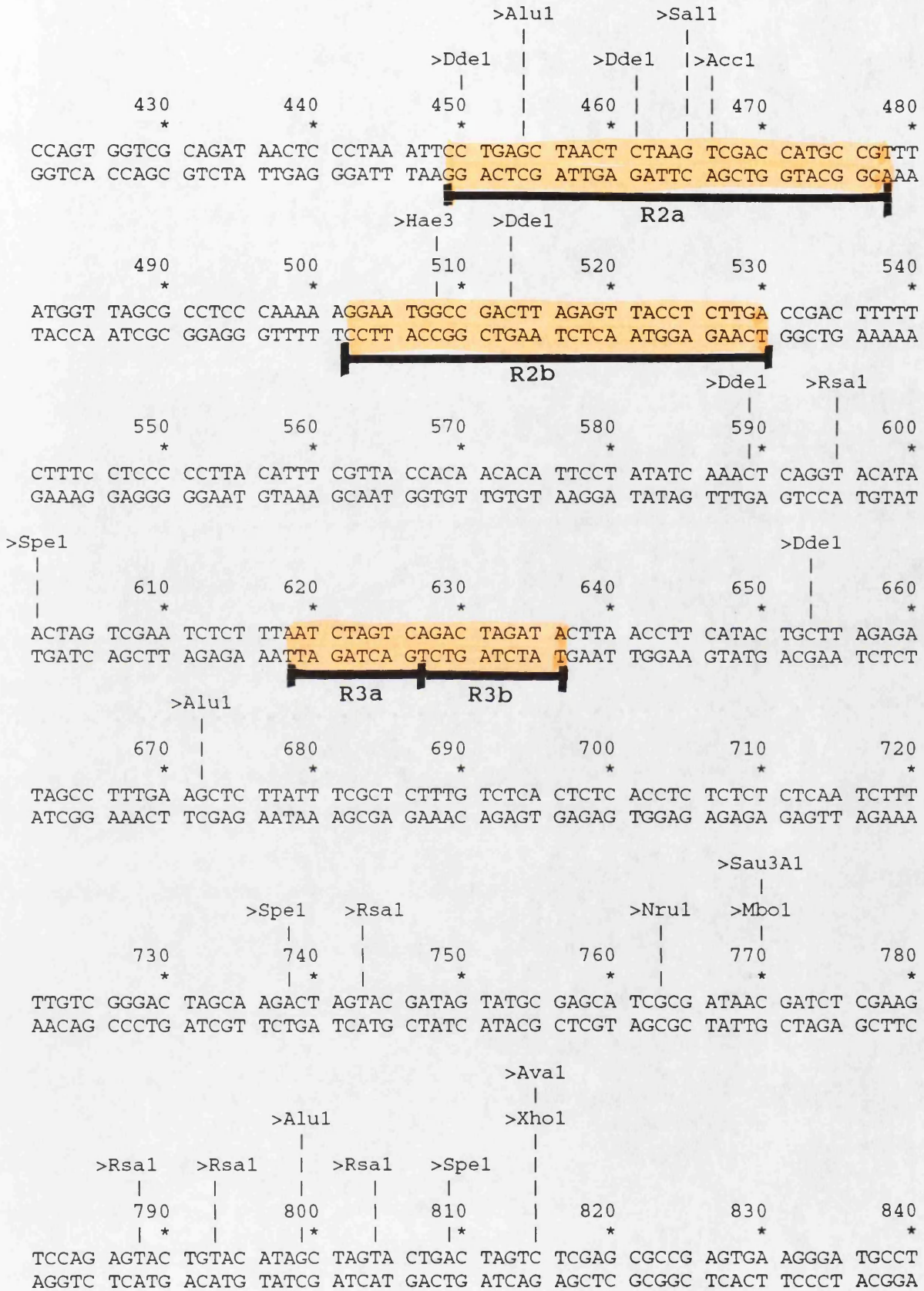


Figure 3.11a: sequence of unique central region and right hand arm of the amal sequence.

R = REPEATED REGIONS = PUC-LIKE REGIONS


>Alu1
>Sal1
>Dde1
>Dde1
>Acc1
430 440 450 460 470 480
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GGTCA CCAGC GTCTA TTGAG GGATT TAAGG ACTCG ATTGA GATTC AGCTG GTACG GCAAA
R2a
>Hae3
>Dde1
490 500 510 520 530 540
ATGGT TAGCG CCTCC CAAAA AGGAA TGGCC GACTT AGAGT TACCT CTTGA CCGAC TTTTT
TACCA ATCGC GGAGG GTTTT TCCTT ACCGG CTGAA TCTCA ATGGA GAACT GGCTG AAAAA
R2b
>Dde1
>Rsa1
550 560 570 580 590 600
CTTTC CTCCC CCTTA CATTT CGTTA CCACA ACACA TTCCT ATATC AAACT CAGGT ACATA
GAAAG GAGGG GGAAT GTAAA GCAAT GGTGT TGTGT AAGGA TATAG TTTGA GTCCA TGTAT
>Spe1
>Dde1
610 620 630 640 650 660
ACTAG TCGAA TCTCT TTAAT CTAGT CAGAC TAGAT ACTTA ACCTT CATAC TGCTT AGAGA
TGATC AGCTT AGAGA AATTA GATCA GTCTG ATCTA TGAAT TGGAA GTATG ACGAA TCTCT
R3a
R3b
>Alu1
670 680 690 700 710 720
TAGCC TTTGA AGCTC TTATT TCGCT CTTTG TCTCA CTCTC ACCTC TCTCT CTCAA TCTTT
ATCGG AAACT TCGAG AATAA AGCGA GAAAC AGAGT GAGAG TGGAG AGAGA GAGTT AGAAA
>Sau3A1
>Spe1
>Rsa1
>Nru1
>Mbo1
730 740 750 760 770 780
TTGTC GGGAC TAGCA AGACT AGTAC GATAG TATGC GAGCA TCGCG ATAAC GATCT CGAAG
AACAG CCCTG ATCGT TCTGA TCATG CTATC ATACG CTCGT AGCGC TATTG CTAGA GCTTC
>Ava1
>Alu1
>Xho1
>Rsa1
>Rsa1
>Rsa1
>Spe1
790 800 810 820 830 840
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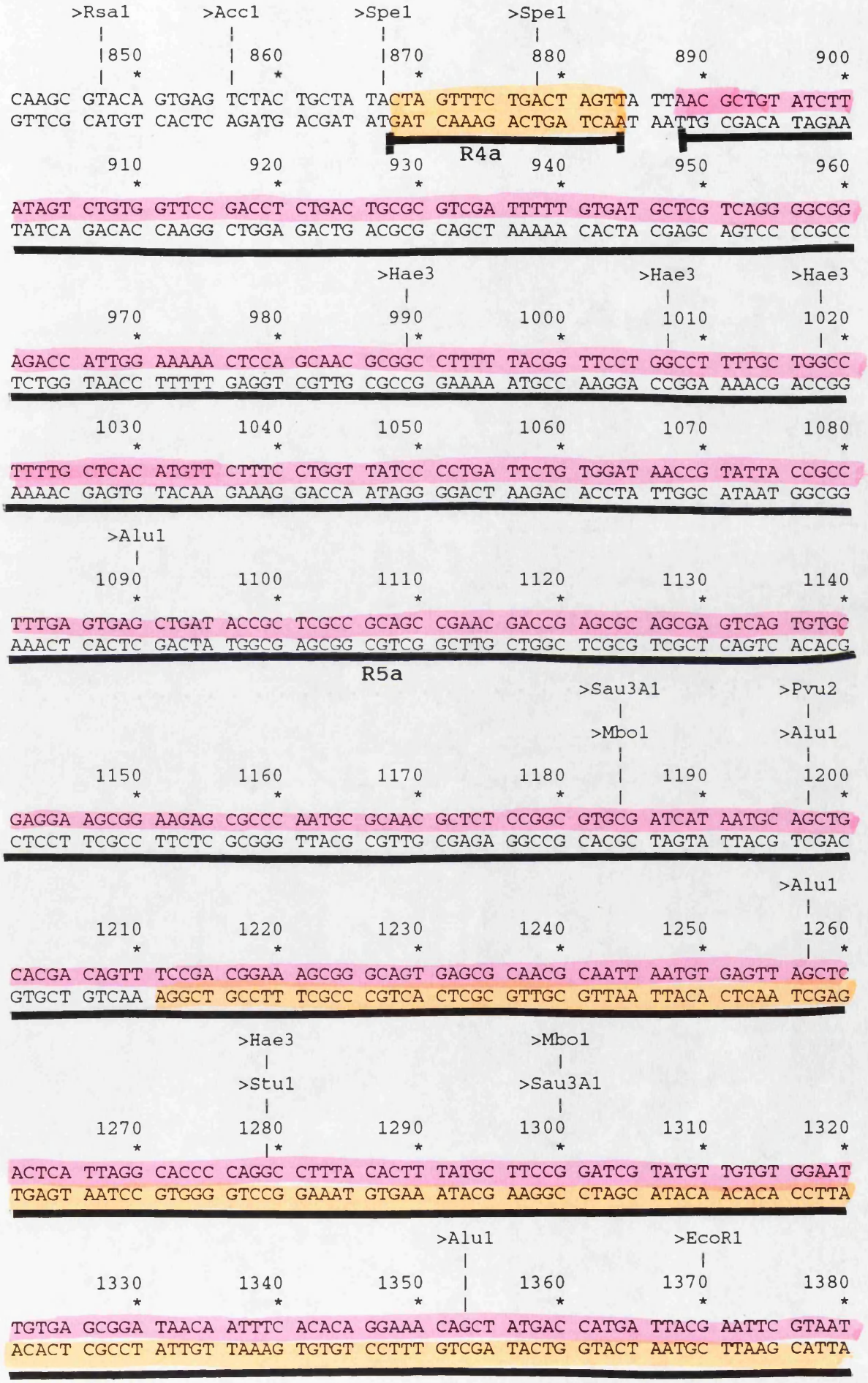
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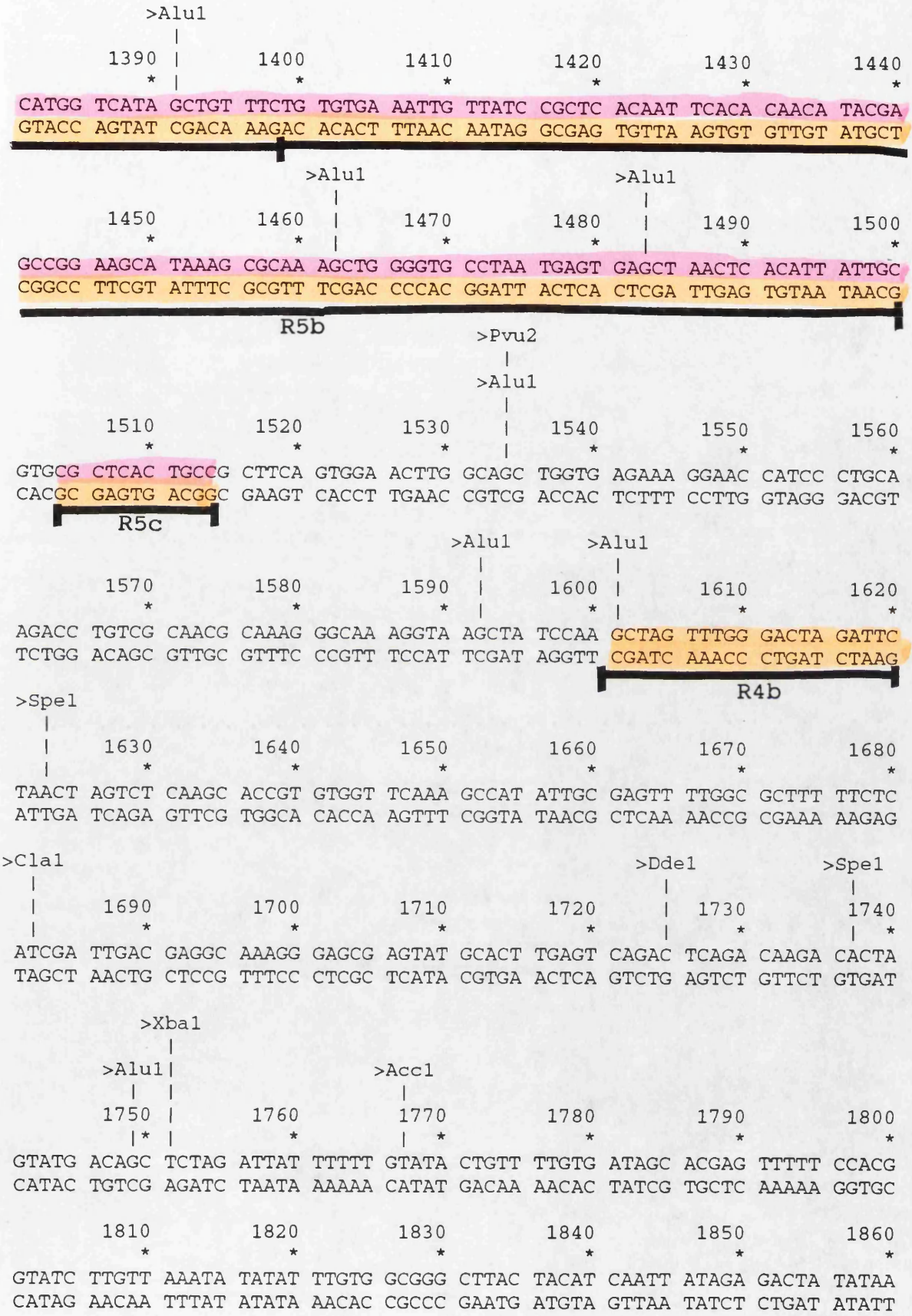
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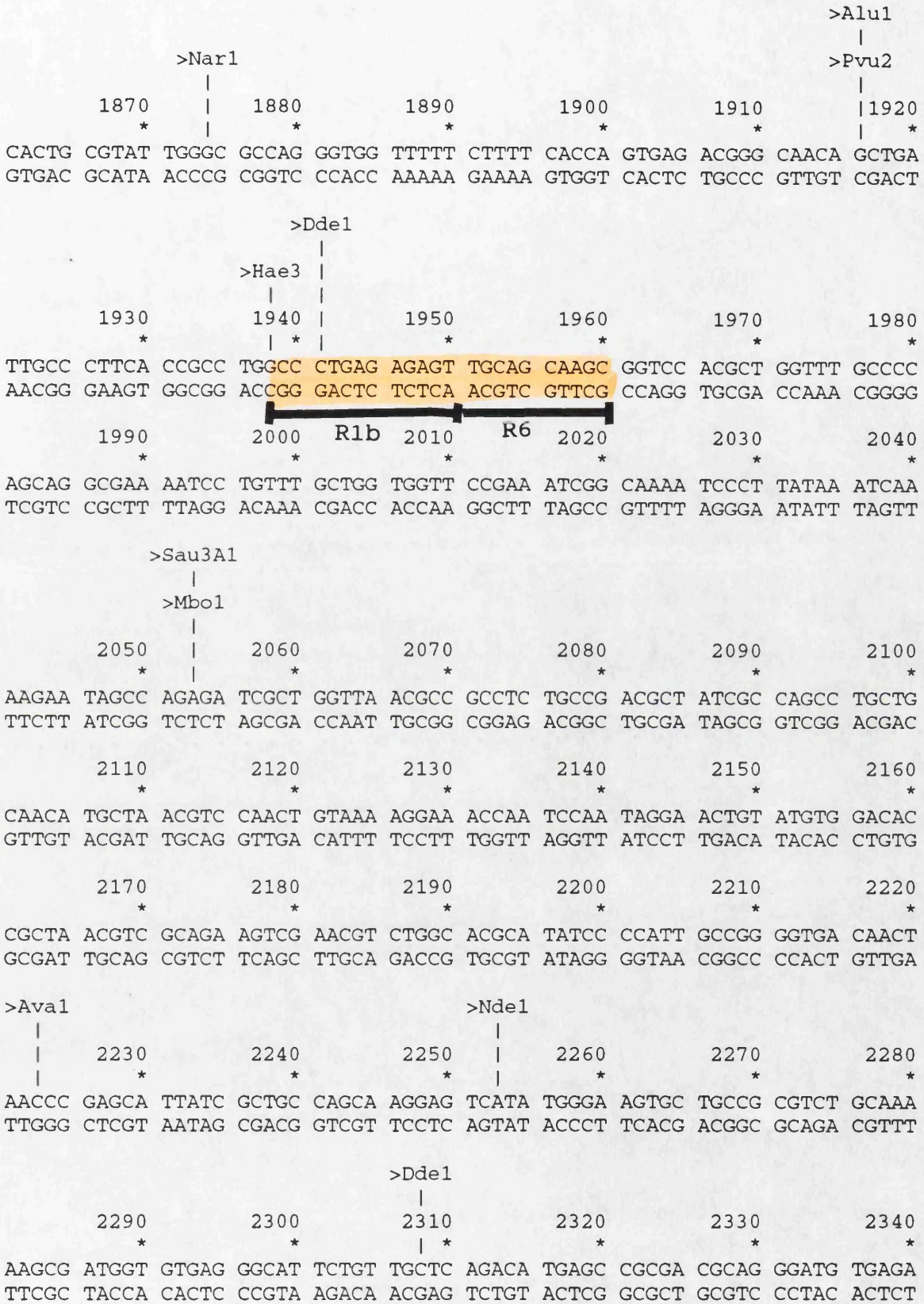
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R1b
R6
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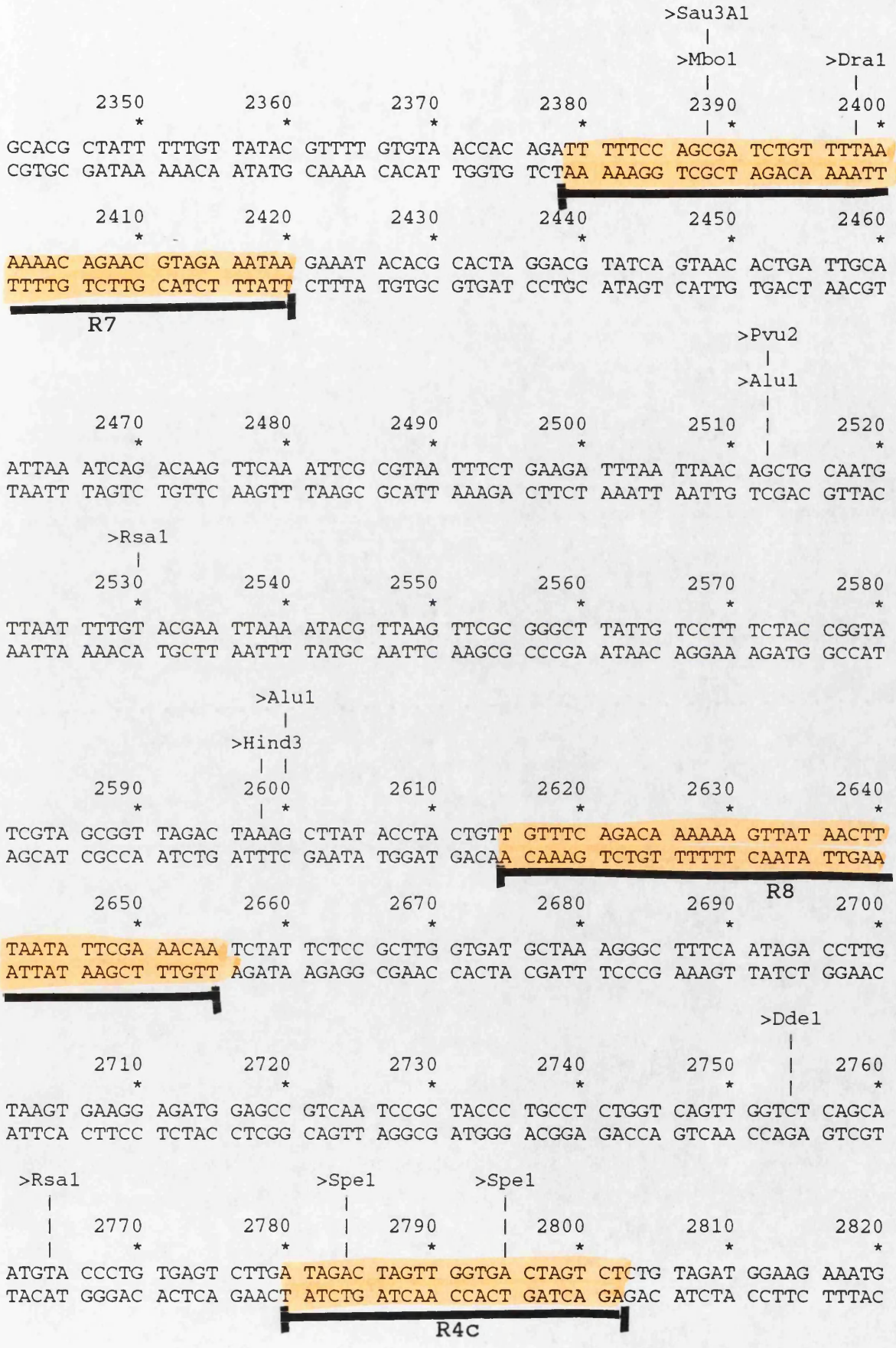
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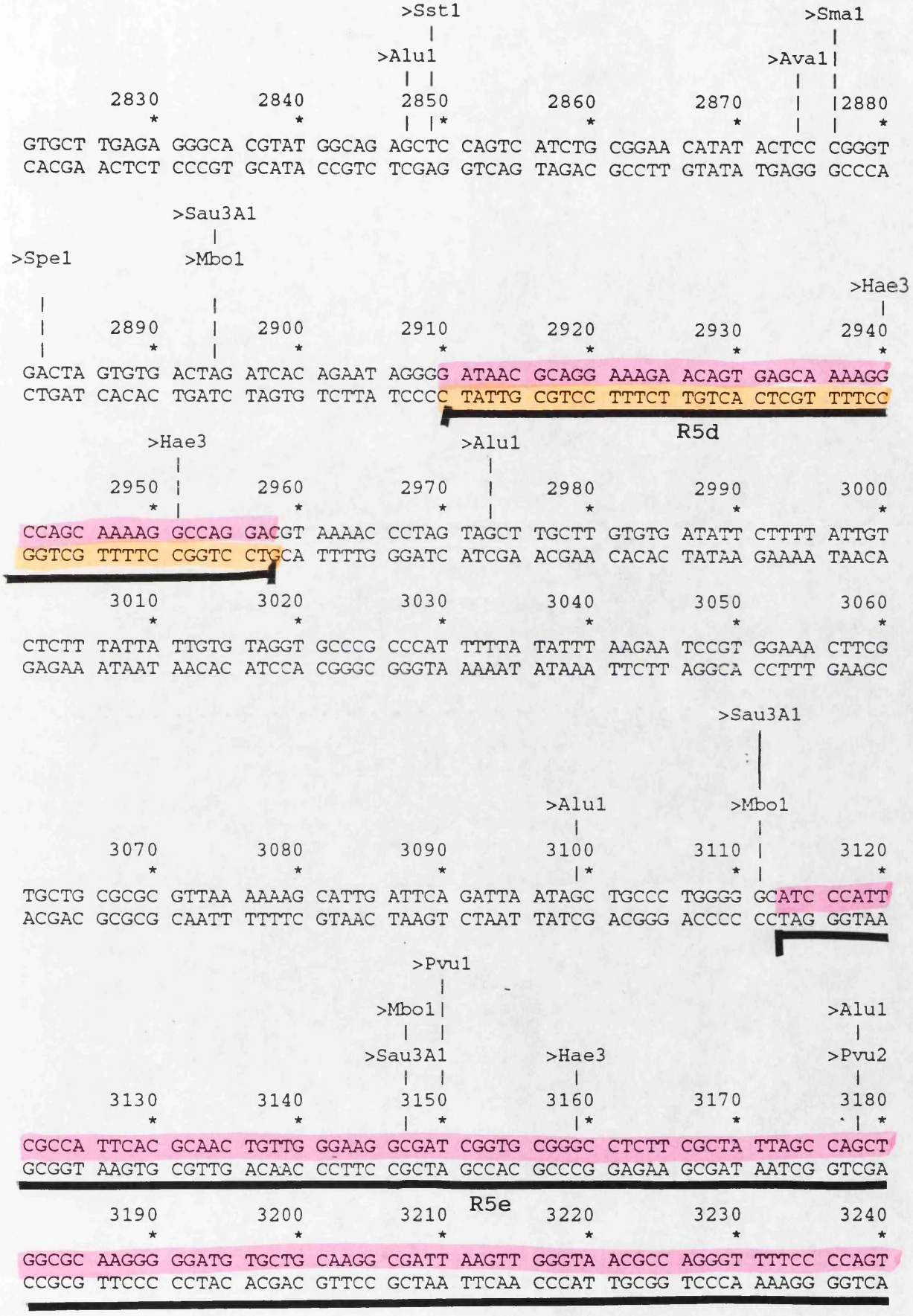
R4c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R5e
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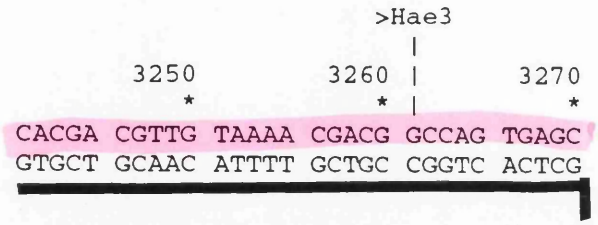
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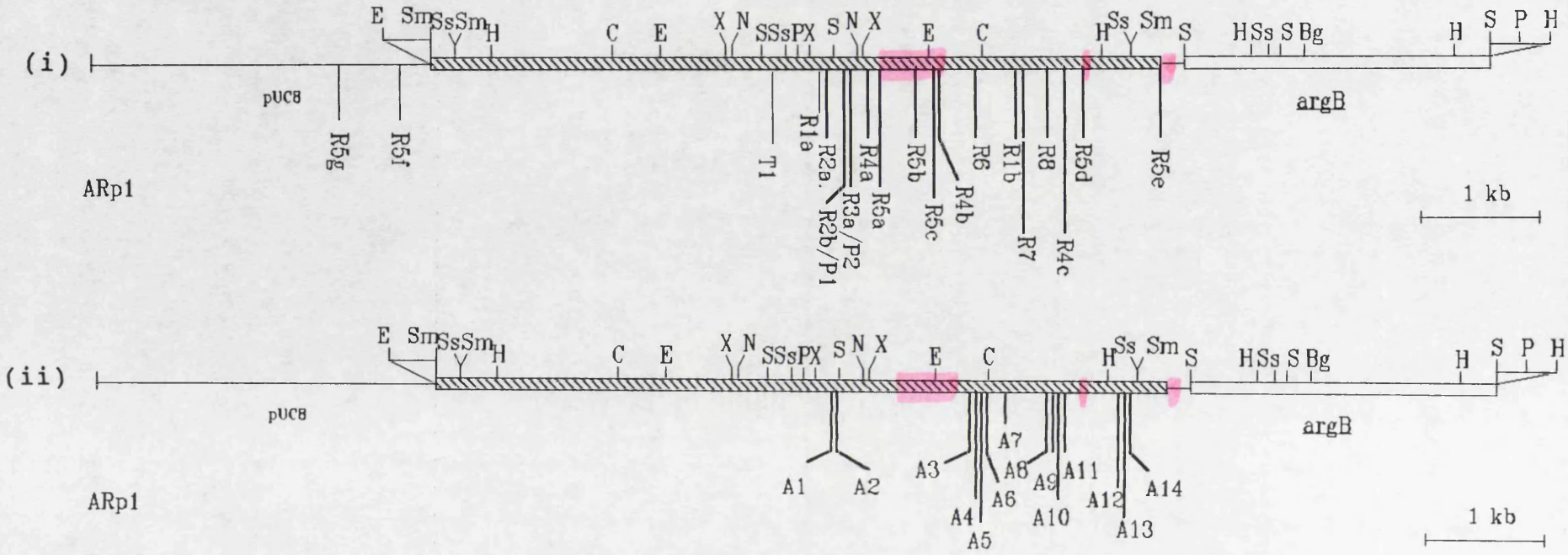

Figure 3.11b: maps of the repeated regions (i) and (ii) A/T rich regions in ama1. All pink highlighted regions mark the location of pUC-like DNA in ama1.

facing page 69i

R+A DESIGNATIONS DETAILED IN FIGURES 3·11c TO 3·11e

REGION	POSITION	LENGTH (bp)	DESCRIPTION.
T1	30-36	6	yeast telomere
R1a	411-420	9	repeat of R1b.
R1b	1937-1950	13	repeat of R1a.
T1	440-446	13	yeast telomere
R2a	448-477	29	repeat of R2b.
R2b	501-530	29	inverted repeat of R2a.
P1	535-561	26	purine rich region.
R3a	618-626	8	repeat of R3b.
R3b	627-636	9	inverted repeat of R3a.
P2	664-722	58	purine rich region.
R4a	867-883	23	repeat of R4b and R4c.
R5a	888-1398	510	ColEI origin and Lac Z.
R5b	1398-1500	102	inverted repeat of R5a.
R5c	1503-1513	10	small part of R5e.
R4b	1600-1620	20	repeat of R4a and R4c.
R6	1948-1960	12	small repeat.
R7	2378-2420	42	inverted repeat.
R8	2614-2655	41	inverted repeat.
R4c	2780-2802	22	repeat of R4a and R4b.
	3111		end of ama1 insert.
R5d	2910-2957	47	part of R5a.

Figure 3.11c: locations and brief description of all regions of interest in ama1. R=repeated region, P=purine rich region, T=telomere

REGION	POSITION	LENGTH (bp)	DESCRIPTION.
R5e	3112-3270	150	Lac Z region.
R5f			Lac Z region in pUC8.
R5g			ColEI origin in pUC8.

Figure 3.11c continued: locations and brief description of all regions of interest in ama1.

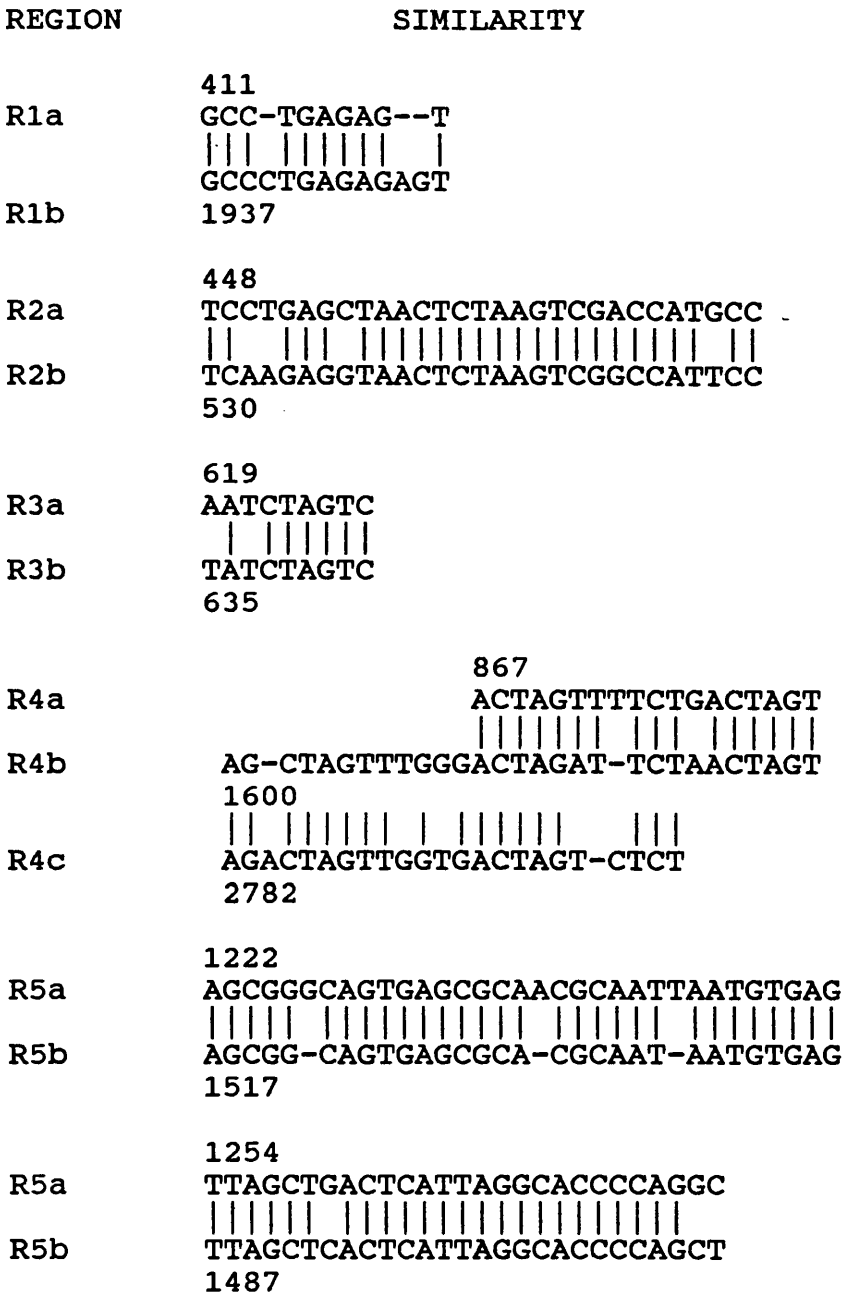

Figure 3.11d: degree of similarity between internal repeated regions of ama1.

```
            2378
R7          TTTTTCCAGCGATCTGTTTT
            | |||| | || ||||||||
            TATTTCTA-CGTTCTGTTTT
            2429

            2614
R8          TTGTTTCAGACAAAAAAGTTAT
            ||||||| |  ||       |||
            TTGTTTC-G--AA------TAT
            2655

            2943
R5d         GATAACGCAGGAAAGAACAG-TGAGCAAAAGGCCAG
            ||||||| ||||||||||  ||||||||||||||||
R5a         GATAACC-AGGAAAGAACATGTGAGCAAAAGGCCAG
            1049

            30
T1          CCCTAA

            440
T1          CCCTAA

            535
P1          CTTTTTCTTTCCTCCCCCTTaCaTTTC

            664
P2          CCTTTgCTCTTaTTTCgCTCTTTgTCTCaCTCTCa

            698
            CCTCTCTCTCTCaaTCTTTTT
```

Figure 3.11d continued: degree of similarity between internal repeated regions of ama1.

REGION	POSITION	LENGTH (bp)	DESCRIPTION.
AT1	534-544	10	AcTTTTTcTTT
AT2	580-590	10	TATATcAAAcT
AT3	1751-1795	45	TcTAgATTATTTTTTgTATAcTg TTTTgTgATAgcAcgAgTTTT
AT4	1802-1822	20	TATcTTgTTAAATATATTT
AT5	1831-1860	29	cTTAcTAcATcAATTATAgAgAc TATATAA
AT6	1885-1895	10	TTTTTcTTTT
AT7	2030-2047	17	TTATAAATcAAAAgAATA
AT8	2390-2426	40	TcTgTTTTAAAAAAcAgAAcgTA gAAATAAgAAATA
AT9	2460-2483	23	AATTAAATcAgTTcAAATT
AT10	2488-2509	21	TAATTTcTgATTTAATTAA
AT11	2521-2552	31	TTAATTTTgTAcgAATTAAAATA cgTTAAgTT
AT12	3003-3012	9	cTcTTTATTATT
AT13	3030-3046	16	TTTTTATATTTAAgAAT
AT14	3072-3098	26	TTAAAAAAgcATTgATT

Figure 3.11e: locations and brief description of all major A/T rich regions in amal.

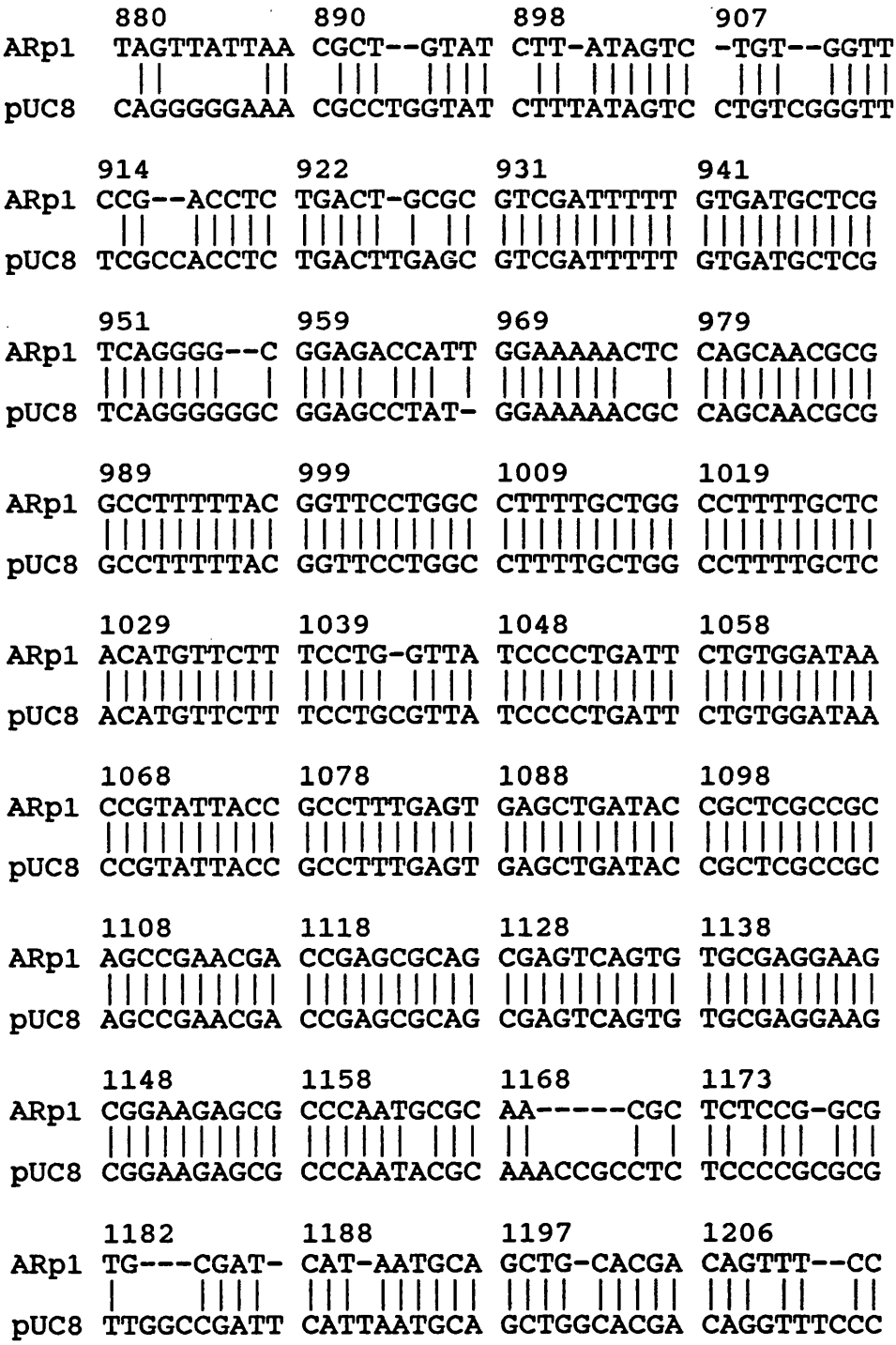

Figure 3.12a: similarity between ama1 and pUC8.

Figure 3.12a continued:

```
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ARp1 GAC-GGAAAG CGGGCAGTGA GCGCAACGCA ATTAATGTGA
     ||| ||||||| |||||||||| |||||||||| ||||||||||
pUC8 GACTGGAAAG CGGGCAGTGA GCGCAACGCA ATTAATGTGA

     1253        1263        1273        1283
ARp1 GTTAGCTCAC TCATTAGGCA CCCCAGGCCT TTACACTTTA
     |||| ||||| |||||||||| ||||||||| | ||||||||||
pUC8 GTTACCTCAC TCATTAGGCA CCCCAGGC-T TTACACTTTA

     1293        1303        1313        1323
ARp1 TGCTTCCGGA TCGTATGTTG TGTGGAATTG TGAGCGGATA
     |||||||||  |||||||||| |||||||||| ||||||||||
pUC8 TGCTTCCGGC TCGTATGTTG TGTGGAATTG TGAGCGGATA

     1333        1343        1353        1363
ARp1 ACAATTTCAC ACAGGAAACA GCTATGACCA TGATTACGAA
     |||||||||| |||||||||| |||||||||| ||||||||||
pUC8 ACAATTTCAC ACAGGAAACA GCTATGACCA TGATTACGAA

     1373        1383        1393        1402
ARp1 TTCGTAATCA TGGTCATAGC TGTTT-CTGT GTGAAATTGT
     |||||||||| |||||||||| ||||| |||| ||||||||||
pUC8 TTCGTAATCA TGGTCATAGC TGTTTCCTGT GTGAAATTGT

     1412        1422        1431        1441
ARp1 TATCCGCTCA CAATT-CACA CAACATACGA GCCGGAAGCA
     |||||||||| ||||| |||| |||||||||| ||||||||||
pUC8 TATCCGCTCA CAATTCCACA CAACATACGA GCCGGAAGCA

      1451       1461        1470        1480
ARp1 TAAAGCGCAA AG-CTGGGGT GCCTAATGAG TGAGCTAACT
     ||||| | || || |||||||| |||||||||| ||||||||||
pUC8 TAAAGTGTAA AGCCTGGGGT GCCTAATGAG TGAGCTAACT

     1490        1499        1508
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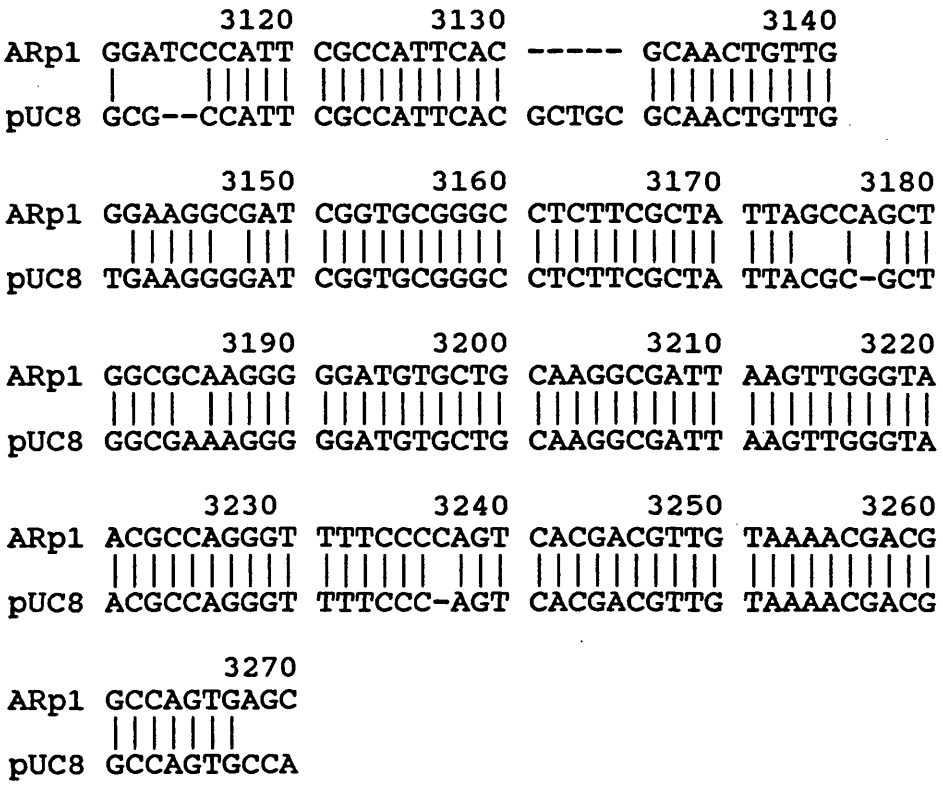


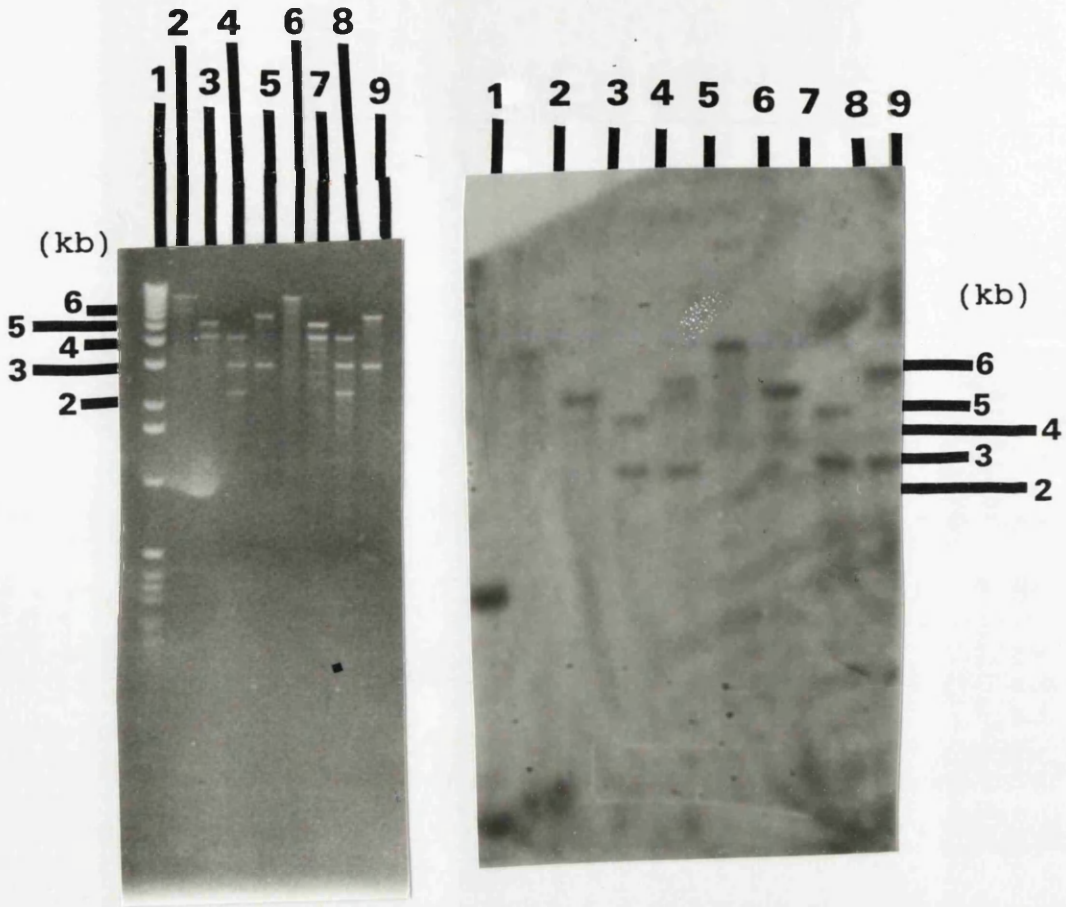
Figure 3.12b: similarity between amal and pUC8.

3.6 Analysis of the ama1 sequence.

The compiled DNA sequence is shown in figure 3.11a and is numbered from 0 to 3270: 0 being the SalI site to the left of the unique central region and 3270 is within the vector DNA on the right hand end of the ama1 sequence; (note that the total sequence ends at position 3328, the terminal 50 bases were not sequenced). The positions of restriction enzyme cutting sites within the DNA sequence for SalI, SstI, PstI, XhoI, NruI, EcoRI, ClaI, HindIII and SmaI, correlate with the enzyme restriction map, (see figure 3.1).

A number of ama1 regions were found to have sequence similarity to pUC8. The regions highlighted in pink show sequence similarity to pUC8, all other regions are assumed to be Aspergillus genome derived DNA; regions in orange are sequences that are repeated more than once. The pink and orange regions can overlap. For clarity all highlighted regions are refered to by a specific number e.g. R1a. These regions are shown in a map in figure 3.11b which shows the location of both repeats and A/T rich regions on the sequenced arm. Figures 3.11c., 3.11d and 3.11e detail the reference numbers and gives a brief description of each region.

The regions R5a, R5c and R5e have strong sequence similarity to pUC8. R5c is a ten bp region which is also found in R5e. R5e is 150bp in size and



LANE	DNA	BAND SIZES (kb)
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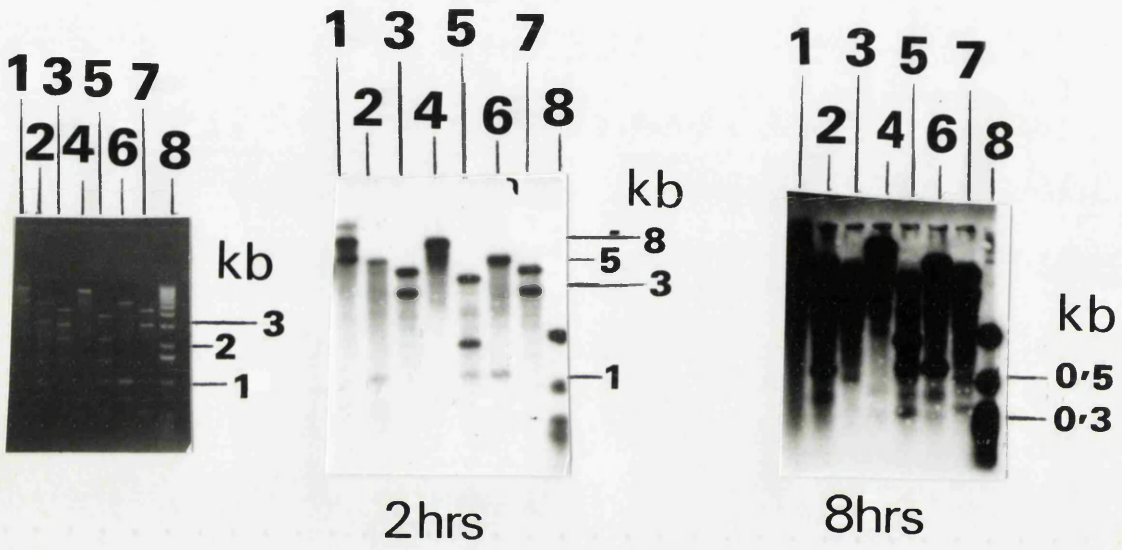
2	uncut pHELP2	-
3	pHELP2/HindIII	5, 4.3
4	pHELP2/EcoRI	4.3, 2.8, 2.2
5	pHELP2/SalI	5.8, 2.9, 0.6
6	uncut pHELP2	-
7	pHELP2/HindIII	5, 4.3
8	pHELP2/EcoRI	4.3, 2.8, 2.2
9	pHELP2/SalI	5.8, 2.9, 0.6

Figure 3.13a: pHELP2 DNA probed with radiolabelled pUC8 DNA.

is identical to a part of the pUC 8 lacZ region. R5a is 510bp in size and also contains an imperfect inverted repeat of part of the pUC8 lacZ and the ColE1 origin, designated as R5b. The similarity between R5a+b and R5e with the lacZ/origin region of pUC 8 is shown in figures 3.12a and 3.12b. R5a+b has 91% similarity with pUC; R5e has 88% similarity with pUC. The presence of this vector-like DNA complicated the assembly of the DNA data but its presence in these locations was confirmed by data from the Southern blots, (see figures 3.13a and 3.13b).

The presence of pUC-like DNA in the ama1 sequence was confirmed by analysis of a plasmid called pHELP2, Gems (1990). The plasmid pHELP2 comprises the 5kb HindIII ama1 fragment ligated into a non-pUC-related plasmid, pACγC184. Duplicate 1μg aliquots of pHELP2 were digested separately with 20 units of HindIII, EcoRI and SalI. The pHELP2 DNA was run out on a 0.8% agarose gel and Southern blotted. The results are shown in figure 3.13a. The distinctive 5kb HindIII ama1-derived fragment, (lanes 3 and 7), hybridises with the radiolabelled pUC DNA. This result confirms that the ama1 sequence contains pUC-like DNA components. However, the 2.2kb ama1-derived EcoRI does not appear to hybridise with the pUC8 probe, suggesting that the central 2.2kb EcoRI ama1 fragment does not contain pUC-like DNA, thereby contradicting the sequence data. The



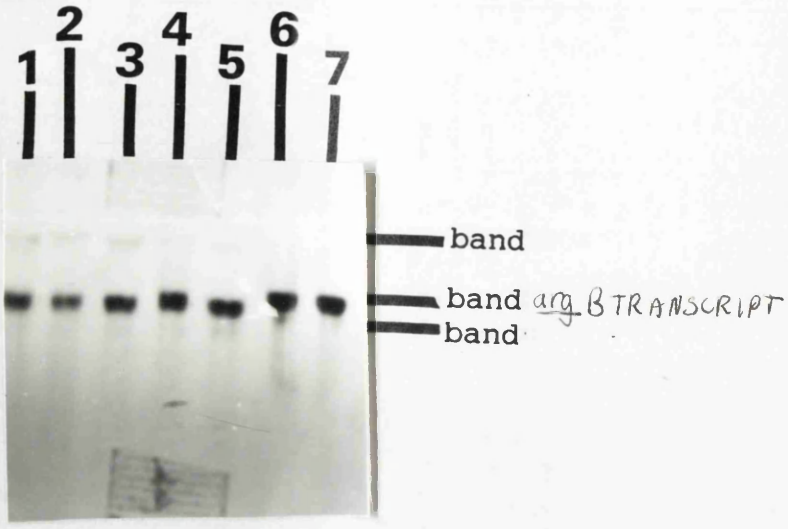
LANE	DNA	BAND SIZES (kb)
1	uncut pHELP2	-
2	pHELP2/ClaI	5.1, 3.3, 0.9
3	pHELP2/EcoRI	4.3, 2.8, 2.2
4	pHELP2/XhoI	8.2, 0.6, 0.5
5	pHELP2/ClaI/EcoRI	3.8, 2.2, 1.5, 1, 0.3x2
6	pHELP2/ClaI/XhoI	6.2, 1x2, 0.6, 0.5
7	pHELP2/EcoRI/XhoI	4.2, 3.1, 0.6x2, 0.6, 0.5
8	1kb marker	

Figure 3.13b: pHELP2 DNA probed with radiolabelled pUC8 DNA and exposed for 2 hours and 8 hours; ___ denotes ama1-derived bands of interest, (see text and also figure 3.16 for restriction sites and relevant fragments).

apparent failure to detect pUC-like DNA in the 2.2 EcoRI fragment could be due to the inverted repeat structure folding-back or snapping-back so that it hybridises to itelf, rather than with the pUC probe. Snap-backs can be a common occurrence with inverted repeat sequences, (Iain Hunter, personal communication). The Snap-backs are more likely to occur in large DNA fragments than in small DNA fragments: in short, large fragments will not hybridise with the pUC8 DNA probe but small fragments will hybridise.

The snap-back hypothesis was tested by digesting pHELP2 with various enzymes. 1µg aliquots of pHELP2 were digested separately with 20 units of ClaI, EcoRI and XhoI. 1µg aliquots of pHELP2 were double digested with EcoRI and ClaI, EcoRI and XhoI, and ClaI and XhoI. All the digested pHELP2 DNA samples were run on a 0.9% agarose gel and Southern blotted. This Southern blot was then probed with radiolabelled pUC8 DNA. The results are shown in figure 3.13b and include a 2 hour and a 8 hour exposure of the Southern blot.

In figure 3.13b, the ama1-derived 3.3kb ClaI fragment and 2.2kb EcoRI fragment do not appear to hybridise with the pUC8 DNA probe, (see 2 hour exposure). Initially, this result suggests that ama1 does not contain any pUC-like DNA within the central 3.3kb ClaI fragment: so either the DNA sequence in figure 3.11a must be incorrect, (see also figure



LANE

1 18hrs

2 20hrs

3 22hrs

4 24hrs

5 26hrs

6 28hrs

7 30hrs

Figure 3.14a: Northern blot of Aspergillus G34 RNA from differently aged cultures, probed with radiolabelled ARp1 and exposed for 12 hours.

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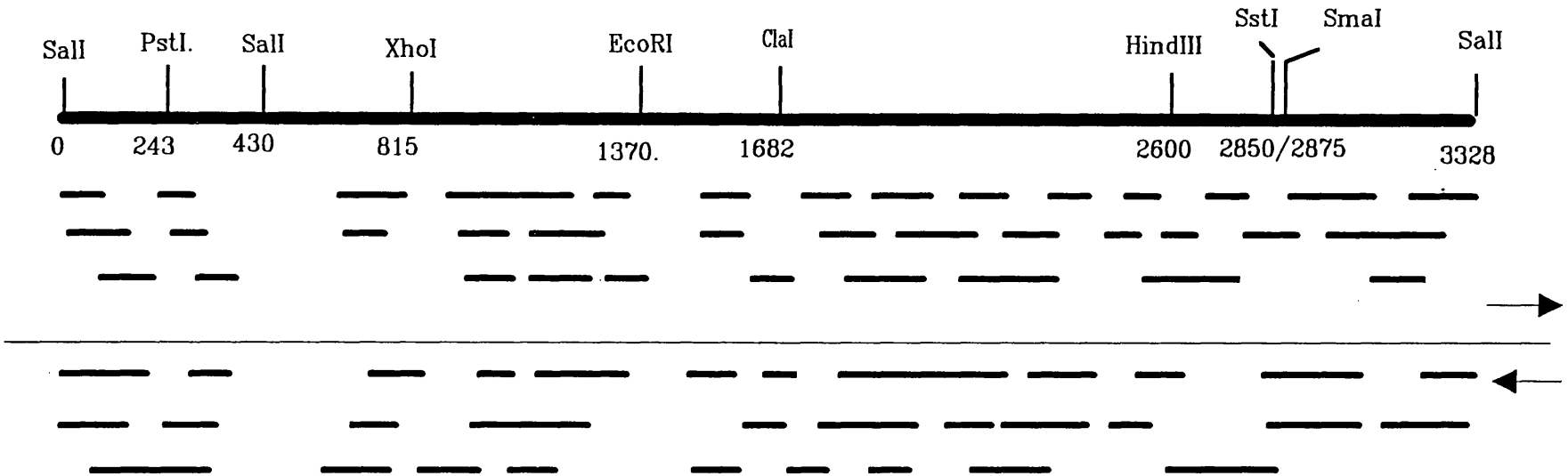


KEY

direction of transcript,
all start/stop postions of all possible ORFs are plotted.

transcript

0.15kb.

Figure 3.14b: start/stop positions and sizes of ama1 open reading frames.

3.16) or snap-backs are present in these fragments.

However, the 0.3kb ama1-derived fragments generated by the ClaI/EcoRI double digest and the 0.6kb ama1-derived fragments produced by the EcoRI/XhoI double digest, do hybridise with the pUC8 DNA probe, (see 8 hour exposure). The ClaI/XhoI double digest (lane 6), (see 2 hour exposure), is ambiguous because a 1kb band also hybridises with a similar intensity in the ClaI/EcoRI double digest (lane 5). Overall, this result suggests that, in fact, the ama1 sequence does contain pUC-like DNA at the locations shown in figures 3.11a and 3.16. In addition, this result suggests that the large ama1-derived fragments do not hybridise with the pUC probe due to the formation of snap-backs.

Northern blots of Aspergillus RNA from differently aged cultures, probed with radiolabelled ama1 suggested that ama1 is not transcribed at all frequently; when the blot was probed with radiolabelled pILJ16 the ArgB transcript was clearly identifiable, (see figure 3.14a); no hybridising band was visible when this blot was probed with radiolabelled ama1; three hybridising bands, one very strong: two faint,were seen when this blot was probed with radiolabelled ARp1. A computer search for open reading frames in ama1 found a total of 85 open reading frames larger than 100bp, (see figure 3.14b). Further analysis of these ORFs failed to identify any related ORFs present in the databases. It is

Mono-Nucleotide Frequencies

Base	Count	Frequency	Measured/Expected	Base	Count	Frequency	Measured/Expected	Base	Count	Frequency	Measured/Expected	Base	Count	Frequency	Measured/Expected
T	874	0.263	1.05	C	759	0.228	0.91	A	866	0.260	1.04	G	828	0.249	1.00

Ambiguous = 0 Total Positions = 3327

Di-Nucleotide Frequencies

Base	Count	Frequency	Measured/Expected	Base	Count	Frequency	Measured/Expected	Base	Count	Frequency	Measured/Expected	Base	Count	Frequency	Measured/Expected
TT	259	0.078	1.13	TC	179	0.054	0.90	TA	208	0.063	0.91	TG	228	0.069	1.05
CT	218	0.066	1.09	CC	162	0.049	0.94	CA	200	0.060	1.01	CG	179	0.054	0.95
AT	200	0.060	0.88	AC	183	0.055	0.93	AA	242	0.073	1.07	AG	241	0.072	1.12
GT	198	0.060	0.91	GC	235	0.071	1.24	GA	216	0.065	1.00	GG	179	0.054	0.87

Ambiguous = 0 Total Positions = 3327

Tri-Nucleotide Frequencies

Base	Count	Frequency	Measured/Expected	Base	Count	Frequency	Measured/Expected	Base	Count	Frequency	Measured/Expected	Base	Count	Frequency	Measured/Expected
TTT	89	0.027	1.48	TTC	48	0.014	0.92	TTA	61	0.018	1.02	TTG	61	0.018	1.07
TCT	56	0.017	1.07	TCC	38	0.011	0.84	TCA	51	0.015	0.98	TCG	34	0.010	0.69
TAT	60	0.018	1.00	TAC	37	0.011	0.71	TAA	55	0.017	0.93	TAG	56	0.017	0.99
TGT	62	0.019	1.09	TGC	58	0.017	1.17	TGA	56	0.017	0.95	TGG	52	0.016	0.96
CTT	51	0.015	0.97	CTC	51	0.015	1.12	CTA	50	0.015	0.98	CTG	66	0.020	1.33
CCT	44	0.013	0.97	CCC	35	0.011	0.89	CCA	45	0.014	1.00	CCG	38	0.011	0.88
CAT	38	0.011	0.78	CAC	41	0.012	0.91	CAA	59	0.018	1.15	CAG	62	0.019	1.26
CGT	33	0.010	0.67	CGC	60	0.018	1.39	CGA	52	0.016	1.06	CGG	34	0.010	0.72
ATT	63	0.019	1.05	ATC	42	0.013	0.81	ATA	54	0.016	0.91	ATG	41	0.012	0.72
ACT	56	0.017	1.08	ACC	35	0.011	0.78	ACA	44	0.013	0.86	ACG	48	0.014	0.98
AAT	53	0.016	0.90	AAC	57	0.017	1.11	AAA	74	0.022	1.26	AAG	58	0.017	1.03
AGT	63	0.019	1.11	AGC	70	0.021	1.42	AGA	61	0.018	1.09	AGG	47	0.014	0.88
GTT	57	0.017	1.00	GTC	38	0.011	0.77	GTA	43	0.013	0.76	GTG	60	0.018	1.11
GCT	62	0.019	1.25	GCC	54	0.016	1.25	GCA	60	0.018	1.22	GCG	59	0.018	1.26
GAT	49	0.015	0.87	GAC	48	0.014	0.98	GAA	54	0.016	0.95	GAG	65	0.020	1.21
GGT	39	0.012	0.72	GGC	47	0.014	1.00	GGA	47	0.014	0.85	GGG	46	0.014	0.90

Ambiguous = 0 Total Positions = 3327

Strand Asymmetry
0.0 = Symmetric strands (purines = pyrimidines on one strand)
1.0 = Asymmetric strands (all purines or all pyrimidines on one strand)

Strand Asymmetry = 0.023

Percent ACGT over a window of 50

poly A/T no. of bases	frequency
4	0.98
5	1.07
6	1.64
7	3.75
8	3.00

Figure 3.15: base composition of sequenced region of amal.

possible that any ama1 transcripts are unstable or have a short half life so will not be identified under the experimental conditions used, (see figure 3.14a), but there is no reason to assume that ama1 is not derived from a transcribed region.

Computer analysis of base composition and distribution in ama1 is shown in figure 3.15. Overall, the nucleotide frequency of A/T compared to G/C is approximately 50/50. Some triplet codon frequencies are significantly higher than expected e.g. the TTT frequency is 1.48 and the AAA frequncy is 1.26, suggesting that ama1 contains A/T rich regions. However, some triplet codons are more rare e.g. the frequency of TCG is 0.69. The figure calculated for the strand asymmetry is 0.023. This low figure indicates that roughly the same number of both purines and pyrimidines are found on the same strand; this is not the case if short regions of ama1 are considered e.g. there is a run of twenty purines between positions 535 and 554 in ama1, see figure 3.11a.

3.7 Discussion.

Johnstone (1985), originally isolated ARp1 from a pILJ16 gene bank. The pILJ16 gene bank was constructed by cloning Sau3A random Aspergillus genomic DNA fragments into pILJ16. The gene bank was then used to transform Aspergillus nidulans

protoplasts and ARp1 was isolated from a slow growing transformant colony.

The database searches failed to identify any recognisable DNA replication origins in ama1 even though a variety of known origin sequences were used as models; the ama1 sequence was tested with the yeast consensus sequence 5'A/TTTTATPuTTT(A/T)3', the Adenovirus sequence ATAATATACC, (Tamanmoi and Stillman 1983), the SV40 origin regions, (Soeda et al 1979). A number of A/T rich regions are present in ama1 as shown in figures 3.11b and 3.11c. The role of such A/T regions is not known although it is possible that these sequences could act as ARSs, but the structure of Aspergillus origins is unknown. Alternatively the A/T rich sequences may be similar to skeletal attachment regions (SAR) which include the "A-box" AATAAAT/CAAA and the "T-box" TTATTTTTT, (Johnson and McKnight 1989). It has also been suggested, Perez-Martin and Espinosa (1991), Wells (1988), that A/T rich regions are flexible so allow localised folding/bending of the DNA helix. What is interesting is the apparent clustering of these AT rich regions as shown in figure 3.11b, both the function and effects on plasmid behaviour, if any, of this clustering is unknown.

In addition, there are two sequences similar to yeast telomeres within ama1, (see figures 3.11c and 3.11d). The function, if any, of these sequences is not clear but it has been shown by Perrot et al

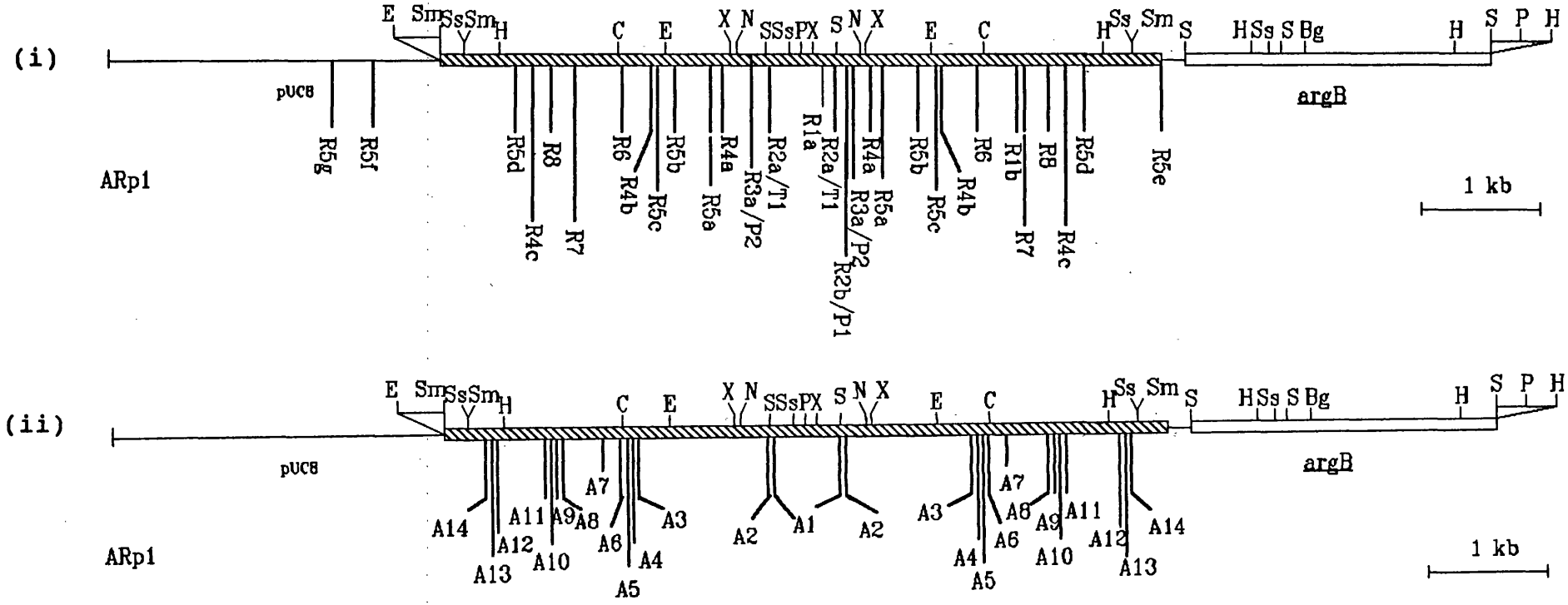


Figure 3.16: maps of all (i) repeated regions and all (ii) A/T rich regions and all pUC-like regions, (high-lighted pink), in the total amal sequence.

facing page 75

(1987), that telomeric sequences significantly increase linear plasmid stability.

The sequenced arm of ama1 contains two purine (C/G) rich regions. Again the role of these sequences in either autonomous replication or plasmid stability is not known, but Jacobs et al (1989) have shown that purine rich regions appear to have an important role in the replication of Sea urchin mitchondrial DNA.

The sequenced arm of ama1 also contains ten Spe1 cutting sites. I would expect a six-cutter like Spe1 to cut ama1 two times. The relevance, if any, of the number of Spe1 sites is not clear.

Ama1 contains a significant amount of related pUC8 DNA. Figure 3.16 shows ARp1 with all the repeats and pUC8 DNA marked in for both arms. The prescence of this pUC related DNA is not due to sequencing errors, the pUC containing regions of ama1 were sequenced separately using different vectors, (see sections 3.2 and 3.3), but the homology was found as a result of other people's errors: not everyone checks their sequence for sequencing vector DNA before putting it in a database. The presence of the pUC-related DNA in the ama1 insert and the overall structure of the ama1 insert can be explained if the original ARp1 parental plasmid from the pILJ16 gene bank underwent some rearrangement process in the transformant colony, thereby giving rise to present structure and composition of ARP1. This hypothesis

is supported by the presence pUC-like DNA found in R5e. In the original pILJ16 library construct, the vector DNA in region R5e was derived from the tet gene of pBR327, Johnstone (1985). The results suggest that the pBR327 DNA has been replaced with pUC-derived DNA via some rearrangement process. Rearrangements of the pUC sequence in this region would also explain the imperfect homology between pUC8 and the ama1 pUC related sequences detailed in figures 3.12a and 3.12b and suggests that this region of ama1 may have been structurally unstable during its formation. The evidence presented in Chapter 4 suggests that ARp1 is probably not rearranged so the ama1 region is likely to be structurally stable.

The question is does this pUC8 DNA have a functional role in ARp1? Powell and Kistler (1990), have isolated an autonomously replicating linear plasmid from Fusarium oxysporium. This plasmid, pFOLT4, is of great relevance because sequencing and subcloning of this plasmid has shown that the ARS consists of pUC DNA in an inverted repeat; the pUC arms are separated by 126bp of fungal DNA. These findings have important implications when considering the possible function of the ama1 pUC DNA: is this pUC DNA the ARS?

One result of the rearrangements in the ama1 pUC regions is that the ColE1 replication origin located between DNA positions 888-970 (see figure 3.11a) is now probably incapable of initiating DNA replication

by its usual method via formation of a DNA/RNA hybrid. Normally, a 600base DNA fragment containing the origin and the promoter for RNAII is required for replication. RNAII is the initiation of replication primer RNA which binds to origin at a CCCCCC sequence (3'-5'). This sequence is critical i.e. single base changes or deletion of any bases in the CCCCCC sequence abolishes replication, (Masukata and Tomizawa, 1990). By looking at figures 3.11a and figure 3.12a it is clear that this vital CCCCCC motif has been replaced by a CCCCGC motif located at position 954 onwards.

In summary, what is responsible for autonomous replication, plasmid maintenance and structural stability in ARp1? These are important questions because no functional ARS sequences and endogenous replicating plasmids have been isolated from or identified in Aspergillus nidulans. The following chapters attempt to answer these questions.

Chapter 4.

Behaviour of ARp1 and derived subclones.

4.1 Introduction.

This chapter addresses some of the questions raised in Chapter 3: the location and number of functional ARS/origin-like sequences, the extent and nature of amal sequence actually required for autonomous replication, plasmid maintenance and structural stability and the role of the inverted repeat. The answers to these questions would also give clues to the mechanism by which ARp1 replicates. Gems (1990), proposed a theoretical model describing ARp1 replication. This model is based on the yeast 2μ circle and is discussed fully in section 4.7. Before attempting to discuss how ARp1 replicates I want to describe in some detail the mechanisms controlling replication and stability in the 2μ circle model.

4.1.1. The yeast 2μ circle.

In many respects the yeast 2μ circle acts like a tiny circular chromosome; it is found in the nucleus, behaves like a nuclear marker in cytoduction crosses and has the same chromatin structure as a yeast chromosome, Gunge (1983) and Volkert et al (1989). The 2μ plasmid origin of replication has a concensus sequence similar to the yeast chromosomal ARS sequences: 5'(A/T)TTTATPuTTT(A/T)3', Kearsey (1984), Broach and Hicks (1988). Initiation of replication

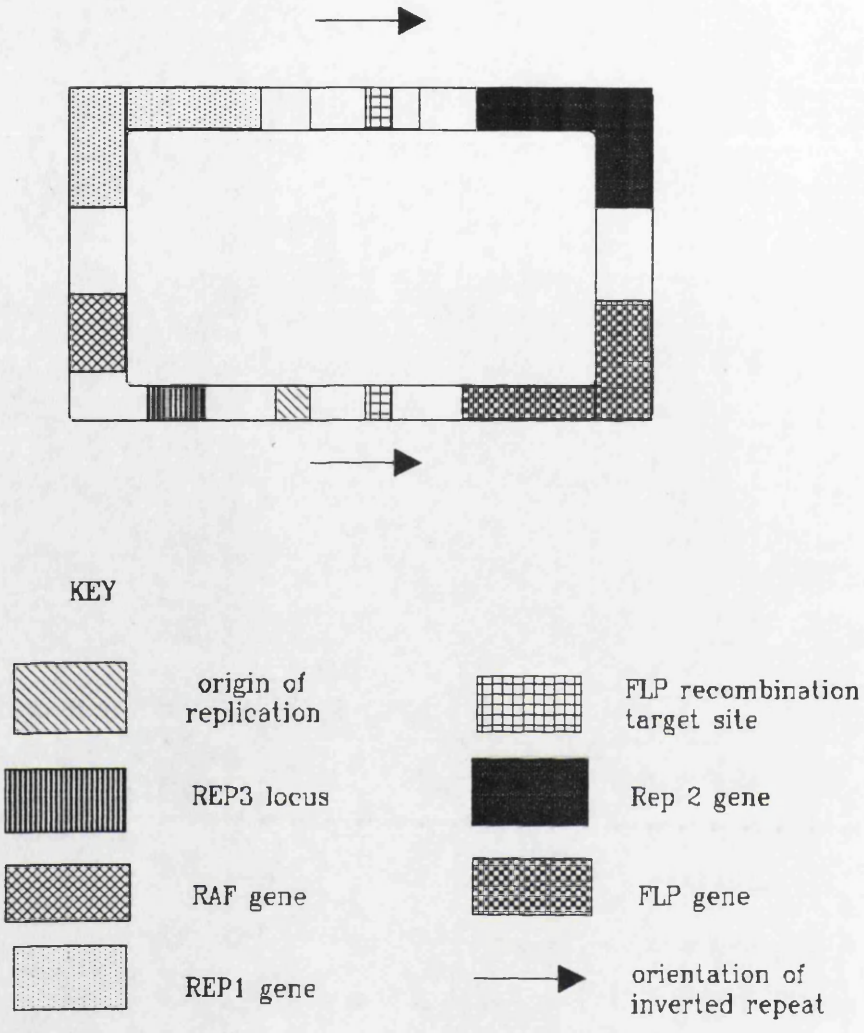


Figure 4.1: structure of the yeast 2μ circle plasmid.

at the plasmid origin occurs at a defined point in the cell cycle. This replication is dependent on a number of gene products that are also required for chromosomal replication.

The 2μ plasmid differs from a yeast chromosome in two main aspects: maintenance of a controllable high copy number and the segregation of the acentromeric plasmids into daughter cells. It is these two factors of copy number and segregation that make the 2μ circle a model system for the study of the behaviour of ARp1 in Aspergillus.

The 2μ circle comprises two unique regions of 2,779 and 2,346bp which are separated by a pair of 599bp inverted repeats, Som et al (1988), (see figure 4.1). Each unique region contains two ORFs which are now designated Rep1, Rep2, RAF and FLP. There is also an additional locus called Rep3.

4.1.2. 2u plasmid instability.

The stability and proper partitioning of the 2μ plasmid is due to the gene products of Rep1 and Rep2, Jayram et al (1983) and Som et al (1988). Subcloning and mutational analysis have shown that the Rep3 locus is essential for partitioning, plasmid stability and proper functioning of Rep1 and Rep2 gene products. The Rep3 locus (also called STB) contains five tandem repeats, although as few as three of these repeats give full stabilisation. This

locus is not yet fully characterised but it does appear that sequences flanking Rep3 affect its activity. Rep1 encodes a 42kDa polypeptide which is known to be localised within the nucleus. This protein resembles such structural proteins as the nuclear lamins A and C. REP1 also contains DNA sequences which appear to be involved in protein-DNA binding and seem to promote protein-protein interactions. REP1 may well be involved in homo- and heterodimerisation. REP2 may also be a nuclear protein. It has been suggested that REP1 is required either for REP2 stability or transport of REP2 to the nucleus. REP1 and REP2 are involved in regulating the expression of the FLP, Rep1 and RAF genes. The FLP gene product is a protein which mediates recombination at a site called FRT, Bruschi and Howe (1988). The FRT site is a 8bp core surrounded by a pair of inverted 13bp repeats. This site specific recombination reaction gives rise to two coexisting and interchangeable forms of the 2μ plasmid. The RAF gene product is involved in gene regulation; this protein counteracts the REP1/REP2 repression of the FLP promoter.

Two models have been proposed to explain how the REP1/REP2 system is involved in the stability of the 2μ plasmid, Volkert et al (1989). Both models are based on the premise that poor segregation of the plasmids is due to a failure in transmitting copies of the plasmid to the daughter cells following

mitosis and cell division. This "transmission failure" could be due to the plasmids becoming "entangled" either with each other or with the nuclear structures as the plasmids diffuse to the respective daughter cells.

The REP1/REP2 system could act as an "anti-entanglement" diffusion system by either active segregation or facilitated diffusion.

In an active segregation mechanism, the REP1/REP2 proteins would promote attachment of the 2μ circle, via the rep3 locus, to some structure such as the chromosomal spindle, which is partitioned equally between the mother and bud cells. In effect, the REP1/REP2 proteins form a bridge between the rep3 locus and the segregating nuclear structure.

In the facilitated diffusion model, the REP1/REP2 proteins could mediate the temporary, localised depolymerisation of the nuclear structures, with the rep3 locus as a trigger, giving the 2μ plasmid "free passage". Alternatively, the REP1/REP2 proteins could act specifically on plasmids containing the rep3 locus, resulting in increased folding, thereby making these super-folded plasmids smaller and more diffusable. There is now some evidence for a transitionary association of the 2μ circle with the nuclear spindle, thereby lending support for the active segregation model, Amati and Gasser (1988), Conrad and Zakian (1989).

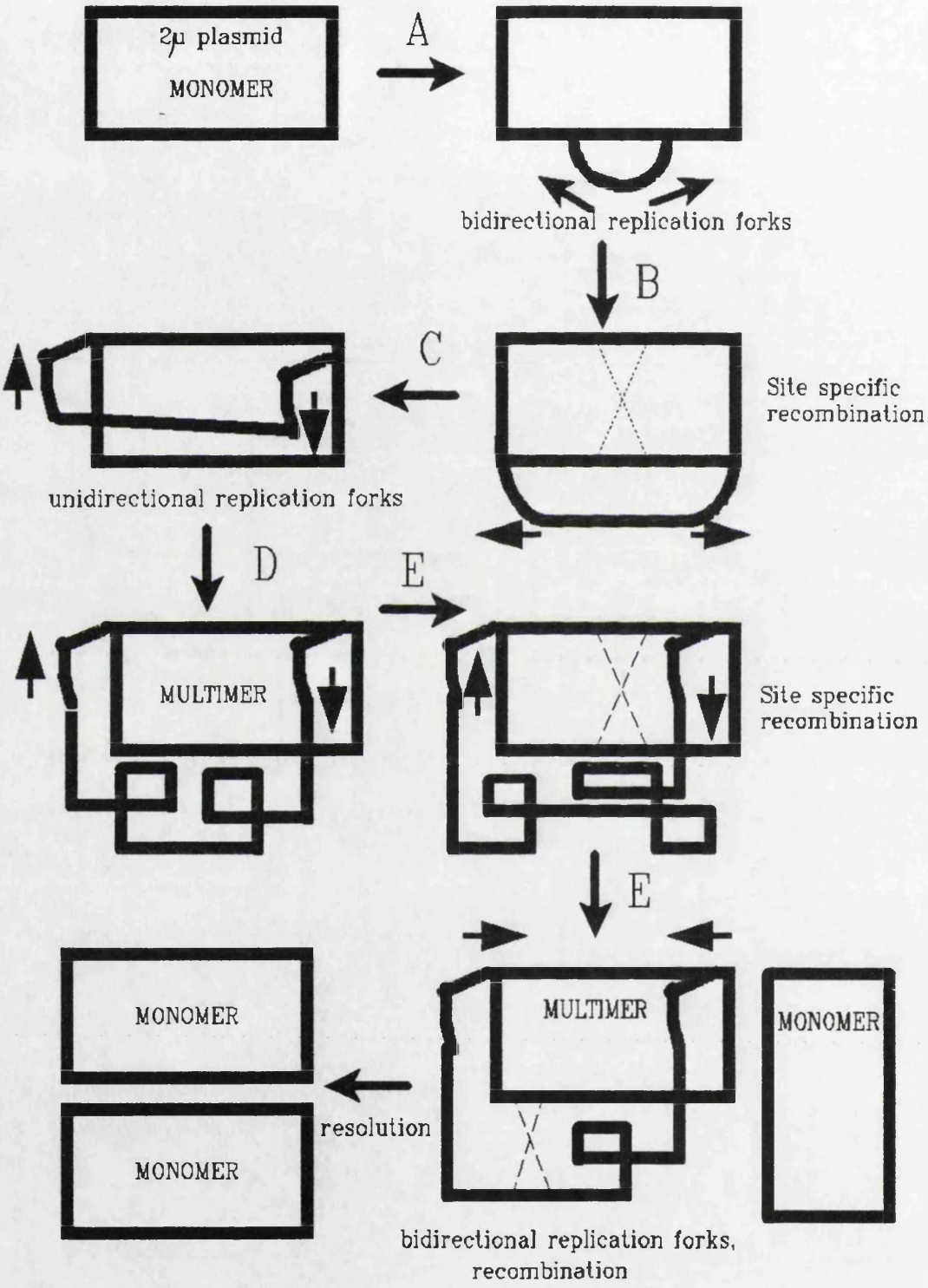


Figure 4.2: Futcher's Double Rolling Circle model of plasmid replication.

4.1.3. 2μ plasmid replication.

Futcher (1986), proposed the double rolling circle (DRC) amplification model to explain how the 2μ circle increased and maintained its copy number. This model is based on the observation that site specific recombination is induced by the FLP protein. This recombination event results in an increase in plasmid copy number without requiring multiple initiation of replication events. Copy number amplification is induced in cells where copy number is low. The model (figure 4.2) proposes that (A) normal semiconservative DNA replication initiates at the plasmid origin and proceeds bidirectionally. (B) FLP acts on the FRT site after one of the diverging replication forks has passed a FRT site but before the other fork has reached the other FRT site. (C) the resulting recombination reorientates the replication forks so that they no longer converge and now move around the plasmid in the same direction. (D) overall, this recombination event allows indefinite DNA chain extension from a single replication event and yields a multimeric replication intermediate. (E) Another FLP recombination event restores the converging orientation of the replication forks and yields both monomer and multimer forms of the 2μ plasmid. This multimer form can be resolved to the monomer form by either FLP-mediated or general recombination events.

4.1.4. The 2μ plasmid and ARp1.

There are clearly a number of important similarities and differences between ARp1 and the 2μ plasmid that I should state from the outset. Both the 2μ circle and ARp1 are capable of autonomous replication. Both plasmids contain inverted repeats. The most important differences are that ARp1 contains genomically derived DNA; ARp1 is unlikely to encode any recombination or copy number control proteins, (see Chapter 3). I will discuss the theoretical models for ARp1 replication and stability in the Discussion section.

4.1.5. Outline of chapter 4.

I tested the properties of ama1 by studying the behaviour of plasmids containing specific regions of the ama1 sequence. I have considered three different aspects in studying ama1 plasmid subclone behaviour: transformation frequency, % instability per asexual generation i.e. the number of progeny that lose the plasmid and finally whether or not the plasmid is rearranged.

Transformation frequency is fairly straight forward as it is a measure of the "efficiency" of

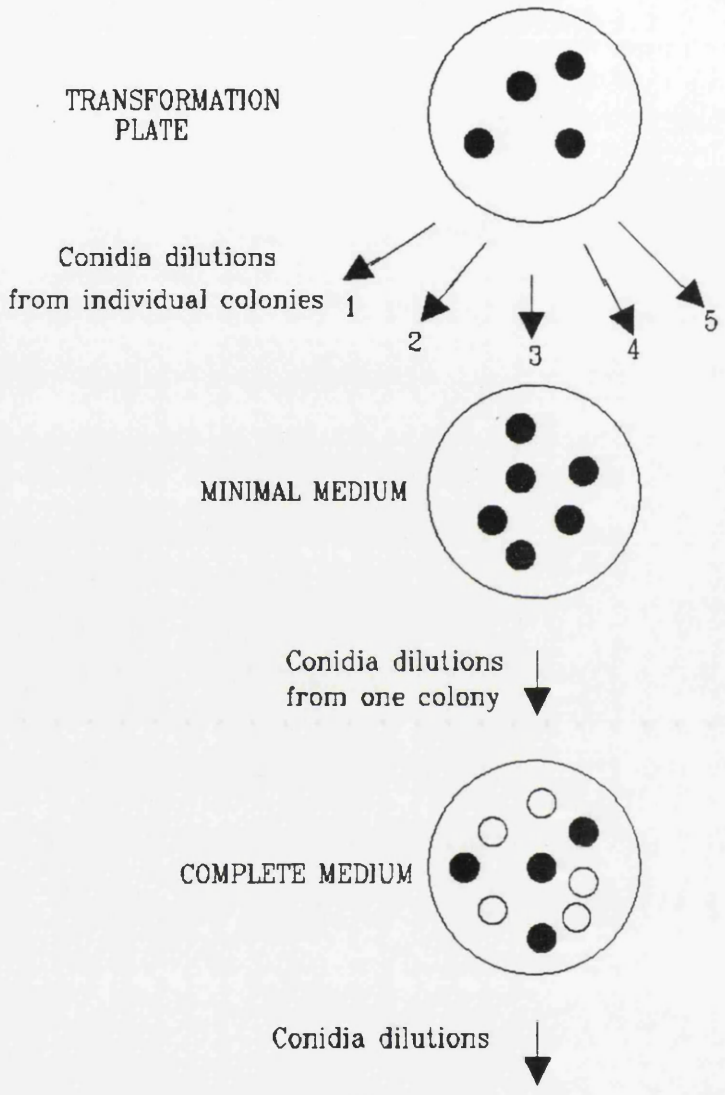


Figure 4.3: Instability tests procedure.

each individual subclone plasmid. This "efficiency" is calculated by counting the total number of colonies derived from a transformation experiment using a known number of protoplasts and a known amount of plasmid DNA. All the transformations described in this chapter were carried out as described in Chapter 2, using Aspergillus nidulans G34 protoplasts and using the same protoplast suspensions, DNA concentrations, protoplast concentrations and solutions for comparison of a series of subclones. This approach means that any differences in plasmid behaviour in any one experiment are due to plasmid properties and not to variations in the quality of the protoplasts and so on. Various controls were included in these experiments: pILJ16 and ARp1 samples and negative controls with no DNA. Protoplast viabilty was also tested.

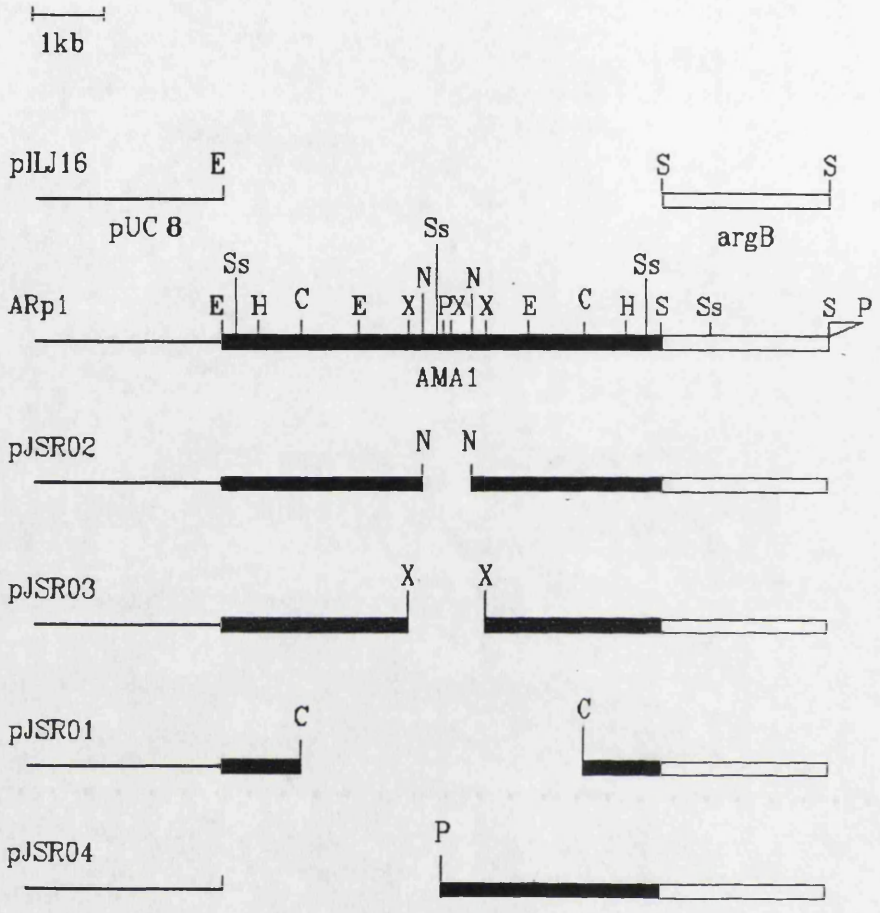
Each transformation experiment with individual plasmids was repeated three times. Rather than describe all the results, from all the experiments, for all the plasmids, I have quoted the results from one of these three transformation experiments for each plasmid. The average transformation frequency and % instability of each plasmid, calculated from three experiments, is quoted in figure 4.42.

Figure 4.3 outlines the protocol for testing the instability of an ARG+ plasmid. Conidia are collected from the initial transformation plate,

diluted then spread onto a selective (no arginine) minimal medium plate. The conidia from these colonies can be either Arg+ (plasmid containing) or Arg- (no plasmid). This plating ensures that only colonies derived from single conidia that contain the plasmid will grow. It is possible for plasmid-less nuclei to be present in the initial transformant colony due to PEG-fusion of transformed and untransformed protoplasts. Once the colonies have grown, conidia are again collected, diluted and spread onto complete medium. Complete medium contains a low level of arginine so that Arg- conidia will give rise to slow growing, non-conidiating white colonies; Arg+ conidia give rise to conidiating yellow colonies, (G34 contains the spore-colour mutant yA2), so the % loss can then be calculated. In all cases, the % instability for each subclone plasmid was calculated by studying 5 individual transformant colonies over four asexual generations.

Gems (1990), transformed Aspergillus with pILJ25, a subclone of ARp1 and found that some of the resulting transformants contained plasmid DNA that appeared to be much larger than pILJ25. Analysis of the rescued plasmid DNA showed that pILJ25 had undergone significant rearrangement. I wanted to test each of the amal containing plasmid subclones for rearrangements in an attempt to fit any rearrangements into a recognisable pattern.

Plasmid DNA was isolated from fungal



C=ClaI E=EcoRI H=HindIII N=NruI
P=PstI S=SalI X=XhoI

Figure 4.4: structure of pJSR series of ARp1 derived subclones.

transformants by so called "plasmid rescues". The plasmid rescues are carried out by first diluting total genomic DNA prepared from fungal transformants. This diluted DNA is then used to transform a suitable E.coli host. The drawback with this procedure is that only plasmids that are capable of replication within the bacterial host are selected. Any plasmid which has either lost the pUC regions in some manner or been rearranged such that it is no longer viable in E.coli will not be isolated. It should therefore be noted that the rescued plasmids described in this and other chapters are only those that could be isolated using the described plasmid rescue protocol.

4.2 Construction of ARp1 subclones.

All the pILJ and pDHG series of ARp1 subclones were constructed by Johnstone (1985) and Gems (1990) respectively.

The plasmids pJSR01, pJSR02 and pJSR03 were made as follows. 5µg aliquots of ARp1 were digested separately with ClaI, NruI and XhoI restriction enzymes. The DNA fragments were then separated on a 0.8% agarose gel, (gel not shown). The 8.4kb ClaI fragment, 10.9kb NruI fragment and the 10.8kb XhoI fragment were excised, prepared using the BioRad Prepagene kit and self-ligated. The resulting plasmid structures are shown in figure 4.4.

ARp1 COLONY		GENERATION 1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	75	106	97	112	390		
	ARG+	45	57	50	63	215	605	64.46
2	ARG-	170	121	103	179	573		
	ARG+	152	101	66	161	480	1053	54.42
3	ARG-	138	91	83	125	437		
	ARG+	119	74	62	101	356	793	55.11
4	ARG-	147	63	197	118	525		
	ARG+	123	50	174	93	440	965	54.40
5	ARG-	141	130	190	74	535		
	ARG+	120	106	161	52	439	974	54.93
subtotal	ARG-	671	511	670	608			
subtotal	ARG+	559	388	513	470	Average		56.66
TOTAL		1230	899	1183	1078	Standard Deviation		3.91
%LOSS		54.55	56.84	56.64	56.40			

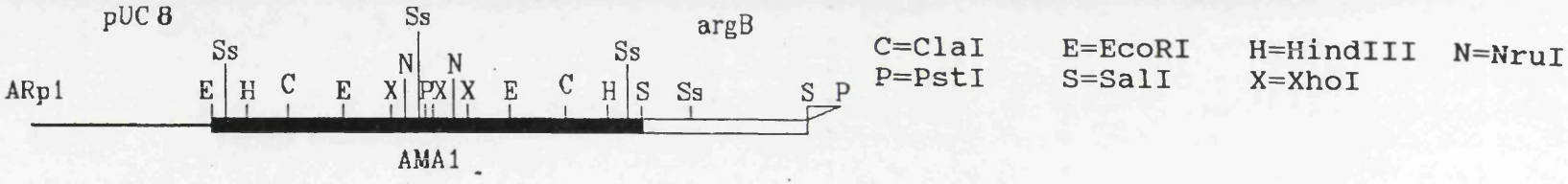


Figure 4.5: ARp1 instability test results.

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The plasmid pJSR04 was made by digesting ARp1 with PstI. This digestion yields two 5.8kb sized fragments; one fragment comprises pUC8 and half of the ama1 sequence, the other fragment contains the remainder of the ama1 sequence and the argB gene. These fragments were cloned into phosphatased, PstI-digested pUC8. The resulting mixed plasmids were used to transform E.coli DS941. DNA was then prepared from individual transformants and digested with SalI. This digestion identifies pJSR04 plasmid (8.8kb) because only this plasmid contains the argB gene which yields two SalI fragments of 0.8kb and 1.8kb in size.

4.3. Plasmid behaviour.

4.3.1.1 ARp1 transformations.

The average transformation frequency/µg DNA for ARp1 is 40,000. This figure is significantly higher than the transformation frequency of the integrative vector pILJ16 which yields 20 transformants/µg DNA. This 2000 fold increase in transformation frequency is due to the ama1 sequence since ARp1 differs from pILJ16 only by possesing the ama1 sequence.

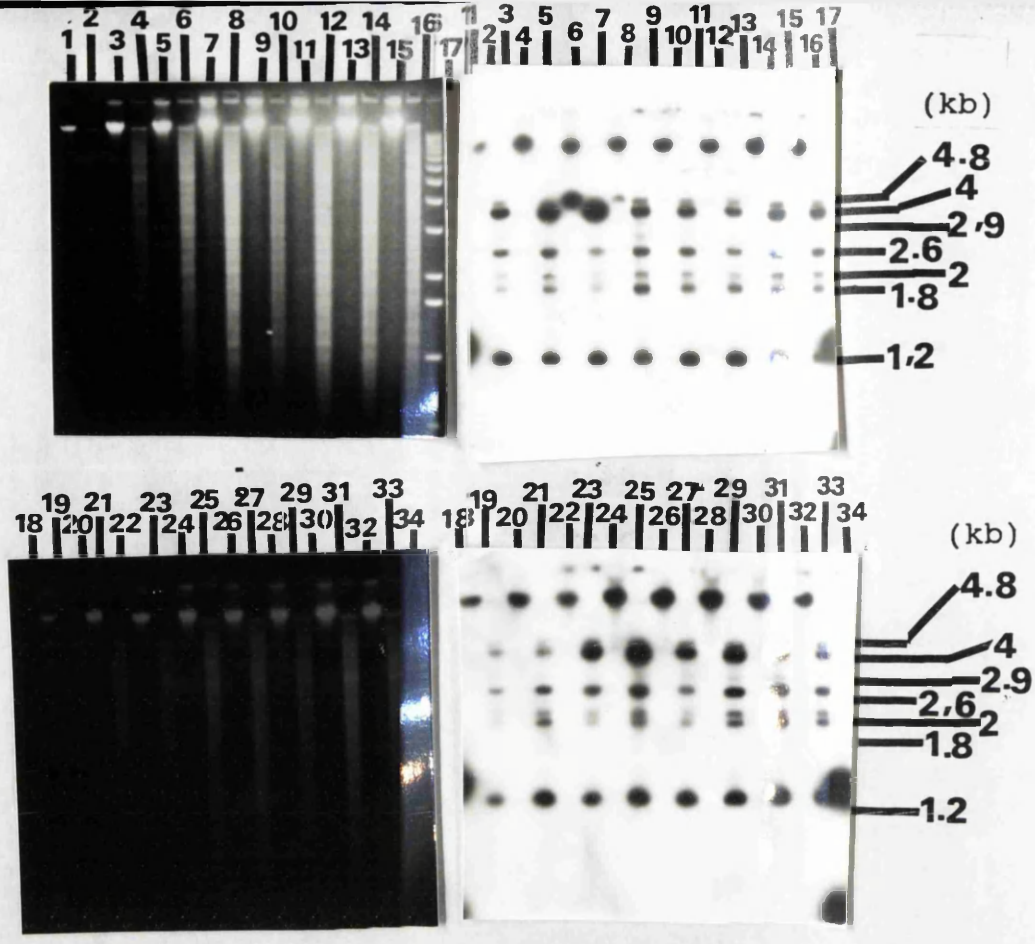
The instability of ARp1 per asexual generation was calculated as described previously. Figure 4.5 shows both the structure of ARp1 and a detailed breakdown of plasmid loss from the progeny of five

individual ARp1 transformants through four generations. All instability results will be presented in this format; ARG- (no plasmid), ARG+ (plasmid). The % loss for progeny derived from each individual, over the four generations, is shown in the extreme right hand column. Below this column is the average % loss and the associated standard deviation. Beneath each generation column I have calculated the % loss for all the progeny in that specific generation.

Examination of figure 4.5 shows that on average, 56.6%±3.9% of progeny derived from ARp1 transformants lose the plasmid. The results also show that plasmid loss is consistent, regardless of whether individual transformants or generations are considered. The one exception is transformant 1; a heterogeneity chi-squared test suggests that the difference between individual 1 and the remainder is significant, (chi-squared=20.37 with 4 degrees of freedom and a 95% confidence level). This result may indicate that ARp1 has undergone a rearrangement in this one individual.

4.3.1.2 Rearrangements of ARp1.

Potential rearrangements of ARp1 were assessed by examining plasmids from 4 individual transformants through the four generations. These transformants were referred to as TARp1/1.1 - TARp1/1.4 (first



LANE		LANE	
1	TARp1/1.1	18	TARp1/3.1
2	TARp1/1.1 SstI	19	TARp1/3.1 SstI
3	TARp1/1.2	20	TARp1/3.2
4	TARp1/1.2 SstI	21	TARp1/3.2 SstI
5	TARp1/1.3	22	TARp1/3.3
6	TARp1/1.3 SstI	23	TARp1/3.3 SstI
7	TARp1/1.4	24	TARp1/3.4
8	TARp1/1.4 SstI	25	TARp1/3.4 SstI
9	TARp1/2.1	26	TARP1/4.1
10	TARp1/2.1 SstI	27	TARp1/4.1 SstI
11	TARp1/2.2	28	TARP1/4.2
12	TARp1/2.2 SstI	29	TARp1/4.2 SstI
13	TARp1/2.3	30	TARp1/4.3
14	TARp1/2.3 SstI	31	TARp1/4.3 SstI
15	TARP1/2.4	32	TARp1/4.4
16	TARp1/2.4 SstI	33	TARp1/4.4 SstI
17	marker	34	marker

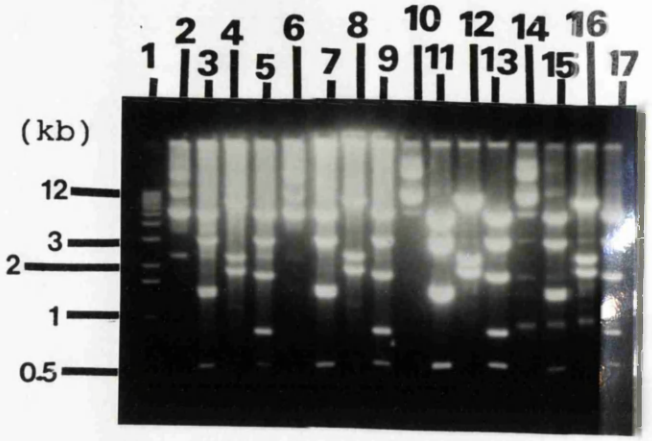
BAND SIZES

Expected	4.8, 2.9, 2.6, 1.2
Observed	4.8, 4.0, 2.9, 2.6, 2.0, 1.8, 1.2

Figure 4.6: SstI digest of ARp1 transformants from different conidial generations. Probed with radiolabelled pUC8 DNA.

conidial generation) to TARp1/4.1 - TARp1/4.4 (fourth conidial generation). Total genomic DNA was prepared from each of the above classes and 3µg of DNA was digested with 20 units of SstI. The digested DNA was run out on a 0.8% agarose gel and Southern blotted. These Southern blots were probed with radiolabelled pUC8 DNA. From the map of ARp1 shown in section 3.1 I would predict that the SstI digest should yield 4 bands which hybridise with the pUC probe. These bands should be 4.8kb, 2.9kb, 2.6kb and 1.2kb in size. The results are shown in figure 4.6. It is clear from these autoradiographs that the banding pattern is identical for all the individual colonies, regardless of generation. However the banding pattern is more complex than predicted: 7 bands hybridise: 4.8kb, 2.9kb, 2.6kb, and 1.2kb as expected and three additional bands of 4.0kb, 2.0kb and 1.8kb. This observation suggests that either ARp1 has been rearranged so that there is more then one type of plasmid present or that these bands are due to contamination with bacterial DNA. Another possibility is that these additional bands are produced by the ARp1 replication mechanism, (see section 4.7.4). If ARp1 is rearranged then any rearrangement must occur in the original transformant colony because all subsequent progeny appear to have identical banding patterns.

Plasmid rescues were then carried out, using the prepared total genomic DNA, to rescue and then map



LANE		LANE	
1	marker	10	uncut TARp1/3.3
2	uncut TARp1/1.1	11	HindIII TARp1/3.3
3	HindIII TARP1/1.1	12	EcoRI TARp1/3.3
4	EcoRI TARp1/1.1	13	SalI TARp1/3.3
5	SalI TARp1/1.1	14	uncut TARp1/4.2
6	uncut TARp1/2.4	15	HindIII TARp1/4.2
7	HindIII TARp1/2.4	16	EcoRI TARp1/4.2
8	EcoRI TARp1/2.4	17	SalI TARp1/4.2
9	SalI TARp1/2.4		

BAND SIZES EXPECTED			BAND SIZES OBSERVED		
HindIII	EcoRI	SalI	HindIII	EcoRI	SalI
5.0	7.3	5.4	5.0	7.3	5.4
3.2	2.3	2.9	3.2	2.3	2.9
1.3	1.9	1.8	1.3	1.9	1.8
1.2		0.8	1.2		0.8
0.5		0.5	0.5		0.5
----	----	----	----	----	----
11.2	11.5	11.4	11.2	11.5	11.2
----	----	----	----	----	----

Figure 4.7: digested ARp1 rescued plasmid DNA, showing table of expected and observed band sizes.

ARp1 plasmids.

4.3.1.3 ARp1 plasmid rescues.

On average, transformation of E.coli SURE cells with each of the total genomic DNA samples yielded 10-30 E.coli transformants. DNA was prepared from three of these E.coli transformants in each class. Single Colony Gels (results not shown) suggested that all the rescued plasmids were approximately 11.5kb in size. Plasmid DNA was prepared from one representative E.coli transformant from each class. 2µg aliquots of this plasmid DNA was then digested separately with 20 units of HindIII, EcoRI and SalI restriction enzymes. The results are shown in figure 4.7. In all the examined cases, with the exception of TARp1/4.2, the restriction band pattern matches with the known map of ARp1. The SalI-digested TARp1/4.2 DNA (lane 17) contains an additional band which may be a contaminating band since it appears in all the TARp1/4.2 lanes, including the uncut DNA lane. It therefore appears that the extra bands seen in figure 4.6 are possibly due either to contamination or replication products or some sort of rearrangement of ARp1, but the rearranged versions of ARp1, if such plasmids exist, were not rescuable.

pILJ25		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	74	101	109	119	403		
	ARG+	19	20	18	19	76	479	84.13
2	ARG-	132	144	133	173	582		
	ARG+	23	25	22	30	100	682	85.34
3	ARG-	137	102	88	118	445		
	ARG+	23	17	14	25	79	524	84.92
4	ARG-	124	126	131	130	511		
	ARG+	21	20	23	21	85	596	85.74
5	ARG-	191	167	169	122	649		
	ARG+	32	30	27	20	109	758	85.62
subtotal	ARG-	658	640	630	662			
subtotal	ARG+	118	112	104	115		Average	85.15
TOTAL		776	752	734	777		Standard Deviation	0.58
%LOSS		84.79	85.11	85.83	85.20			

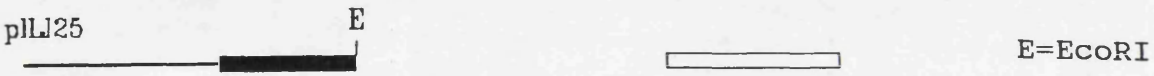


Figure 4.8: pILJ25 instability test results.

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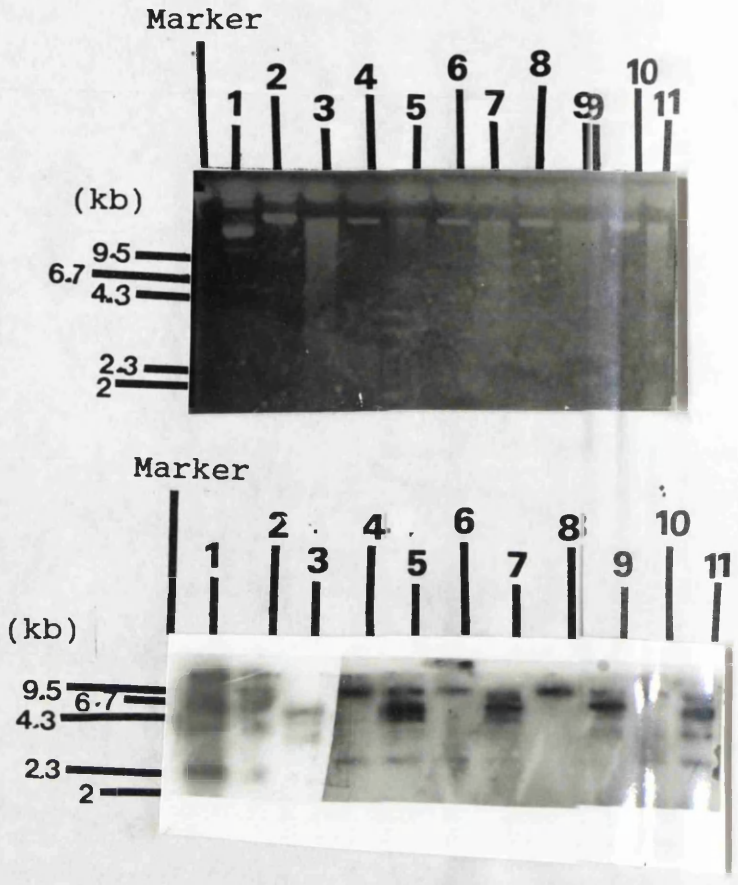
4.3.2.1 pILJ25 transformations.

The plasmid pILJ25 is 7.4kb in size and comprises the 1.9kb EcoRI fragment from one arm of ama1 cloned into pILJ16, (see figure 4.8). The transformation frequency obtained using pILJ25 was 100 transformants per µg of plasmid. Plasmid instability was tested as previously described; the transformant colonies were referred to as T25/01 to T25/05.

The results of the instability tests are shown in figure 4.8. These results show that 85% of progeny derived from pILJ25 transformants lose the plasmid. This instability value is consistent for all five individuals over four generations. That 85% of pILJ25 progeny lose the plasmid suggests that pILJ25 is more "unstable" than ARp1 which displays 56% instability. The differences in plasmid instability between ARp1 and pILJ25 are statistically significant.

4.3.2.2 Rearrangements of pILJ25.

Total genomic DNA was made from five transformant colonies. Approximately 2µg of this genomic DNA was digested with 20 units of SstI, run on a 0.8% agarose gel, Southern blotted and probed with radiolabelled pUC8. The results of this blot



LANE	CONTENTS	BAND SIZE (kb)
1	TARp1/1.1	-
2	T25/01	-
3	T25/01 SstI	4.2, 3.8, 2.2, 2.1
4	T25/02	-
5	T25/02 SstI	6.3, 3.8, 0.8
6	T25/03	-
7	T25/03 SstI	6.3, 4.0, 0.8
8	T25/04	-
9	T25/04 SstI	8, 6, 2.5, 2.3
10	T25/05	-
11	T25/05 SstI	8, 5.5, 2.2, 0.8

Figure 4.9: Southern blot of undigested and SstI-digested genomic DNA from 5 pILJ25 derivatives. Probed with radiolabelled pUC8 DNA.

are shown in figure 4.9. Free plasmid can be clearly seen as fast-running bands in the TARp1/1.1 control lane and in the undigested T25/01, T25/02, T25/03 and T25/05 lanes. No free plasmid is apparent in the undigested T25/04 lane. A variety of bands hybridise in the SstI digested lanes. There are 4 bands in the digested T25/01 lane: 4.2kb, 3.8kb, 2.2kb and 2.1kb. The T25/02 digest lane contains three bands of 6.3kb, 3.8kb and 0.8kb in size. The T25/03 digest lane contains three bands of 6.3kb, 4.0kb and 0.8kb in size. There are four bands in the digested T25/04 lane: 8.0kb, 6.0kb, 2.5kb and 2.3kb. The T25/05 digest lane contains 4 bands of 8.0kb, 5.5kb, 2.2kb and 0.8kb in size. The wide difference in the banding patterns strongly suggests that pILJ25 has undergone a number of rearrangement events in individual transformant colonies.

4.3.2.3 Rescue of pILJ25 derived plasmids.

The pILJ25 derivatives were rescued out of the fungal genomic DNA samples as previously described. No plasmids were rescuable from samples T25/01: these plasmids must be rearranged such that they lack viable pUC sequences and/or their overall structure is not viable in the E.coli host. The TARp1/1.1, T25/02, T25/03 and T25/04 gave rise to 30, 3, 16 and 7 E.coli SURE transformant colonies respectively. A pILJ25 derivative (pUAT25/09) recovered from a

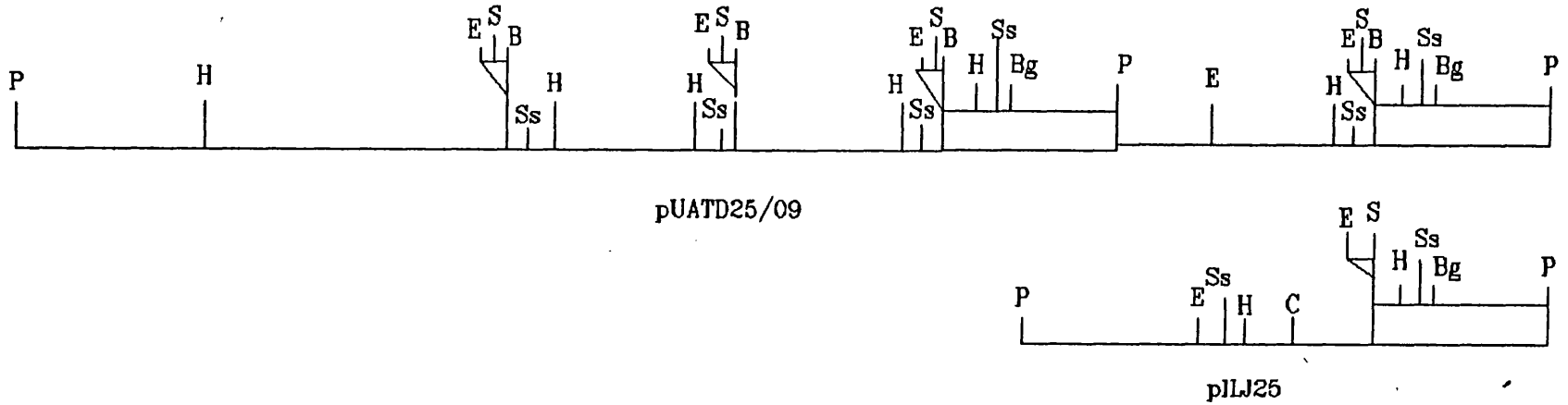


Figure 4.10: restriction map of pUAT25/09, a rearranged pILJ25 derivative (from D.H.Gems).

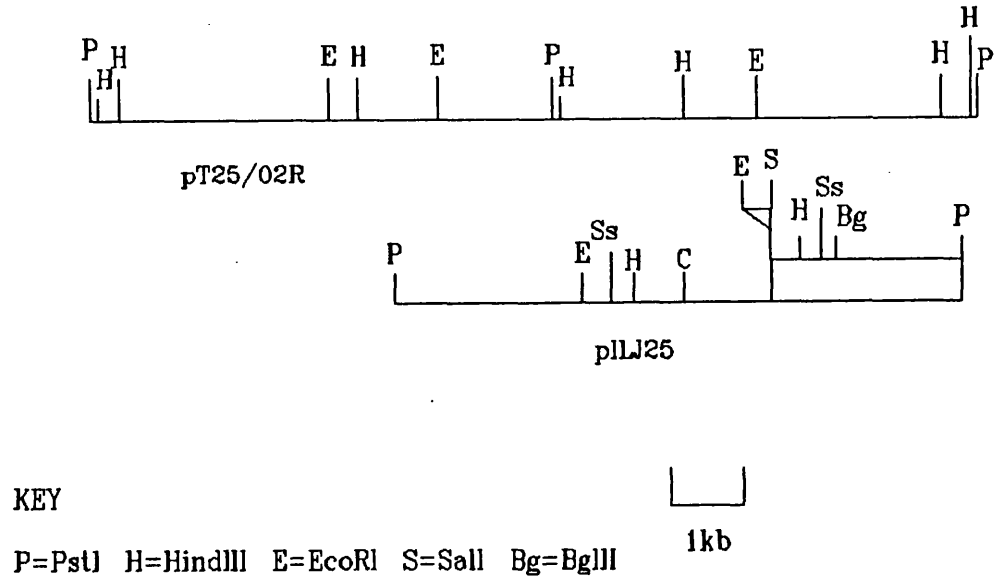
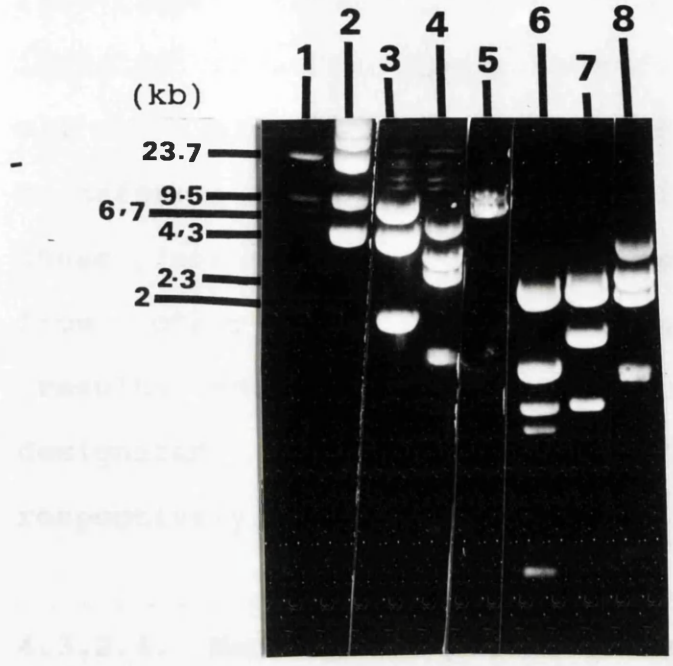


Figure 4.11: restriction map of pT25/02R, a rearranged pIIJ25 derivative.

facing page 94b



LANE	ENZYME(S)	BAND SIZES (kb)
1	marker	-
2	uncut DNA	-
3	EcoRI, (E)	6, 4, 1.8
4	HindIII, (H)	4.1, 3.2, 2.6, 1.5, 0.4, 0.2
5	PstI, (P)	6, 6
6	E/H	3, 2.4, 1.6, 1.5, 1.1, 1.1, 0.7, 0.4, 0.2
7	E/P	3, 3, 3, 1.8, 0.9
8	H/P	4.1, 3.2, 2.6, 1.5, 0.4, 0.2

Figure 4.11: restriction mapping of pT25/02R showing banding pattern on a 0.8% gel and a list of fragment sizes.

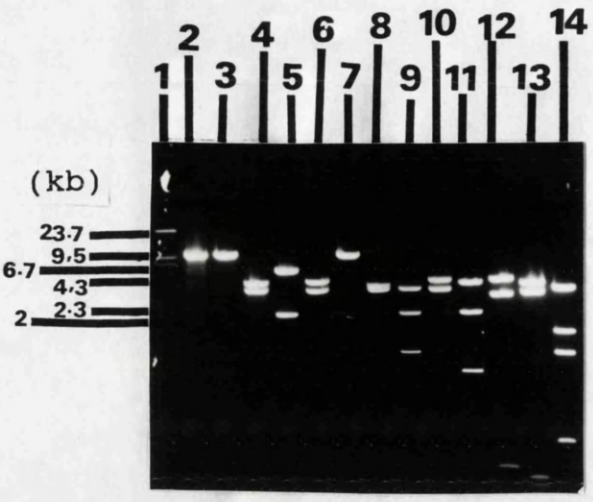
transformant by David Gems, has been mapped by Gems, (see figure 4.10). Single Colony gels of plasmids isolated from the E.coli transformants showed that all the plasmids isolated from same Aspergillus transformant colony were identical to each other, but these plasmids were different from plasmids isolated from other Aspergillus transformant colonies, (results not shown). The rescued plasmids were designated pT25/02R, pT25/03R and pT25/04R respectively.

4.3.2.4. Mapping the pILJ25 derivatives.

The restriction map of pUAT25/09, (David Gems 1990), with the pILJ25 map for comparison, is shown in figure 4.10. This plasmid is 22kb in size and appears to contain two copies of the ArgB gene. The plasmids pT25/02R, pT25/03R and pT25/04R were mapped by digesting 1µg aliquots of the plasmid DNAs with a number of different restriction enzymes. Only the argB gene sequences were clearly identified, no regions within pUAT25/09 were clearly identified as being derived from either pUC8 or amal.

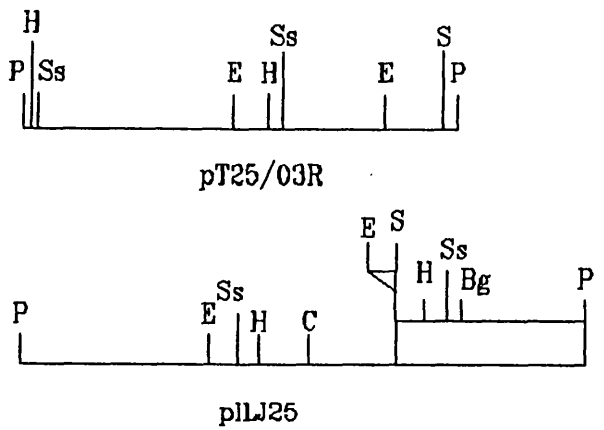
pT25/02R restriction map.

Figure 4.11 shows the restriction digest banding pattern for pT25/02R, the list of fragment sizes and the actual map of the plasmid, with the pILJ25 map



LANE	ENZYME(S)	BAND SIZES (kb)
1	marker	-
2	PstI, (P)	6.2
3	SalI, (S)	6.2
4	SstI, (Ss)	3.2, 3.0
5	EcoRI, (E)	4, 2.2
6	HindIII, (H)	3.2, 3.0
7	P/S	5.9, 0.3
8	P/Ss	3.1, 3.0, 0.1
9	P/E	3.0, 2.2, 1
10	P/H	3.2, 3.0
11	S/E	3.2, 2.2, 0.8
12	S/H	3.2, 2.7, 0.3
13	Ss/H	3.1, 2.7, 0.2, 0.1
14	E/H	3.0, 1.6, 1.1, 0.5

Figure 4.12: restriction mapping of pT25/03R showing banding pattern on a 0.8% gel and a list of fragment sizes.



KEY

P=PstI H=HindIII S=SalI E=EcoRI Ss=SstI

1kb

Figure 4.12: restriction map of pT25/03R, a rearranged pILJ25 derivative.

for comparison. The plasmid map was assembled as follows. PstI produces a 6-7kb doublet. The EcoRI/PstI double digest produces 3 bands of 0.9kb, 1.8kb, 3.0kb triplet: the 0.9kb and one 3.0kb are the result of a single PstI site in the EcoRI 4.0kb fragment, while the other two 3.0kb fragments are the result of a single PstI site in the 6.0kb EcoRI fragment. The EcoRI/HindIII double digest produces 9 fragments: the 3.0kb, 0.4kb and 0.2kb fragments are produced by two HindIII sites within the EcoRI 6.0kb fragment; the 0.7kb and 1.1kb fragments are the result of one HindIII site within the 1.8kb EcoRI fragment; the 1.6kb, 1.5kb and 1.1kb fragments are produced by two HindIII sites within the 4.0kb EcoRI fragment. The HindIII/PstI double digest is indistinguishable from the HindIII digest so the PstI sites must map to within 100 bases of two of the HindIII sites.

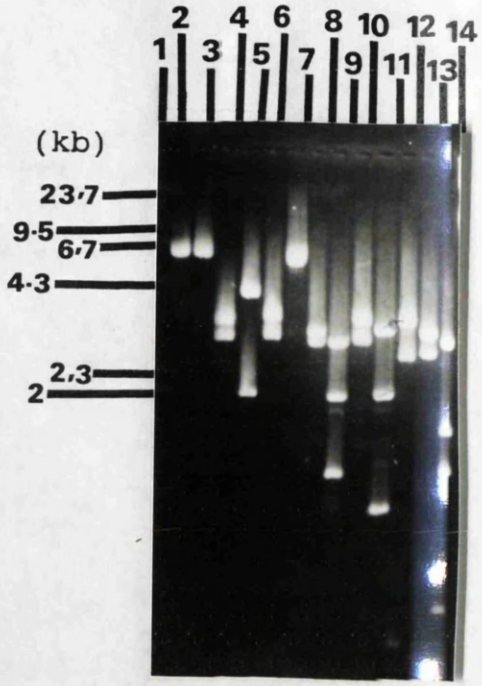
In conclusion, the plasmid pT25/02R is 12.2kb in size and pUC, argB and ama1 sequences are not recognisable in this plasmid.

pT25/03R restriction map.

This plasmid was mapped in a similar fashion to pT25/02R. Figure 4.12 shows the restriction digest gel, the banding pattern and the assembled plasmid map. The PstI digest yields a single 6.2kb fragment so pT25/03R is 6.2kb in size. The PstI/SalI double

digest produces 2 bands: the 5.9kb and the 0.3kb bands are produced by a SalI site within 0.3kb of the PstI site. The PstI/SstI double digest produces 3 bands of 3.1kb, 3.0kb and 0.1kb, so one of the SstI sites is within 0.1kb of the PstI site. The PstI/EcoRI digest produces 3 bands of 3.0kb, 2.2kb and 1.0kb, so the PstI site is within the 4.0kb EcoRI fragment. The PstI/HindIII double digest is identical to the HindIII digest, so the PstI site maps very closely to one of the SstI sites. The SalI/EcoRI double digest produces 3 bands: the 3.2kb and 0.8kb bands are the result of a SalI site within the 4kb EcoRI fragment. In the SalI/HindIII double digest the 2.7kb and 0.3kb bands are produced by a SalI site within the 3.0kb HindIII fragment. The SstI/HindIII digest produces 4 bands: the 2.7kb and 0.3kb bands are produced by a single SstI site within the 3.0kb HindIII fragment; the 3.1kb and 0.1kb bands are produced by a single SstI site within the 3.2kb HindIII fragment. Finally, the EcoRI/HindIII digest produces 4 bands: the 3.0kb and the 1.0kb bands are produced by a single HindIII site within the 4.0kb EcoRI fragment; the 0.5kb and the 1.7kb fragments are produced by a single HindIII site within the 2.2kb EcoRI fragment.

By aligning the pILJ25 and pT25/03R maps it is possible to identify what appears to be the pUC region and part of the <u>ama1</u> region by the position of the EcoRI, HindIII and SstI restriction sites, (see



LANE	ENZYME(S)	BAND SIZES (kb)
1	marker	-
2	PstI, (P)	6.2
3	SalI, (S)	6.2
4	SstI, (Ss)	3.2, 3.0
5	EcoRI, (E)	4, 2.2
6	HindIII, (H)	3.2, 3.0
7	P/S	5.9, 0.3
8	P/Ss	3.1, 3.0, 0.1
9	P/E	3.0, 2.2, 1
10	P/H	3.2, 3.0
11	S/E	3.2, 2.2, 0.8
12	S/H	3.2, 2.7, 0.3
13	Ss/H	3.1, 2.7, 0.2, 0.1
14	E/H	3.0, 1.6, 1.1, 0.5

Figure 4.13: restriction mapping of pT25/04R showing banding pattern on a 0.8% gel and a list of fragment sizes.

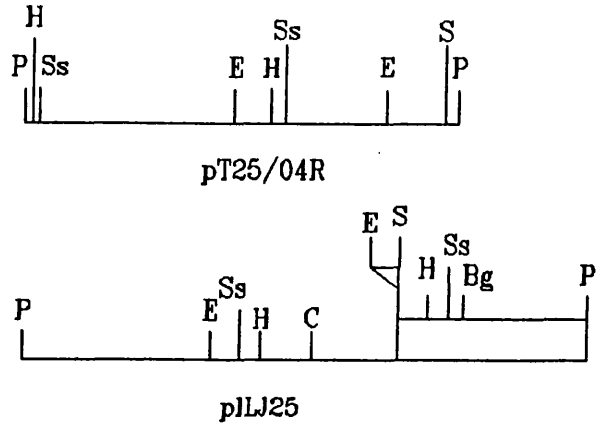


Figure 4.13: restriction map of pT25/04R, a rearranged pILJ25 derivative.

figure 4.12).

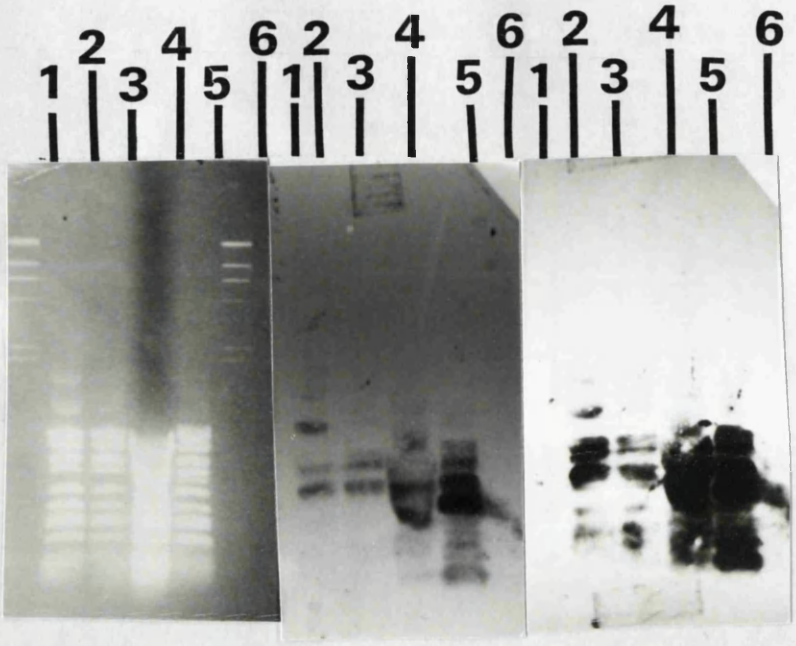
pT25/04R restriction map.

This plasmid was mapped in the same way as pT25/02R and pT25/03R. The restriction digest gel, list of band sizes and the derived plasmid map are shown in figure 4.13. From the restriction digest pattern it appears that pT25/03R and pT25/04R are identical.

4.3.2.5 Sau3A digests of pILJ25 derivatives.

It is possible that the increase in size pUAT25/09 and pT25/02R compared to the pILJ25 parent is due to the rearranged plasmids integrating into the chromosome, then excising in such a way that additional chromosomal DNA is present on the now independent plasmid. A similar mechanism of aberrant plasmid excision could explain the reduction in size of pT25/03R and pT25/04R.

One way of testing for the presence of additional chromosomal DNA picked up in this way, is to partially digest the plasmids with Sau3A and then probe with labelled ARp1. Any bands which do not hybridise with the probe must be additional, chromosomal DNA. This experiment was set up by



5 minute exposure

10 minute exposure

LANE	DNA
1	marker
2	pUATD25/09R
3	pT25/02R
4	pT25/03R
5	pT25/04R
6	marker

Figure 4.14: Sau3A-digested plasmid T25 series DNA, probed with DIG-labelled ARp1 DNA.

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digesting 3µg of the pT25 series plasmids and pUAT25/09 with 0.1 units of Sau3A for 10 minutes. The enzyme was inactivated by the addition of 50mM EDTA. The resulting DNA ladders were run out on a 1% agarose gel, Southern blotted and probed with non-radioactively labelled ARp1. Figure 4.14 shows the results of this experiment. It is clear from the 10 minute exposure plate that while all the bands hybridise, they do so to different extents. This result is not suprising since ARp1 is itself repetitive i.e. certain regions are represented more than once. The result of this experiment is therefore inconclusive but suggests that all components of the rescued plasmids are probably represented in ARp1.

4.3.2.6 Transformations with pILJ25 derivatives.

An important question to be answered in this section is whether or not the rescued pILJ25 derivatives will behave as autonomously replicating plasmids when used to transform Aspergillus. The plasmids pT25/03R and pT25/04R are of particular interest because of their small size. Both these plasmids are approximately 6kb in size; pILJ16 is 5kb in size and contains only pUC8 and the argB gene. Therefore, if both pT25/03R and pT25/04R are capable of autonomous replication and assuming that these plasmids contain at least functional pUC8 and argB

PLASMID	TRANSFORMATION FREQUENCY/µg DNA	%LOSS
pILJ25	100	85
pUATD25/09	650	54
pT25/02R	600	53
pT25/03R	260	58
pT25/04R	780	51

Figure 4.15a: transformation frequencies and %loss values for the pILJ25 derivatives.

pUATD25/09		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	119	27	120	115	381		
	ARG+	98	18	101	97	314	695	54.82
2	ARG-	123	23	134	125	405		
	ARG+	110	12	120	113	355	760	53.29
3	ARG-	117	17	145	117	396		
	ARG+	89	9	129	94	321	717	55.23
4	ARG-	151	60	132	141	484		
	ARG+	139	49	117	128	433	917	52.78
5	ARG-	125	25	118	130	398		
	ARG+	109	19	103	117	348	746	53.35
subtotal	ARG-	635	152	649	628			
subtotal	ARG+	545	107	570	549		Mean	53.89
TOTAL		1180	259	1219	1177		Standard Deviation	0.95
%LOSS		53.81	58.69	53.24	53.36			

Figure 4.15b: pUATD25/09R instability test results.

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pT25/02R		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	43	102	112	79	336		
	ARG+	38	89	94	68	289	625	53.76
2	ARG-	56	132	120	124	432		
	ARG+	48	111	114	119	392	824	52.43
3	ARG-	83	143	133	99	458		
	ARG+	79	133	128	91	431	889	51.52
4	ARG-	47	117	171	113	448		
	ARG+	43	100	159	90	392	840	53.33
5	ARG-	51	131	138	129	449		
	ARG+	47	124	121	119	411	860	52.21
subtotal	ARG-	280	625	674	544			
subtotal	ARG+	255	557	616	487	Average		52.65
TOTAL		535	1182	1290	1031	Standard Deviation		0.80
%LOSS		52.34	52.88	52.25	52.76			

Figure 4.15c: pT25/02R instability test results.

pT25/03R		GENERATION						
COLONY		1	2	3	4	subtotal	TAL	%LOSS
1	ARG-	133	129	148	129	539		
	ARG+	88	90	109	88	375	914	58.97
2	ARG-	135	141	135	131	542		
	ARG+	91	101	91	101	384	926	58.53
3	ARG-	130	137	140	146	553		
	ARG+	72	93	114	99	378	931	59.40
4	ARG-	138	156	143	115	552		
	ARG+	99	113	109	88	409	961	57.44
5	ARG-	121	142	144	132	539		
	ARG+	89	111	95	94	389	928	58.08
subtotal	ARG-	657	705	710	653			
subtotal	ARG+	439	508	518	470		Average	58.48
TOTAL		1096	1213	1228	1123		Standard Deviation	0.68
%LOSS		59.95	58.12	57.82	58.15			

Figure 4.15d: pT25/03R instability test results.

facing page 99c

pT25/04R		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	18	73	141	137	369		
	ARG+	16	69	130	129	344	713	51.75
2	ARG-	44	58	132	110	344		
	ARG+	41	55	123	102	321	665	51.73
3	ARG-	20	39	130	138	327		
	ARG+	19	37	126	127	309	636	51.42
4	ARG-	20	35	137	100	292		
	ARG+	18	32	132	97	279	571	51.14
5	ARG-	25	42	101	113	281		
	ARG+	23	41	97	104	265	546	51.47
subtotal	ARG-	127	247	641	598			
subtotal	ARG+	117	234	608	559		Average	51.50
TOTAL		244	481	1249	1157		Standard Deviation	0.23
%LOSS		52.05	51.35	51.32	51.69			

Figure 4.15e: pT25/04R instability test results.

sequences, (the argB transcript is approximately 1kb in size), then these plasmids must contain a minimum of 1kb of amal derived DNA. Such a 1kb sequence must then be the minimium required for autonomous replication.

The same transformation conditions were used as described previously. For the sake of clarity the transformation frequencies and instability figures for all the pILJ25 derivative plasmids are grouped together in figure 4.15a, along with ARp1, pILJ16 and pILJ25 controls. The data in figure 4.15a clearly show that all the rearranged derivatives transform at a higher rate and are more stable than pILJ25. The transformation frequencies vary from a two fold increase over pILJ25 for pT25/03R (260 transformants/ µg DNA), to a seven fold increase for pT25/04R (780 transformants/ µg DNA).

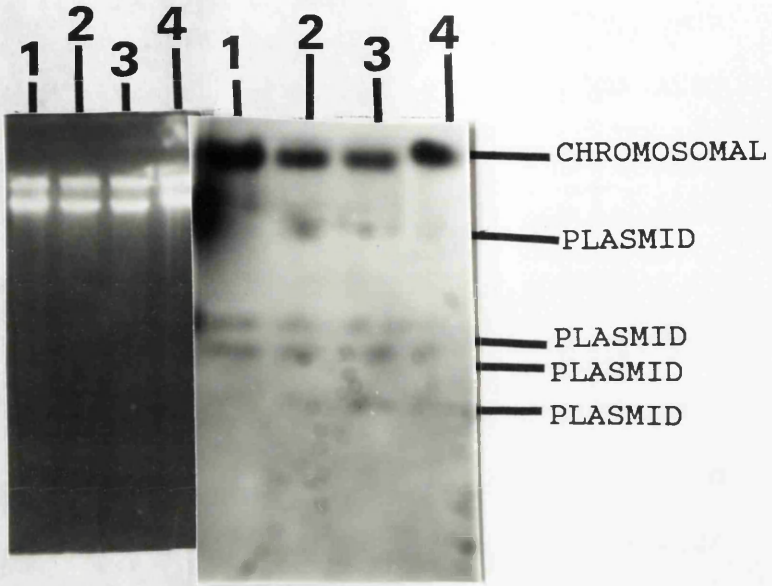
The % instability data for all four plasmids are shown in figures 4.15b, 415c, 4.15d and 4.15e. From the data in figures 4.15a to 4.15e it is clear that in all cases, with the exception of pUATD25/09, the % instability for each individual plasmid does not vary much from the calculated average value. In the case of the second generation of pUATD25/09 the difference in the actual % instability (58.6%), from the average % instability (53.8%), is not statistically significant. The plasmid pILJ25 shows a % instability of approximately 85% while all the % instability values for the rearranged derivatives are

noticeably lower and vary from approximately 51% to 58%. Statistical tests (paired t-test), show that the differences in % instabililty between pT25/02R, pT25/03R, pT25/04R and pUATD25/09 are not significant, but the differences between pILJ25 and each of the rearranged plasmids are significant.

These results lead to two important conclusions. The first conclusion is that plasmid behaviour is not simply dependent on plasmid size: both pT25/03R and pT25/04R are half the size of pT25/02R and approximately a quarter the size of pUATD25/09, yet the behaviour of the smaller plasmids is comparable with that of the larger plasmids. The second conclusion has important implications when considering how ARp1 originated: the data suggests that a small precursor plasmid can be rearranged to give a more efficient transformer or replicator which can be either larger e.g. pUATD25/09 or smaller e.g. pT25/03R. Therefore, plasmid rearrangements can be advantageous.

4.3.2.7 Further rearrangements of pILJ25 derivatives.

The next question was whether or not these rearranged plasmids underwent further rearrangements. Total genomic DNA was prepared from 5 transformant colonies (transformed with the rearranged pILJ25 derivatives) from conidial generation 5 for each plasmid class, these transformants were labelled



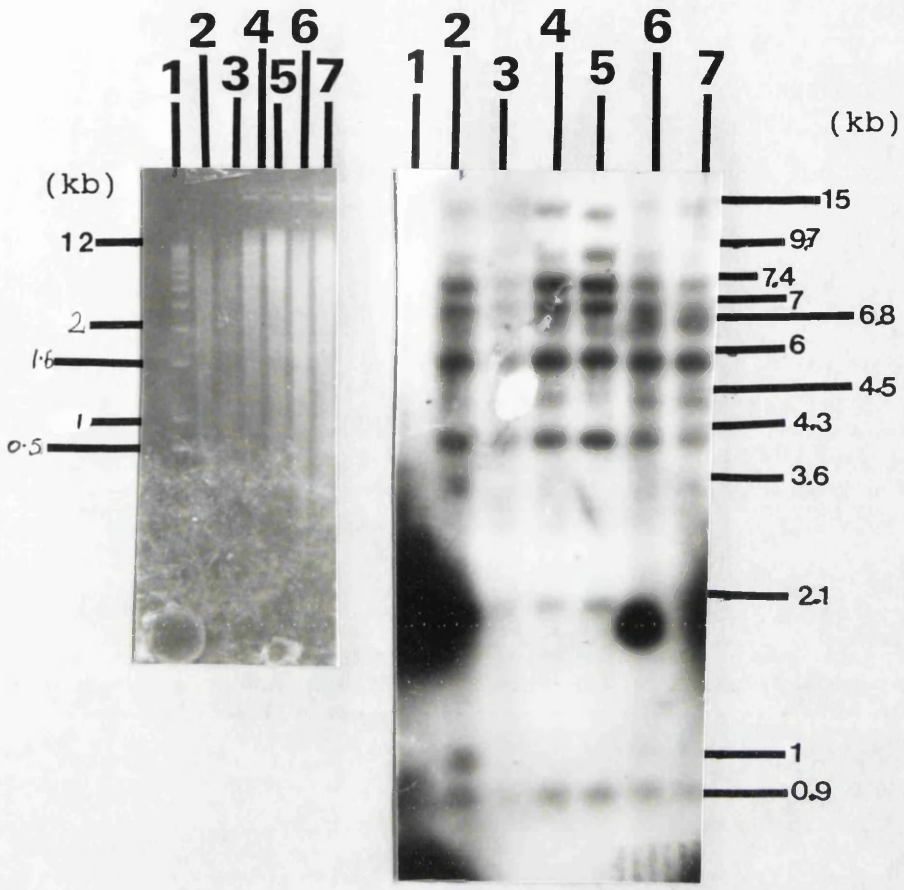
LANE	DNA
1	T25/02R/01
2	T25/03R/01
3	T25/04R/01
4	pUATD09R/01

Figure 4.16: undigested genomic DNA from Aspergillus pT25 series transformants. Probed with radiolabelled pUC8 DNA.

T25/02R/01 to T25/02R/05, T25/03R/01 to T25/03R/05, T25/04R/01 to T25/04R/05 and pUATD25/09/01 to pUATD25/09/05. Plasmid rescues into E.coli were attempted with each of the total genomic DNA samples. In no case was a pILJ25 derivative plasmid rescued; the TARp1.1/04 control sample yielded 35 ARp1-containing transformant E.coli colonies, so the bacterial host was transformable. This result implies that the pILJ25 derivatives have either been rearranged and as a result are no longer viable in E.coli or that the plasmids had in fact integrated into the fungal genome.

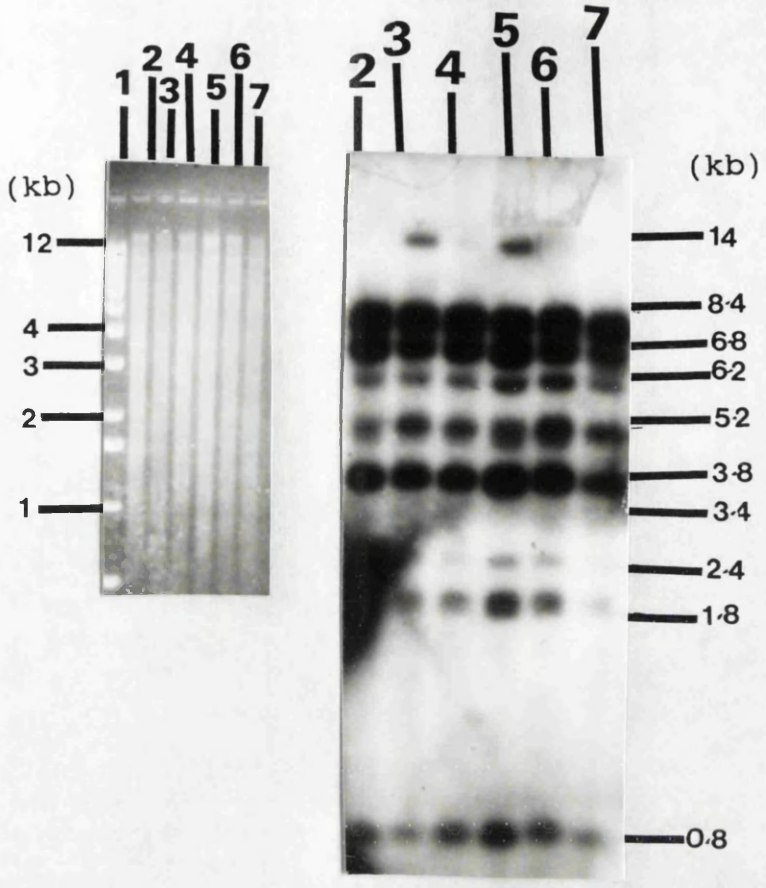
A Southern blot of 10µg of undigested total genomic DNA from T25/02R/01, T25/03R/01, T25/04R/01 and pUATD09/01 is shown in figure 4.16. The blot was probed with radiolabelled pUC DNA. This blot, although overloaded, clearly shows that while some form of free plasmid is present, the chromosomal bands also hybridise with the probe suggesting that either pUC material has integrated or else plasmid DNA is entangled with chromosomal material. Southern blots of DNA from the remaining DNA samples show the same result (not shown).

2µg aliquots of DNA from T25/02R/01, T25/03R/01, T25/03R/05, T25/04R/01, T25/04R/05 and pUATD25/09/01 were digested separately with BglII and double digested with EcoRI and SalI. The BglII cuts once in the argB gene so was expected to linearise the plasmid DNA, providing that only one copy of the argB



LANE	DNA
1	marker
2	T25/02R/01
3	T25/03R/01
4	T25/03R/04
5	T25/04R/01
6	T25/04R/03
7	TUATD09R/01

Figure 4.17a: genomic DNA from pT25 series Aspergillus transformants, digested with BglII. Probed with radiolabelled pUC8 DNA. Hybridising band sizes are listed on the right hand side of the blot.

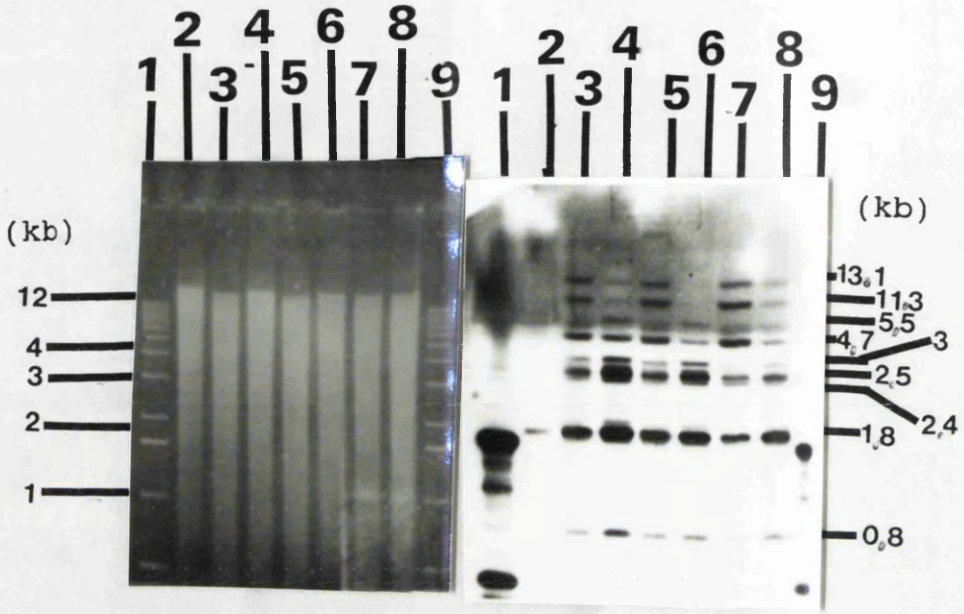


LANE	DNA
1	marker
2	T25/02R/01
3	T25/03R/01
4	T25/03R/04
5	T25/04R/01
6	T25/04R/03
7	TUATD09R/01

Figure 4.17b: genomic DNA from pT25 series Aspergillus transformants, digested with EcoRI and SalI. Probed with radiolabelled pUC8 DNA. Hybridising band sizes are listed on the right hand side of the blot.

gene is present. The EcoRI/SalI double digest should produce a number of fragments including intact 3.0kb pUC8 plasmid DNA. The digested DNA was run on a 0.8% gel, Southern blotted and probed with radiolabelled pUC8. These Southern blots are shown in figures 4.17a and 4.17b. It is obvious that each lane containing BglII digested DNA contains very similar banding patterns and that more than one hybridising band is present: there are nine common bands of 7.4kb, 7.0kb, 6.8kb, 6.0kb, 4.5kb, 4.3kb, 3.6kb, 2.1kb and 0.9kb. The EcoRI/SalI double digest, (figure 4.17b), produces 9 bands: 8.6kb, 6.8kb, 6.2kb, 5.2kb, 3.8kb, 3.4kb, 2.4kb, 1.8kb and 0.8kb; there is no band of 3.0kb which corresponds to unrearranged pUC8 DNA; there is an additional 14kb band in lanes 3 and 5. It is unlikely that these bands are the partial digest products because each lane contains this identical banding pattern with exceptions in intensity. It is clear that the plasmids have undergone the same rearrangement process that results in the formation of a complicated plasmid. The approximate size of this plasmid can be calculated by adding together the sizes of the various bands. The BglII banding pattern suggests a plasmid size of 42kb; the double digest banding pattern suggests a plasmid size of 39kb.

A further Southern blot was prepared from the same DNA samples and a control T16/01 sample. All



LANE	DNA
1	marker
2	pILJ16 transformant
3	T25/02R/01
4	T25/03R/01
5	T25/03R/04
6	T25/04R/01
7	T25/04R/03
8	TUATD09R/01
9	marker

Figure 4.18: genomic DNA from pT25 series Aspergillus transformants, digested with SalI. Probed with radiolabelled argB DNA. Hybridising band sizes are listed on the right hand side of the blot.

DNA was digested with SalI. The Southern blot was probed with radiolabelled argB gene. A SalI digest of this DNA, probed with argB, should produce a 0.8kb fragment and a 1.8kb fragment which are derived from an unrearranged argB gene. The argB DNA was prepared by digesting pILJ16 with SalI and then gene-cleaning the argB 0.8kb and 1.8kb fragments, these fragments were then pooled. The results of this blot are shown in figure 4.18. The expected 0.8kb and 1.8kb bands are present in all lanes, probably produced by the chromosomal copy of the argB gene. However, additional bands of 13.1kb, 11.3kb, 5.5kb, 4.7kb, 3.0kb, 2.5kb and 2.4kb are present in all lanes except the pILJ16 transformant control; the total plasmid size is estimated at 44kb. Therefore, the rearrangements of the pILJ25 derivatives include multiple duplications of the argB gene.

What is of considerable interest is the fact that pT25/02R, pT25/03R, pT25/04R and pUATD25/09, originally 12.2kb, 6.2kb, 6.0kb and 22kb in size and all derived from one common plasmid, have been rearranged in very similar ways i.e. the banding pattern is nearly identical for all DNA samples in both blots.

pILJ23		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	50	86	87	106	329		
	ARG+	7	14	16	18	55	384	85.68
2	ARG-	121	190	255	30	596		
	ARG+	20	32	42	5	99	695	85.76
3	ARG-	199	205	245	107	756		
	ARG+	32	33	40	19	124	880	85.91
4	ARG-	114	79	200	168	561		
	ARG+	20	13	34	27	94	655	85.65
5	ARG-	115	133	179	201	628		
	ARG+	19	23	29	33	104	732	85.79
subtotal	ARG-	599	693	966	612			
subtotal	ARG+	98	115	161	102	Average		85.76
TOTAL		697	808	1127	714	Standard Deviation		0.09
%LOSS		85.94	85.77	85.71	85.71			

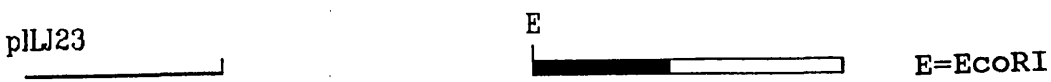


Figure 4.19: pILJ23 instability test results.

facing page 104d

pJSR04		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	118	120	43	170	451		
	ARG+	80	76	26	102	284	735	61.36
2	ARG-	93	151	143	132	519		
	ARG+	56	92	86	80	314	833	62.30
3	ARG-	114	170	156	163	603		
	ARG+	69	102	94	98	363	966	62.42
4	ARG-	120	83	144	131	478		
	ARG+	75	50	85	75	285	763	62.65
5	ARG-	141	124	128	131	524		
	ARG+	81	74	75	77	307	831	63.06
subtotal	ARG-	586	648	614	727			
subtotal	ARG+	361	394	366	432	Average		62.36
TOTAL		947	1042	980	1159	Standard Deviation		0.56
%LOSS		61.88	62.19	62.65	62.73			

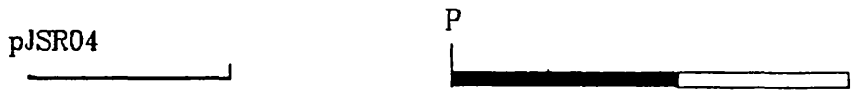


Figure 14.20: pJSR04 instability test results.

4.3.3 pILJ23 transformations.

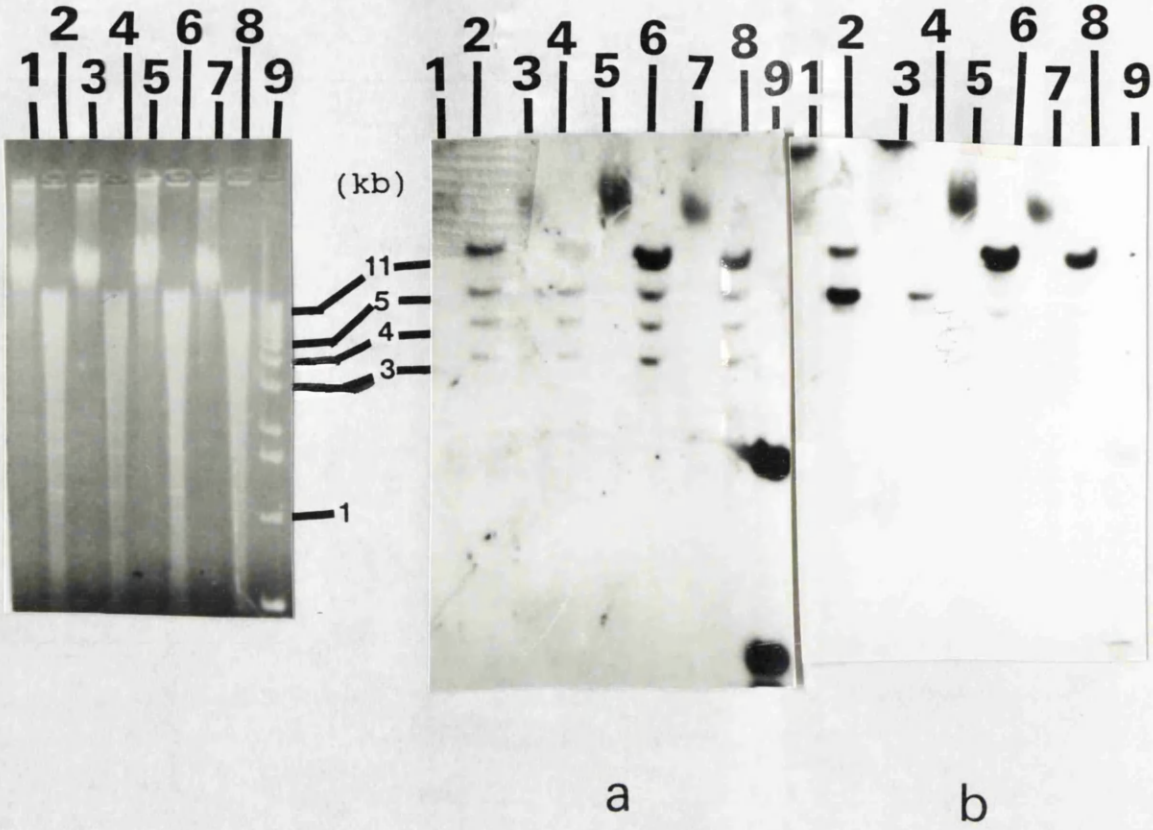
The plasmid pILJ23 contains the 1.9kb EcoRI ama1 fragment, pUC8 and the argB gene, (see figure 4.19). The structure of pILJ23 is similar to pILJ25, the difference is that the ama1 fragment in pILJ23 is in the opposite orientation compared to the pILJ25 ama1 fragment. The plasmid pILJ23 has a transformation frequency of 100 transformants/μg DNA.

The % instability results are shown in figure 4.19. The % instability per asexual generation is 85%. This figure is consistent for all 4 generations and for all the individuals examined. There is no appreciable difference between the transformation frequencies and % instability for pILJ23 and pILJ25 (see section 4.3.2). Similar experiments by Gems (1990), showed that pILJ23 was rearranged after transformation. It appears that pILJ23 and pILJ25 behave similarly, therefore the orientation of the ama1 sequence does not affect the behaviour of the plasmid.

4.3.4. pJSR04 transformations.

This plasmid is 8.8kb in size. Its construction is detailed in section 4.2 and its structure is shown in figure 4.20. It contains one complete arm and half the unique central region of ama1.

The transformation frequency of pJSR04 is 780



LANE	DNA	BAND SIZES pUC8 probe	argB probe
1	TJSR04.1		
2	TJSR04.1 CUT	11.5, 5.5, 4, 3.1	11.5, 5.2
3	TJSR04.2		
4	TJSR04.2 CUT	11.5, 5.5, 4, 3.1	5.2
5	TJSR04.3		
6	TJSR04.3 CUT	11.5, 5.5, 4, 3.1	11.5, 4
7	TARp1/4.4		
8	TARp1/4.4 CUT	11.5, 5.5, 4, 3.1	11.5
9	marker		

Figures 4.21a and 4.22b: pJSR04 fungal transformant genomic DNA digested with BglII and probed with (a) radiolabelled pUC8 DNA and (b) radiolabelled argB DNA.

transformants per µg of DNA. The original transformant colonies were designated T04/01 to T04/05. The % instability results are shown in figure 4.20. From the table it appears that 62% of asexual progeny per generation lose pJSR04.

Total genomic DNA was made from T04/01 to T04/05. The DNA samples prepared from T04/04 and T04/05 were badly degraded. 3µg aliquots of DNA from T04/01, T04/02, T04/03 and a TARp1 control sample were digested with BglII and run out on a 0.8% agarose gel along with uncut DNA samples. The gel was then Southern blotted and probed separately with DIG-labeled pUC8 and argB DNA. These probes should each produce only one identical band of 11.5kb which corresponds to linearised ARp1 plasmid. The results of these probes are shown in figures 4.21a (pUC8 probe) and 4.21b (argB probe).

Figure 4.21a, the pUC8 specific probe, shows that 4 identical bands hybridise in all the lanes, including the TARp1 control (lane 8). The top band is 11.5kb in size; this band is most intense in the T04/03 and TARp1 lanes. The remaining bands are 5.5kb, 4kb and 3.1kb in size; these bands differ in intensity from lane to lane e.g. these bands are most intense in lane 6 and faint in lane 4. It is impossible to explain away these additional bands as the products of partial digestion because BglII only cuts ARp1 once and ARp1 is not generally rearranged.

Fortunately an explanation is possible when

figure 4.21b, the argB specific probe, is examined. Again, only a single 11.5kb band was expected; in fact in the T04/01 lane there are two equally intense bands of 11.5kb and 5.2kb; in the T04/02 lane there is one band of 5.2kb; in the T04/03 lane there is an intense 11.5kb and a much less intense 4kb band; in the TARp1 lane there is only one 11.5kb band.

Taken together, the results described above show that plasmid DNA in T04/01, T04/02 and T04/03 has been rearranged to some extent and that these rearrangements have affected both the pUC8 and argB sequences. What about ARp1? In the case of the TARp1 lane all of the bands contain pUC8 DNA but only the 11.5kb band contains any argB DNA. This result can be explained by reference to the ARp1 proposed mechanism of replication. This mechanism is analogous to the 2μ circle model of replication discussed in section 4.1.3, (see also section 4.7.4).

In all cases it is impossible to identify any free plasmid DNA in any of the undigested DNA lanes. The resolution of free plasmid DNA from the genomic DNA appears to be determined by the voltage used when running the gel.

Plasmid rescues were attempted with the DNA samples prepared from T04/01, T04/02 and T04/03. No E.coli transformants were obtained from these samples but 23 E.coli transformants, containing ARp1 were obtained from the TARp1 control DNA (results not shown).

pDHG24		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	39	112	73	111	335		
	ARG+	19	83	31	80	213	548	61.13
2	ARG-	26	151	133	170	480		
	ARG+	3	111	110	131	355	835	57.49
3	ARG-	10	189	101	107	407		
	ARG+	1	139	74	81	295	702	57.98
4	ARG-	43	201	155	172	571		
	ARG+	21	99	101	129	350	921	62.00
5	ARG-	25	163	160	193	541		
	ARG+	2	121	120	140	383	924	58.55
subtotal	ARG-	143	816	622	753			
subtotal	ARG+	46	553	436	561	Average		59.43
TOTAL		189	1369	1058	1314	Standard Deviation		1.80
%LOSS		75.66	59.61	58.79	57.31			

Figure 4.22: pDHG24 instability test results.

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4.3.5. pDHG24 transformations.

The plasmid pDHG24 is 9.8kb in size and contains approximately three quarters of the amal sequence. It contains all of the right hand amal arm, all of the central unique region and 1.7kb of the left hand amal arm, (see figure 4.22).

The transformation frequency for pDHG24 is 10,000 per µg of DNA. The % instability data are shown in figure 4.22. On average, 59% of progeny derived from pDHG24 transformants lose the plasmid. The one exception is the results from the first generation for all the individuals. This result suggests that the % instability is 75.6%, much higher than the average calculated value. This result is statistically significant. These results are almost "ARp1"-like. The table below compares the behaviour of pDHG24 and ARp1.

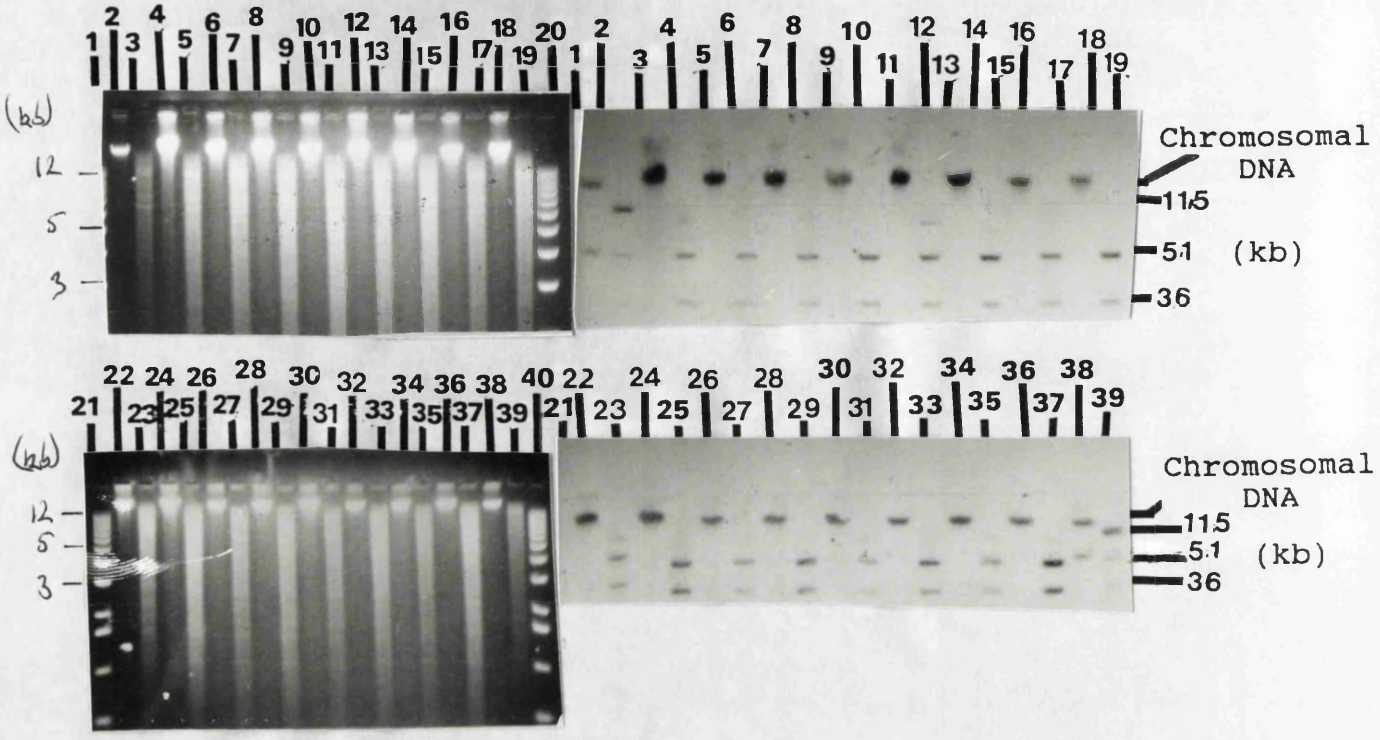
PLASMID	TRANSFORMATION FREQUENCY/µg DNA	% INSTABILITY
pDHG24	10,000	59%
ARp1	40,000	56%

The data in the above table suggests that pDHG24 contains the minimium amount of amal DNA required for comparatively high frequency of transformation and

plasmid stability: 1µg of pJSR04 yields 780 transformants and 62% of progeny lose this plasmid; 1µg of pILJ25 yields 100 transformants and 85% of progeny lose this plasmid. The decrease in transformation could be explained if the deleted 1.7kb EcoRI fragment contained a transformation frequency enhancer element. It is probable that amal contains more than one transformation frequency enhancer element, (see sections 4.5 and 4.6). Another possibility is that pDHG24 may undergo a rearrangement process after transformation, generating the ARp1 plasmid, thereby explaining the plasmid behaviour. The data in the above table does not of course give any indication as to whether or not pDHG24 is rearranged.

Therefore, total genomic DNA preparations were made from four individual transformants taken from the initial transformation plates, and DNA was made from individual colonies from the first, second and third generation conidial progeny. These DNA samples were designated T24/0.1 to T24/3.4. Approximately 3µg of this genomic DNA was digested with BglII, along with a TARp1 control and run out on a 0.8% agarose gel, Southern blotted and probed with DIG-labelled pUC DNA. The BglII digest should linearise any plasmid DNA. The results of this blot are shown in figure 4.23.

In the TARp1 control lane it is possible to see a single 11.5kb band. In the pDHG24 transformant



LANE	DNA	LANE	DNA
1	marker	21	marker
2	TARp1/1.3	22	TDHG24/3.1
3	TARp1/1.3 CUT	23	TDHG24/3.1 CUT
4	TDHG24/1.1	24	TDHG24/3.2
5	TDHG24/1.1 CUT	25	TDHG24/3.2 CUT
6	TDHG24/1.2	26	TDHG24/3.3
7	TDHG24/1.2 CUT	27	TDHG24/3.3 CUT
8	TDHG24/1.3	28	TDHG24/3.4
9	TDHG24/1.3 CUT	29	TDHG24/3.4 CUT
10	TDHG24/1.4	30	TDHG24/4.1
11	TDHG24/1.4 CUT	31	TDHG24/4.1 CUT
12	TDHG24/2.1	32	TDHG24/4.2
13	TDHG24/2.1 CUT	33	TDHG24/4.2 CUT
14	TDHG24/2.2	34	TDHG24/4.3
15	TDHG24/2.2 CUT	35	TDHG24/4.3 CUT
16	TDHG24/2.3	36	TDHG24/4.4
17	TDHG24/2.3 CUT	37	TDHG24/4.4 CUT
18	TDHG24/2.4	38	TARp1/1.3
19	TDHG24/2.4 CUT	39	TARp1/1.3 CUT
20	marker	40	marker

Figure 4.23: genomic DNA from pDHG24 Aspergillus transformants from 4 different conidial generations, digested with BglII. Probed with radiolabelled pUC8 DNA.

lanes the banding pattern is similar. It is immediately clear from this blot that all the hybridising bands in the pDHG24 transformant lanes are identical, regardless of which generation or individual the sample was prepared from. There appear to be two main bands present in these lanes. The band sizes are 5.1kb and 3.6kb. An additional band of 8.7kb can be seen in lane T24/4.1. This band is probably a partial digest product.

It appears then that pDHG24 has undergone some sort of rearrangement. The results presented in figure 4.23 suggest that this rearrangement must have taken place in the original transformants. It was not possible to rescue any plasmids from the genomic DNA samples but the TARp1 control yielded 24 ARp1 containing transformant E.coli colonies. This result implies that the rearrangement has not produced an ARp1 structure and that the ama1 sequence contained in pDHG24 is not the minimum required for a stable, unrearranged plasmid capable of high frequency transformation.

4.3.6. pILJ20 transformations.

This plasmid contains the 2.5kb central EcoRI inverted repeat fragment, (see figure 4.24). The transformation frequency of pILJ20 was calculated to be 11 transformant colonies per µg DNA, so pILJ20 does not appear markedly different from pIL16.

pILJ20		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	29	27	13	17	86		
	ARG+	115	111	77	54	357	443	19.41
2	ARG-	0	0	0	0	0		
	ARG+	127	119	121	100	467	467	0.00
3	ARG-	0	0	0	0	0		
	ARG+	151	114	101	171	537	537	0.00
4	ARG-	0	0	0	0	0		
	ARG+	210	103	139	155	607	607	0.00
5	ARG-	0	0	0	0	0		
	ARG+	113	97	143	148	501	501	0.00
subtotal	ARG-	29	27	13	17			
subtotal	ARG+	716	544	581	628	Average	0 OR 20	
TOTAL		745	571	594	645			

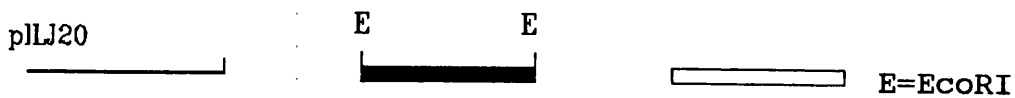


Figure 4.24: pILJ20 instability test results.

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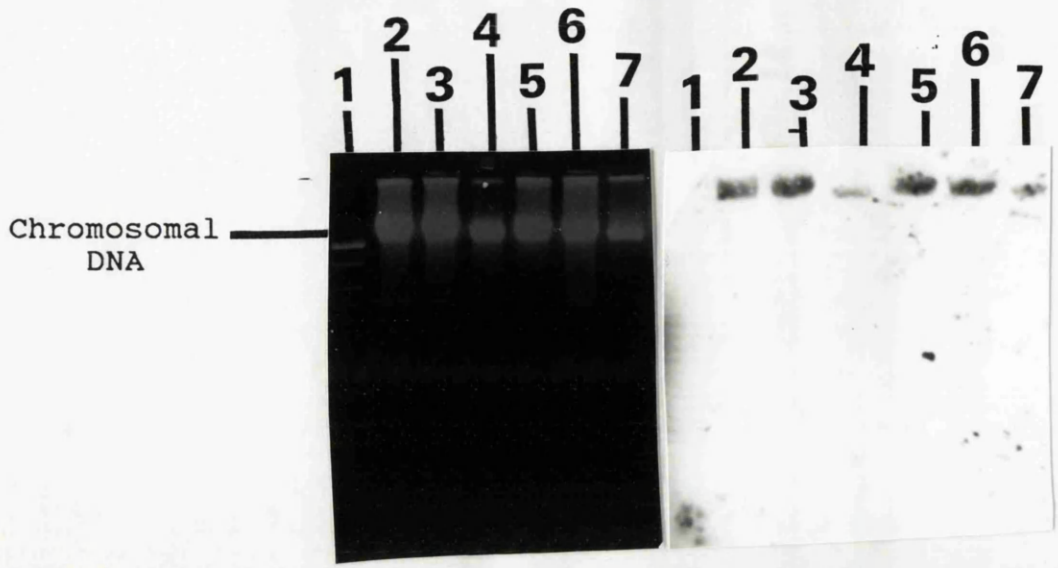
The plasmid % loss results shown in figure 4.24 suggest that pILJ20 usually integrates into the genome because there is no indication of plasmid instability for colonies 2-5. Therefore it appears that pILJ20 is an integrative plasmid. However, individual 1 is the exception. The results show that 20% of the progeny lose the plasmid, implying that the plasmid or some rearranged version of the plasmid is capable of autonomous replication in some transformants. Another explanation is that pILJ20 has first integrated into the genome and then been excised, the subsequent loss of the excised plasmid gives the appearance of the loss of a replicating plasmid. If these results are due to plasmid integration and excision then the excision event must be rare: only one in five of the original transformants appears to contain an unstable plasmid and only 20% of the progeny show signs of instability, but this figure is consistent over 5 generations. The control transformations with pILJ16 and ARp1 gave the expected results as did the control instability tests, so the results shown in figure 4.24 are not simply due to faulty experimental procedure.

Total genomic DNA was made from the original 5 pILJ20 transformants, these samples were labelled T20/01 to T20/05. 3µg samples of this uncut DNA, along with a TARp1 control were run on 0.8% agarose gel, Southern blotted and probed with radiolabelled



LANE	DNA
1	marker
2	TARp1/2.3
3	T20/01
4	T20/02
5	T20/03
6	T20/04
7	T20/05

Figure 4.25a: undigested genomic DNA from Aspergillus pILJ20 transformants. Probed with radiolabelled pUC8 DNA.

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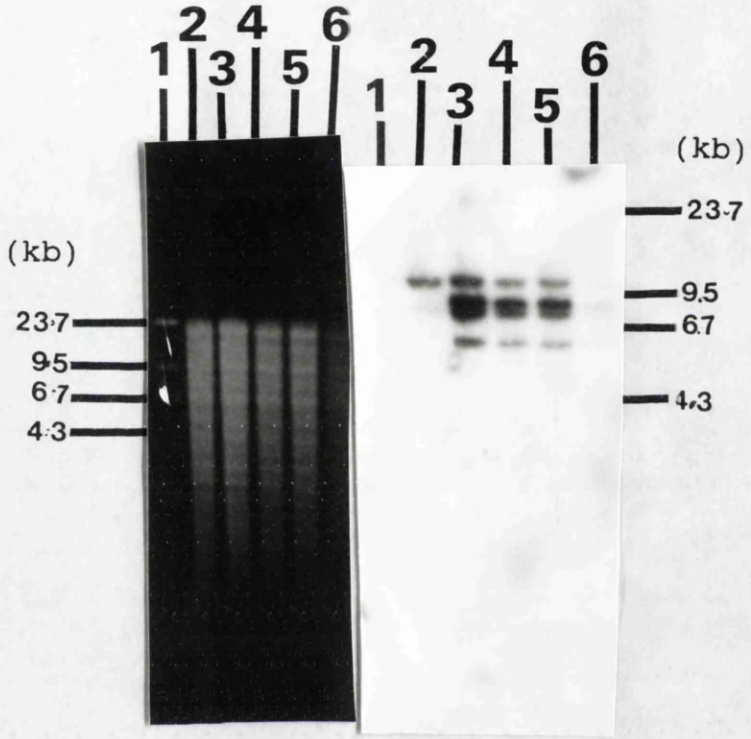

LANE	DNA	BAND SIZES (kb)
1	marker	-
2	TARp1/2.3	11.5,
3	T20/01	11.5, 8.7, 8, 6.1
4	T20/02	11.5, 8.7, 8, 6.1
5	T20/03	11.5, 8.7, 8, 6.1
6	T20/04	as above but very faint

Figure 4.25b: BglII digested genomic DNA from Aspergillus pILJ20 transformants. Probed with radiolabelled pUC8 DNA.

pUC DNA. The results of this blot are shown in figure 4.25a. No free plasmid is distinguishable in any lane, even TARp1. Further 3µg samples of the T20/01 to T20/04 DNA were digested with BglII and run out on a 0.8% agarose gel with a TARp1 control, Southern blotted and probed with radiolabelled pUC DNA, see figure 4.25b. The TARp1 lane contains the expected single 11.5kb band. All the other lanes contain 4 identical bands of 11.5kb, 8.7kb, 8.0kb and 6.1kb; suggesting that the pILJ20 derived DNAs have an identical structure. The Southern blot, (figure 4.25b), shows that the pILJ20 DNA has been extensively rearranged. It is therefore possible to explain the results shown in figure 4.25b by refering to plasmid rearrangements. As shown in section 4.3.2 it is possible for a plasmid e.g. pILJ25 to be rearranged to give a variety of different derivatives with different transformation properties . Likewise, the results in figure 4.24 are due to plasmid rearrangements, which in transformants T20/02 to T20/05 led to the formation of either an integrative plasmid or a replicative plasmid with low transformation capability but with radically increased plasmid stability. In T20/01 the rearrangements produced a replicating plasmid with low transformation capability but increased plasmid stability. It was not possible to rescue any plasmids from the T20 DNA samples so it was impossible to test the above theories.

pDHG25		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	128	140	138	171	577		
	ARG+	86	94	93	114	387	964	59.85
2	ARG-	113	121	125	137	496		
	ARG+	76	83	85	93	337	833	59.54
3	ARG-	176	89	131	143	539		
	ARG+	119	60	87	78	344	883	61.04
4	ARG-	144	136	139	125	544		
	ARG+	98	92	94	85	369	913	59.58
5	ARG-	123	118	113	132	486		
	ARG+	83	80	79	87	329	815	59.63
subtotal	ARG-	684	604	646	708			
subtotal	ARG+	462	409	438	457	Average		59.93
TOTAL		1146	1013	1084	1165	Standard Deviation		0.57
%LOSS		59.69	59.62	59.59	60.77			

Figure 4.26: pDHG25 instability test results.

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It is interesting to compare the pILJ20 results with the results described in section 4.6, which are based on plasmids containing a smaller inverted repeat than the one in pILJ20. These small plasmids show an increase in transformation frequency and are unstable; both criteria implying that these small plasmids are genuine, autonomously replicating plasmids. The section 4.5 results add support for the idea that pILJ20 results are due to plasmid rearrangements rather than simple plasmid integration and excision events.

4.3.7. pDHG25 transformations.

The plasmid pDHG25 contains the 5kb HindIII ama1 fragment and is 10kb in size, (see figure 4.26). The transformation frequency of this plasmid is 1000 colonies per µg DNA. The % loss data is shown in table 4.26. On average, 59% of progeny derived from pDHG25 transformants lose the plasmid. Gems (1990), has characterised pDHG25 transformants and shown that the plasmid is not rearranged. This result implies that the 5kb HindIII ama1 fragment is in fact the minimum amount of ama1 DNA that ensures an unrearranged, relatively stable plasmid with fair transformation capability.

pJSR01		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	133	76	181	102	492		
	ARG+	199	114	271	153	737	1229	40.03
2	ARG-	122	130	131	115	498		
	ARG+	180	201	196	173	750	1248	39.90
3	ARG-	147	102	85	93	427		
	ARG+	230	245	137	136	748	1175	36.34
4	ARG-	100	132	170	79	481		
	ARG+	139	198	250	120	707	1188	40.49
5	ARG-	129	130	111	67	437		
	ARG+	194	195	170	99	658	1095	39.91
subtotal	ARG-	631	570	678	456			
subtotal	ARG+	942	953	1024	681	Average		39.33
TOTAL		1573	1523	1702	1137	Standard Deviation		1.51
%LOSS		40.11	37.43	39.84	40.11			

Figure 4.27a: pJSR01 instability test results.

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pJSR02		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	76	114	100	207	497		
	ARG+	31	67	59	181	338	835	59.52
2	ARG-	166	150	137	140	593		
	ARG+	101	98	73	78	350	943	62.88
3	ARG-	198	173	143	112	626		
	ARG+	69	79	60	43	251	877	71.38
4	ARG-	123	119	73	166	481		
	ARG+	47	29	10	98	184	665	72.33
5	ARG-	89	135	129	183	536		
	ARG+	48	60	41	117	266	802	66.83
subtotal	ARG-	652	691	582	808			
subtotal	ARG+	296	333	243	517		Average	66.59
TOTAL		948	1024	825	1325		Standard Deviation	4.89
%LOSS		68.78	67.48	70.55	60.98			

Figure 4.27b: pJSR02 instability test results.

pJSR03		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	47	117	111	100	375		
	ARG+	53	120	102	96	371	746	50.27
2	ARG-	50	112	103	171	436		
	ARG+	47	105	97	160	409	845	51.60
3	ARG-	23	89	114	122	348		
	ARG+	26	85	107	115	333	681	51.10
4	ARG-	111	143	109	133	496		
	ARG+	104	135	102	125	466	962	51.56
5	ARG-	76	98	131	146	451		
	ARG+	72	92	123	126	413	864	52.20
subtotal	ARG-	307	559	568	672			
subtotal	ARG+	302	537	531	622	Average		51.35
TOTAL		609	1096	1099	1294	Standard Deviation		0.64
%LOSS		50.41	51.00	51.68	51.93			

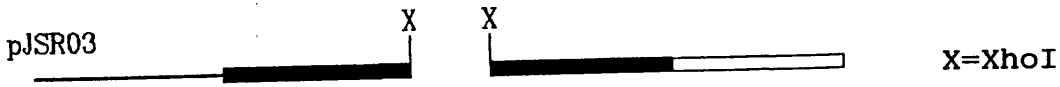

Figure 4.27c: pJSR03 instability test results.

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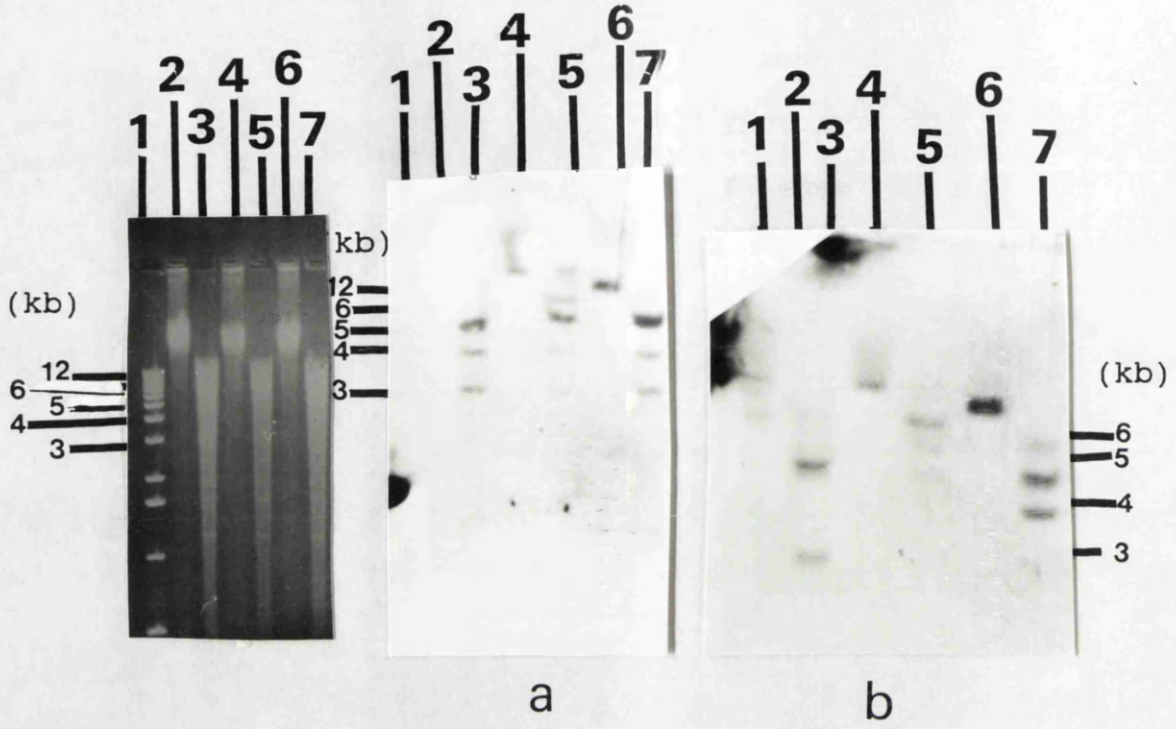
4.3.8. pJSR01, pJSR02 and pJSR03 transformations.

The construction of plasmids pJSR01, pJSR02 and pJSR03 are described in section 4.2, (see also figure 4.4). The calculated transformation frequencies for pJSR01, pJSR02 and pJSR03 are 790, 1170 and 1830 colonies per µg DNA respectively. The % plasmid loss per generation are shown in figures 4.27a, 4.27b and 4.27c.

Figure 4.27a shows that 39% of pJSR01 derived progeny lose the plasmid. This figure is fairly consistent for all the individuals tested. Figure 4.27b shows that 66% of pJSR02 derived progeny lose the plasmid. This time however the calculated values are more variable. The total % loss for individual 1 is 59%; for individual 4 the total % loss is 72%. Similarly, the total % loss for all individuals in generation 3 is 70%; the figure for all the individuals in generation 4 is 60%. These differences are statistically significant. Therefore, these results could suggest that pJSR02 has undergone rearrangements which have produced a number of plasmids with varying properties. Figure 4.27c shows that 51% of pJSR03 derived progeny lose the plasmid. There is no noticeable differences in the calculated values for different individual transformants.

It was not my intention to make an exhaustive study of the rearrangements, if any, of the pJSR



LANE	DNA	BAND SIZES pUC8 probe	argB probe
1	marker	-	-
2	TJSR01	-	-
3	TJSR01 CUT	5.5, 4, 3.1	5, 2.9
4	TJSR02	-	-
5	TJSR02 CUT	13, 7, 6.3, 4,	6.3
6	TJSR03	-	-
7	TJSR03 CUT	5.5, 4, 3.1	4.8, 3.8

Figures 4.28a and 4.28b: genomic DNA from Aspergillus pJSR series transformants, digested with BglII. Probed seperately with (a) DIG-labelled pUC8 DNA and (b) DIG-labelled argB DNA.

series. I only needed to know if plasmid rearrangements had occurred. Therefore, total genomic DNA was made from one of the original transformant colonies for each of the pJSR series. The samples were labelled TJSR01/0.4, TJSR02/0.4 and TJSR03/0.4.

DNA from samples TJSR01/0.4, TJSR02/0.4 and TJSR03/0.4 were digested with BglII, run on a 0.8% agarose gel, Southern blotted and probed seperately with DIG-labelled pUC DNA and argB DNA. The results are shown in figures 4.28a and 4.28b. These Southern blots were prepared at the same time and using the same TARp1 control as the blots presented in figures 4.21a and 4.12b. The TARp1 control is described in detail in section 4.3.4.1, figure 4.21b. The digested TJSR01/0.4 lane contains 3 hybridising bands: an intense 5.5kb band and a 4kb and a 3.1kb band. The digested TJSR02/0.4 lane contains 4 hybridising bands: 13kb, 7kb, an intense 6.3kb band and a 4kb band. The digested TJSR03/0.4 lane contains 3 bands of similar size to those in the TJSR01/0.4 lane. Free plasmid can be seen in the undigested TJSR02/0.4 lane. When this blot was probed with the argB probe, a different banding pattern was evident. Two bands of 5.0kb and 2.9kb in size hybridise in the digested TJSR01/0.4 lane. A single band, 6.3kb in size hybridises in the digested TJSR02/0.4 lane. Two bands hybridise in the digested TJSR03/0.4 lane: 4.8kb and 3.8kb. The suspicion

remains that the repeated series of pUC-hybridising bands are due to contamination, (probably of the DNA-extraction buffer), but the distinct pattern of arg-hybridising bands indicate that all three pJSR plasmids have undergone different rearrangements.

4.4. Transformations with linear DNA.

This set of experiments attempts to study the effect, if any, of transforming Aspergillus with linear DNA. The pJSR series were used in this set of experiments. The plasmid DNA was prepared as described in section 4.2: 3µg aliquots of ARp1 were digested separately with ClaI, NruI and XhoI, each of which would cut ARp1 twice, effectively deleting a central fragment of varying size from amal. The digested DNAs were then run on a 0.8% agarose gel and the required bands gene-cleaned. 2µg of each of these digested samples were then self-ligated after the Geneclean step. The remaining 1µg of digested DNA aliqouts were not self-ligated; overall, this procedure produces linear and circular versions of the pJSR01, pJSR02 and pJSR03 plasmids. 1µg of ARp1 was linearised by a BglII digest which effectively cuts the centre of the argB gene. To measure the effect, if any, of cutting within the argB gene I digested 1µg of circularised pJSR03 with BglII. Approximately $2x10^7$ protoplasts were transformed separately with 1µg of each of the plasmids in both

PLASMID	RESTRICTION ENZYME	TRANSFORMATION FREQUENCY/µg DNA		%LOSS	
		C	L	C	L
pJSR01	ClaI	800	750	39	40
pJSR02	NruI	1090	973	67	66
pJSR03	XhoI	1785	1651	50	52
pJSR03	BglII	-	345	-	51
ARp1	BglII	40,000	8600	56	56

Figure 4.29: transformation frequencies and % loss for linear and circular plasmids.

C=circular DNA

L=linear DNA

circularised and linear forms. The transformation frequencies and average % instability values are shown in figure 4.29.

From figure 4.29. it is clear that there is no real difference in transformation frequency when circular and linear plasmid DNA is compared. In the pJSR series the difference is approximately 100 colonies, so can be considered to be slight. The most marked difference is the comparison between circular and linear forms of ARp1 and the BglII digested pJSR03 DNA. In this case there is a 5-fold decrease in transformation frequency when linear DNA is used. This decrease is probably due to the BglII digest which effectively cuts the argB gene centrally. There appears to be no difference in plasmid loss (% instability) when these values are compared for linearised and circularised plasmid.

In summary, there is no real difference in linearised or circularised plasmid behaviour where the argB gene is undamaged. The results also imply that Aspergillus has an efficient DNA repair system which can recognise and repair linear plasmid DNA. These results independently confirm similar results obtained by Gems (1990), using different plasmids. No further work was carried out on this topic.

4.5 Cotransformations of *Aspergillus* with pILJ16 and the deletion series.

When *Aspergillus* is cotransformed with an integrative plasmid and an ARS-containing plasmid the resulting cointegrate plasmid transforms at a higher frequency than the integrative vector and is unstable, Gems (1990). By cotransforming *Aspergillus* with pILJ16 and the deletion series of pBLUESCRIPT plasmids used for sequencing, as described in Chapter 3, I hoped to identify functionally, minimal regions of *amal* that act as ARS sequences. The *argB* gene is only on pILJ16: none of the deletion series plasmids contain a fungal selectable marker. The construction of the deletion series is detailed in section 3.4. Plasmid structures are detailed in section 3.4, figures 3.9a and 3.9b.

4.5.1 Cotransformations with the KpnI/XhoI deletion series, NotI/SmaI deletion series and pILJ16.

Protoplasts were prepared as usual and $1x10^7$ protoplasts were cotransformed with a mixture of 1µg of pILJ16 and 1µg of each plasmid from the pSEQ01 KpnI/XhoI deletion series. A similar experiment was set up in which plasmids from the pSEQ01 NotI/SmaI deletion series were used. Experimental controls included protoplast aliquots transformed with ARp1, pILJ16, pILJ16 plus pUC8 and protoplasts with no DNA.

PLASMID	TRANSFORMATION FREQUENCY/μg DNA	COORDINATES OF INSERT
pSEQ01.1KX	20	815-2600
pSEQ01.3KX	18	994-2600
pSEQ01.5KX	121	1142-2600
pSEQ01.7KX	23	1378-2600
pSEQ01.9KX	15	1601-2600
pSEQ01.11KX	111	1790-2600
pSEQ01.13KX	17	1995-2600
pSEQ01.15KX	21	2236-2600
pSEQ01.17KX	115	2488-2600
PSEQ01.19KX	25	2563-2600
pILJ16	23	-
pILJ16/pBLUESCRIPT	21	-
ARp1	40,000	-

Figure 4.30a: transformation frequencies for both pSEQ01.KX/pILJ16 cotransformations and control plasmids. Co-ordinates give the start position on the sequenced region of amal, (see figure 3.10), for each subclone.

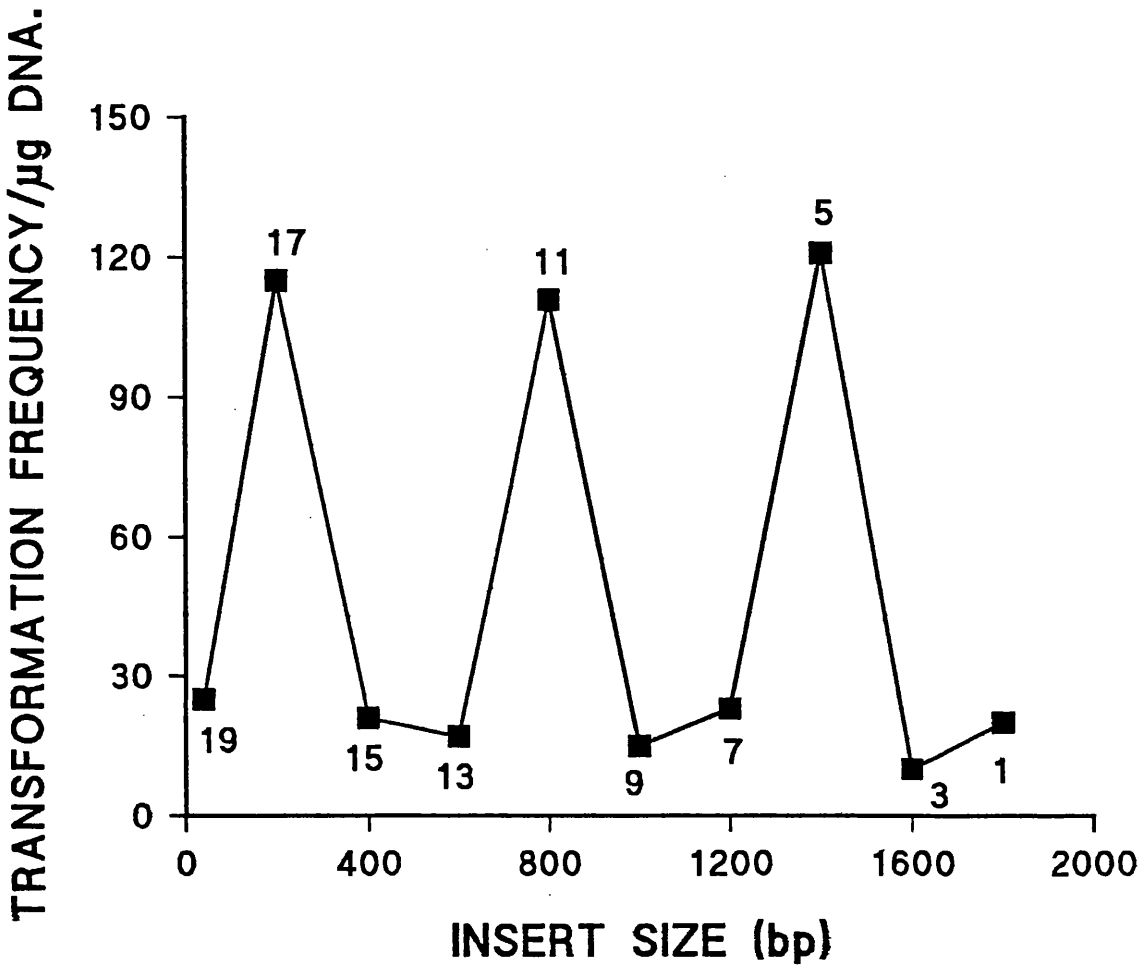


Figure 4.30b:–

Graph showing results of pSEQ01/plLJ16 cotransformation using Kpn1/Xho1 deletion series.
Numbers refer to specific subclones.

PLASMID	TRANSFORMATION FREQUENCY/μg DNA	COORDINATES OF INSERT
pSEQ01.1NS	116	2600-465
pSEQ01.3NS	125	2470-465
pSEQ01.5NS	20	2257-465
pSEQ01.7NS	20	2054-465
pSEQ01.9NS	102	1833-465
pSEQ01.11NS	24	1640-465
pSEQ01.13NS	16	1382-465
pSEQ01.15NS	144	1167-465
pSEQ01.17NS	30	987-465
pSEQ01.19NS	24	822-465
pSEQ01.21NS	80	647-465
pSEQ01.23NS	20	511-465
pILJ16	28	-
pILJ16/pBLUESCRIPT	19	-
ARp1	35,000	-

Figure 4.31a: transformation frequencies for both pSEQ01.NS/pILJ16 cotransformations and control plasmids. Co-ordinates give the start position on the sequenced region of ama1, (see figure 3.10), for each subclone.

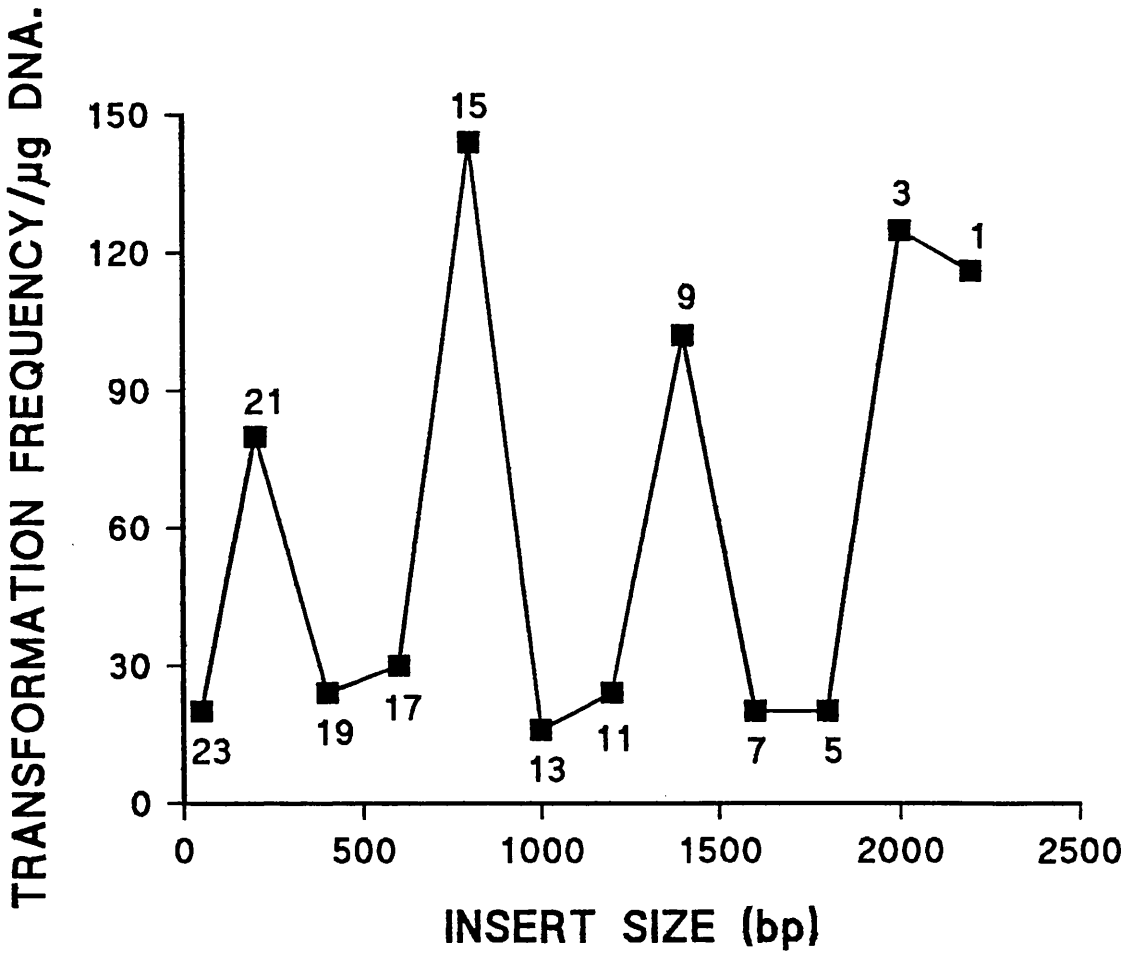


Figure 4.31b:–
Graph showing results of pSEQ01/plLJ16 cotransformation using Not1/Sma1 deletion series.
Numbers refer to specific subclones.

The protoplasts were then plated out. Transformant colonies were clearly visible after 3 days. The transformation frequencies were then calculated.

The results for these two experiments are described in figure 4.30a (KpnI/XhoI) and figure 4.31a (NotI/SmaI). These figures show the cotransformation frequency of each deletion plasmid with pILJ16. Also included is a graph for each experiment, figures 4.30b and 4.31b, on which the transformation frequency is plotted against amal insert size for each deletion plasmid. The numbers on each plotted point refer to the specific plasmid e.g. on the NotI/SmaI graph the number 1 refers to plasmid pSEQ01.1, number 3 refers to plasmid pSEQ01.3 and so on. No cotransformations were carried out with the pSEQ02 series, pSEQ03 or pSEQ04.

The peaks and troughs appear to be regularly spaced at 400bp intervals. This was not the result that was expected. The following plasmids i.e. the peaks in figures 4.30b and 4.31b, appear to behave as autonomously replicating, transformation frequency enhancing plasmids: pSEQ01.5KX (121 transformants), pSEQ01.11KX (111 transformants), pSEQ01.17KX (115 transformants), PSEQ01.1NS (116 transformants), pSEQ01.3NS (125 transformants), pSEQ01.9NS (102 transformants), pSEQ01.15NS (144 transformants) and pSEQ01.21NS (80 transformants).

It is clear from both sets of graphs that no single region can be identified as being a site of

autonomous replication. Moreover, plasmids containing larger fragments of amal do not necessarily transform more efficiently than those with smaller inserts.

4.5.2 Cotransformations with reisolated pSEQ01.5, pSEQ01.21 and pILJ16.

To confirm that enhanced transformation frequencies are not directly related to plasmid size a further experiment was set up in which two of the NotI/SmaI deletion series plasmids were reisolated for both pSEQ01.5NS and pSEQ01.21NS. The plasmid pSEQ01.5NS, the largest plasmid with no apparent ARS function i.e. low transformation frequency and pSEQ01.21NS, the smallest plasmid with ARS function i.e enhanced transformation frequency, in the NotI/SmaI deletion set, were recovered from the original deletion reactions from four separate E.coli DS941 colonies. Plasmid identity was confirmed by restriction enzyme analysis, (gel not shown). Plasmid DNA was prepared using the miniprep method.

Approximately 1µg aliquots of the reisolated plasmid DNAs were then used in cotransformations with 1µg of pILJ16; 2.3×10^7 protoplasts prepared from Aspergillus nidulans G34 were used in each transformation. The control DNAs pILJ16 and ARp1 were also prepared using the miniprep method. Transformant colonies were visible after 3 days.

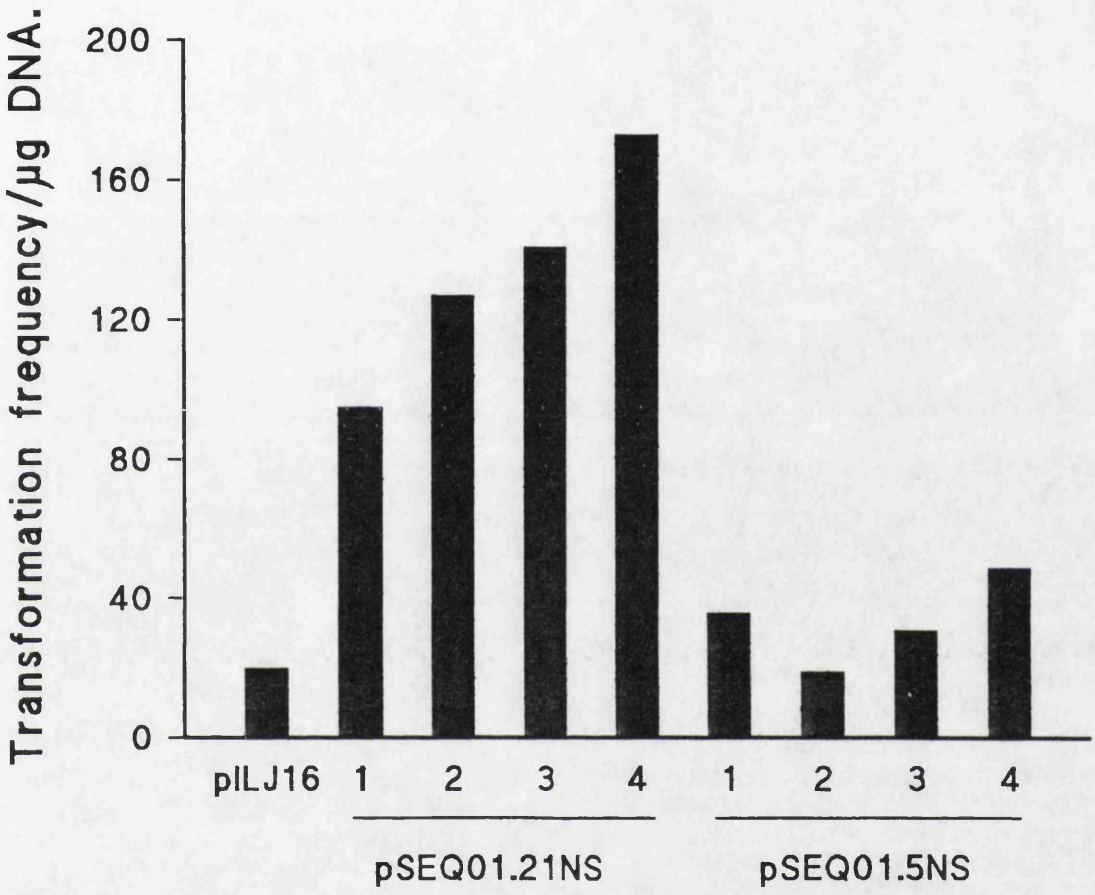


Figure 4.32: cotransformations with pSEQ01 plasmids.
1,2,3,4=experiment number.

pSEQ01.5/16		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	80	43	85	122	330		
	ARG+	4	1	12	32	49	379	87.07
2	ARG-	38	41	116	109	304		
	ARG+	47	8	9	21	85	389	78.15
3	ARG-	25	83	121	78	307		
	ARG+	37	4	16	7	64	371	82.75
4	ARG-	57	33	93	112	295		
	ARG+	12	6	7	19	44	339	87.02
5	ARG-	75	56	134	110	375		
	ARG+	13	9	29	14	65	440	85.23
subtotal	ARG-	275	256	549	531			
subtotal	ARG+	113	28	73	93		Average	84.04
TOTAL		388	284	622	624		Standard Deviation	3.34
%LOSS		70.88	90.14	88.26	85.10			

Figure 4.33: pSEQ01.5/16 instability test results.

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pSEQ01.21/16		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	37	64	98	101	300		
	ARG+	12	16	29	32	89	389	77.12
2	ARG-	60	100	75	84	319		
	ARG+	13	22	12	21	68	387	82.43
3	ARG-	35	89	100	40	264		
	ARG+	7	31	33	11	82	346	76.30
4	ARG-	60	109	67	89	325		
	ARG+	26	70	19	23	138	463	70.19
5	ARG-	56	78	57	97	288		
	ARG+	20	8	16	30	74	362	79.56
subtotal	ARG-	248	440	397	411			
subtotal	ARG+	78	147	109	117		Average	77.12
TOTAL		326	587	506	528		Standard Deviation	4.07
%LOSS		76.07	74.96	78.46	77.84			

Figure 4.34: pSEQ01.21/16 instability test results.

Figure 4.32 shows the transformation frequency results for each of the cotransformation experiments. The average transformation frequencies are as follows: pSEQ01.5NS/pILJ16 yields 33 transformants, pSEQ01.21NS/pILJ16 yields 134 transformant colonies. The difference in transformation frequency between the pSEQ01.5NS/pILJ16 cotransformation and the pSEQ01.21NS/pILJ16 cotransformation is statistically significant; paired T-test results: with 6 degrees of freedom and a 95% confidence level, T=5.757 which is significant at 0.0012.

The % instability results are shown in figures 4.33 and 4.34. The % instability results for pSEQ01.5NS/pILJ16, (figure 4.33), are variable, ranging from 70% in the first generation to 90% in the second generation. This variability is statistically significant. The calculated average % loss of 84% correlates with the behaviour of similar sized plasmids e.g. pILJ25 and pILJ23. The data in figure 4.34, describing the % loss of pSEQ01.21NS/pILJ16 clearly shows that although this plasmid contains a very small amount of ama1, it is still capable of autonomous replication. The average % loss of this plasmid is 77%.

It is clear from this set of experiments that cotransformations with pSEQ01.5NS do not lead to an increase in transformation frequency. In contrast, cotransformations with pSEQ01.21NS do lead to an increase in transformation frequency and significant

plasmid instability. The results again indicate that pSEQ01.21NS acts as an autonomously replicating plasmid. However the % instabilty results for pSEQ01.5NS suggest that this plasmid can also replicate autonomously but without any apparent increase in transformation frequency.

I conclude from these results that the ama1 sequence clearly plays a central role in transformation capability, but the results with plasmid pSEQ01.5NS suggest that the relationship between autonomous replication and high transformation frequency is not simple. This theme is discussed in detail in section 4.7.

4.6.1 pY184 and pILJ16 cotransformations.

The plasmid pY184 was obtained from Iain Johnstone. This plasmid consists of three molecules of pBLUESCRIPT KS+ and a 7kb Nematode genomic DNA insert. The structure is shown in figure 4.35. The pBLUESCRIPT molecules are in inverted repeat orientation i.e. like ama1. An experiment was designed to test pY184 in cotransformation experiments with pILJ16 and then test for autonomous replicating plasmids. If such plasmids were isolated then this result would indicate that ama1, although required for high frequency of transformation was not essential for autonomous replication. Such a result would imply that the pUC sequences are in fact the

origin of replication. Powell and Kistler, (1990), have shown that the pUC origin is acting as an ARS in Fusarium oxysporum. Some workers have also reported that in some rare transformants, pILJ16 can be unstable, suggesting a limited ability to replicate autonomously, although this apparent instability is short-lived, (Reddy, personal communication). This instability could be due to sequences either in the pUC DNA or the argB gene. Experiments by Gems (1990) have shown that the behaviour of ama1 derived sequences is consistent, regardless of the linked gene e.g. replacement of argB with either trpC or oliC does not appear to have a significant effect on plasmid transformation frequency.

In addition, an experiment by Gems (1990), suggests that the pUC origin is of some importance. Gems cloned the 5kb HindIII ama1 fragment into a plasmid, pAYAC184, which does not contain any pUC DNA. This plasmid construct was called pHELP2 and is equivalent to pDHG25 in terms of ama1. Subsequent cotransformations with pHELP2 showed that this plasmid was unstable, but the transformation frequency was at least 20 times lower than that for related plasmids containing pUC8 and the same ama1 fragment, suggesting that the pUC8 DNA has a role to play in ARp1 function.

pY184/16		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	58	126	22	101	307		
	ARG+	41	96	11	94	242	549	55.92
2	ARG-	39	96	85	134	354		
	ARG+	33	100	79	127	339	693	51.08
3	ARG-	52	97	100	106	355		
	ARG+	37	82	54	98	271	626	56.71
4	ARG-	30	34	79	95	238		
	ARG+	25	25	70	87	207	445	53.48
5	ARG-	24	40	132	129	325		
	ARG+	20	32	125	113	290	615	52.85
subtotal	ARG-	203	393	418	565			
subtotal	ARG+	156	335	339	519	Average		54.01
TOTAL		359	728	757	1084	Standard Deviation		2.06
%LOSS		56.55	53.98	55.22	52.12			

Figure 4.37: pYI84/16 instability test results.

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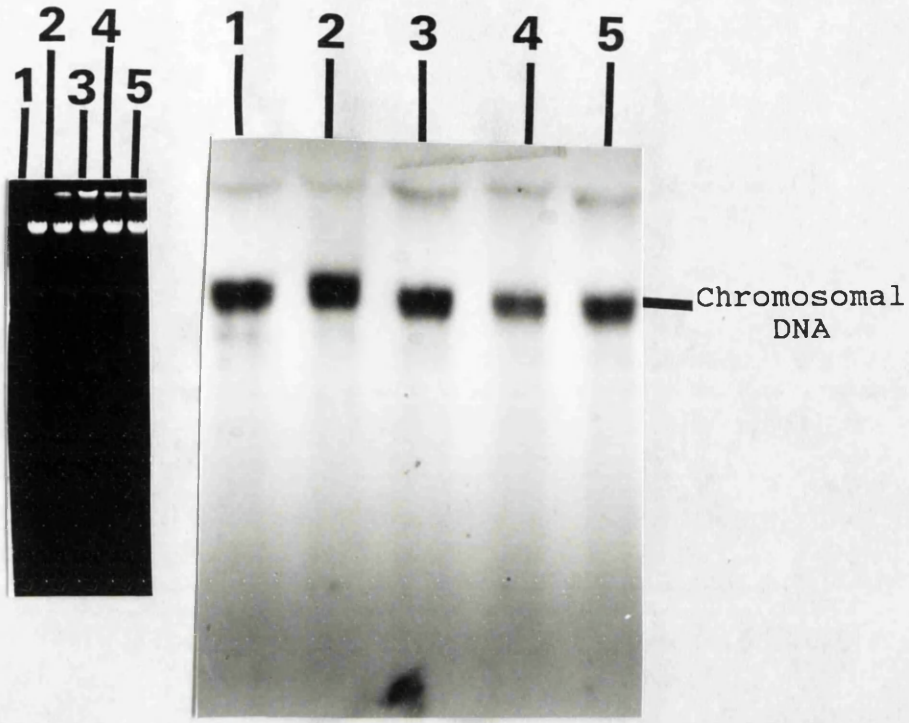
4.6.2 pYI84/pILJ16 cotransformation results.

The transformations were set up as previously described using $2x10^7$ protoplasts per transformation. 1µg aliquots of pY184 were digested separately with 20 units HindIII and PstI and gel-purified to remove the Nemotode genomic DNA insert. The enzymes were heat inactivated and the pY184 DNA was mixed with 1µg of pILJ16 for the cotransformations. These enzymes produce two different linear versions of the Bluescript dimer, (see figure 4.35). The transformation results are given in figure 4.36. It is immediately clear from the results that the pY184/pILJ16 cotransformations result in a 5 fold increase in transformation frequency when compared to pILJ16 and the pILJ16/pBLUESCRIPT control. The pY184/pILJ16 cotransformations yields approximately 160 colonies: pILJ16 yields 25 and the pILJ16/pBLUESCRIPT control yields 30. This enhancement in transformation frequency is comparable to the results obtained with the deletion series plasmids described in section 4.5 onwards.

Plasmid instability tests were carried out as previously described on 5 individual colonies from the PstI digested pY184 cotransformation. The results of these tests are shown in figure 4.37. The pY184/pILJ16 cointegrate shows that 54% of progeny derived from the original transformants lose the plasmid. These results suggest that the cointegrate plasmid is capable of autonomous replication.



LANE	DNA
1	T184/16R/01
2	T184/16R/02
3	T184/16R/03
4	T184/16R/04
5	T184/16R/05

Figure 4.38: uncut genomic DNA from Aspergillus pY184/pILJ16 cotransformants. Probed with radiolabelled pUC8 DNA.

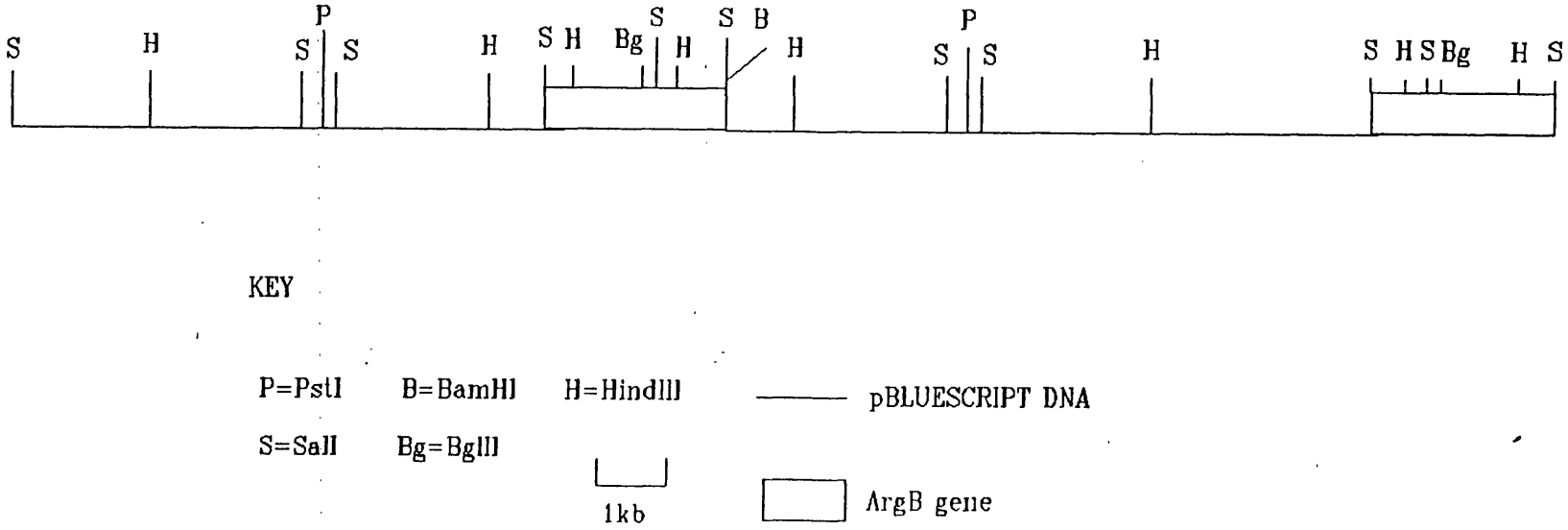


Figure 4.39: map of pT184/16R rescued plasmid.

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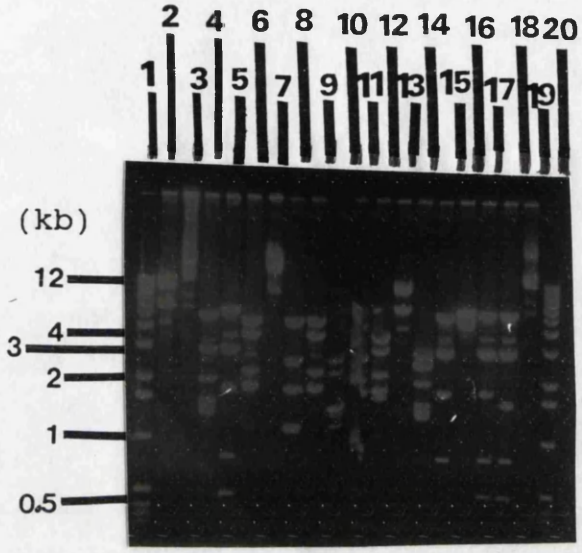


LANE	ENZYME	BAND SIZES (kb)
1	marker	-
2	P	13., 9.
3	B	22
4	H	5, 5, 3 2., 2.6, 1.5, 1.5, 1.4, 1.3
5	S	5-6., 4 (, 3 1, 3 L, 1.8, 1.8, 0.8, 0.8, 0.5, 0.5
6	E	-
7	Bg	11, 11
8	E/P	-
9	E/B	-
10	E/H	-
11	E/S	-
12	E/Bg	-
13	P/B	13, .6, 3
14	P/H	3 4., 2 5., 2 5., 2 5, 2.5, 1.6, 1.6, 1.6, 1.6, 1.1
15	P/S	5.2, 3.9, 3, 2.8, 1.8, 1.8, 0.8, 0.8, 0.2, 0.2, 0.2, 0.2
16	P/Bg	6.3, 5.7, 4.4, 4.3
17	S/B	5.2, 3.9, 3, 2.8, 1.8, 1.8, 0.8, 0.8, 0.5, 0.5
18	S/Bg	5.2, 3.9, 3, 2.8, 1.5, 1.5, 0.8, 0.8, 0.5, 0.5, 0.3, 0.3
19	uncut	-
20	marker	-

Figure 4.39 continued: table of band sizes.

Total genomic DNA was made from the 5 original transformants. 3µg samples were run out on a 0.8% agarose gel and Southern blotted. This blot was then probed with DIG-labelled pUC8 DNA, the results are shown in figure 4.38. No free plasmid DNA is visible in any lane. Three plasmid rescues were attempted with the genomic DNA preparations. Plasmid DNA was rescued from only one of the genomic DNA samples. The rescued plasmid was called pT184/16R. The other samples appear to contain free plasmid which must have been rearranged in such a way that the plasmids are no longer viable in E.coli, (see previous section).

4.6.3 Mapping of pT184/16R.

The plasmid was mapped by digesting the DNA with a variety of different enzymes. The digested DNA was then run out on a 0.8% agarose gel. This gel and the derived plasmid map are shown in figure 4.39.

PstI produces two bands of 13kb and 9kb in size. BamHI produces a single band of 22kb. The HindIII digests results in 8 bands of 5kb, 5kb, 3.2kb, 2.6kb, 1.5kb, 1.5kb, 1.4kb and 1.3kb in size. The SalI digest produces 10 bands: 5.6kb, 4.1kb, 3.1kb, 3.1kb, 1.8kb, 1.8kb, 0.8kb, 0.8kb, 0.5kb and 0.5kb. The 1.8kb and 0.8kb fragments are characteristic of the argB gene. The fact that the 1.8kb and 0.8kb SalI

fragments are present as doublets implies that there are two copies of the argB gene. The BglII digest produces 2 bands of 11kb in size, supporting the idea that pT184/16R contains two copies of the argB gene.

The PstI/BamHI double digest produces three fragments of 13kb, 3kb and 6kb, so the BamHI site is present on the 9kb PstI fragment. The PstI/HindIII double digest produces 10 fragments, the four 2.5kb bands are the result of one PstI site present on each of the 5kb HindIII bands. The PstI/SalI double digest produces 10 bands. The 0.5kb SalI fragments have been digested to produced the visible 0.3kb bands, so there is one PstI site in each of the 0.5kb SalI fragments. The PstI/BglII digest produces four fragments of 6.5kb, 6.5kb, 5kb and 4kb, suggesting that there is one BglII site in each of the PstI fragments. The SalI/BamHI digest is identical to the SalI digest, so the BamHI site maps very closely to one of the SalI sites. The SalI/BglII digest produces 12 bands; the 0.3kb and 1.5kb fragments are the result of one BglII site within each of the SalI 1.8kb fragments.

Overall, two copies of the argB can be clearly identified.

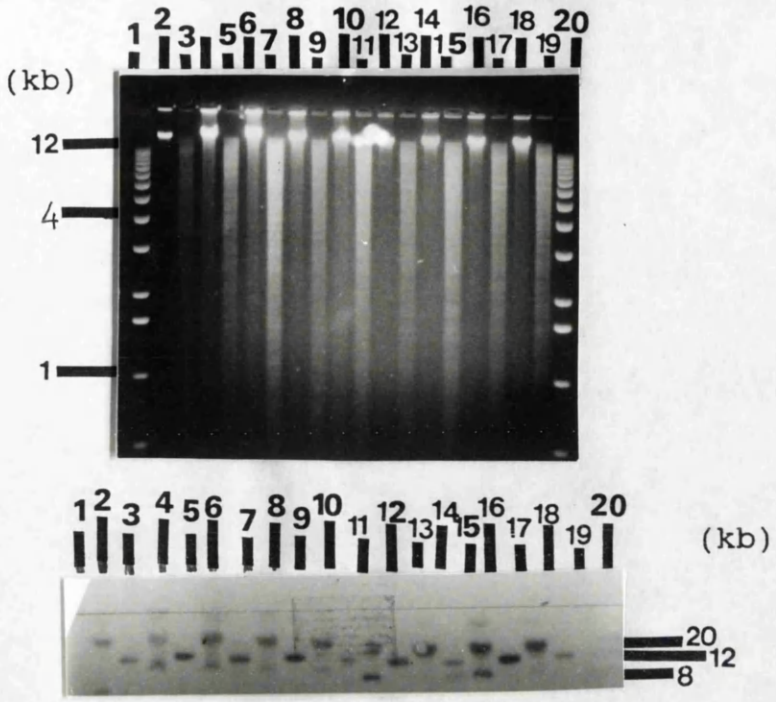
4.6.4 pT184/16R transformations.

The transformations were carried out using the same conditions described in section 4.7.2.

pY184/16R		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	12	122	125	120	379		
	ARG+	10	101	102	107	320	699	54.22
2	ARG-	21	134	137	113	405		
	ARG+	17	119	108	98	342	747	54.22
3	ARG-	23	109	119	137	388		
	ARG+	20	87	106	121	334	722	53.74
4	ARG-	38	78	131	106	353		
	ARG+	30	70	117	89	306	659	53.57
5	ARG-	44	99	131	111	385		
	ARG+	37	83	118	92	330	715	53.85
subtotal	ARG-	138	542	643	587			
subtotal	ARG+	114	460	551	507		Average	53.92
TOTAL		252	1002	1194	1094		Standard Deviation	0.26
%LOSS		54.76	54.09	53.85	53.66			

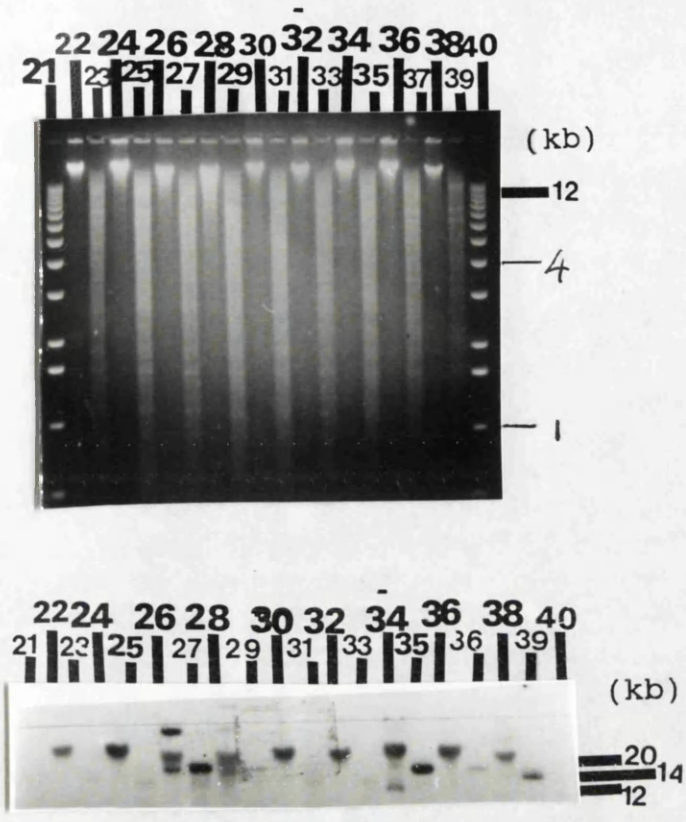
Figure 4.40: pY184/16R rescue instability test results.

facing page 126a



LANE	DNA	BAND SIZE (kb)
1	marker	-
2	TARp1/1.1	-
3	TARp1/1.1 CUT	11.5
4	T184/16R/1.1	-
5	T184/16R/1.1 CUT	8
6	T184/16R/1.2	-
7	T184/16R/1.2 CUT	20
8	T184/16R/1.3	-
9	T184/16R/1.3 CUT	20
10	T184/16R/1.4	-
11	T184/16R/1.4 CUT	8
12	T184/16R/2.1	-
13	T184/16R/2.1 CUT	8
14	T184/16R/2.2	-
15	T184/16R/2.2 CUT	20
16	T184/16R/2.3	-
17	T184/16R/2.3 CUT	20
18	T184/16R/2.4	-
19	T184/16R/2.4 CUT	8
20	marker	

Figure 4.41: genomic DNA from pY184/16R Aspergillus transformants, conidial generations 1 and 2, digested with BglII. Probed with radiolabelled pUC8 DNA.



LANE	DNA		BAND SIZE (kb)
21	marker		-
22	T184/16R/3.1		-
23	T184/16R/3.1	CUT	14
24	T184/16R/3.2		-
25	T184/16R/3.2	CUT	12
26	T184/16R/3.3		-
27	T184/16R/3.3	CUT	20
28	T184/16R/3.4		-
29	T184/16R/3.4	CUT	20
30	T184/16R/4.1		-
31	T184/16R/4.1	CUT	12
32	T184/16R/4.2		-
33	T184/16R/4.2	CUT	12
34	T184/16R/4.3		-
35	T184/16R/4.3	CUT	20
36	T184/16R/4.4		-
37	T184/16R/4.4	CUT	20
38	TARp1/2.3		-
39	TARp1/2.3	CUT	11.5
40	marker		

Figure 4.41 continued: genomic DNA from pY184/16R Aspergillus transformants, conidial generations 3 and 4, digested with BglII. Probed with radiolabelled pUC8 DNA.

Approximately $1x10^7$ protoplasts were transformed separately with 2µg aliquots of pT184/16R, pILJ16 and ARp1. The transformation frequencies were as follows: pT184/16R yielded 400 colonies, pILJ16 gave rise to 28 colonies and ARp1 yielded approximately 40,000 colonies. The transformation frequency for pT184/16R shows a 20-fold increase when compared to pILJ16 and a 2-fold increase when compared to the original pY184/pILJ16 cotransformation.

Instability tests were carried out as described previously on 5 individual pT184/16R colonies. The results of these tests are shown in figure 4.40. It is clear from these results that pT184/16R is acting as an autonomously replicating plasmid.

Total genomic DNA was made from colonies from each generation. These colonies were designated pT184/16R1.1 to pT184/16R1.4 (first generation) to pT184/16R4.1 to pT184/16R4.4 (fourth generation).

2µg aliquots of these DNA samples and an ARp1 control were digested with 20 units of BglII. These samples and 2µg aliquots of undigested DNA were run out on a 0.8% agarose gel, Southern blotted and probed with radiolabelled pUC8 DNA.

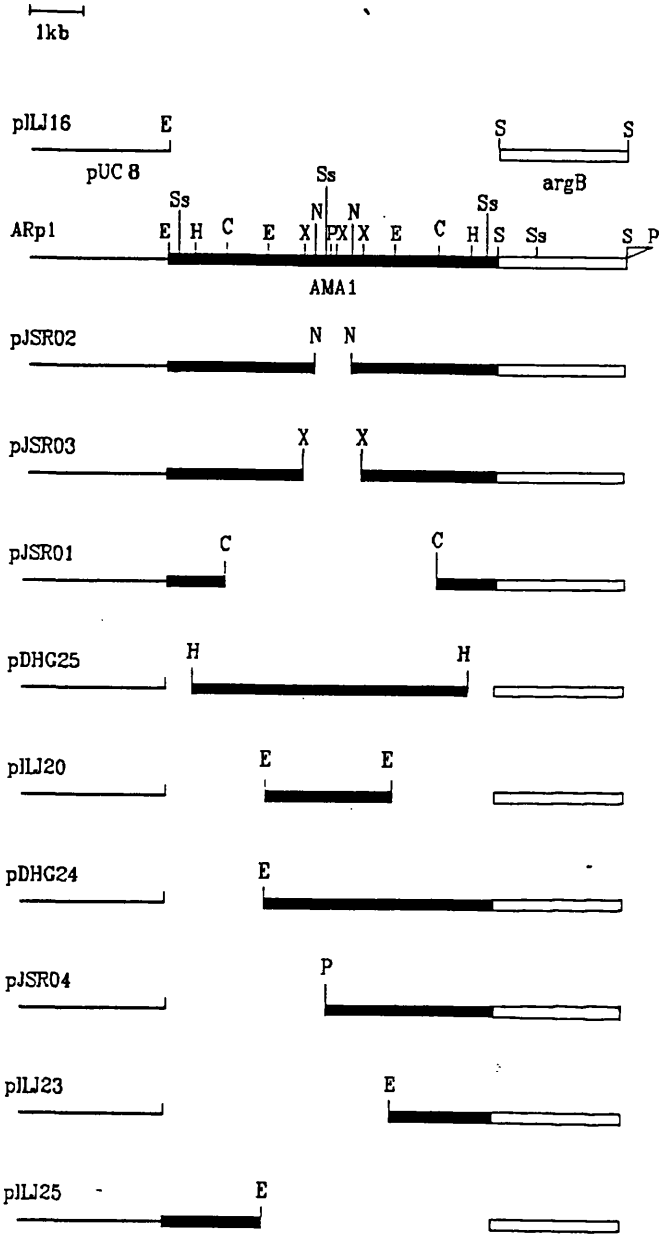
The results of this blot are shown in figure 4.41. It is clear that free plasmid is present in many of the undigested DNA lanes. The digested DNA lanes show that a variety of differently sized plasmids are present, e.g. lanes 11 and 13 contain a 8kb band, lanes 7 and 9 contain a 20kb band. The

digested TARp1 lanes (lanes 3 and 39), contain a single 11.5kb band. The hybridising bands in the digested DNA lanes appear to vary in size from generation to generation, indicating that the plasmid(s) are undergoing rearrangements. The fact that the undigested T184/16R lanes contain free plasmid bands suggests that pT184/16R is replicating autonomously.

Overall, the results indicate that pT184/16R is capable of autonomous replication.

Therefore, my conclusions are that a component of pUC8, possibly an origin, either the bacterial ColE1 or the M13 phage origin, is acting as an origin of replication in Aspergillus. This statement could be tested further by cloning the pUC8 origin into pDHG29 which transforms at a lower frequency than plasmids based on the pUC8 vector. An increase in transformation frequency would then be due to the presence of the pUC origin.

It is also likely that the structure of the pUC DNA and not just the number of potential origins is important for replication; only the pY184/pILJ16 cotransformation showed an increase in transformation frequency, the pILJ16/pBLUESCRIPT cotransformation did not. It is unlikely that the rearranged pUC origins present in the small inverted repeat within amal, (see chapter 3), are critical for replication because the plasmid pSEQ01.21NS can still transform at a relatively high frequency, even though these



	TRANSFORMATION FREQUENCY /pg DNA	INSTABILITY / ASEXUAL GENERATION	REARRANGED
pILJ16	20	0%	NO
ARp1	40, 000	56%	NO
pJSR02	1170	67%	YES
pJSR03	1830	51%	YES
pJSR01	790	39%	YES
pDHG25	1000	60%	NO, (N/T)
pILJ20	11	0 TO 20%	N/T
pDHG24	10, 000	60%	YES
pJSR04	780	62%	YES
pILJ23	100	86%	YES
pILJ25	100	85%	YES

N/T=Not Tested

Figure 4.42: behaviour of ARp1 derived subclones.

sequences have been deleted. In short, some pUC8 sequence appears to have a role in ARp1 replication.

4.7 Discussion.

The main results: structures, transformation frequencies, average % instability values and rearrangement behaviour of ARp1 and associated subclones are summarised in figure 4.42.

4.7.1 Amal and transformation frequency.

Analysis of the data summarised in figure 4.42 suggests that there is no clear relationship between the size of the amal insert and transformation frequency. Nor can any single component of amal be identified as critical for replication.

For example, pDHG24 contains approximately 4kb of amal DNA and has a transformation frequency of 10,000 colonies/µg DNA: pDHG25 contains approximately 5kb of amal DNA and has a transformation frequency of 1000 colonies/µg DNA.

One possible explanation for this decrease in transformation efficiency when pDHG24 and pDHG25 are compared is that pDHG25, although it contains 5kb of amal DNA, lacks the terminal 720bp HindIII/SalI fragment which is present in the relatively efficient plasmids pDHG24 and pJSR04.

The plasmids pJSR02 and pJSR03 contain 5.1kb and 5kb of ama1 DNA respectively and both plasmids contain the 720bp HindIII/SalI fragment but lack the central unique region; yet the transformation frequencies are 10 fold lower compared to pDHG24. This result implies that the central unique region is important when considering transformation frequency.

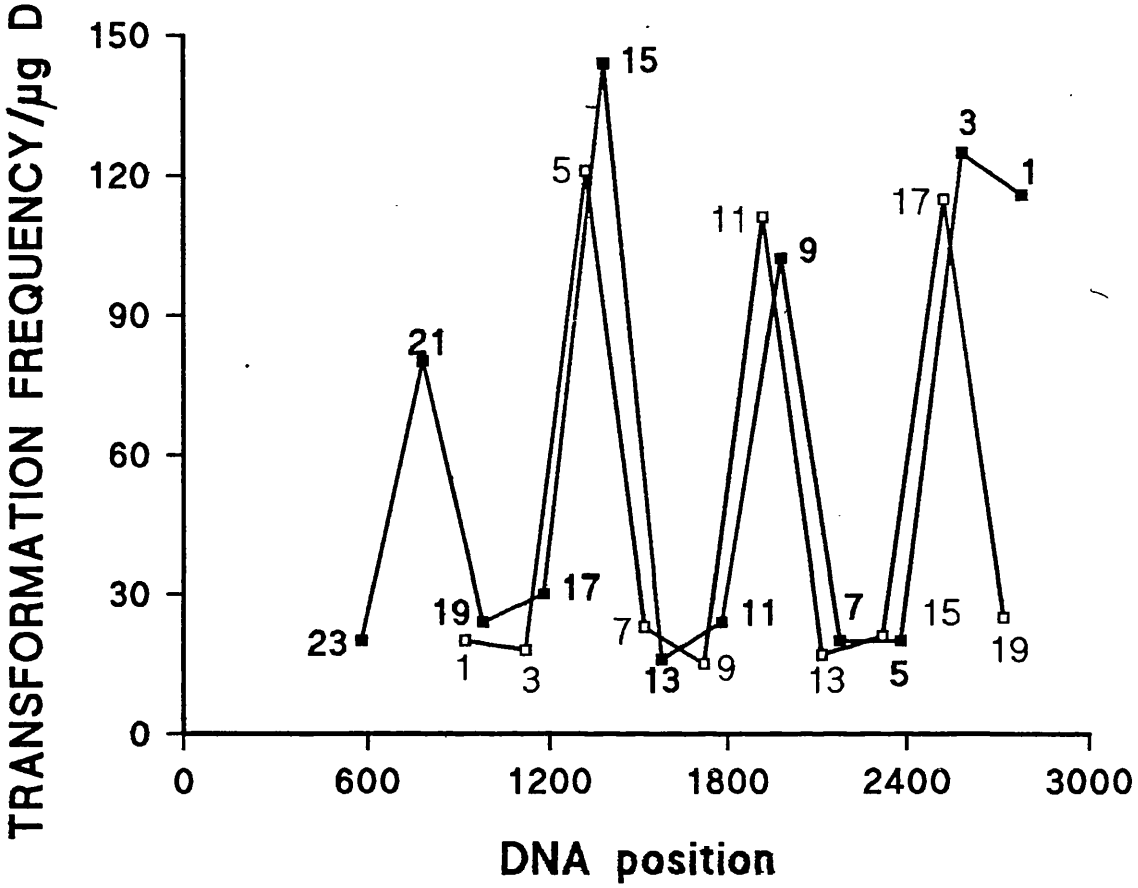
The transformation results detailed in figures 4.30, 4.31, 4.32 and 4.42 suggest that a variety of regions in the ama1 sequence, regardless of size and orientation, can enhance the ability to replicate autonomously and increase the frequency of transformation.

It is possible to explain the results in figure 4.42 in one of two ways; either ama1 contains multiple, discrete sequences, any of which can enhance the frequency of transformation or alternatively, ama1 function is dependent on a combination of DNA structure and regional interactions. The database searches described in Chapter 3 failed to identify any ama1 sequences that exactly fit the Yeast ARS-concensus sequence. However as described previously, ama1 may contain sequences which enhance the frequency of transformation. I will call these hypothetical sequences TFEs: Transformation Frequency Enhancers.

As previously described in section 4.5, the sequencing subclones were used in cotransformation

Figure 4.43:-

Graph showing results of both sets of pSEQ01/plLJ16 cotransformations with DNA position.



KEY

1 ■ Not/Sma deletion series.

1 □ Kpn/Xho deletion series.

Numbers refer to specific subclones.

experiments. The data from section 4.5.2, figures 4.31b and 4.32b has been redrawn and summarised in figure 4.43. The graph shows the transformation frequencies of both the pSEQ01 deletion series cotransformations plotted against the DNA sequence presented in figure 3.11a. It is clear from figure 4.43 that the locations of the peaks and troughs match remarkably well.

The data presented in figure 4.43 can be explained using two different models. Model 1 attempts to describe the results summarised in figure 4.43 by speculating that discrete ARS-like sequences are switched either on or off by control sequences. Model 2 is a version of model I; model 2 attempts to describe the data in figure 4.43 by postulating that transformation frequency is influenced by positional effects via ama1 DNA interactions.

MODEL 1.

The data in figure 4.43 can be interpretated in a one of two ways; either the troughs represent the location of a TFE which is identified by the loss of transformation efficiency when this region is deleted or the peaks represent the switching on of a TFE at this location by the deletion of some sort of negative control region.

There is no obvious difference in the comparative strength of the individual peaks and

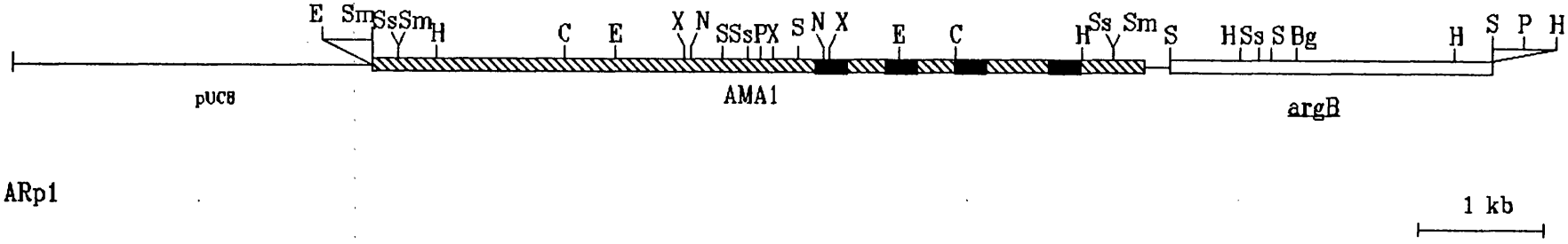


Figure 4.44: approximate positions of peaks and trough regions on sequenced ama1 arm. ▨ = TROUGH ■ = PEAK

facing page 131a

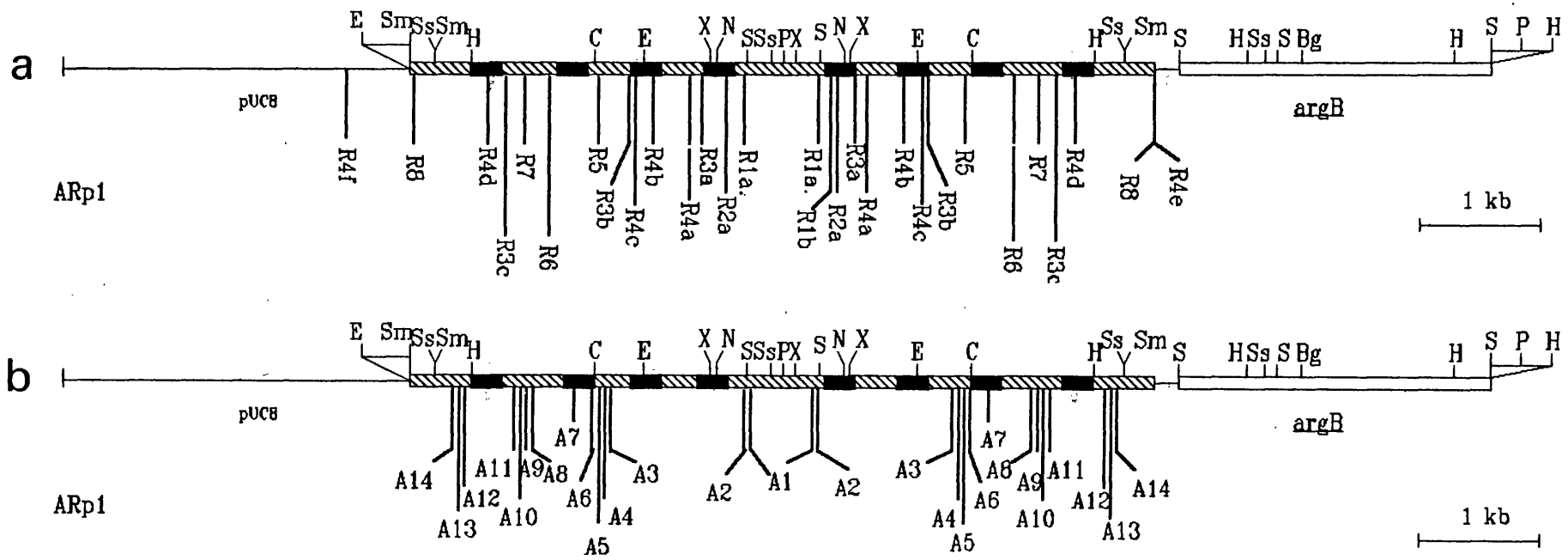


Figure 4.45: location of all (a) repeated regions and (b) A-T rich regions with respect to both the peaks and trough regions and pUC8-like DNA. pUC8-like sequences

troughs, but note that the complete ama1 sequence in ARp1 is approximately 250 times more efficient than any of pSEQ01 series of subclones. It is possible that the peaks and/or the troughs may act additively in the whole ama1 sequence.

The peaks and troughs are spaced at regular 400base intervals which is roughly 40 turns of the DNA helix, so it is possible that any control of the TFEs is structural and that the TFEs are switched on by localised unwinding of the DNA helix. Figure 4.44 shows ARp1 with the approximate positions of the peaks, (black regions) and troughs, (hatched regions), marked in on the sequenced arm.

Figure 4.45a shows the location of all the repeated regions with respect to the locations of the peaks and troughs. It is known that the origins of SV40 consist of a combination of a number of palindromic regions, 17bp A/T rich sequences, 3 copies of a 21bp repeat and 2 copies of a 72bp repeat. However, there appears to be no simple correlation between the position of the repeats and peaks and troughs in ama1 i.e. deletion of any repeat and any number of repeats does not affect the overall transformation frequency. Similar ly, when the A/T rich regions are plotted against the location of the peaks and troughs as in figure 4.45b, there is no apparent relationship between the location of the A/T regions and peaks and troughs, except that all the A/T regions appear to map in the trough regions.

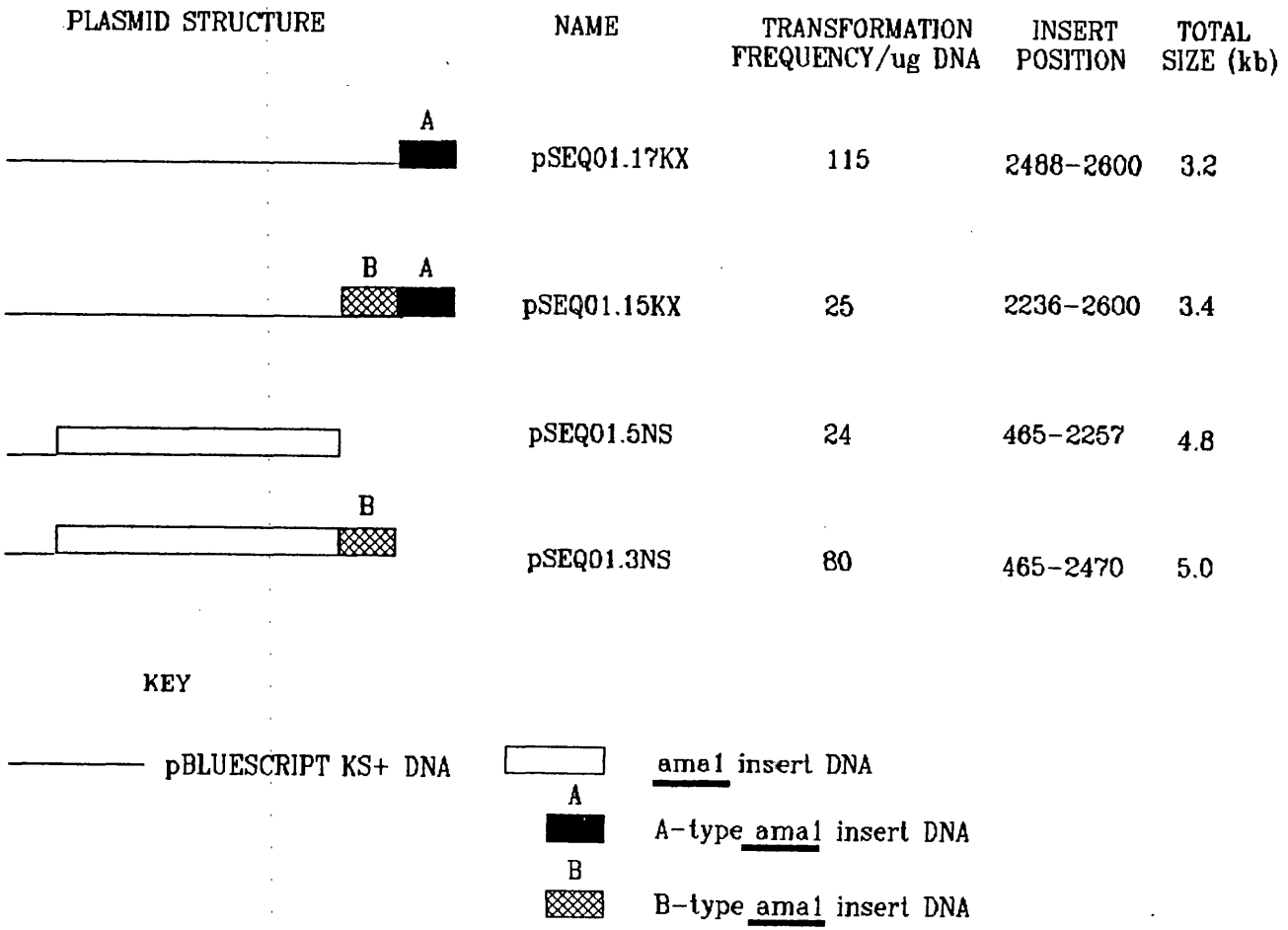


Figure 4.46: behaviour of A-type and B-type amal regions.

facing page 132

A/T rich regions may be involved in the relaxation of negative supercoils, (see later).

At no time have I convincingly shown that any of the peaks/troughs/TFEs are acting as the plasmid origin of replication or that such discrete sequences do exist. This theory could be tested very simply by cloning each 200bp ARS-like fragment into pILJ16 and then looking for free, autonomously replicating plasmid. In fact, results from section 4.6 suggest that pUC8 DNA may be important when considering ARp1 replication and behaviour.

MODEL 2.

As outlined previously, the data presented in figure 4.43 is open to another interpretation, that the behaviour of ama1 is dependent not on discrete, identifiable small sequences, (as described above), but is controlled by the positional effects via interactions of two types of regions within ama1. In this version of the model, these hypothetical regions are referred to as A and B type regions.

The transformation frequency data used to plot figure 4.43 is presented in a different format in figure 4.46 which shows the structure of two plasmids from each of the KpnI/XhoI and NotI/SmaI deletion series and the transformation frequency obtained with each plasmid.

From figure 4.46 it is clear that the 112bp ama1

fragment in pSEQ01.17KX enhances transformation frequency; in this case the insert, described as containing an A-type region, is acting as a TFE. The 364bp ama1 fragment in pSEQ01.15KX does not enhance transformation frequency although it contains the same A-type sequence as pSEQ01.17KX; so it must contain both an A-type and a B-type sequence. The additional 252bp sequence present in pSEQ01.15KX is designated as a B-type sequence and in this plasmid acts as an anti-TFE.

The reverse is true when a second pair of plasmids are considered. The pSEQ01.5NS plasmid contains a 1792bp ama1 fragment which does not enhance transformation frequency. However, addition of the 213bp ama1 sequence from position 2257 to 2470 which comprises approximately, the B-type sequence described for pSEQ01.15KX, gives pSEQ01.3NS which gives a high transformation frequency. The B-type region is therefore in this case acting as a TFE.

From these results it is possible to put forward the hypothesis that ama1 is composed of A-type and B-type sequences and it is the balance of A-types and B-types which determines transformation frequency.

An experimental test of the theory outlined above would be to clone A-type and B-type ama1 regions into plasmids and measure the transformation frequency effects of different combinations of A and B-types.

4.7.2.*ama1* and plasmid instability.

It is extremely difficult to correlate either the plasmid instability or rearrangement behaviour described in figure 4.42 with the size of the *ama1* sequence, nor is it possible to relate any specific region of *ama1* with plasmid stability. Both pILJ23 and pILJ25 are unstable and rearranged so the orientation of the *ama1* sequence does not appear to be a factor.

It is possible to explain the different plasmid losses outlined in figure 4.42 in general terms of plasmid rearrangements. These rearrangements affect the mechanism(s) involved in plasmid partitioning, leading to either increased plasmid stability, e.g. pJSR01 or to decreased plasmid instability e.g. pILJ25. The questions are: (1) How might ARp1 and its derivatives segregate? and (2) how could rearrangements affect this mechanism?

I outlined the yeast 2μ plasmid segregation mechanisms in section 4.1.2. There are two possible segregation mechanisms that are applicable to ARp1, assuming that plasmid loss is due to a failure in "transmitting" copies of the plasmid to the daughter nuclei during mitosis. The two possible models are either an active mechanism or a passive mechanism (facilitative diffusion). A review by Nordstrom (1989), details various aspects of plasmid segregation mechanisms.

In the active mechanism, which is identical to the proposed 2μ circle model, copies of ARp1 bind to the nuclear scaffold and are then shared out to the daughter nuclei during mitosis. This binding to the nuclear scaffold requires either centromeric-like sequences or Skeletal Attachment Regions, (Amati and Gasser, 1988). S.cerevisiae centromeric sequences (CEN) have been characterised. Skeletal Attachment Regions or SARs have been characterised in Drosophila, (Harden and Ashburner 1990). Both CEN and SAR sequences are A/T rich. As disscussed in chapter 3, the A/T rich regions within ama1 could be either SARs or CEN-like sequences. This hypothesis could be tested by using ARp1 and Aspergillus cell extracts in gel retardation experiments which assess the protein binding capability of specific DNA sequences. More specific binding studies could then be carried out using purified nuclear spindle protein and testing whether or not ARp1 will bind such proteins.

The SAR sites could be modified by plasmid rearrangements. Such rearrangements could increase plasmid stability by increasing the number of SAR sites by duplication. Alternatively, plasmid rearrangements could decrease plasmid stability by reducing the number of SAR sites or by altering the base compositon of such sites. This hypothesis could be tested by sequencing rescued, rearranged plasmids such as the pILJ25 derivatives and comparing their

sequence to ARp1.

There are no such specialised protein sites required in the passive segregation model. In this model, plasmid segregation is random e.g. if there is only one copy of the plasmid present then there is 50% probability of either of the daughter nuclei inheriting the plasmid, . The chances of inheriting the plamid increase with plasmid copy number. the probability of a 20:0 split are low (0.5^{20}). Plasmid rearrangements which lead to an increase in copy number lead to an increase in plasmid stability (decrease in plasmid loss). The probability of inheriting a plasmid is decreased when plasmid copy number decreases. Such decreases could be due to rearrangements which affect the plasmid replication system and hence reduce plasmid copy number. Plasmid copy number can also be reduced by the plasmids recombining to form dimers and larger forms e.g. 20 copies of the plasmid means that there are 20 available, inheritable plasmid "units"; if these plasmids form dimers then there are only 10 available "units"; if the plasmids form tetramers there are only 5 available "units" and so on. Similarly, recombination could also lead to resolution of the plasmid multimers and so increase copy number. Extensive work has been carried out on resolution of plasmid multimers in bacteria, (see Stirling et al 1988, and Chapters 5 and 9).

It could be possible to test whether or not

plasmid copy number influences plasmid loss by comparing plasmid copy numbers in, for example, pILJ16 (0% loss), ARp1 (59% loss), pJSR01 (40% loss) and pILJ25 (85% loss) transformed nucleus. From this data it should be clear as to whether or not there is a relationship between copy number and stability and plasmid rearrangements. Gems (1990) has estimated from bulk genomic DNA Southern blots, prepared from two ARp1 Aspergillus transformants, that the average ARp1 copy number is between 10-20 copies per transformed nuclei.

4.7.3. Ama1 structure, the inverted repeats and plasmid rearrangements.

It is possible to describe both plasmid instability and plasmid rearrangements and subsequent transformation frequencies in terms of ama1 structure. All the plasmids in figure 4.42 appear to be rearranged with one exception: pDHG25. The Southern blots in figures 4.6, 4.23 and 4.41 show the presence of additional, unexpected ARp1 bands; these bands are either a product of ARp1 replication (see later), or produced by rearrangement of ARp1. Gems (1990) found no evidence for ARp1 rearrangement. If ARp1 is rearranged then the rearranged versions of ARp1 are not rescuable, (see section 4.3.1.3). It may be possible to isolate ARp1 plasmid DNA, regardless of rearrangements by preparing plasmid DNA

directly from Aspergillus ARp1 transformants, (see Chapter 5.)

Both ARp1 and pDHG25 contain the 5kb HindIII amal fragment. No other plasmid shown in figure 4.42 contains an amal sequence with this structure. The plasmids pJSR01, pJSR02 and pJSR03 contain an amal inverted repeat but lack the central region. The transformation results obtained with the pJSR series suggests that the central region is important for efficient transformation.

All the plasmids described in figure 4.42 contain pUC8 and argB which are extensively rearranged on transformation with some of the plasmid derivatives, notably pILJ25, (see section 4.3 onwards). The results in section 4.6 show that a pUC derived inverted repeat, pY184, is rearranged. It is possible that the pUC8 and argB sequences are also modified in ARp1, and that these modifications contribute to transformation frequency and plasmid stability.

This possibility can be discounted by looking at the structure and behaviour of pILJ23 and pILJ25. The plasmid pILJ23 consists the self-ligated ARp1 7.3kb EcoRI fragment; so this plasmid contains ARp1-derived pUC8 and argB DNA. The plasmid pILJ25 consists of the ARp1 1.7kb EcoRI fragment cloned into EcoRI-digested pILJ16; so this plasmid contains E.coli-derived pUC8 and argB DNA. Both pILJ23 and pILJ25 display very similar transformation

frequencies and plasmid stabilities.

The question as to whether or not ARp1 does contain any rearranged pUC and argB DNA can be answered by sequencing the ARp1 pUC8 and argB regions.

It is reasonable to assume that if ARp1 and pDHG25 are not rearranged then it is because they contain an inverted repeat structure which is separated by a 345bp unique region. These two plasmids are not rearranged; either because they contain the ama1 structure described or because they contain the unique central region. This distinction could be tested by cloning the 345bp unique region into the pYI84 inverted repeat, (section 4.6) and then looking for rearrangements. Similarly, the unique region could be cloned back into the pJSR series plasmids, (section 4.2), and then tested for rearrangements.

The gross inverted repeat structure of ama1 appears to be involved in the process of autonomous replication as well as rearrangement because although pYI84 contains no ama1 DNA it has a limited ability to replicate autonomously, (see sections 4.6. and 4.7.4)

Transcription of a DNA template produces both positive and negative supercoils in the DNA. The supercoiling can be relaxed by Topoisomerases I and II, Lui and Wang (1987). Negative supercoils in circular DNA can also be partially relieved by the

creation of localised denatured regions such as hairpin (cruciform) loops within A/T rich regions and inverted repeats. Ama1 contains a number of A/T rich regions and internal inverted repeats, (see Chapter 3). Hairpin stability of inverted repeats is increased by the length of the repeat unit and is decreased by the length of the central nonrepeat unit, Lilley (1980).

It is possible that the internal ama1 inverted repeats are involved in the relaxation of negative supercoiling, which may be produced by transcription of either the argB gene or any of the potential reading frames identified in ama1, (see Chapter 3). Lilley (1980), suggests that inverted repeats may act as protein-recognition sites and may be involved in expression of nearby genes.

The internal ama1 pUC8-like inverted repeats may be involved in both inter- and intra-molecular recombination. Gems (1990) found evidence that the ama1 arms can be exchanged via recombination. This recombination event appears to be centured on the two pUC8-like inverted repeats on both arms. Internal recombination between either of these two inverted repeats and any other ARp1-derived pUC8 DNA could lead to rearrangements of some of the ARp1 subclones.

Gems (1990), constructed a plasmid called pHELP2 for use in cotransformation experiments. This plasmid consists of the 5kb HindIII ARp1-derived fragment cloned into pACYC184.

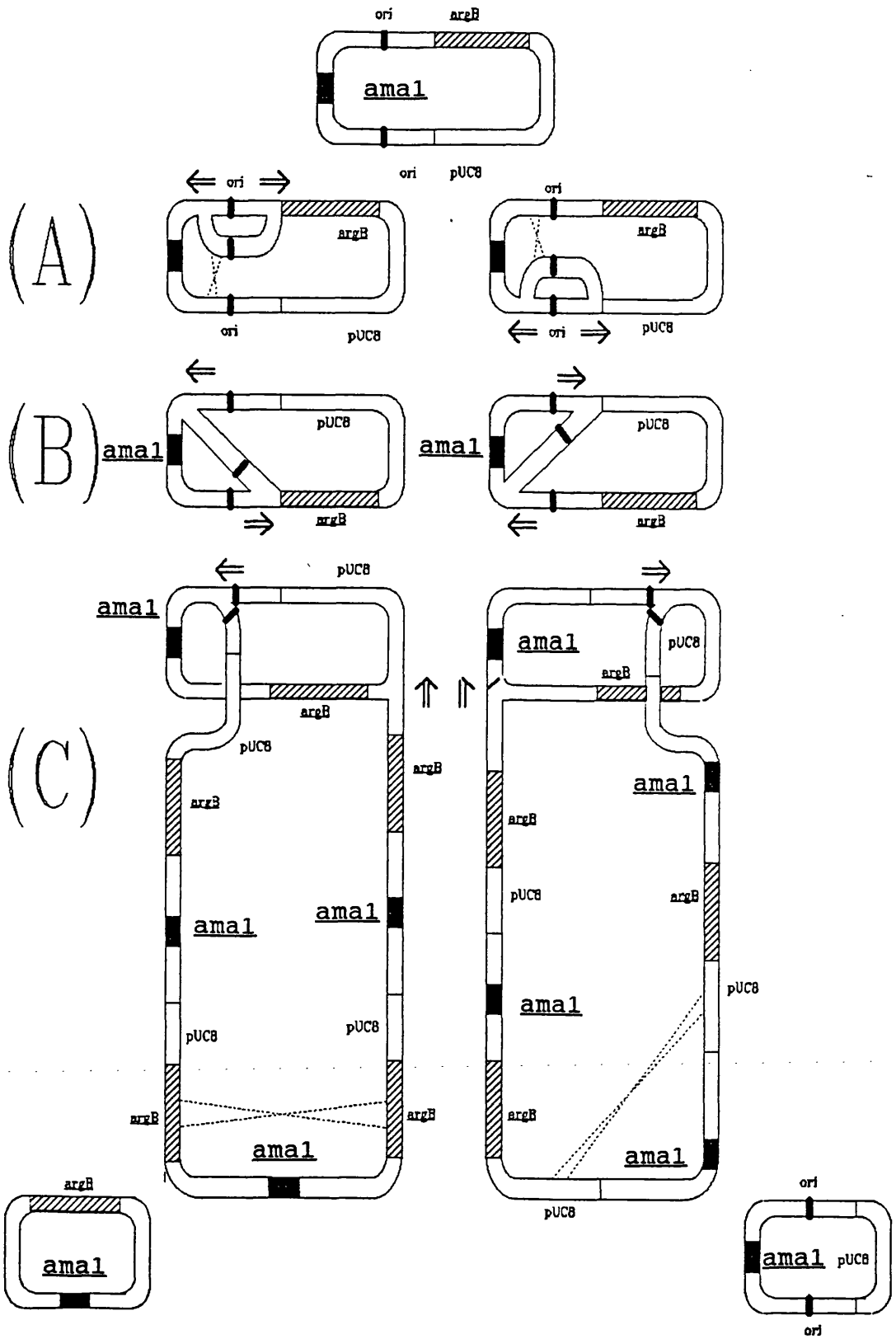


Figure 4.47: theoretical model of ARp1 replication, (Gems 1990).

Gems (1990), noted that a cointegrate plasmid, designated pCOT2, was the product of non-homologus recombination between pILJ16 and pHELP2. It is likely however, that pCOT2 was in fact the product of homologus recombination between the pUC8 DNA in pILJ16 and the ama1 pUC8-like DNA in pHELP2.

4.7.4 ARp1 replication.

This model describing ARp1 replication was proposed by Gems (1990) and is based on the yeast 2u circle mechanism outlined in section 4.1. Figure 4.47 shows the overall mechanism.

The proposed mechanism is as follows. (A) replication is initiated at an origin of replication somewhere on ARp1, which is shown here for the sake of arguement on each of the two ama1 arms. The replication forks diverge, meanwhile an internal recombination event between the two rearranged pUC sequences within the arms takes place. (B) The replication forks are now following one another resulting in a double rolling circle molecule. (C) Further rounds of replication can produce an indefinitely large molecule which can be resolved by further recombination events into a variety of products e.g. if the entire ama1 sequence is not pesent then the recombination event marked produces an ARp1 derivative without any pUC8 sequences or without any argB DNA, (see figure 4.21b).

Recombination events can produce ARp1 molecules by resolution of the multimeric forms.

The examples mentioned above illustrate how internal recombination within a large, complex molecule can produce a variety of derivatives. This mechanism also explains the production of ARp1 derivatives (as seen on the Southern blots, figure 4.21), which lack the argB gene. These recombination events may be responsible for the diversity of both the pILJ25 derivatives and the other plasmid rearrangements noted in section 4.3 onwards.

The hypothesis that ARp1 replicates via a double rolling circle model can be tested in three ways. (1) Electron microscopy of plasmid DNA prepared directly from Aspergillus transformants, (see chapter 5), would visualise large molecules, theta forms and the small derivatives which may not be viable in E.coli. (2) Isolate plasmid DNA directly from Aspergillus and map it by restriction digestion. The best candidate for this type of experiment would be pDHG25 which contains a unique BamHI site. The double rolling circle model produces large molecules, any one of which would contain a certain number of BamHI sites depending on how far replication had progressed. These large plasmids could then be mapped and sized using BamHI. All the plasmids listed in figure 4.42 including ARp1 could be characterised in the same way using BglII which cuts once within the argB gene so that fragment sizes

could be predicted. (3) Replication via a Double Rolling Circle mechanism would result in variability of ARp1 copy number between individual nuclei. This variability could be examined by PCR analysis. Such an experiment would involve making protoplasts from germinating conidia because the germ tubes can be treated with hydroxyurea which inhibits DNA replication to produce uninucleate germtubes. After protoplasting, DNA would then be extracted from a number of individual nuclei. This total DNA would contain some plasmid DNA which could then be amplified using PCR. If a qualitative PCR assay could be developed, this approach allows the plasmid copy number of individual nuclei and hence variability of copy number per nucleus to be calculated.

The mechanism(s) which produce the plasmid rearrangements are unknown although I have speculated that these rearrangements are due to recombination events. Rearrangements of autonomously replicating plasmids have been reported by Barnes and MacDonald (1988), and Powell and Kistler (1990). Such rearrangements could also be produced by the various DNA repair systems. These ideas could be tested by transforming recombination and repair deficient mutants with plasmids known to be liable to rearrangement. It should then be possible to isolate plasmid DNA from these transformants and characterise any rearrangements and the mechanisms by which these rearrangements take place.

Chapter 5.

Electron microscopy of ARp1.

5.1 Introduction.

The proposed model of ARp1 replication and the evidence for it was examined in detail in chapter 4. Two questions need to be answered; (1) Does ARp1 replicate without chromosomal integration i.e. can theta (replicating) plasmid forms be seen, (Cairns 1966)? (2) Does ARp1 replicate via the double rolling circle model i.e. can giant plasmid forms be seen, (Fuchter 1986)? The most direct way to visualise such structures, if they do exist, is by electron microscopy.

In addition, it might also be possible to calculate the relative abundance of monomers and multimers by simply counting the numbers of such molecules. To ensure that such a count is fair it is necessary to do such counts using a number of randomly chosen grid sections, otherwise there is a tendency to only count the most impressive and photogenic molecules.

The ARp1 DNA was prepared from a single Aspergillus nidulans ARp1 transformant grown under selective conditions. The supercoiled plasmid DNA samples were prepared as described in chapter 2, section 2.21 via CsCl gradient ultracentrifugation. Approximately 1µg of apparently pure ARp1 plasmid was recovered from 1 litre of culture. The samples were examined in a Transmission Electron Microscope (TEM). All plasmid sizes were measured from photographs,

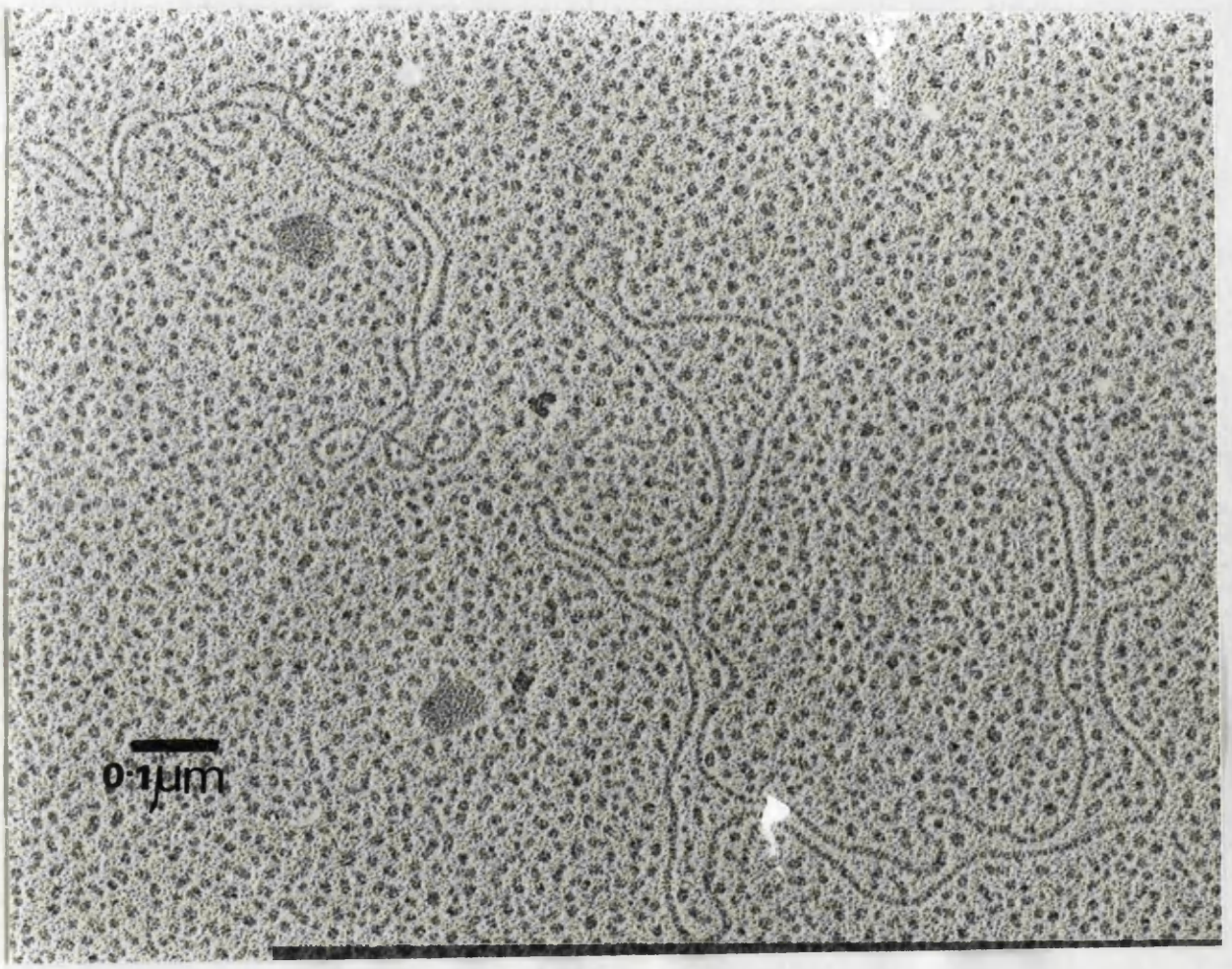
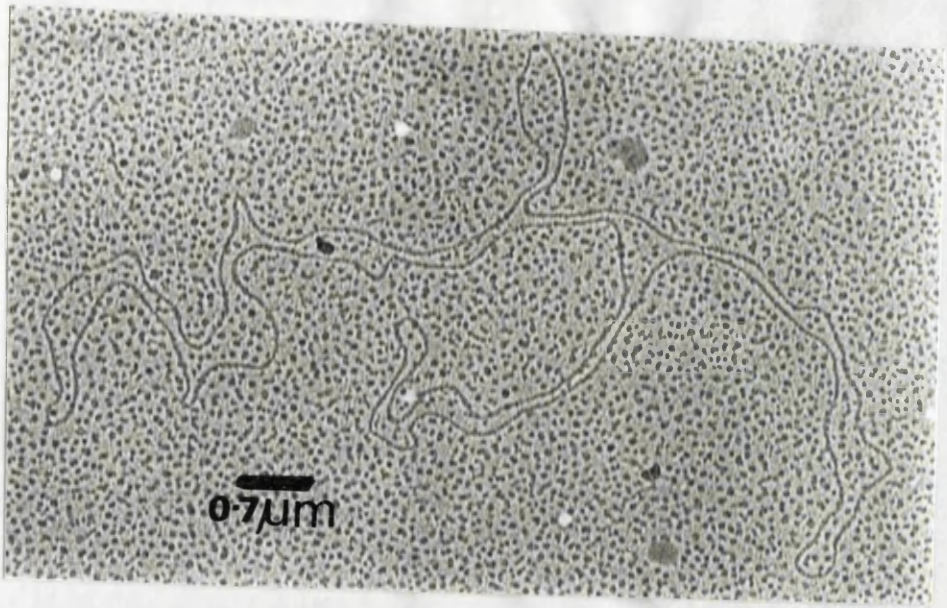
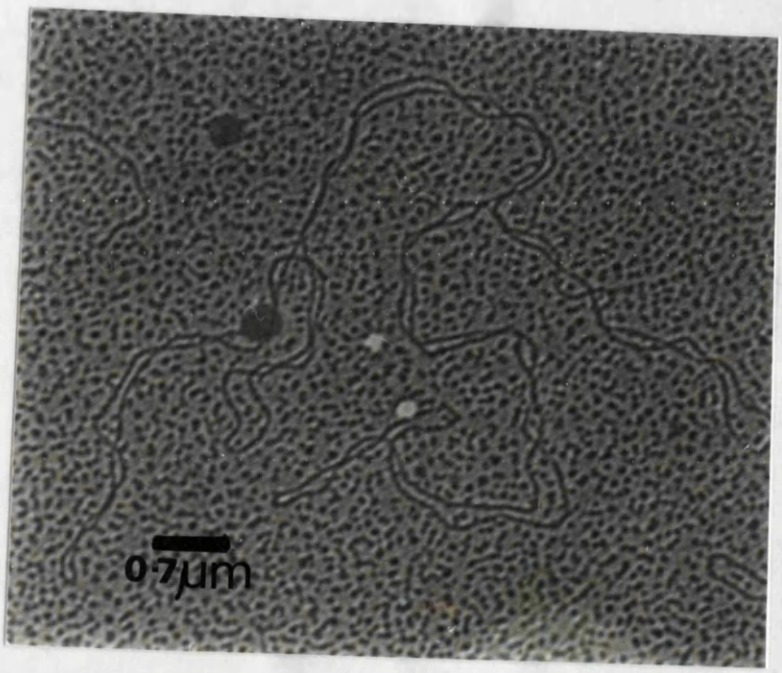


Figure 5.1: ARp1 monomer and dimer, approximate sizes are 11.5kb and 23kb respectively.

a



b



Figures 5.2a and 5.2b: ARp1 trimer, approximate size is 34kb; ARp1 tetramer, approximate size is 44kb.

Figure 5.3: complex molecule, approximate size is 100kb+.

using a PC computer and digitiser.

5.2 ARp1 multimers.

ARp1 DNA was prepared using formamide which relaxes the structure of CCC plasmids as described in chapter 2. This material was then examined. Figure 5.1 shows an ARp1 monomer of 11.5kb in size and an ARp1 dimer of approximately 23kb in size. Figure 5.2a shows an ARp1 trimer of approximately 34kb in size. Figure 5.2b shows an ARp1 tetramer of approximately 44kb in size. The scale in figure 5.1 is 3cm=1kb, the black bar represents 0.1µm; the scale in figures 5.2a and 5.2b is 1cm=500bp, the black bars represent 0.7µm.

Figure 5.3 is difficult to decipher but the DNA molecule(s) are at least 100kb+ in size, the scale in this figure is 1cm=1kb, the black bar represents 0.34µm. The exact nature of the DNA in figure 5.3 is unknown, it could be a large Double Rolling Circle molecule. Alternatively, it could be the result of ARp1 molecules aggregating around a protein contaminant. Also present in the sample, although not photographed, were long linear molecules of 100kb+ in size. Again, these large molecules could be sheared Double Rolling Circle molecules or simply genomic DNA contaminants. All ARp1 plasmid DNA used in this study was prepared by CsCl ultracentrifugation, so there should be only

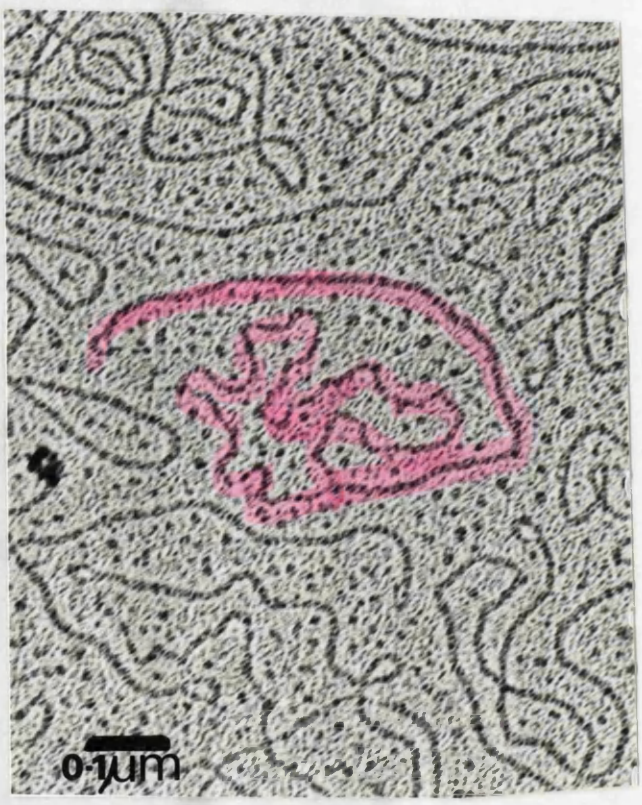


Figure 5.4: analysis of reannealed ARp1 DNA.

supercoiled plasmid DNA present.

5.3 Examination of reannealed ARp1.

ARp1 DNA was melted as described in chapter 2. When the DNA is melted it forms single stranded DNA molecules. As the DNA solution cools, sections of homologous DNA reanneal to form double stranded regions; non homologous DNA remains single stranded. Low DNA concentrations were used to ensure that single stranded molecules would self reanneal rather than anneal with other molecules.

It is then possible to check that the ama1 arms are identical to each other. If the arms are similar in sequence then the reannealing should produce a molecule which contains a 3kb, continuous, double stranded region consisting of ama1 and a 5kb single stranded region consisting of both argB and pUC8. If the arms are not sufficiently similar in sequence then the 3kb ama1 region will consist of some double stranded sections, (homologous DNA) and some single stranded "bubbles", (non-homologous DNA). Double stranded regions appear slightly thicker than single stranded regions in the photographs.

This experiment was carried out, an example of the results is shown in figure 5.4, the scale is approximately 3cm=1kb; the black bar represents 0.1µm, note that this size refers only to double stranded DNA. There is an ARp1 monomer highlighted

CLASS	TOTAL	%ABUNDANCE
monomer	65	26%
dimer	61	24%
trimer/tetramer	126	50%
total identical plasmids	252	
100kb+ DNA	18	--

Table 5.5: relative abundance of different forms of ARp1 plasmid from 50 random grid squares.

in figure 5.4. There are three distinct regions in the ARp1 monomer. The small, single stranded loop is approximately 300 bases in size. The double stranded "stem" is 3kb in size. The large, single stranded loop is aproximately 5kb in size. From both its size and location the small loop must be the 345bp central unique region which separates the amal arms. The 3kb "stem" must therefore be the amal arms which appear to be entirely homologous. The large loop must consist of ArgB and pUC8.

5.4 Abundance of ARp1 multimers.

Calculating the relative abundance of the different forms of ARp1 was carried out by choosing random squares on the grid, then simply counting all the plasmids that could be clearly identified; approximately 10% of the total DNA on any one grid could be clearly identified. Monomers and dimers are easily distinguishable so were counted separately. Trimers and tetramers were counted together as one class because it is difficult to distinguish between these forms unless each plasmid is photographed and individually measured. The extremely large, intact molecules, see figure 5.3, were also counted but are not included in the final % abundance because the large linear molecules were not counted at all. The results of these counts are shown in table 5.5. Table 5.5 shows the total number of plasmids counted

in 50 random squares. From these results it appears that 26% of ARp1 molecules are present as monomers: 74% of ARp molecules are present as multimers.

5.5 Discussion.

The results described in this Chapter strongly suggest that ARp1 monomers, dimers and multimers are present in Aspergillus. There is no unambiguous evidence that the large molecules noted here are the product of Double Rolling Circle replication. Dimers, trimers and larger plasmid forms may be the result of recombination between monomers. The existence of DRC molecules could be tested by carrying out the experiments discussed in detail in the Chapter 4 discussion section.

The calculated relative abundace of the different forms are interesting. The results shown in table 5.5 suggest that multimer forms of ARp1 are very common. It is unlikely that ARp1 benefits from a plasmid specific recombination system that resolves multimers to monomers. Such systems have been studied in detail e.g. the yeast 2μ circle (see Chapter 4) and the cer system found in the E.coli plasmid, ColE1, Stirling et al (1988).

The cer system consists of a 279bp non-coding region designated cer, the xerA and xerB genes. Unlike the yeast 2μ circle raf, flp and rep genes the argR, xerA and xerB are all chromosomal genes. The

xerA gene product appears to be indentical to the argR gene product. The argR protein acts as a repressor of the arginine biosynthetic genes. The argR protein binds to a specific site within the cer region, recombination then takes place at a specific site within the DNA-protein complex and the plasmid dimers are resolved. The mechanism of dimer resolution is not fully understood but argR protein does not act as a recombinase. The xerB gene has been mapped close to the argR and pyrB genes and appears to encode a transpeptidase.

The fact that ARp1 multimers are so prevalent needs to be taken into account when considering plasmid copy number/plasmid instability. Plasmid recombination is discussed in more detail in Chapter 9.

Chapter 6.

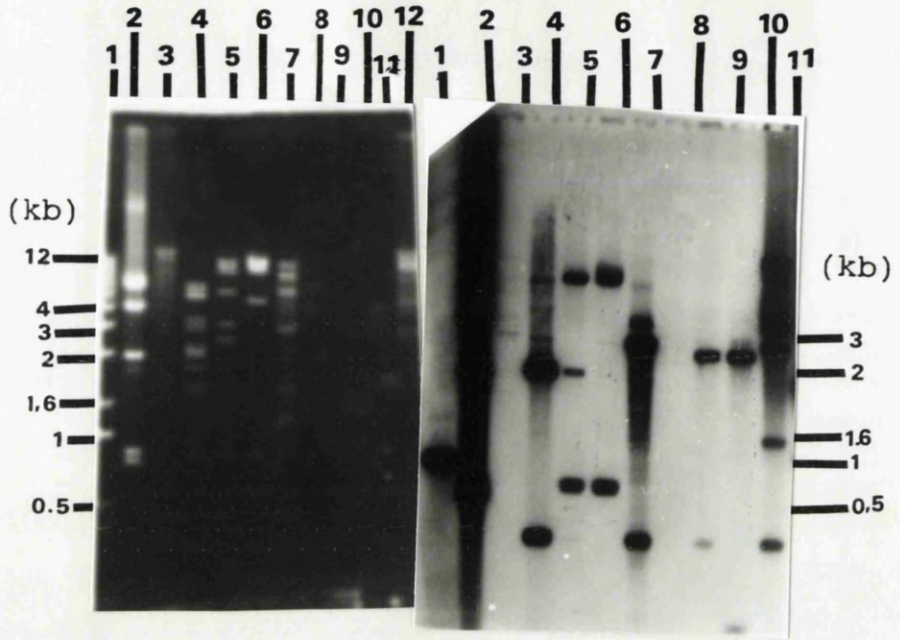
Genomic *ama1*-like sequences in *Aspergillus nidulans*.

6.1 Introduction.

Yelton et al (1985), constructed an Aspergillus nidulans gene bank in a cosmid vector called pKBY2. Each cosmid contains approximately 40kb of A.nidulans DNA. H. Brody, (personal communication), probed this gene bank with the ARp1 derived amaI sequence and identified 13 cosmid clones which hybridise with this probe.

Further work by Brody i.e. probing a CHEF gel, suggests that each of the 8 A.nidulans chromosomes contain at least one copy or one component of an amaI-like sequence, (see Chapter 3). Brody's CHEF gel experiments, (personal communication), show that the ARp1-derived amaI sequence hybridises most strongly to chromosome 4, moderately to chromosomes 1, 7 and 8 and weakly to the remaining chromosomes.

Nine of these cosmids were obtained from Yelton et al (1985), with the intention of testing these cosmids to see if the amaI-like sequence could be related to the ARp1-derived amaI sequence, (see chapter 3). In addition, I wanted to use these cosmids in cotransformation experiments to see if the cosmid amaI-like sequences would confer increased frequency of transformation and plasmid instability.



LANE	2	3	4	5	6	7	8
COSMID	ARp1	L25F11	L26F10	L30E9	L6H12	C8C1	L31C1
band	-	5.1	9.1	9.3	9.3	4.6	-
size		3.8	4.9	2.2	0.9	3.5	
(kb)			3.1	0.9		0.5	
			2.2				
			0.3				

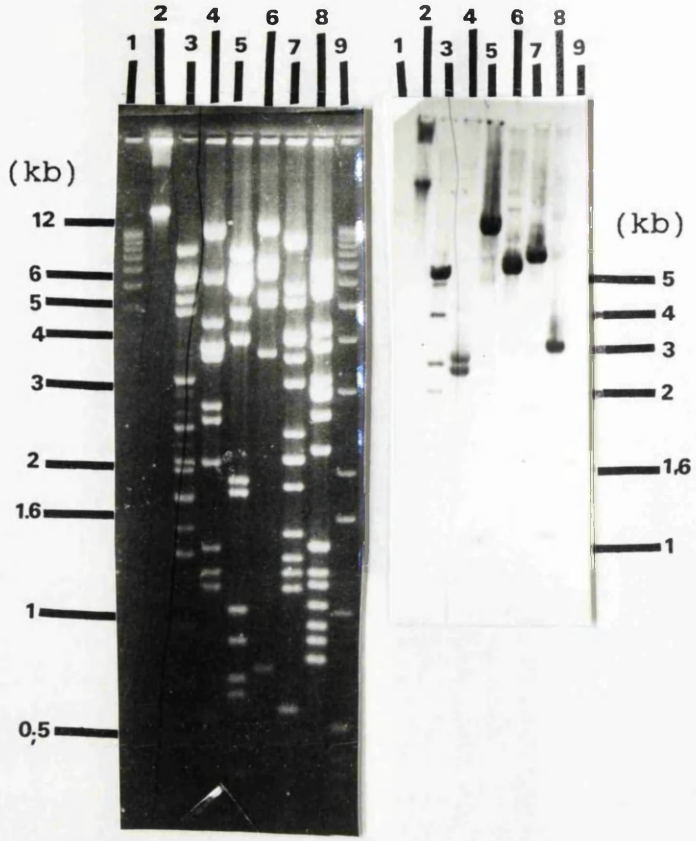
LANE	9	10	11	1+12
COSMID	L31D4	L25G2	L25F9	marker
band	3.1	3.1	4.5	-
size	0.5	0.3	3.2	
(kb)			1.6	
			0.5	

Figure 6.1: cosmid DNA digested with HindIII. Probed with radiolabelled ARp1-derived <u>ama1</u> DNA.

6.2.1 Characterisation of cosmids.

DNA from each of the cosmids was prepared from small scale *E.coli* cultures, digested with 10 units of HindIII and run on a 0.8% agarose gel. The gel was then Southern blotted and probed with the 5kb HindIII *ama1* DNA fragment. It was reasoned that if *ama1* was derived directly from the *Aspergillus* chromosome then the HindIII digest of the appropriate cosmid DNA should produce a characteristic 5kb fragment. Note that this experiment was done before sequencing, (see Chapter 3), revealed the composite nature of *ama1*.

The results are shown in figure 6.1. Only bands which hybridise with the *ama1* probe are listed. It is clear from figure 6.1 that only cosmids L25F11 and L26F10 (lanes 3 and 4), produce bands of approximately 5.1kb and 4.9kb in size respectively. Cosmid L8C1 (lane 7), produces a 4.6kb band. Cosmid L13C1 (lane 8), doesn't appear to hybridise with *ama1*. From these results it appears that at the very least cosmids L25F11 and L26F10 are worth further investigation and that the other cosmids contain *ama1* homologues with different structures compared to the ARp1 derived *ama1* sequence.



LANE	1	2	3	4	5	6	7	8	9
ENZYME	mk	uncut	E	B	X	Sm	S	H	mk
band size (kb)			4.8 4.3 3.3 2.7 2.3	2.8 2.6	7.5	5.2	5.8 2.2	5.8 3.0 1.1	

KEY

E=EcoRI B=BamHI X=XhoI S=SalI H=HindIII
mk=size marker, 1kb ladder

Figure 6.2: cosmid L25F11 DNA digested with EcoRI, BamHI, XhoI, SmaI, SalI and HindIII. DIG-labelled with ARp1-derived ama1 DNA.

6.2.2 Characterisation of L25F11.

2µg aliquots of cosmid L25F11 were digested separately with 20 units of EcoRI, BamHI, XhoI, SmaI, SalI and HindIII. The digested DNA was then run on a 0.8% agarose gel and Southern blotted. The blot was then probed with DIG-labelled ARp1 derived ama1 DNA. The results of this blot are shown in figure 6.2. Only bands which hybridise with ama1 are listed.

As can be seen from figure 6.2, (in the table comparing banding patterns), the banding pattern for ama1-like bands is very different from the banding pattern for the ARp1-derived ama1 sequence, (see section 4.3). For example, there is no strongly hybridising 5kb HindIII band in the HindIII digested L25F11 lane. Similarly, XhoI digested ARp1, probed with ama1 yields two ama1 specific bands of 0.8kb and 0.7kb in size; these bands are not present in the XhoI digested L25F11 lane.

In conclusion, L25F11 does contain ama1 homologous DNA, but the structure of the cosmid ama1 homologue is very different from the ARp1 derived ama1 sequence.

6.2.3 Characterisation of L26F10.

2µg aliquots of cosmid L26F10 were digested with a variety of different enzymes, (see figure 6.3a). The digested DNA was run on a 0.8% agarose gel and

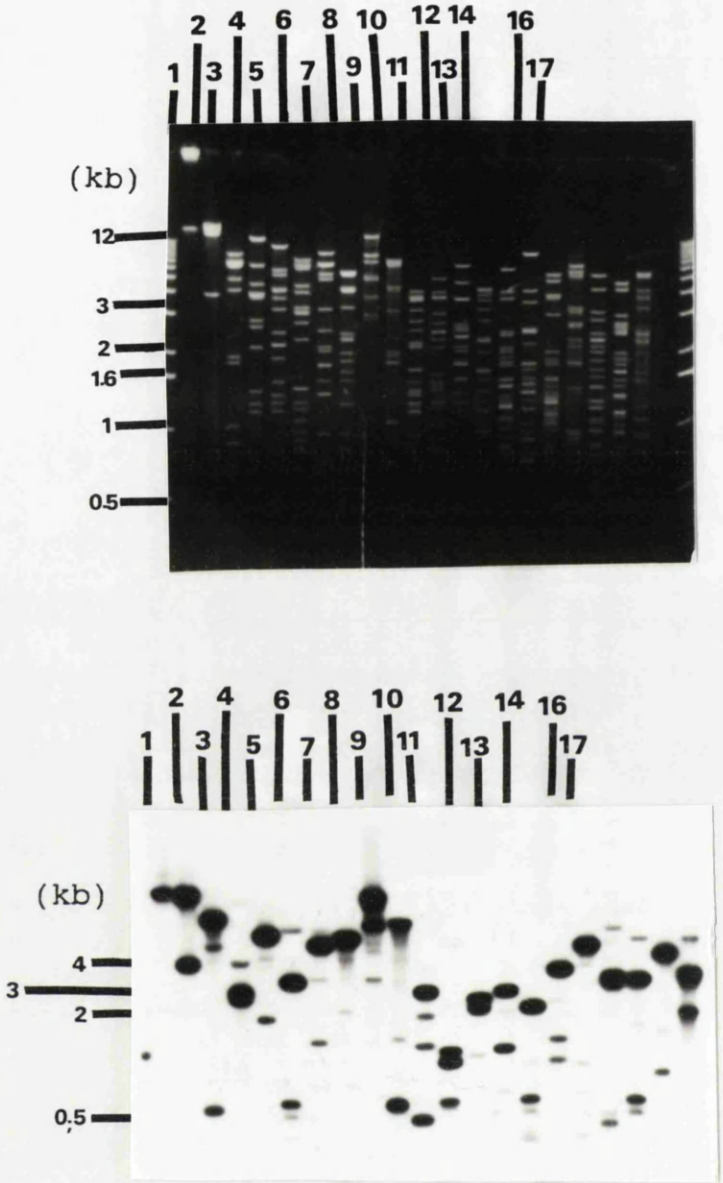


Figure 6.3a: digested cosmid L26F10 DNA, probed with radiolabelled ARp1-derived amal DNA.

LANE	1	2	3	4	5	6	7	8
ENZYME	mk	uncut	C	X	E	S	H	B
band sizes (kb)			-	-	11	5.8	6.2	6.2
					4.4	5.4	5.8	5.2
					3	4.6	3.4	3.6
					2.2	3.6	1.1	2
						2.4	0.8	0.8

LANE	9	10	11	12	13	14	16	17
ENZYME	P	Ss	C/X	E/S	E/H	E/B	S/H	S/B
band sizes (kb)	-	-	-	4.2	5.4	3.2	4.3	4.4
				3.4	3	2.6	4.2	3.8
				2.2	1.3	2.2	2.6	2.8
				1.8	1.3	2	1.7	2
				0.8	0.8	1.2	1.2	1.8
						0.8	1.1	1.4
							0.8	1

KEY

C=ClaI	X=XhoI	E=EcoRI	S=SalI	mk=marker
B=BamHI	P=PstI	Ss=SstI	H=HindIII	

Figure 6.3a continued: table of band sizes for relevant enzymes, (see figure 6.3b).

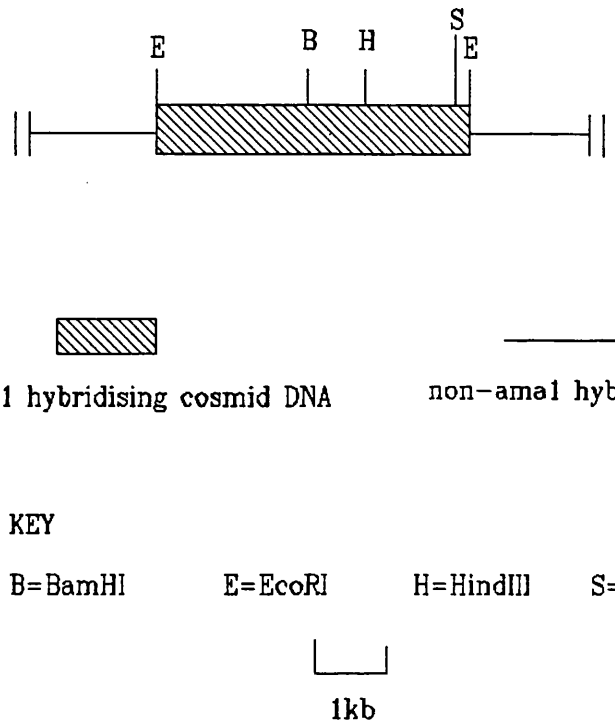


Figure 6.3b: structure of region of L26F10 which hybridises with ARp1-derived ama1 probe. Please note that the non-hybridising cosmid DNA is not included in this diagram.

Southern blotted. The filter was then probed with radiolabelled amal DNA. The results are shown in figure 6.3a, only bands which hybridise are listed. From these results it was possible to map the strongly hybridising 4.4kb EcoRI fragment.

However, only the positions of the BamHI, EcoRI and HindIII sites are shown on the map, (see figure 6.3b). Only these restriction sites are relevant to the experiments described in section 6.4.

It is clear from figures 6.3a and 6.3b that L26F10 does contain amal homologous DNA, but that the cosmid amal homologue is very different in structure from the ARp1 derived amal sequence e.g. the cosmid amal DNA does not appear to contain inverted repeats.

6.3. Cotransformations with pILJ16 and undigested cosmids.

Cotransformation experiments with the cosmids and pILJ16 were set up as described in chapter 2. Approximately $1.4x10^7$ A.nidulans strain G34 protoplasts were transformed with 1µg of pILJ16 plus 1ug of each of the cosmid DNAs in turn. Control transformations with uncut DNA, pILJ16, ARp1, a pILJ16/pSEQ01.21 cotransformation and a pKBY2 based cosmid containing A.nidulans DNA with no amal homologue were included. The cosmid control, (called CGG01), was included to ensure that any

DNA	TRANSFORMATION FREQUENCY/µg DNA	% INSTABILITY/ ASEXUAL GENERATION
no DNA	0	-
pILJ16	31	0%
ARp1	30,000	55%
L25F11/pILJ16	470	0%
L26F10/pILJ16	480	0%
L30E9/pILJ16	210	0%
L6H12/pILJ16	260	0%
L8C1/pILJ16	200	0%
L13C1/pILJ16	45	0%
L31D4/pILJ16	580	0%
L25G2/pILJ16	490	0%
L25F9/pILJ16	400	0%
pSEQ01.21 /pILJ16	115	89%
CGG01/pILJ16	28	0%

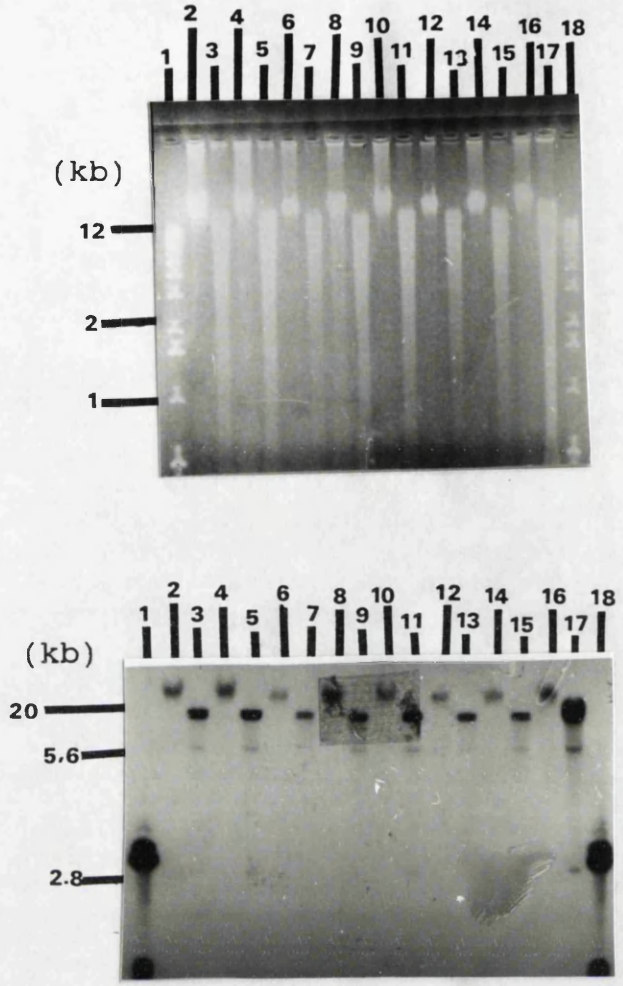
Figure 6.4: transformation frequency and % instability results for intact cosmids and pILJ16 cotransformations.

increase in transformation frequency or plasmid instability is due to ama1-like sequences and not due to either the pKBY2 DNA or a general property of cosmid-based-A.nidulans DNA. Instability tests were carried out as described previously in chapter 4.

The results are shown in figure 6.4. It is clear from the results that all the cosmids, with the exception of L13C1 and the control cosmid, led to at least a 10 fold increase in transformation frequency. For example, the pILJ16/L31D4 cotransformation yields 580 transformants/µg DNA; the pILJ16/L8C1 cotransformation yields 200 transformants/µg DNA.

The instability values are shown in figure 6.4. There is no suggestion of any plasmid instability in any of the cotransformations except the pILJ16/pSEQ01.21 control. The fact that the pILJ16/pSEQ01.21 behaves as expected suggests that the plasmids have formed a cointegrate, so equally, the cosmids and pILJ16 should also be able to form cointegrates.

The most likely conclusion from these results is that the argB gene had integrated into the genome. The increase in transformation frequencies could then be explained if a pILJ16/cosmid cointegrate formed and the cosmid DNA either acted as an integration enhancer due to the amount of homologous A.nidulans DNA present on each cosmid, or else the cointegrate was capable of transient replication. Alternatively, cointegrate formation may be irrelevant, and the



LANE	DNA	LANE	DNA
1	marker	10	L8C1
2	L25F11	11	L8C1 CUT
3	L25F11 CUT	12	L31D4
4	L26F10	13	L31D14 CUT
5	L26F10 CUT	14	L25G2
6	L30E9	15	L25G2 CUT
7	L30E9 CUT	16	L25F9
8	L6H12	17	L25F9 CUT
9	L6H12 CUT	18	marker

Figure 6.5: NruI digested DNA from Aspergillus cosmid/pILJ16 cotransformants. Probed with radiolabelled pUC8 DNA.

cosmid DNA may act in the same way as additional DNA which increases transformation rates in many organisms, (John Clutterbuck, personal communication).

To test whether or not the pILJ16 DNA had integrated, I made total genomic DNA preparations from one individual cotransformant with each cosmid. 3µg aliquots of these DNAs were digested with 20 units of NruI and run on a 0.3% agarose gel, along with 3µg aliquots of undigested DNA. This gel was then Southern blotted and probed with DIG-labelled pUC DNA. NruI does not cut pILJ16. The results are shown in figure 6.5.

In figure 6.5 there is no free plasmid present in the undigested lanes and the banding pattern is identical in all the digested lanes. The three bands which hybridise in the digested DNA lanes are 20kb, 5.6kb and 2.8kb in size. The 2.8kb band is faint and is the same size as pUC8. Lane 17 appears to contain a band larger than 20kb but this additional band may be a partial digest product or due to sample overloading and smearing. The fact that multiple bands of the same size hybridise in all cases suggests that rearrangements of pUC8 DNA may have occurred.

In three separate attempts, no plasmid rescues were possible using the genomic DNA samples; it was possible to rescue DNA from the TARp1 control. Overall, the results suggest that the cointegrate DNA

has integrated into the genome.

6.4 Cotransformations with pILJ16 and digested L26F10.

The results of the cosmid cotransformations with undigested DNA suggest that the argB gene had integrated into the genome due to the size of the cosmid DNA. By digesting the cosmid DNA and then using this digested DNA in cotransformations, it might then be possible to test whether or not the ama1 hybridising fragments from L26F10 are capable of enhancing transformation frequency and produce autonomously replicating cointegrate plasmids. 1µg aliquots of cosmid L26F10 were digested separately with 20 units of EcoRI and HindIII. The enzymes were heat-inactivated after two hours and the DNAs used in cotransformation experiments as described in section 6.3.

Cotransformant colonies obtained using the EcoRI cosmid DNA were designated TL26F10/16E: transformants obtained from the HindIII digested cosmid DNA were designated TL26F10/16H. On average, the EcoRI digested cosmid DNA yielded 1000 transformants/µg DNA in cotransformations with pILJ16; the HindIII digested cosmid DNA yielded on average 870 transformants/µg DNA under the same conditions. The digested cosmid DNA produced approximately a 50-fold increase in transformation frequency when compared to the integrative vector

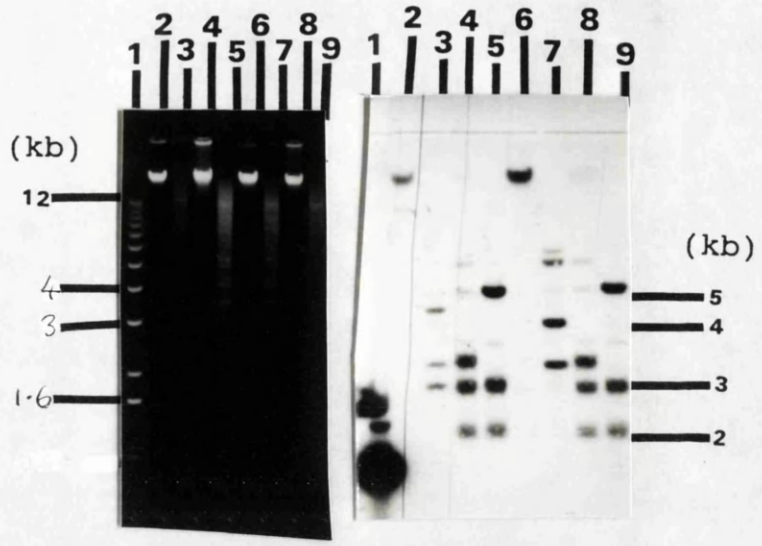
pTL26F10/16E		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	109	127	56	45	337		
	ARG+	42	78	13	9	142	479	70.35
2	ARG-	77	133	128	141	479		
	ARG+	24	91	76	68	259	738	64.91
3	ARG-	177	34	141	110	462		
	ARG+	92	6	102	56	256	718	64.35
4	ARG-	112	97	130	129	468		
	ARG+	55	24	64	67	210	678	69.03
5	ARG-	124	110	140	137	511		
	ARG+	99	73	89	77	338	849	60.19
subtotal	ARG-	599	501	595	562			
subtotal	ARG+	312	272	344	277	Mean		65.76
TOTAL		911	773	939	839	Standard Deviation		3.62
%LOSS		65.75	64.81	63.37	66.98			

Figure 6.6a: pTL26F10/pILJ16 (EcoRI) instability test results.

facing page 159a

pTL26F10/16H		GENERATION						
COLONY		1	2	3	4	subtotal	TOTAL	%LOSS
1	ARG-	75	112	79	89	355		
	ARG+	34	76	28	45	183	538	65.99
2	ARG-	99	210	121	128	558		
	ARG+	48	155	87	69	359	917	60.85
3	ARG-	64	101	120	134	419		
	ARG+	40	77	62	98	277	696	60.20
4	ARG-	53	91	89	81	314		
	ARG+	24	42	50	53	169	483	65.01
5	ARG-	38	63	91	118	310		
	ARG+	17	29	64	80	190	500	62.00
subtotal	ARG-	329	577	500	550			
subtotal	ARG+	163	379	291	345	Mean		62.81
TOTAL		492	956	791	895	Standard Deviation		2.29
%LOSS		66.87	60.36	63.21	61.45			

Figure 6.6b: pTL26F10/pILJ16 (HindIII) instability test results.



LANE	DNA
1	marker
2	TL26/16/01H
3	TL26/16/01H CUT
4	TL26/16/02H
5	TL26/16/02H CUT
6	TL26/16/03
7	TL26/16/03H CUT
8	TL26/16/04
9	TL26/16/04 CUT

GEL	LANE	BAND SIZES (kb)
	3	4.5, 3.8, 3.2, 2.9
	5	5.2, 3.4, 2.9, 2.1
A	7	7, 6.1, 5.2, 3.8, 3.2
	9	5.2, 3.4, 2.9, 2.1

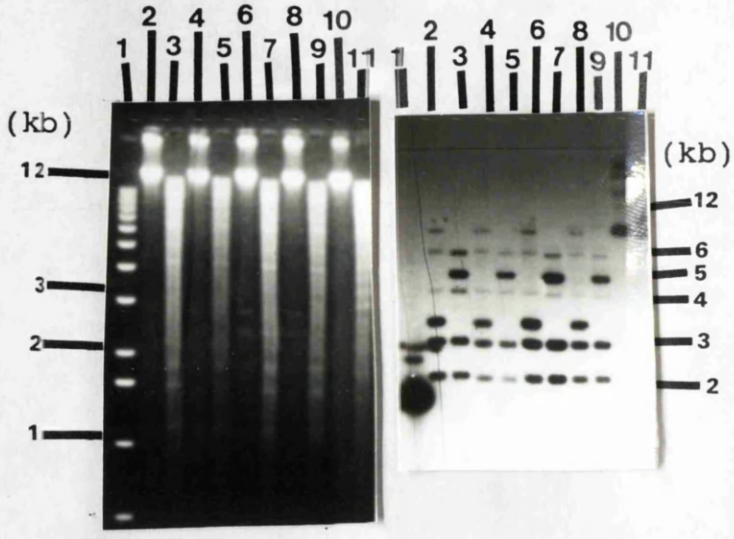
Figure 6.7a: BglII digested genomic DNA from Aspergillus pTL26F10/pILJ16 (HindIII) cotransformants. Probed with radiolabelled pUC8 DNA.

pILJ16 alone.

The % instability results for TL26F10/16E individuals, 65%±3.6% and TL26F10/16H individuals, 62%±2.2%, are show in figures 6.6a and 6.6b respectively. The results show that the digested cosmid DNA has formed unstable cointegrate plasmids with pILJ16.

Total genomic DNA was made from 4 transformant colonies from each class. 2µg aliquots of this genomic DNA were digested with 20 units of BglII and run on a 0.8% agarose gel along with 2µg of uncut genomic DNA and aliquots of DNA from an ARp1 transformant. The gel was then Southern blotted and probed with radiolabelled pUC DNA. The results are shown in figures 6.7a (EcoRI digested cosmid) and 6.7b (HindIII digested cosmid).

In figure 6.7a, the genomic band is visible in lanes 2 and 6: this band is less intense in lanes 4 and 8. Conversely, the undigested plasmid bands are more intense in lanes 4 and 8 but much less intense in lanes 2 and 6. The banding patterns in the digested DNA lanes 3, 5, 7 and 9 suggests that there are three types of plasmid present in the DNA samples, the banding patterns in lanes 5 and 9 are identical. Although the plasmids appear to have different banding patterns a number of bands appear to be common: the 5.2kb band is common to lanes 5, 7 and 9, but is less intense in lane 7; both the 3.8kb and 3.2kb bands are common to lanes 3 and 7, but



LANE	DNA
1	marker
2	TL26/16/01E
3	TL26/16/01E CUT
4	TL26/16/02E
5	TL26/16/02E CUT
6	TL26/16/03E
7	TL26/16/03E CUT
8	TL26/16/04E
9	TL26/16/04E CUT
10	TARp1/1.4
11	TARp1/1.4 CUT

		band sizes kb
	3	6.2, 5.1, 4, 3, 2.2
	5	6.2, 5.1, 4, 3, 2.2
B	7	6.2, 5.1, 4, 3, 2.2
	9	6.2, 5.1, 4, 3, 2.2
	11	12, 11.5, 9, 5.8

Figure 6.7b: BglII digested genomic DNA from Aspergillus pTL26F10/pILJ16 (EcoRI) cotransformants. Probed with radiolabelled pUC8 DNA.

lane 7 contains other, larger additional bands; the 2.9kb band is common to lanes 3, 5 and 9, but this band is less intense in lane 3.

In figure 6.7b the genomic DNA band is visible only in the TARp1 control lane. The banding patterns in the undigested DNA lanes 2, 4, 6 and 8 show a degree of similarity. As shown in the table of band sizes in figure 6.7b there are 5 bands that are common to all the digested DNA lanes. There are a number of uncut bands which are common to both cut and uncut lanes in figures 6.7a and 6.7b e.g. in figure 6.7a, the 2kb and 3kb bands in lane 4 are present in the digested DNA lane 5. The 3.3kb band in lane 4 is present in lane 5 as a 5.5kb band. These results indicate that the 2kb and 3kb bands are not digestable with BglII so probably do not contain unrearranged argB DNA. The 3.3kb band does appear to contain at least one BglII site. Overall, these results suggest that there is more than one type of plasmid present.

It was not possible to recover any plasmid DNA from the genomic DNA samples, although three separate attempts to rescue plasmids were carried out. It was possible to rescue ARp1 from the control DNA sample.

6.5 Discussion.

It is clear from the cotransformation results in section 6.5 that some component of the ama1 related DNA in cosmid L26F10 both enhances transformation frequency and leads to plasmid instabilty and hence the ability to replicate autonomously. The behaviour of these cointegrate plasmids is in line with the known behaviour of ARp1 subclones, (see Chapter 4, figure 4.42). The other cosmid-borne ama1 DNA may also behave in a similar fashion, such a theory would have to be tested by carrying out similar experiments to those described in section 6.4, with each of the remaining cosmids.

The Southern blots in figure 4.7 suggest that the cointegrate plasmids have been either rearranged to produce a variety of plasmids or that the cosmid L26F10 DNA contains a number of ama1 related sequences which give rise to a number of plasmids with different structures; some of these plasmids are smaller than pILJ16 and lack the argB gene, or at least its BglII site. The cosmid-borne ama1 related DNA region was not identified due to the inability to rescue any cointegrate plasmid DNA, due possibly to plasmid rearrangements.

The structure of the ARp1-derived ama1 appears to be a mixture of pUC and Aspergillus genomic DNA. Therefore, it may be that the ARp1 ama1 sequence(s) may be derived from any or all of the cosmid-borne

ama1-related regions, so the ARp1 *ama1* sequence(s) may be located on any or all of the eight chromosomes.

It could be of interest to test the relatedness of the ARp1 *ama1* sequence to the cosmid-borne *ama1*-like regions by comparing the sequence composition of each region with ARp1.

Chapter 7.

Ama1-like sequences in *Penicillium chrysogenum* and *Cephalosporium acremonium*.

7.1.1 Introduction.

Chapters 3, 4, 5 and 6 examined the ARp1-derived amal sequence and amal-like sequences found in Aspergillus nidulans. ARp1 is the ideal model system to study both amal-like sequences from other filamentous fungi and to develop species-specific autonomously replicating vectors.

I now want to address a number of questions: (1) Are amal-like sequences to be found in other filamentous fungi or are such sequences unique to A.nidulans? Replication origins must exist in other fungi. (2) Do sequences of similar composition to amal, but derived from other fungi, promote plasmid replication in either the parental fungi or Aspergillus?

Work carried out by Gems, (1990), has shown that ARp1 replicates autonomously, increases transformation frequencies and is unstable in both Aspergillus niger and Aspergillus oryzae. J.R. Kinghorn, (personal communication), has found that ARp1 behaves in a similar fashion in Aspergillus giganteus. I wanted to concentrate on two species of industrially important filamentous fungi, namely: Penicillium chrysogenum and Cephalosporium acremonium.

Penicillium and Cephalosporium are classed as hyphomycetes in the Deuteromycotina or Fungi Imperfecti.

Both Penicillium and Cephalosporium are important antibiotic producers. These fungal species produce a variety of different antibiotics such as Penicillins F, K, N, V, G and Cephalosporin C. In addition, hedonists will note that Penicillium roqueforti and Penicillium camemberti are, as the names suggest, used in cheese making.

Until recently, the only way of improving the antibiotic yield of industrial fungal strains, (strain improvement), was by successive rounds of random mutation followed by selection for specific mutations, Rowlands (1984). This approach, although very successful, is both time and labour intensive. A potentially much faster, hence cheaper way, is to increase antibiotic yield by expressing multiple copies of the genes involved. Such an approach requires transformation systems and plasmid vectors. A number of integrative vectors, (see below), have been developed for both Penicillium chrysogenum and Cephalosporium acremonium. These vectors are based on three types of selectable markers: dominant markers such as antibiotic resistance e.g. oligomycin and hygromycin; utilisation of a novel nutrient e.g. acetamide; complemention of an auxotrophic mutation e.g. ArgB and niaD mutations.

There are advantages and disadvantages with each type of marker. Antibiotic resistance selection ensures that there is little or no background growth and that no abortive colonies can grow. It is

relatively expensive and so is not practical for industrial scale-up. Selection by complemention of an auxotrophic mutation is much more attractive but requires that specific mutant strains are available, and that the auxotrophy does not interfere with the industrial usage.

7.1.2 *Cephalosporium* transformation systems.

Integrative transformation systems using a variety of different selectable markers have been reported. Isogoni *et al* (1987), developed a vector called pCYG97 which confers Kanamycin resistance. This vector was of interest because it contained a 1kb *Cephalosporium* genomic fragment that was shown to act as an ARS in yeast. This genomic fragment was sequenced and found to have 91% homology with the yeast ARS core sequence, (see chapter 1). Further analysis showed that this genomic fragment had no ARS activity in *Cephalosporium* and that the plasmid integrated into the genome. The plasmid pCYG97 yields 10 transformants/ µg DNA.

Whitehead *et al* (1990), utilised the *A.nidulans niaD* gene to transform a nitrate reductase mutant of *Cephalosporium* with a plasmid called pSTA700 and obtained a transformation frequency of 40 transformants/µg DNA. Both Whitehead *et al* (1990) and Skatrud *et al* (1987) have utilised the hygromycin resistance gene hyg^B as a selectable marker.

7.1.3 *Penicillium* transformation systems.

A number of different integrative vector systems have been developed for use in *Penicillium*. The *AmdS* gene, derived from *A.nidulans*, has been used by a number of groups, namely: Beri and Turner (1987), Koler *et al* (1988), Skatrud *et al* (1988) and Geisen and Leistner (1989). The *AmdS* gene codes for acetamidase and allows the utilisation of acetamide. The transformation frequencies of integrative plasmids based on *AmdS* varies between 2 and 20 transformants/µg DNA.

Whitehead *et al* (1989), used the *A.niger niaD* gene as a selectable marker on a plasmid called pSTA10. A similar plasmid called pST12 contained the *niaD* gene and the *ans-1* sequence, (Ballance and Turner, 1985). The transformation frequencies of both plasmids were approximately 5 transformants/µg DNA; the *ans-1* sequence did not enhance the transformation frequency in this case, but see below.

Cantoral *et al* (1987), used a plasmid containing the *ans-1* sequence and the *N.crassa pyr4* gene to transform *Penicillium* uracil auxotrophic mutants. In this case the *ans-1* sequences appears to enhance the transformation frequency to 2000 transformants/µg DNA: in comparison a plasmid containing only the *pyr4* gene yields 5 transformants/ µg DNA.

Finally, Bull *et al* (1988), used the OliC gene,

(Ward et al 1986, 1988), to select for oligomycin resistant transformants. The transformation frequency for plasmids carrying this gene was approximately 5-10 transformants/µg DNA.

7.1.4 Experimental approach.

There were, at the start of these experiments, no autonomously replicating vectors for use in Penicillium or Cephalosporium. The transformation systems outlined in sections 7.1.2 and 7.1.3 show that Aspergillus-derived selectable markers can function in both Penicillium and Cephalosporium transformants. Therefore, it is feasible that plasmids containing ARp1-derived amal sequences may replicate autonomously in Penicillium and Cephalosporium.

I took two approaches in trying to construct ARVs for use in Penicillium and Cephalosporium. One approach was to construct plasmids based on the ARp1-derived amal sequence. The other approach was to use amal as a probe to identify homologous sequences in both Penicillium and Cephalosporium. If such sequences were identified I intended to construct Lambda phage libraries, clone these sequences into plasmids and then attempt transformations with these plasmids.

I decided initially to use the hygromycin gene for Cephalosporium transformations and the OliC gene

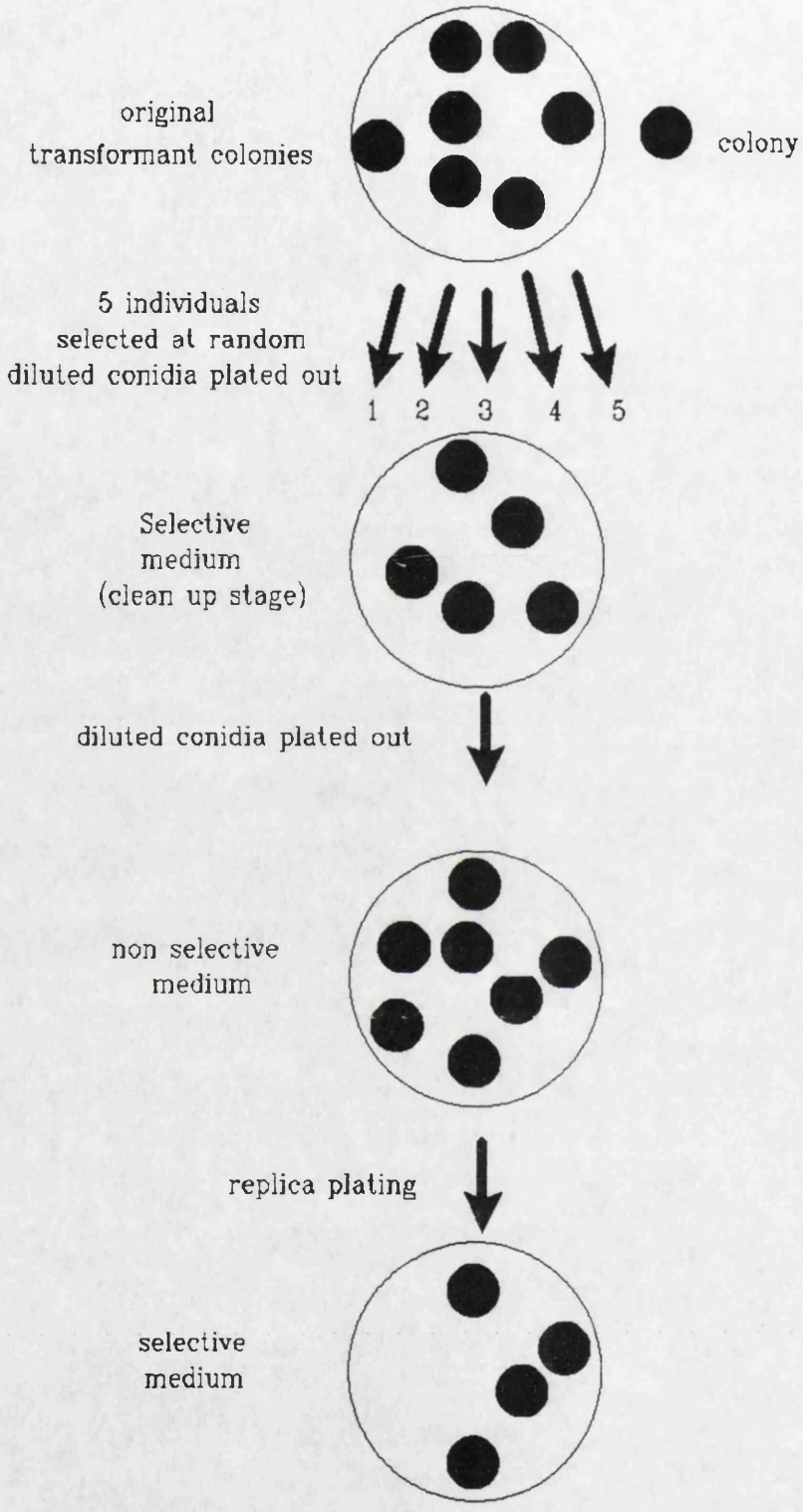


Figure 7.1: Oligomycin and hygromycin instability tests

for Penicillium transformations. The choice of markers was determined by the availability of GlaxoChem fungal ancestral strains.

Instability tests described in the following sections were similar to those described in chapter 4. As before, conidia were collected from individual transformants and plated onto selective minimal media such that each colony which grows is derived from a single spore. The antibiotic resistance tests were then carried out as outlined in figure 7.1. Conidia were again collected but spread out onto non selective media which allows both antibiotic resistant and antibiotic sensitive colonies to grow. After incubation the number of colonies on these plates are counted. The colonies are then replicated, using velvet, onto selective plates. Only antibiotic resistant, (plasmid containing), colonies will grow; sectored or semi-resistant colonies were scored as plasmid containing. The number of such colonies is counted and gives a measure of plasmid loss.

7.2.1 Construction of pJSR10 and pJSR11.

The plasmids pJSR10 and pJSR11 were constructed as follows. The 5kb HindIII amal fragment from ARp1 was purified and cloned into the HindIII site of plasmids pMWI4 and pIH4. Plasmid pMWI4 carries the OliC gene and pIH4 carries the HygB gene. The

	TRANSFORMATION FREQUENCY/ug DNA			%INSTABILITY/ ASEXUAL GENERATION		
attempt	1	2	3	1	2	3
PLASMID						
no DNA	0	0	0	-	-	-
pMW14	5	7	7	0	0	0
pJSR10	10	15	11	55	57	60

Figure 7.2: Penicillium transformations with pJSR10, an amal-containing plasmid and pMW14, an integrative plasmid.

plasmid pJSR10 contains the *OliC* gene and the 5kb HindIII *amal* fragment; pJSR11 contains the *HygB* gene and the the 5kb HindIII *amal* fragment. The composition of both pJSR10 and pJSR11 was checked by digesting purified plasmid DNA with HindIII and then separating the bands on a 0.8% agarose gel, (results not shown).

7.2.2 *Penicillium* transformations with pJSR10.

Penicillium protoplasts were prepared as described in chapter 2. Approximately $1x10^7$ protoplasts were transformed separately with 20µg of pJSR10 (9kb) and pMW14 (4kb). A NO-DNA control transformation was included.

The transformation frequencies and % plasmid instability from three experiments of both pJSR10 and pMW14 is shown in figure 7.2. On average, pMW14 yields 5 transformants/µg DNA and integrates into the genome (0% plasmid instability): pJSR10 yields 12 transformants/ µg DNA and 57% of conidia-derived progeny lose pJSR10.

The results suggest that pJSR10 can replicate autonomously in *Penicillium*, therefore *amal* appears to be functional, but there is little difference in transformation frequency between the integrative plasmid, pMW14 and the supposedly autonomously replicating plasmid, pJSR10. It is possible that pJSR10 is not replicating autonomously but is simply

	TRANSFORMATION FREQUENCY/µg DNA			%INSTABILITY/ ASEXUAL GENERATION		
attempt	1	2	3	1	2	3
PLASMID						
no DNA	0	0	0	-	-	-
pIH4	3	5	3	0	0	0
pJSR11	0.4	4	2	0	0	0

Figure 7.3: Cephalosporium transformations with pJSR11, an amal- containing plasmid and pIH4, an integrative plasmid.

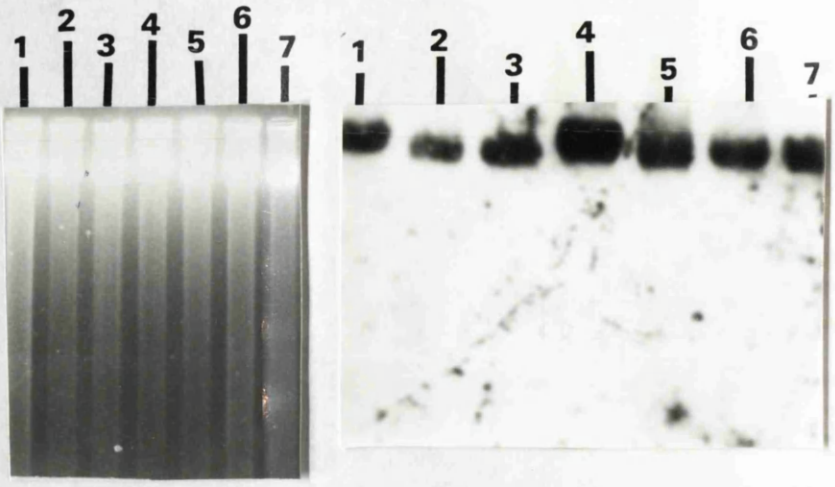
integrating into, then excising from the genome; giving the impression of an unstable, autonomously replicating plasmid.

The only way to differentiate between these two possibilities is to prepare genomic DNA from pJSR10 transformant colonies, separate samples on an agarose gel, prepare Southern blots and then probe with labelled DNA and look at the positions of the bands which hybridise. The result of one such experiment is shown in figure 7.11b, lanes 2 and 3, which suggest that pJSR10 has not integrated. Plasmid rescues with these pJSR10 DNA samples yielded two E.coli colonies which contain only pJSR10 plasmid. A HindIII digest of isolated plasmid gave two bands of 4 kb and 5kb in size, indicating that pJSR10 is unrearranged, (gel not shown). Taken together, these results suggest that pJSR10 replicates autonomously, hence ama1 promotes autonomous replication in Penicillium.

7.2.3 Cephalosporium transformations with pJSR11.

Protoplasts from Cephalosporium acremonium M8650 were prepared as described in chapter 2. Approximately 1.6×10^7 protoplasts were transformed separately with 40µg of both pIH4 (7.1kb) and pJSR10 (12.1kb). The results from three such experiments are shown in figure 7.3.

The results from figure 7.3 indicate that pJSR11



LANE	DNA
1	TIH4/01
2	TIH4/02
3	TIH4/03
4	TJSR11/01
5	TJSR11/02
6	TJSR11/03
7	TILJ16/01

Figure 7.4: undigested genomic DNA from Cephalosporium transformants. Probed with radiolabelled pUC8 DNA.

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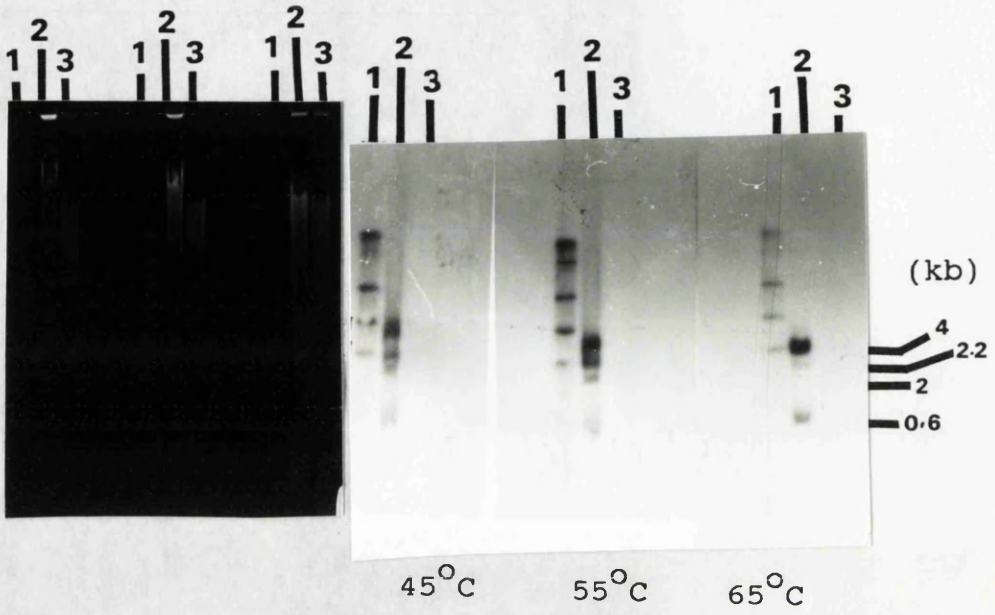
has integrated into the chromosome. There is no indication of plasmid instability in any of the pJSR11 transformants. The average transformation frequencies of both pIH4 and pJSR11 are 4 and 2 transformants/μg DNA respectively. Overall, these results suggest that pJSR11 does not replicate autonomously, hence amal does not promote autonomous replication in Cephalosporium.

To confirm this result I made genomic DNA from three pIH4 and three pJSR11 transformants. 5μg aliquots of these genomic DNAs, along with 5μg of DNA from an Aspergillus pILJ16 transformant, were run on a 0.8% agarose gel and Southern blotted; 5μg of DNA were used in an attempt to identify any free plasmid that might be present. The Southern blot was probed with radiolabelled pUC DNA. The results are shown in figure 7.4. It is clear from this blot that no free plasmid can be seen in any of the pJSR11 transformant lanes. It was not possible to isolate any plasmid DNA from pJSR11 transformants by plasmid rescues.

To sum up, there is no evidence that amal is capable of promoting autonomous plasmid replication in Cephalosporium.



LANE	DNA
1	Aspergillus wild type
2	Penicillium wild type
3	Cephalosporium wild type

Figure 7.5: Aspergillus, Penicillium and Cephalosporium wild type genomic DNA, digested with BamHI. Probed with radiolabelled ARp1-derived ama1 DNA at 45°C, 55°C and 65°C.

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7.3 *Amal* homologues in *Penicillium* and *Cephalosporium*.

Total genomic DNA was made from wild type *Aspergillus nidulans*, *Cephalosporium acremonium* and *Penicillium chrysogenum*. 2µg aliquots of the *Cephalosporium* and *Penicillium* and 500ng of the *Aspergillus* DNA were digested in triplicate with BamHI and run out on a 0.8% agarose gel. The gel was divided into three parts and Southern blotted. Each Southern blot contains the same DNA and was probed with radiolabelled ARp1-derived *ama1* DNA, but each hybridisation was carried out at a different temperature: 45°C, 55°C and 65°C. I could not predict how closely related the *Aspergillus ama1* sequence might be to any such sequence in *Cephalosporium* and *Penicillium*; by hybridising at different temperatures I could modulate the stringency of each hybridisation e.g.45°C is the least stringent and 65°C is the most stringent. The results are shown in figure 7.5.

It is clear from the results in figure 7.5. that under the experimental conditions used *Cephalosporium* does not appear to contain any *ama1* related sequences but *Penicillium* does. At 65°C, the *Penicillium* lane contains three hybridising bands, at 55°C the *Penicillium* lane contains four such bands. The four bands which do hybridise 'are approximately 4kb, 2.2kb, 2kb and 0.6kb in size. This result suggests

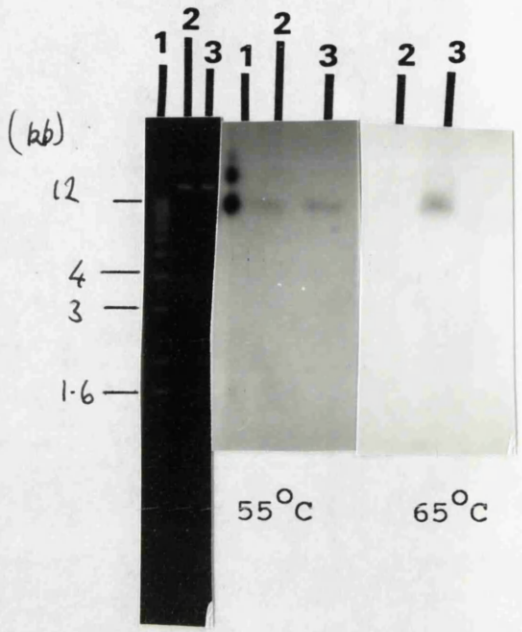
that the 4kb band is less closely related to the amal sequence than the other three sequences. The Aspergillus lane contains much less DNA than the other lanes, but multiple bands hybridise, including a 4kb band. The next step was to clone these Penicillium hybridising bands.

7.4.1 Construction and screening of Penicillium EMBL3 phage library.

The library was constructed in EMBL3 which is a Lambda replacement vector. DNA fragments of 9-23kb can be cloned into this vector.

Genomic DNA from wild type Penicillium was prepared as described in Chapter 2. The optimal conditions for digestion of genomic DNA with Sau3A to generate fragments of 15-22kb were determined by setting up pilot digests. These pilot digests involved digesting 1µg aliqouts of the genomic DNA with increasingly diluted aliquots of Sau3A enzyme. The digests were then incubated at 37°C for 30 minutes, the enzyme was inactivated and the digests run on a 0.4% agarose gel, (gel not shown).

The large scale digests of the genomic DNA were carried out with 300ug of DNA and 2 units of Sau3A for 1 hour at 37°C. A small aliquot was then removed and run on a 0.4% agarose gel to check that the digestion was adequate, (gel not shown). The DNA was extracted with phenol/chloroform, EtOH-precipitated



LANE	DNA
1	marker
2	Lambda PEN02
3	Lambda PEN01

Figure 7.6: Lambda PEN01 and PEN02 DNA, probed with radiolabelled ARp1-derived <u>ama1</u> DNA at 55°C and 65°C.

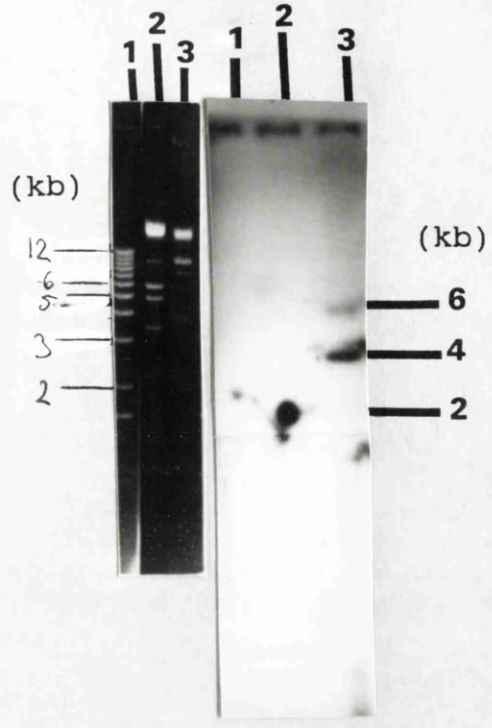
and the DNA samples were resuspended in 500µls of TE.

Approximately 1µg of genomic DNA Sau3A fragments was ligated with 0.5µg of EMBL3 arms in a total volume of 5µls. The ligation mix was left at room temperature for three hours. The ligated DNA was then packaged as described in Chapter 2. The packaged phage were then grown up and plated out as described in Chapter 2. The amount of Penicillium genomic DNA cloned was calculated to be approximately equivalent to 66 genomes.

A sample of the phage library, approximately 50,000 clones, was then plated out and blotted. The blots were probed with radiolabelled amal DNA. Plaques which hybridise with the amal probe were picked, grown up, plated out and probed again. This process was carried out until single, well spaced individual plaques could be identified. In this way two plaques were identified and designated Lambda PEN01 and Lambda PEN02.

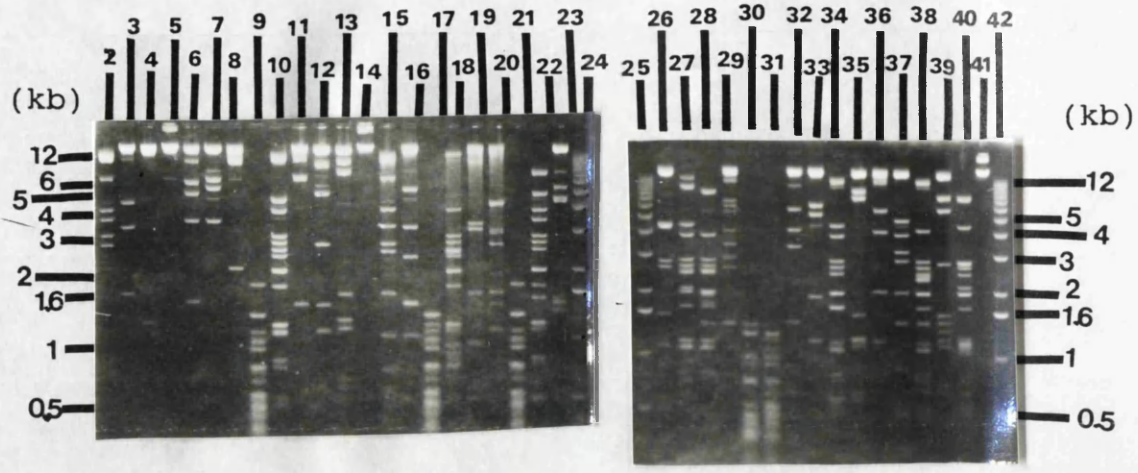
7.4.2 Identification of Lambda PEN01 and Lambda PEN02.

DNA was prepared from both Lambda PEN01 and Lambda PEN02 as described in Chapter 2. 1µg aliquots of these DNAs were run out on a 0.4% agarose gel and Southern blotted. This blot was probed with radiolabelled amal DNA at 55°C and then at 65°C. The results are shown in figure 7.6. At 55°C both Lambda



LANE	DNA
1	marker
2	Lambda PEN02 CUT
3	Lambda PEN01 CUT

Figure 7.7: SalI digested Lambda PEN01 and PEN02 DNA, probed with radiolabelled ARp1-derived ama1 DNA at 55°C. Hybridising band sizes are noted on the right hand side of the Southern blot.



LANE	ENZYME	LANE	ENZYME
1	marker	22	H/P
2	ClaI (C)	23	H/SI
3	XhoI (X)	24	marker
4	EcoRI (E)	25	marker
5	SmaI (Sm)	26	H/SII
6	SalI (S)	27	P/SI
7	HindIII (H)	28	P/SII
8	BamHI (B)	29	SI/SII
9	HaeII (Ha)	30	P/Ha
10	PstI (P)	31	SI/Ha
11	SstI (SI))	32	E/S
12	SstII (SII)	33	E/H
13	BglII (Bg)	34	E/P
14	NotI (No)	35	E/Bg
15	C/X	36	E/B
16	X/H	37	S/H
17	X/Ha	38	S/P
18	X/P	39	S/Bg
19	X/SI	40	Bg/P
20	X/SII	41	uncut
21	H/Ha	42	marker

Figure 7.8a: mapping of Lambda PEN01 showing restriction banding pattern and list of band sizes.

facing page 176b

ENZYME(S)	BAND SIZES (kb)	TOTAL(kb)
XhoI	20,13.8,5.6,3.6,3.6,2	48.6
EcoRI	20,16,11,1.2	48.2
SalI	20,9,7.3,6.9,4.4,1.4	49.0
HindIII	20,7.6,6.5,5.7,4.8,4.4	49.0
BamHI	20,10.8,7,4.2,3.5,3.4	48.9
PstI	16.4,12,5.4,4.6,3.4,3.2,3.2	48.2
SstI	21.2,11.6,7.2,6.8,1.6	47.8
BglII	21,9.4,8.7,7.5,2.2	48.8
X/H	20,7.6,5.6,4.4,2.8,2,1.8,1.8,1.8,0.8	48.6
X/P	16.4,12,3.2,3.2,2.6,2.4,2.4,1.8,1.2, 1.2,0.9,0.8	48.1
X/Ss	20,11.8,4,3.2,3,2,2,1.6,0.6,0.4	48.0
H/P	16.4,7.4,4.4,4.4,3.2,3,3,2.6,2.2,0.8	47.1
H/Ss	20,7,5.7,4.6,4.4,2.1,1.8,1.4,1,0.4	48.5
E/S	20,9,7.3,4.6,3.8,2,0.8,0.6,0.4	48.5
E/H	20,6.6,5.7,4.8,4.8,4.4,1.2,1,0.4	48.9
E/Bg	20.5,9.4,7.5,7,1.6,1.6,0.6,0.6,	48.4
E/B	20,10.8,7,3.4,3,2.4,1.2,0.4,0.4	48.6
S/H	20,4.6,4.6,4.4,4.4,3.6,3,2,1.2,1	48.8
S/P	16.4,9,3.5,3.2,3.2,3.2,2.8,2.8,1.4, 1.4,1,0.6	48.3
S/Bg	20,9,6.4,5,2.4,2.2,2.1.2,0.4	48.6
Bg/P	16.4,9.4,4,3.7,3.2,3.2,2.8,2.6,1.4, 0.8,0.4	47.9
B/H	20,6.4,4.4,4.4,4,3.4,2.4,2,1.2	48.3
B/S	20,9,5.6,3.4,3.1.9,1.8,1.4,1.4,1.4	48.9

Figure 7.8a continued: digested Lambda PEN01 fragment sizes.

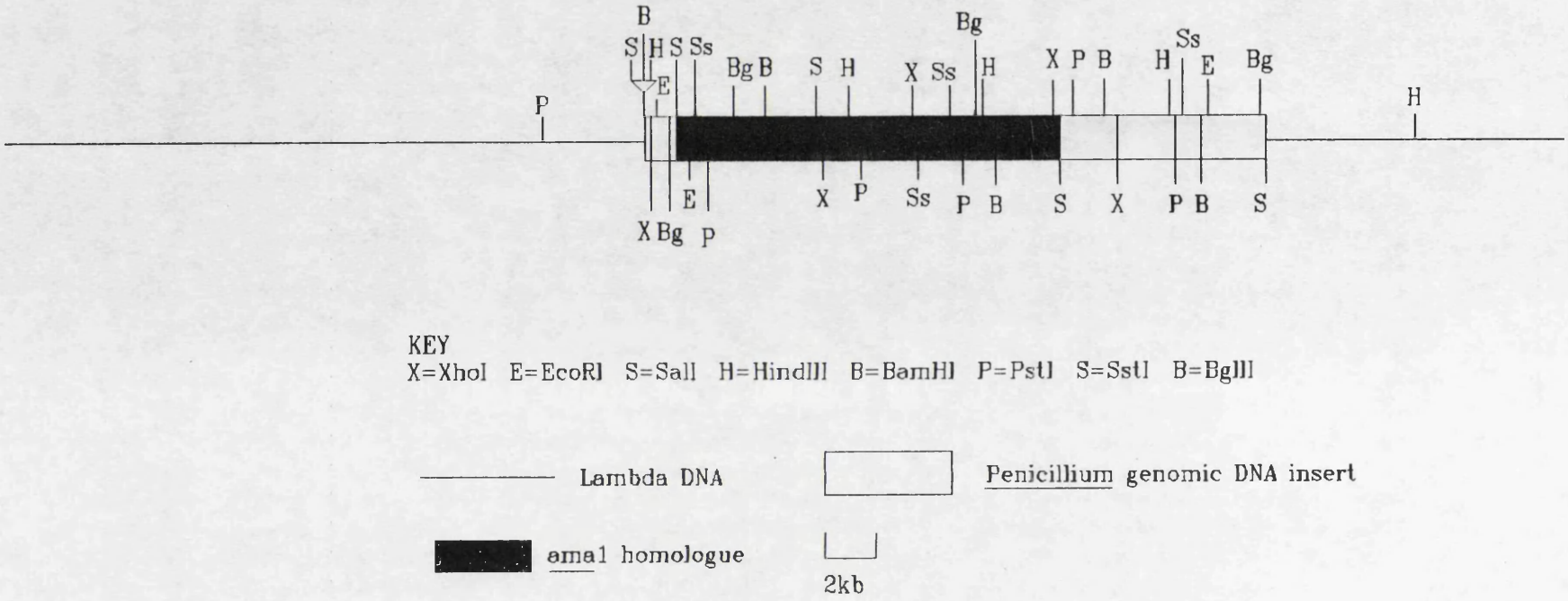


Figure 7.8b: structure of Lambda PEN01, as mapped.

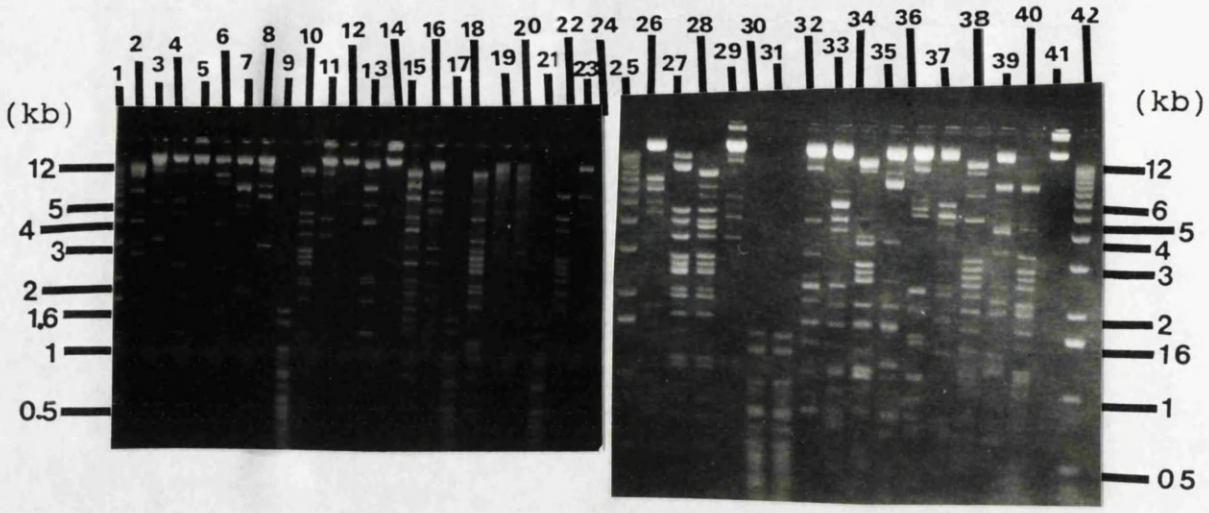
facing page 176

clones hybridise with the amal probe, but at 65°C only Lambda PEN01 hybridises. It appears therefore that amal has more sequence similarity with Lambda PEN01 than with Lambda PEN02.

2µg samples of Lambda PEN01 and Lambda PEN02 DNA were digested with 10 units of SalI and run out on a 0.8% agarose gel. The gel was Southern blotted and probed with radiolabelled amal DNA at 55°C. The results are shown in figure 7.7. Two bands in the Lambda PEN01 lane hybridise. These bands are 6kb and 4kb in size. One band of 2.4kb hybridises in the Lambda PEN02 lane.

7.4.3 Mapping of Lambda PEN01.

1µg aliquots of Lambda PEN01 DNA were digested with the enzymes listed in figure 7.8a. The digests were run out on a 0.8% agarose gel as shown in figure 7.8a. The band sizes are also listed. A map of the Penicillium DNA contained in this clone was constructed using the data in figure 7.8b. The map was assembled by following the same logic used to compile the plasmid maps described in Chapter 4. The amal-related DNA identified in figure 7.7 i.e. the 6kb and 4kb SalI fragments, are marked in. There is no evidence of amal-like inverted repeat structures present in this Lambda clone.



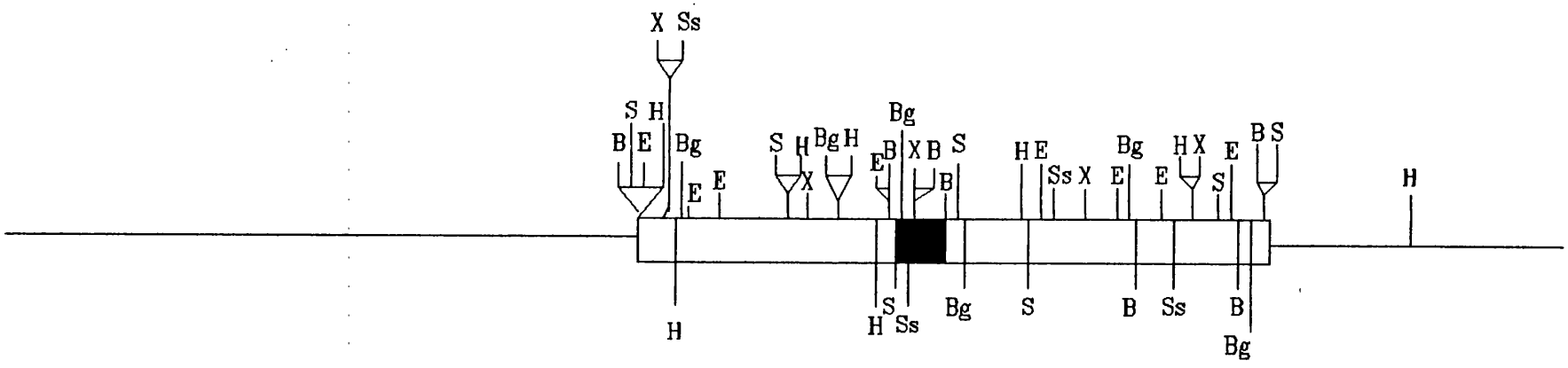
LANE	ENZYME	LANE	ENZYME
1	marker	22	H/P
2	ClaI (C)	23	H/SI
3	XhoI (X)	24	marker
4	EcoRI (E)	25	marker
5	SmaI (Sm)	26	H/SII
6	SalI (S)	27	P/SI
7	HindIII (H)	28	P/SII
8	BamHI (B)	29	SI/SII
9	HaeII (Ha)	30	P/Ha
10	PstI (P)	31	SI/Ha
11	SstI (SI))	32	E/S
12	SstII (SII)	33	E/H
13	BglII (Bg)	34	E/P
14	NotI (No)	35	E/Bg
15	C/X	36	E/B
16	X/H	37	S/H
17	X/Ha	38	S/P
18	X/P	39	S/Bg
19	X/SI	40	Bg/P
20	X/SII	41	uncut
21	H/Ha	42	marker

Figure 7.9a: mapping of Lambda PEN02 showing restriction banding pattern and list of band sizes.

facing page 177a

ENZYME(S)	BAND SIZES (kb)	TOTAL(kb)
XhoI	20.4,11.4,5.8,4.9,3.7,3.2	49.6
EcoRI	20,10.2,5.4,5.1,2.6,2.4,1.4,1.4, 1.0	49.5
SalI	20,9.2,8,4.6,3.7,2.1,2.1	49.7
HindIII	20,6.8,5.7,4.8,4.4,3.9,1.8,1,0.8	49.2
BamHI	20,9,8,6.3,3.5,1.1,0.8,0.6	49.3
SstI	20.5,11.8,7.9,4.8,4.0	49.0
BglII	21,9.2,5.4,5.1,4.2,2.2,2	49.1
X/H	20,6.8,4.4,3.8,3.7,3.6,2.1,1.1, 1,1,0.8,0.4,0.2,	48.9
X/Ss	20.4,11.4,4.9,4.6,3.1,2.9,1.2, 0.8,0.1	49.4
H/Ss	20,6.8,4.4,4,3.9,3.8,1.8,1.1,1 ,1,0.6,0.5,0.2	49.1
E/S	20,9.2,3.2,2.6,2.2,2.1,2.1,1.8, 1.4,1.4,1,1,0.4,0.4,0.2	49.0
E/H	20,5.4,4.6,4.4,2.6,2.1,1.8,1.4, 1.2,1,1,1,0.8,0.6,0.6,0.3	48.8
E/Bg	20,9.4,3.8,2.6,2.4,2.4,2,1.6,1 ,1,1,0.8,0.4,0.4,0.4	49.2
E/B	20,9.3,5.4,3.1,2.6,2.4,1.4,1.1, 1,1,0.8,0.6,0.5,0.2	50.2
S/H	20,5.6,4.6,4.4,3.9,2.1,2,1.8,1.4 ,1,0.8,0.8,0.6,0.2	49.2
S/Bg	20,9.2,3.6,3.4,3,2,2,1.8,1.6,1.2, 1,0.2,0.2,0.2	49.4
B/H	20,4.6,4.4,3.9,3.8,2.6,2,1.8,1.6, 1,0.8,0.8,0.6,0.2	48.1
B/S	20,9.2,4.6,3.6,3.2,2.8,2.1,0.8, 0.6,0.6,0.4,0.2	48.1

Figure 7.9a continued: digested Lambda PEN02 fragment sizes.



KEY

X=XhoI E=EcoRI S=SalI H=HindIII B=BamHI Ss=SstI Bg=BglII

Lambda phage DNA

Penicillium genomic DNA insert

amaI homologue

2kb

Figure 9.9b: structure of Lambda PEN02, as mapped.

facing page 177

7.4.4 Mapping of Lambda PEN02.

1µg aliquots of Lambda PEN02 DNA were digested with a variety of enzymes then run out on a 0.8% agarose gel. The gel and the resulting bands are shown in figure 7.9a. A map of this clone was constructed using the data in figure 7.9b and by following the logic used used to construct the plasmid maps detailed in Chapter 4. The ama1 related DNA identified in figure 7.7 i.e. the 2.4kb SalI fragment, is marked in. There is no evidence of ama1-like inverted repeat structures present in this clone.

7.5.1 Cotransformations of Pencillium with the Lambda clones and pMW14.

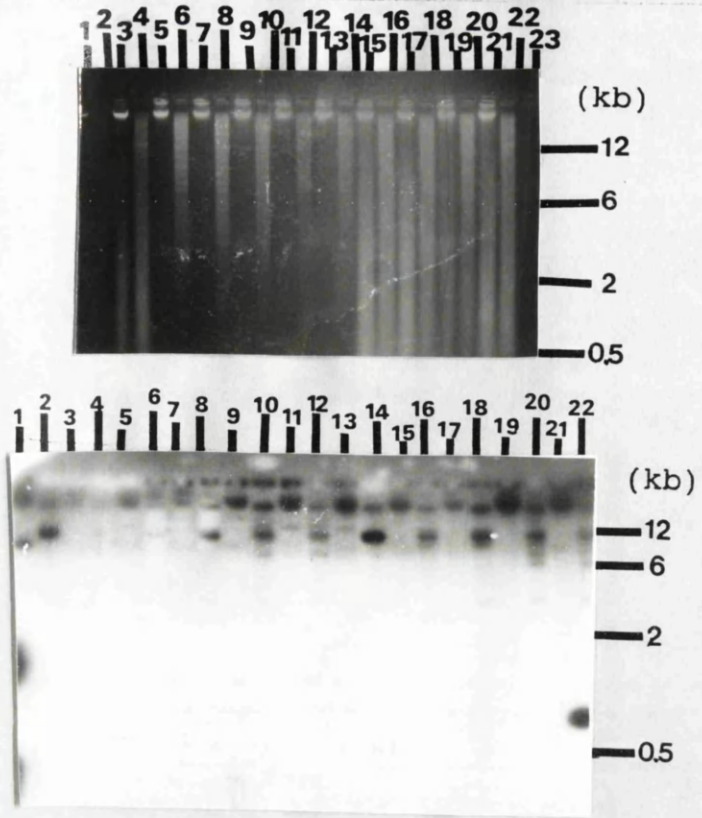
15µg of Lamda PEN01 and Lambda PEN02 were digested with 20 units of SalI. The enzyme was heat inactivated. The digested Lambda DNAs were mixed with 15µg aliquots of pMW14. These DNA mixes were used to transform $1x10^8$/ml Penicillium protoplasts. Transformations with pMW14, pJSR10 and a pHELP1/pMW14 cotransformation control were included. The plasmid pHELP1 consists the 5kb HindIII ama1 fragment and pIC20, but it does not contain any fungal selectable markers. Also included was a EMBL3/pMW14 cotransformation which measures the effect, if any, of Lambda DNA in cotransformations. The results for

	TRANSFORMATION FREQUENCY/µg DNA			%INSTABILITY/ ASEXUAL GENERATION		
attempt	1	2	3	1	2	3
PLASMID						
no DNA	0	0	0	-	-	-
pMW14	10	13	5	0	0	0
pJSR10	35	53	61	58	56	53
pMW14/ pHELP1	59	50	72	61	57	60
PENO1/ pMW14	51	135	67	65	68	61
PENO2/ pMW14	11	17	25	70	75	72
Lambda/ pMW14	9	13	11	0	0	0

Figure 7.10: Penicillium cotransformations with pMW14, pHELP1 and both Lambda PENO1 and PENO2.

each experiment are detailed in figure 7.10.

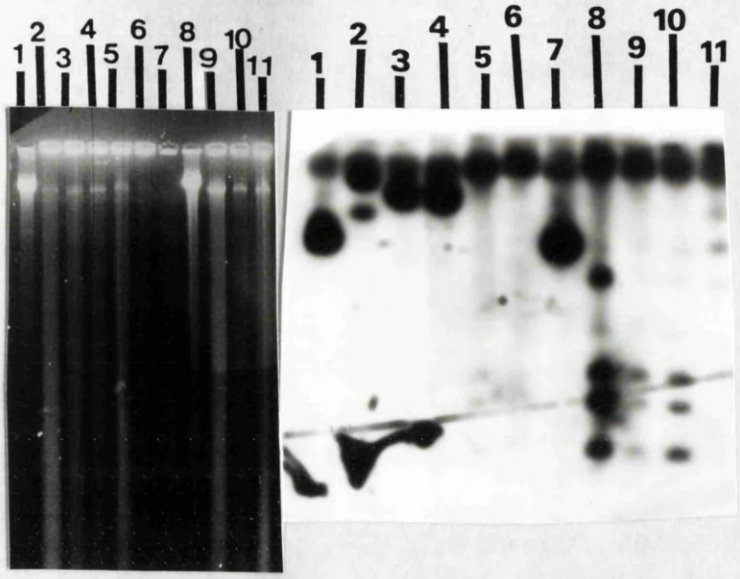
It is clear from the results in figure 7.10 that integrative vector pMW14 behaves as expected i.e. low frequency of transformation, 5-10 transformants/µg DNA and completely stable, 0% plasmid loss. Similarly the autonomously replicating vector pJSR10 gives an increased frequency of transformation, 55-61 transformants/ug DNA and is unstable, 56% of progeny derived from pJSR10 transformants lose the plasmid. These results are similar to the results described in section 7.2.2. The pMW14/pHELP1 cotransformation results suggest that pMW14 and pHELP1 have formed an autonomously replicating cointegrate plasmid i.e. the average transformation frequency is 60 transformants/ µg DNA and 59% of progeny derived from these transformants lose the cointegrate plasmid. Therefore the cointegrate plasmid is behaving in a similar fashion to pJSR10. It appears that cotransformations with both Lambda PEN01 and PEN02 result in the formation of unstable cointegrates: 65% and 72% of progeny derived from Lambda PEN01/pMW14 and Lambda PEN02/pMW14 transformants lose the cointegrate plasmid respectively. Lambda PEN01/pMW14 shows an increase in transformation frequency: 84 transformants/µg DNA, but there is only a slight increase with Lambda PEN02/pMW14: 18 transformants/ µg DNA. The Lambda/pMW14 control shows no increase in transformation frequency or any plasmid instability, therefore it is likely that the results



LANE	DNA	LANE	DNA
1	TARp1/1.1	13	TPEN01/14/1.1
2	TARp1/1.1 CUT	14	TPEN01/14/1.1 CUT
3	T14/1.1	15	TPEN01/14/1.2
4	T14/1.1 CUT	16	TPEN01/14/1.2 CUT
5	T14/1.2	17	TPEN02/14/1.1
6	T14/1.2 CUT	18	TPEN02/14/1.1 CUT
7	TJSR10/1.1	19	TPEN02/14/1.2
8	TJSR10/1.1 CUT	20	TPEN02/14/1.2 CUT
9	TJSR10/1.2	21	TPEN02/14/1.3
10	TJSR10/1.2 CUT	22	TPEN02/14/1.3 CUT
11	THELP1/14/1.1	23	marker
12	THELP1/14/1.1 CUT		

LANE	band sizes (kb)
2	11,
4	11,
6	11,
8	20, 11, 4,
10	20, 11, 7, 4,
12	20, 11, 7, 4.8, 4,
14	11, 4,
16	11, 9,
18	11, 9, 4,
20	11, 9, 7, 6.4, 4,
22	11, 6.4, 4, 0.6

Figure 7.11a: genomic DNA from various Penicillium transformants, digested with BglII. Probed with radiolabelled pUC8 DNA.



LANE	DNA
1	TARp1/1.3
2	TJSR10/1.1
3	TJSR10/1.2
4	THELP1/14/1.1
5	THELP1/14/1.2
6	TPEN01/14/1.1
7	TPEN01/14/1.2
8	TPEN01/14/1.3
9	TPEN02/14/1.1
10	TPEN02/14/1.2
11	TPEN02/14/1.3

Figure 7.11b: uncut genomic DNA from various Penicillium transformants. Probed with radiolabelled pUC8 DNA. The TARp1/1.3 DNA was prepared from an Aspergillus transformant.

with the Lambda clones may be due to functional ama1-like Pencillium DNA sequences.

Total genomic DNA was made from 4 individuals from each of the transformant classes; total genomic DNA had been previously prepared from pJSR10 transformants, see section 7.2.2. 1µg samples of some of these total genomic DNAs were run on a 0.8% agarose gel along with a control sample of genomic DNA from an ARp1 Aspergillus transformant. The gel was Southern blotted and probed with radiolabelled pUC DNA. The results are shown in figure 7.11a; free plasmid is present in a number of lanes: lane 12 contains 2 bands, one of which is identical to the single band in lane 11; lane 21 contains 5 bands, 3 of these bands are also present in lane 19. The banding pattern and band sizes are listed in figure 7.11a. As can be seen from this list, a number of bands are common to two or more lanes: all the digested DNA lanes contain a 11kb band; lanes 8, 10, 14, 18, 20 and 22 all contain a 4kb band, lane 16 lacks this 4kb band but this lane contains a 9kb band which is also present in lanes 18 and 20.

Total genomic DNA samples were made from a further 4 individual transformant colonies from each class. 2µg aliquots of these DNA samples were run uncut on a 0.8% agarose gel and Southern blotted. This blot was probed with radiolabelled pUC DNA. The results of this blot are shown in figure 7.11b, and with the exception of lane 6, free plasmid can be

seen in all lanes. The TPEN02/14 lanes, (9, 10 and 11) contain three bands in common; lanes 9 and 10 also contain 3 additional larger bands. Lanes 8, 9 and 10 all contain similar bands, but these bands vary in intensity and some of these bands are fast running. Lanes 3 and 4 contain a single common band.

7.5.2. Plasmid rescues with Lambda/pMW14 transformant genomic DNAs.

I made three separate attempts to rescue Lambda clone-derived free plasmids into an E.coli host. In each attempt I could rescue ARp1 DNA from the control DNA from an average of 4 E.coli transformant colonies, (gel not shown). I isolated unrearranged pJSR10 from a single E.coli transformant colony, (gel not shown) but I did not obtain any plasmids from any of the other genomic DNA samples.

This result indicates that either the plasmids have been rearranged and are no longer rescuable or else the competence of the E.coli hosts was simply not high enough. The fact that only 4 ARp1 E.coli transformant colonies were generated, strongly suggests that one problem lies with the competence of the E.coli host cells. The problems of plasmid rescues are discussed more fully in chapter 8.

	TRANSFORMATION FREQUENCY/µg DNA			%INSTABILITY/ ASEXUAL GENERATION		
attempt	1	2	average	1	2	average
PLASMID						
ARp1	-	-	32,000	55	58	56
no DNA	0	0	0	-	-	-
pILJ16	30	18	24	0	0	0
PEN01/ pILJ16	120	200	160	65	63	64
PEN02/ pILJ16	70	11	40	83	87	85

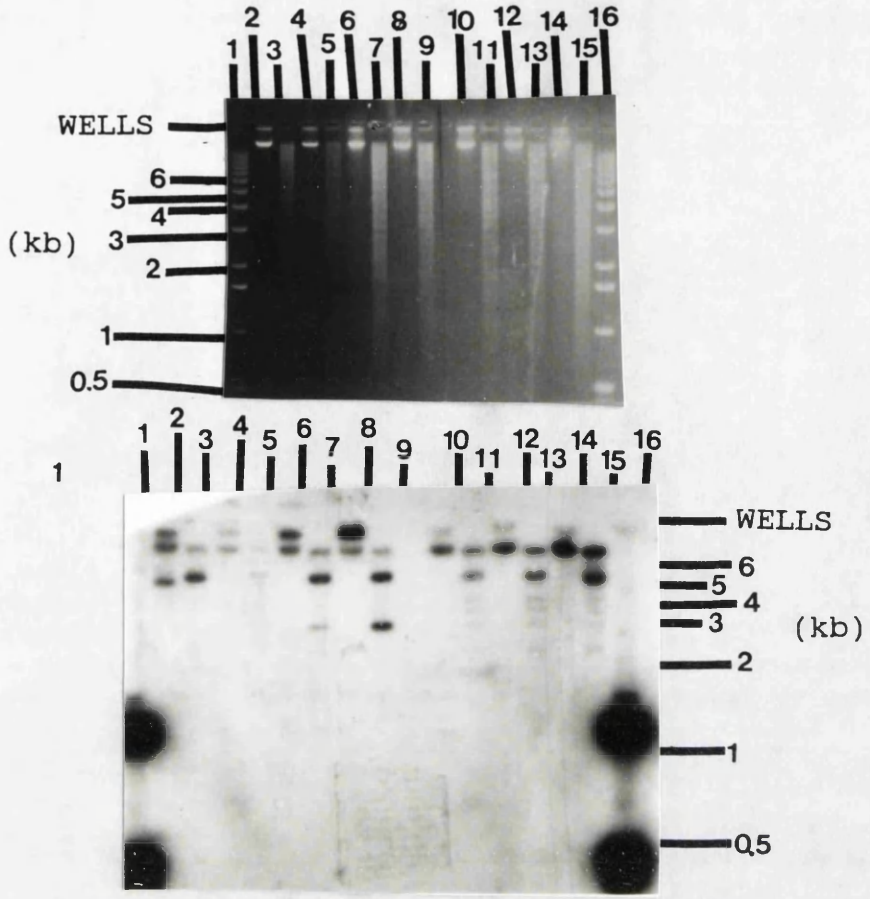
Figure 7.12: Aspergillus cotransformations with pILJ16 and the Lambda clones PEN01 and PEN02; with ARp1 and pILJ16 controls.

7.6.1 Cotransformations of *Aspergillus* with the Lambda clones and pILJ16.

The results in the previous sections suggest that the ARp1-derived *ama1* sequence is functional in *Penicillium*. The question I now wanted to ask was whether the *Penicillium* Lambda PEN01 and PEN02 sequences would be functional in *Aspergillus*.

Therefore, I digested 1µg of each of the Lambda PEN clones with 10 units of SalI. The enzyme was heat inactivated and the digested DNA mixed with 1µg of pILJ16 and used to transform 1.9×10^7 *A.nidulans* G34 protoplasts. ARp1, pILJ16 and NO-DNA control transformations were included. The results of two independent experiments are summarised in figure 7.12.

The Lambda PEN01/pILJ16 cotransformation yields on average, 160 transformants/µg DNA, a 6-fold increase when compared to pILJ16. Also, 64% of progeny derived from Lambda PEN01/pILJ16 transformants lose the plasmid. These results indicate that the Lambda PEN01/pILJ16 cointegrate is capable of autonomous replication. In contrast, the Lambda PEN02/pILJ16 cotransformation leads to a two-fold increase in transformation frequency: 40 transformants/µg DNA. However, 88% of progeny derived from these transformants lose the cointegrate. The results for the Lambda PEN01/pILJ16 and Lambda PEN02/pILJ16 cotransformations mirror the



LANE	DNA	LANE	DNA
1	marker	10	TPEN02/16/1.1
2	TARp1/4.3	11	TPEN02/16/1.1 CUT
3	TARp/4.3 CUT	12	TPEN02/16/1.2
4	TPEN01/16/1.1	13	TPEN02/16/1.2 CUT
5	TPEN01/16/1.1 CUT	14	TPEN02/16/1.3
6	TPEN01/16/1.2	15	TPEN02/16/1.3 CUT
7	TPEN01/16/1.2 CUT	16	marker
8	TPEN01/16/1.3		
9	TPEN01/16/1.3 CUT		

LANE	3	5	7	9	12	14	16
band sizes (kb)	6	7	6	6	6	6	5.8
					4.6	4.6	4.6
					4.2	4.2	4.2
			3.5	3.5	3.7	3.7	3.7
					3	3	3

Figure 7.13: genomic DNA from Aspergillus pILJ16/Lambda PEN cotransformants, digested with PstI. Probed with radiolabelled pUC8 DNA.

results described in section 7.5.1. These results indicate that the Penicillium-derived clones are capable of replicating autonomously in Aspergillus.

Total genomic DNA was made from two Lambda PEN01 /pILJ16 and two Lambda PEN02/pILJ16 transformant colonies. 2µg aliquots of these DNAs were digested with 20 units of BglII. The digested DNA and 2µg aliquots of undigested DNA were run on a 0.8% agarose gel, Southern blotted and probed with radiolabelled ArgB DNA. The results are shown in figure 7.13. It is clear from this blot that with the exception of the TARp1 control no free plasmid is visible either above or below the chromosomal band; note that some DNA is stuck in the wells. The PstI digested TARp1 control, (lane 3), produces a 6kb doublet band. The digested LAMBDA PEN01/16 DNA samples, (lanes 5,7 and 9), produce two types of banding pattern: lanes 7 and 9 are identical. The LAMBDA PEN02/16 DNA samples, (lanes 12, 14 and 16), also produce two types of banding pattern: lanes 12 and 14 are identical. These results suggest that the cointegrate plasmids have undergone rearrangements in different transformant colonies. The next step was to attempt to rescue plasmids from these DNA samples.

7.6.2. Plasmid rescues with Lambda/pILJ16 transformant genomic DNAs.

Three separate attempts were made to rescue plasmid from these DNA samples. Results similar to those noted in section 7.4.3 were obtained. It was possible to rescue and isolate ARp1 from two E.coli transformant colonies, from three attempts. No E.coli transformants were obtained with the Lambda/pILJ16 DNA samples.

7.7 Discussion.

The transformation results with pJSR10 in section 7.2.2 show that the Aspergillus amal sequence is capable of autonomous replication in Penicillium. It is immediately clear that the transformation frequency of pJSR10, (5 transformants/µg DNA), is 200-fold less than a comparable plasmid containing the same amal sequence: pDHG25, (1000 transformants/ µg DNA). This difference could simply be due to variations in DNA binding site recognition between the two species. The amal sequence apparently does not promote autonomous replication in Cephalosporium. These results may be due to the degree of relatedness between Aspergillus, Penicillium and Cephalosporium, as shown by the Southern blot in figure 7.5. Pencillium sequences hybridise with the amal probe; Cephalosporium DNA does not. Aspergillus is more

closely related to Penicillium than to Cephalosporium, Peberdy (1985).

The results presented in this chapter describe two specific Penicillium genomic DNA sequences which are capable of promoting autonomous replication in both Penicillium and Aspergillus. These DNA sequences are present on 6kb, 4kb and a 2.4kb SalI fragments, (sections 7.4.1 to 7.4.3.). It also appears that cointegrate plasmids containing these Penicillium sequences are rearranged in Aspergillus.

In keeping with the designation of the ARp1-derived ama1 sequence, the 6kb and 4kb fragments were called pam1a and pam1b; the 2.4kb fragment was called pam2: pam refers to Penicillium Autonomous Maintenance (sequence). The 6kb pam1 SalI fragment has since been cloned into pMW14; the resulting plasmid was called PRp1: Penicillium Replicative plasmid 1, (details not given).

The differences in transformation frequency between pam1a, pam1b and pam2 could be due to either the differences in size between the sequences or to differences in base composition. As shown in chapter 4, differences in autonomous plasmid behaviour are complicated and appear to be due to the composition of the sequences involved in autonomous replication, positional effects and interactions between these sequences; ARp1 clearly displays its use as a model system to explain the behaviour of autonomously replicating plasmids in fungi other than Aspergillus.

The next step is to sequence pam1a, pam1b and pam2 and compare the sequence data with that of ama1. The gross structure of pam1a, pam1b and pam2 are unlike ama1 i.e. there is no apparent inverted repeat structure. Therefore, it is probably more accurate to consider pam1a, pam1b and pam2 as equivalent to the cosmid derived ama1-like sequences described in Chapter 6, since the sequence derived from cosmid L26F10 allows autonomous replication but has a low transformation frequency. Another obvious experiment is to use the pam1 and pam2 sequences as probes to try to identify Cephalosporium DNA which is homologous to the pam sequences. The rearrangemnts already mentioned could be examined in greater detail by looking at Southern blots of genomic DNA from successive generations of transformant colonies.

Chapter 8

An Instant Gene Bank.

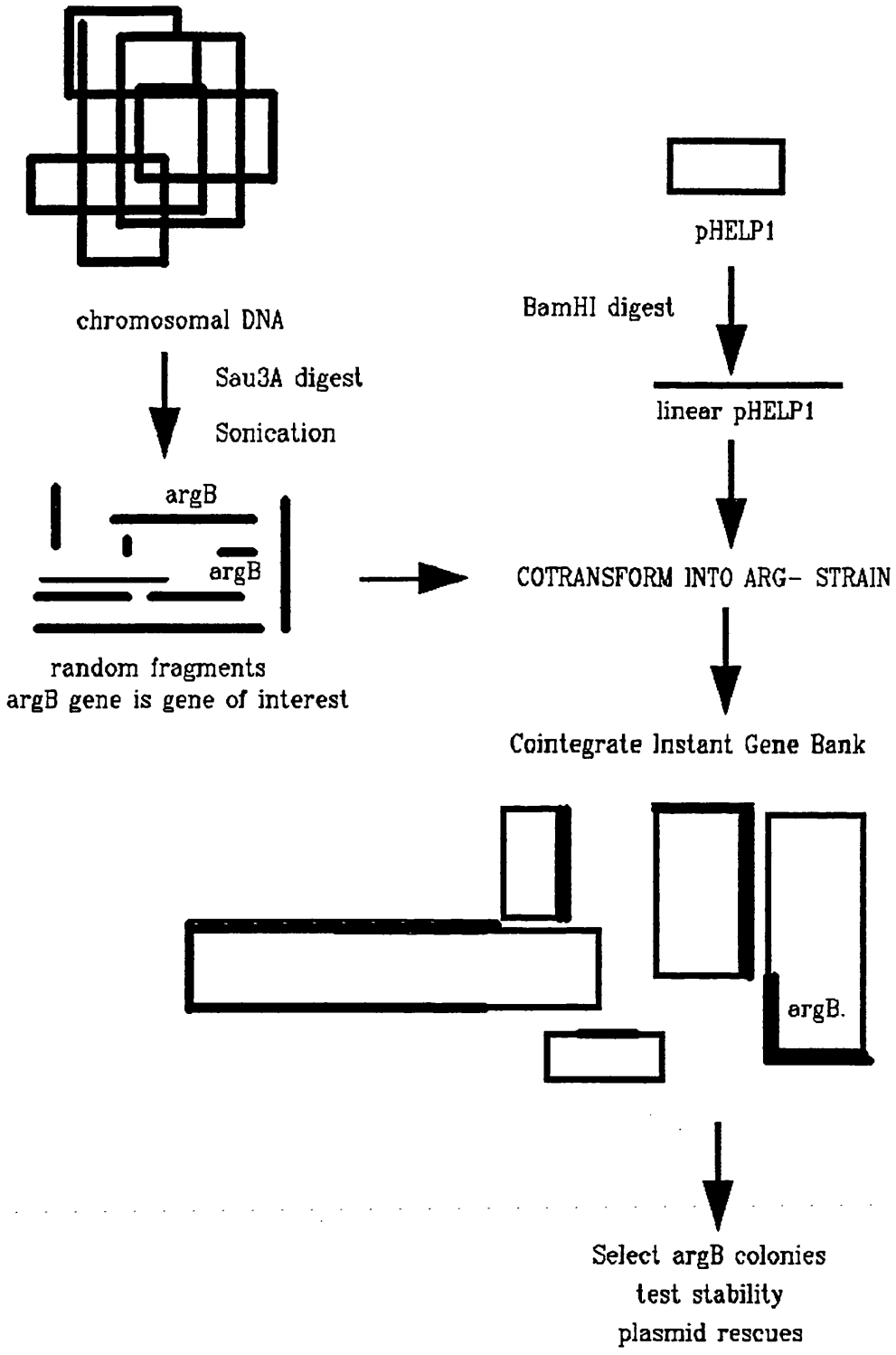


Figure 8.1: basic scheme of the Instant Gene Bank.

8.1.1 Introduction.

The original idea of the instant gene bank was put forward by David Gems (1990). Gems reasoned that it should be possible to clone genes by utilising the properties of cotransformation.

The basic experiment is outlined in figure 8.1, using the argB gene as an example. Random fragments are first generated from wild type, fungal genomic DNA. Fragments large enough to contain intact and fully functional genes e.g. 10kb, are then mixed with a plasmid capable of autonomous replic ation; this replicative plasmid does not contain any fungal selectable markers. This DNA mixture is then used to transform protoplasts made from an auxotrophic mutant i.e an Arg- strain. The gene of interest, argB, is then cloned by plating out the transformed protoplasts onto non-arginine containing (selective) media. Only colonies containing the argB gene will grow.

These transformant colonies are of two distinct types. One type should contain a cointegrate plasmid consisting of the autonomously replicating plasmid and a genomic DNA fragment containing the argB gene. This cointegrate plasmid is produced by recombination. The other type of transformant is produced by a straight forward integration of the random fragment into the genome. These two types of transformant are easily distinguished by plasmid

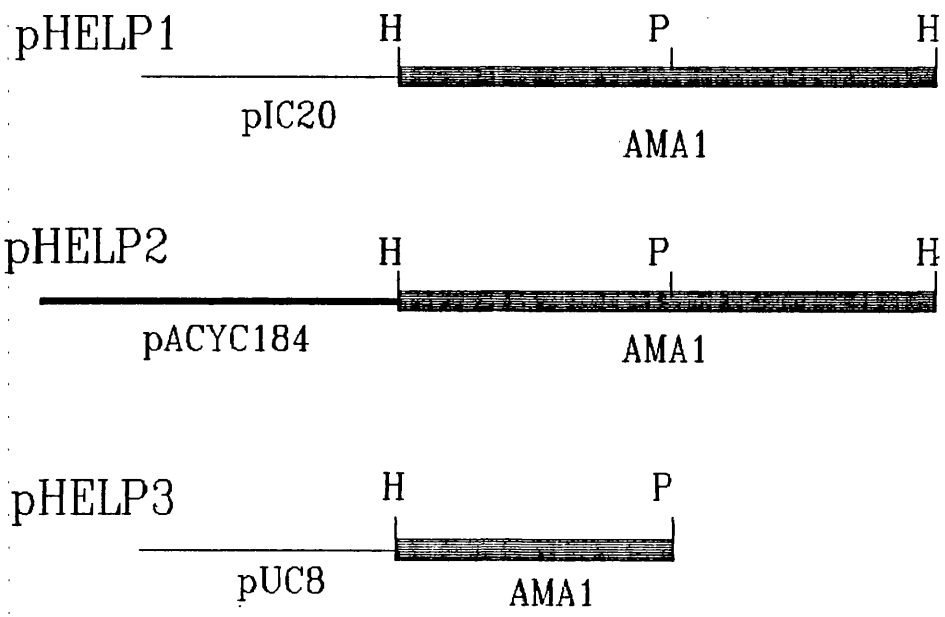


Figure 8.2: structures of pHELP plasmid series. (Gems, 1990)

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instability tests; if the ARG+ phenotype appears to be unstable then the gene is on a cointegrate plasmid; if the ARG+ phenotype is not unstable then the gene has integrated. The cointegrate plasmid is then isolated by plasmid rescue.

Gems constructed three plasmids containing amal DNA and no selectable marker. These plasmids were designated HELPER plasmids. Figure 8.2 shows the stucture of pHELP1, pHELP2 and pHELP3. Both pHELP1 (7.9kb) and pHELP2 (9.1kb), contain the 5kb HindIII amal fragment; these plasmids differ only in the bacterial vector. The plasmid pHELP3 is 5.8kb in size and consists of the HindIII/PstI fragment of ARp1: in effect this fragment is the entire left hand arm of the amal sequence and the pUC8 DNA, (see figure 3.1).

Using pHELP3, Gems successfully cloned the A.nidulans adC-adD genes which code for the adenine biosynthetic enzyme AIR carboxlyase. However, Gems found that the structure of the rescued cointegrate plasmid varied from E.coli transformant to E.coli transformant. The cointegrate plasmid structure also appeared highly variable in the original Aspergillus transformants. It is reasonable to assume that the cointegrates were being rearranged. This result is no suprise, the work described in Chapter 4 shows that any plasmid which does not have the intact amal inverted repeat as defined by the 5kb HindIII fragment will be rearranged, so the cotransformation

would be better carried out using either pHELP1 or pHELP2.

8.1.2 Experimental approach.

The experiments in this chapter describe attempts to isolate the Penicillium argB, niaD and nirA genes using cotransformation and selection in argB, niaD and nirA deficient Aspergillus mutants. The genotypes of the Aspergillus strains used are described in Chapter 2.

Based on both the experimental results by Gems (1990), and my results described in Chapter 4, I decided to use pHELP1 in the cotransformation; the structure of this plasmid should minimise any rearrangements. In addition, Gems (1990), demonstrated that pHELP1 cotransforms with a higher efficiency than pHELP2; the efficiency appears to be dependent on the composition of the bacterial DNA on the plasmid. The frequency of cotransformation in this sort of cloning experiment is critical since the number of transformant colonies produced by the cotransformation will be very low. The transformation frequency will be reduced because the selected piece of DNA will be rare.

I cotransformed using cut and uncut pHELP1. Experiments by Gems (1990), demonstrated that cointegrate plasmids are produced by recombination between homologous DNA sequences or, with lower

frequency, by non-homologous recombination. In using linear HELPER plasmid I attempted to "force" recombination to occur at a known site, thereby simplifying characterisation of the resulting cointegrate. In this situation, a replicating plasmid will be produced by ligation in the fungus of this linear DNA.

The plasmid instability tests for the argB gene were identical to those described in Chapter 4. The instability tests for both the niaD and nirA genes were similar to those carried out for the argB gene but with one difference. The conidia to be tested were plated on minimal medium rather than complete medium, where wild type colonies grow and conidiate as normal; colonies which develop from spores which are either niaD or nirA also grow, but very sparsely.

Therefore, marker- colonies can be clearly differentiated from marker+, plasmid instability values can then be calculated. It should be noted that only colonies which were conidiating, (marker+), were selected from the original transformant plates and these were subsequently streaked out onto minimal medium to obtain colonies derived from single conidia before testing for plasmid stability.

8.2.1 Preparation of wild type Penicillium DNA.

DNA was prepared from 3-day old cultures grown in 10x200 mls of liquid complete media described in Chapter 2. This genomic DNA was then digested in small-scale, pilot Sau3A digests. Large-scale digests were then set up: 0.2 units Sau3A/ µg DNA, this process is described in detail in Chapter 7. These large-scale digests were left for 1 hour and generated fragments of 10-15kb in size. The enzyme was inactivated by addition of EDTA and the DNA preparation was purified with extractions by phenol/chloroform and chloroform and then ethanol precipitation. The genomic DNA was finally resuspended, the concentration measured and the DNA solution stored in 10ug aliquots at $4^{o}C$.

8.2.2 Preparation of pHELP1 and control DNA.

Approximatly 5µg of pHELP1 was digested with 20 units of BamH1 for 3 hours, extracted with phenol/chloroform and chloroform and then ethanol precipitated. In addition, 5µg aliquots of control DNA i.e. pILJ16, ARp1 and pHELP1, were extracted and precipitated as described above, these aliquots were not digested with BamH1.

The plasmids were resuspended in 30µl TE and the concentration measured by running 3µl samples on a 0.8% gel, (gel not shown).

Experiment	Strain	Gene	total no. of colonies		Transformation frequency µg/DNA		Instability (%loss) /asexual generation		no. of E.coli colonies from rescues	
			uncut pHELP1	cut pHELP1	uncut pHELP1	cut pHELP1	uncut pHELP1	cut pHELP1	uncut pHELP1	cut pHELP1
1	G34	argB	21	20	10	10	63	64	6	5
	G833	nirA	2	3	1	1	67	70	4	5
	G0125	niaD	10	8	5	4	65	69	2	5
2	G34	argB	2	4	1	2	65	63	0	0
	G833	nirA	35	39	17	20	68	66	0	0
	G0125	niaD	30	29	15	15	65	64	0	0
3	G34	argB	38	20	19	10	64	65	1	3
	G833	nirA	10	21	5	10	60	62	6	5
	G0125	niaD	11	13	5	6	62	62	2	2

Figure 8.3: Instant Gene Bank results showing the total number of fungal transformants, transformation frequencies, cointegrate plasmid instabilities obtained from three experiments.

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8.2.3 Cotransformation of *Aspergillus* with *Penicillium* genomic DNA and pHELP1.

Protoplasts were made from *Aspergillus* strains G34 (*yA2; methH2; argB2-*), G833 (*yA2; pyroA4; nirA1-*) and G0125 (*biA1; niaD17-*). 1µg aliquots of digested and undigested pHELP1 were mixed separately with 1µg aliquots of the prepared *Penicillium* genomic DNA fragments. Approximately $1.8x10^7$ protoplasts were transformed with each of the DNA mixes. ARp1 and pILJ16 transformations of G34 protoplasts were included as controls. NO-DNA controls were also included. All transformations were repeated three times with fresh protoplasts and DNA aliquots.

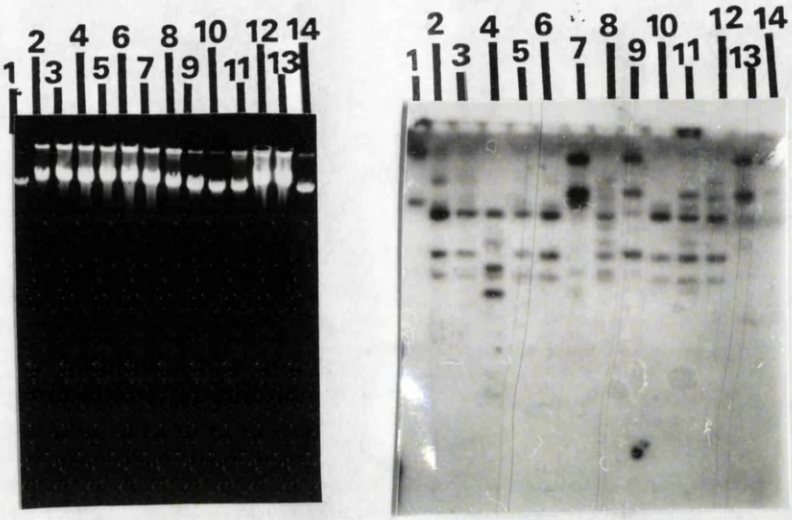
The results of these experiments are detailed in figure 8.3. The table shows the total number of transformants obtained per transformation and the calculated transformation frequency. The % plasmid loss was calculated from 5 individual transformants over 5 generations, but only the total average % loss figures are shown in the table.

It is immediately clear that the *argB*, *nirA* and *niaD* genes have been cloned because colonies were obtained, whereas no colonies grew on the NO-DNA plates. As expected, (see section 8.1.2), the transformation frequencies are low, ranging from 1 to 20 transformants/ µg DNA, regardless of the gene under selection. There is no real difference in transformation frequency when cut and uncut pHELP1

samples are compared e.g. the largest difference is found in experiment 3; cloning the argB gene, in which the uncut pHELP1 cotransformation yields 9 more transformants than the cut pHELP1 cotransformation. These results support the observations detailed in Chapter 4, that linear DNA is as efficient as circular DNA in fungal transformation.

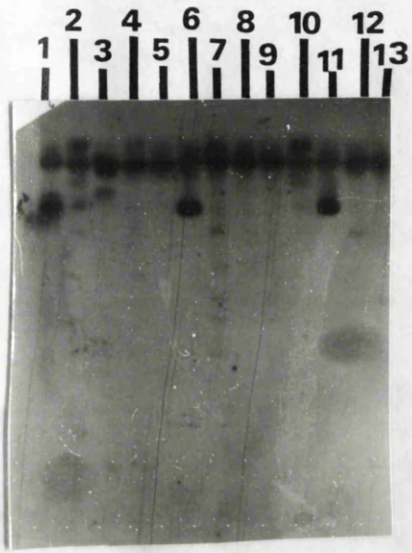
It is also clear from the plasmid % instability results in figure 8.3 that the genes under selection have been incorporated into a replicating cointegrate plasmid. In all cases the gene under selection is unstable, indicating that the gene is plasmid-borne. Again, there is little difference in the behaviour of uncut pHELP1 compared to cut pHELP1 e.g. the largest difference in % instability is found in experiment 1; cloning the niaD gene, in which the variation in behaviour between linear and circular pHELP1 is 4%.

Total genomic DNA was made from 5 individuals of the fifth conidial generation of each class, from all three experiments. The genomic DNA was made from the fifth generation because these individuals should only contain cointegrate plasmids carrying the gene of interest, any other cointegrate plasmid should have been lost during the successive subculturings. 3µg aliquots of the genomic DNAs and an ARp1 transformant control, were run on 0.8% agarose gels and Southern blotted. These blots were then probed with radiolabelled pUC DNA. The results are shown in figures 8.4, 8.5 and 8.6. The origin of each DNA



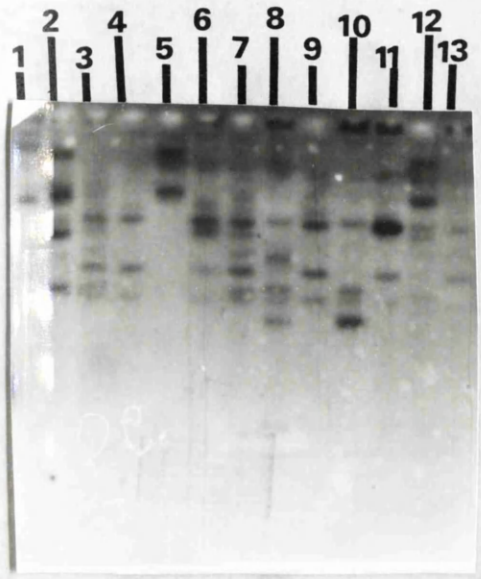
LANE	DNA	LANE	DNA
1	TARp1	9	TNIR1.3
2	TARG1.1	10	TNIR1.4
3	TARG1.2	11	TNIR1.5
4	TARG1.3	12	TNIA1.1
5	TARG1.4	13	TNIA1.2
6	TARG1.5	14	TNIA1.3
7	TNIR1.1		
8	TNIR1.2		

Figure 8.4: Southern blot of undigested genomic DNA from Instant gene bank transformants, (experiment 1). TARG are *argB*$^+$ transformants, TNIR are *nirA*$^+$ transformants and TNIA are *niaD*$^+$ transformants.



LANE	DNA	LANE	DNA
1	TARp1	9	TNIR2.4
2	TARG2.1	10	TNIA2.1
3	TARG2.2	11	TNIA2.2
4	TARG2.3	12	TNIA2.3
5	TARG2.4	13	TNIA2.4
6	TNIR2.1		
7	TNIR2.2		
8	TNIR2.3		

Figure 8.5: Southern blot of undigested genomic DNA from Instant gene bank transformants, (experiment 2). TARG are *argB*$^+$ transformants, TNIR are *nirA*$^+$ transformants and TNIA are *niaD*$^+$ transformants.



LANE	DNA	LANE	DNA
1	TARp1	9	TNIR3.4
2	TARG3.1	10	TNIA3.1
3	TARG3.2	11	TNIA3.2
4	TARG3.3	12	TNIA3.3
5	TARG3.4	13	TNIA3.4
6	TNIR3.1		
7	TNIR3.2		
8	TNIR3.3		

Figure 8.6: Southern blot of undigested genomic DNA from Instant gene bank transformants, (experiment 3). TARG are *argB*$^+$ transformants, TNIR are *nirA*$^+$ transformants and TNIA are *niaD*$^+$ transformants.

sample is marked in each of the figures.

The results in figure 8.5 suggest that multiple plasmid classes were not obtained in experiment 2, although free plasmid DNA is visible in lanes 1, 2, 3, 6, 7, 10, 11 and 12.

It is clear from figures 8.4 and 8.6, (experiments 1 and 3), that there are multiple plasmids present in the DNA samples even in one transformant.

The multiplicity of plasmids obtained in experiments 1 and 3 can be interpretated in a number of ways. It is possible that different cointegrates each contain the gene under selection, but have different structures and DNA compositions, thereby producing cointegrates of different sizes. Alternatively, only a small number of the cointegrates contain the gene under selection; the other plasmids do not contain the gene but are fortuitously retained in the transformant. A third possibility is that cointegrates are formed by multimerisation, these cointegrates are then resolved or fragmented, producing multiple banding patterns on the blots.

The theories mentioned above can be tested by rescuing the plasmids into a recombination deficient E.coli host e.g. E.coli SURE cells. A recombination deficient host avoids the possibility of the cointegrate plasmid being resolved in the host.

The possibilty that cointegrates recombine to

form larger or more complicated cointegrates is intriguing. Intramolecular recombination could lead to either resolution of a co integrate plasmid or to rearrangements of DNA within the cointegrate. More complex interactions such as rearrangement followed by resolution could occur. In short, cointegrates could be complex, which might make plasmid rescue and characterisation of the rescued plasmid difficult.

8.2.4 Plasmid rescues.

All of the DNA samples mentioned in section 8.2.4 were used in plasmid rescues. Unless otherwise stated, the host E.coli were made competent using the TSB method described in Chapter 2. In an effort to increase the proportion of plasmid in the fungal total genomic DNA samples, I pretreated 1ug aliquots with unbuffered phenol, (G.Griffith, personal communication). CCC plasmid should remain in the aqueous phase but linear DNA i.e. genomic DNA would be lost into the organic layer, thus giving overall enrichment for plasmid in the samples.

Untreated genomic DNA from experiment 1 was used to transform both E.coli SURE cells and DS941 cells. Genomic DNA from experiment 2 was treated with unbuffered phenol to enrich for plasmid DNA and then used to transform both E.coli SURE cells and DS941 cells. The SURE cells are deficient for a variety of recombination functions; the DS941 cells are recA$^-$,

(see Chapter 2). Finally, genomic DNA from experiment 3 was treated with unbuffered phenol to enrich for plasmid DNA and then used to transform Stratagene high competence E.coli SURE cells. No further rescues were attempted.

The results for the plasmid rescues for each attempt are outlined in the final column of figure 8.3. It should be noted that control transformations with total genomic DNA from an ARp1 transformant were carried out. On average, these control transformations yielded 30 E.coli transformants which further analysis showed contained ARp1, (results not shown). The results in figure 8.3 show that plasmid rescues were not possible from any DNA samples, (except the ARp1 control), from experiment 2. This result suggests that these cointegrate plasmids are not viable in E.coli. The reason for this inability to rescue these plasmids is not clear but could be due to either plasmid structure, rearrangements, deletions or any combination of these possibilities.

It was however possible to rescue plasmids from the remaining DNA samples. The results show that there was no difference in rescue efficiency between either DS941 and SURE cells or TSB prepared host cells and expensive, commercially prepared high competence cells, (results not shown).

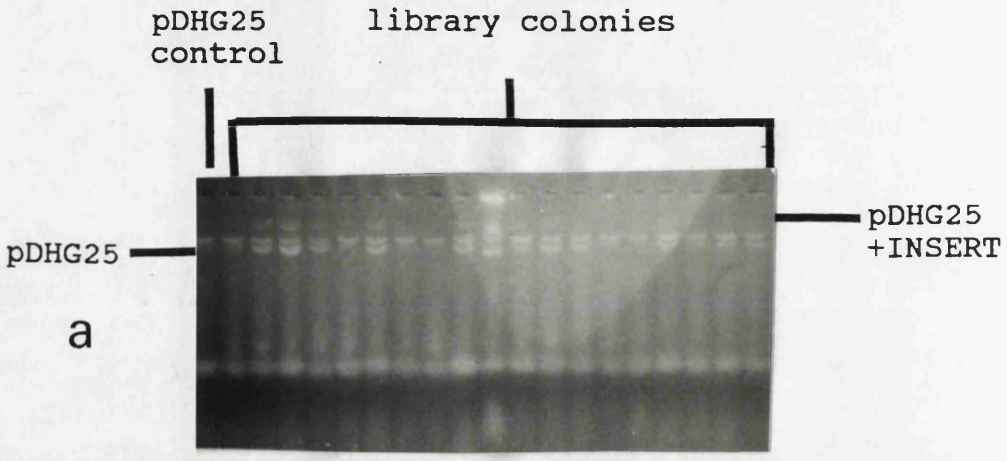
Single Colony Gel analysis, (results not shown), of plasmid DNA prepared from all of the E.coli colonies showed that in all cases the rescued

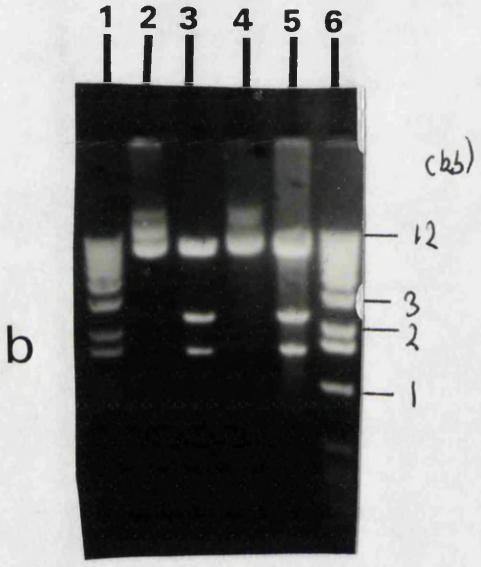
plasmids were smaller than 5kb in size. The plasmid pHELP1 is 7.9kb in size. A pHELP1/gene cointegrate should be at least 10kb in size.

Therefore, it appeared unlikely that the rescued plasmids were large enough to contain amal DNA, bacterial plasmid DNA and the gene under selection. This hypothesis was confirmed by transforming Aspergillus protoplasts of appropriate strains with three of the largest rescued plasmids from argB, niaD and nirA transformants. In no case, with the exception of pILJ16 and ARp1 controls, were Aspergillus prototrophic transformants obtained, (results not shown).

The plasmid rescues failed to isolate plasmid containing the genes of interest, despite the fact that plasmid cointegrates containing these genes appear to be present in the Aspergillus transformants. Factors affecting plasmid rescues are discussed in the next section.

In summary, there is reason to believe that Penicillium argB, niaD and nirA genes were introduced into Aspergillus by cotransformation and that they were active in the recipient species. It was however not possible to isolate these genes by plasmid rescue.





LANE	DNA
1	1kb ladder
2	pDHG25 uncut
3	pDHG25 EcoRI
4	pDHG25 gene bank uncut
5	pDHG25 gene bank EcoRI
6	1kb ladder

Figures 8.7a and 8.7b: Single Colony Gel analysis of E.coli containing Penicillium library inserts (8.7a) and restriction digest analysis of purified Penicillium library plasmid DNA (8.7b).

total no. of *E.coli* colonies from library ligation

$=1x10^5$

% of transformants with insert $=(14/20)x100$

$=70\%$

total no. of *E.coli* colonies with an insert

$=1x10^5 x 70\%$
$=7x10^4$

average insert size (10-15kb) $=13kb$
$=13,000bp$

total amount of *Penicillium* genomic DNA cloned
=average insert size x total colonies with insert

$=13,000x7x10^4$
$=9x10^8 bp$

size of *Penicillium* genome $=3x10^7 bp$

no. of genome equivalents cloned
=total DNA cloned/genome size

$=9x10^8 bp/3x10^7 bp$
$=30$

Figure 8.7c: *Penicillium* library calculations.

8.3 pDHG25 *Penicillium* gene bank .

Following the failure to isolate the *argB*, *niaD* and *nirA* genes using the Instant Gene Bank I decided to construct a conventional gene bank in the ARp1 derivative pDHG25, (see Chapter 4). This plasmid contains a unique BamHI site which is ideal for use with Sau3A random digest fragments. It was hoped that ligation into a defined site using compatible ended fragments and a *recA*- *E.coli* host would give more predictable results, including ability to rescue transforming plasmids back into *E.coli*.

The library was constructed as follows: 10µg of pDHG25 was digested with 20 units of BamHI for three hours, the DNA was phosphatased as described in Chapter 2. The digested plasmid DNA was extracted with phenol/choloroform, chloroform and then EtOH-precipitated.

10µg of the prepared plasmid DNA was mixed with 50µg of Sau3A-digested *Penicillium* genomic DNA, (see section 8.2.1); the genomic DNA fragments were 10-15kb in size. The ligation was carried out in a total volume of 2mls.

The ligated DNA was used to transform *E.coli* SURE cells, thereby amplifying the library; the cells were made competent using the TSB method. The transformed *E.coli* cells were plated onto 2 ampicillin containing, 20cmx20cm LB-agar plates. The plates were incubated at 37^{o}C overnight. 20 colonies

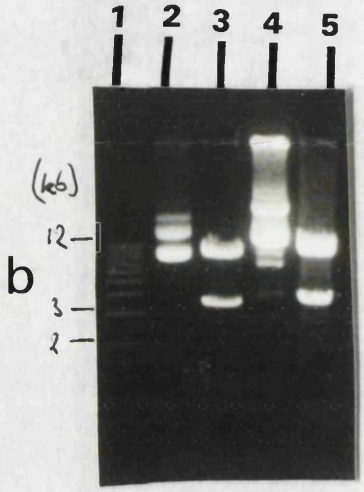
were subcultured onto fresh L-amp plates, (see below) and the remaining colonies were washed off the plates and plasmid DNA was made as described in Chapter 2.

To determine how many E.coli transformants contained a plasmid with insert, the 20 subcultured colonies were analysed by Single Colony Gel electrophoresis. The results of this experiment are shown in figure 8.7a. This gel shows that 14 out of 20 transformants contain plasmid with an insert.

1µg aliqouts of pDHG25 and the pDHG25 library were digested with 20 units of BamHI and run on a 0.8% gel along with 1µg aliquots of undigested DNA. The results are shown in figure 8.7b. The smearing in the digested library lane when there is no such smearing in the control digested pDHG25 lane, confirms that the purified plasmid DNA contains genomic DNA inserts. Figure 8.7c shows how the library efficiency was calculated: approximately 30 genomes worth of Penicillium DNA has been cloned. Note that to date, this library has not been used.

8.4 pDHG25 Aspergillus gene bank.

An Aspergillus pDHG25 gene bank was made in a similar fashion to the Penicicillium gene bank. The number of plasmids containing an insert was calculated as described in section 8.3. The results of the Single Colony gel, figure 8.8a, show that 7 out of 13 transformant colonies contain plasmids with



LANE	DNA
1	1kb ladder
2	pDHG25 uncut
3	pDHG25 EcoRI
4	pDHG25 gene bank uncut
5	pDHG25 gene bank EcoRI

Figures 8.8a and 8.8b: Single Colony Gel analysis of E.coli containing Aspergillus library inserts (8.8a) and restriction digest analysis of purified Aspergillus library plasmid DNA (8.8b).

total no. of *E.coli* colonies from library ligation

$=2x10^5$

% of transformants with insert $=(7/13)x100$

$=54\%$

total no. of *E.coli* colonies with an insert

$=2x10^5 x 54\%$
$=1.08x10^5$

average insert size (10-15kb) $=13kb$
$=13,000bp$

total amount of *Aspergillus* genomic DNA cloned
=average insert size x total colonies with insert

$=13,000 x 1.08x10^5$
$=1.4x10^9 bp$

size of *Aspergillus* genome $=3x10^7 bp$

no. of genome equivalents cloned
=total DNA cloned/genome size

$=1.4x10^9 bp / 3x10^7 bp$
$=46$

Figure 8.8c: *Aspergillus* library calculations.

inserts. Figure 8.8b suggests that the digested library DNA, (lane 4), contains genomic DNA inserts. Overall, 46 genomes worth of Aspergilllus DNA has been cloned in this library, see figure 8.8c. Note that to date, this library has not been used.

The best way to confirm that the pDHG25 libraries are functional is to use these libraries to clone genes by transforming fungal mutants and selecting genes, similar to the Instant Gene Bank.

8.5 Discussion.

The results in this Chapter show clearly that it is possible to transform Aspergillus with DNA from Penicillium, supporting the results presented in Chapter 7. The cotransformations with genomic DNA and pHELP1 show that linearisation of plasmid DNA had no effect on cotransformation efficiency, this observation supports the results with the pJSR series in Chapter 4.

It seems highly likely from the results, (section 8.2.3), that functional Penicillium argB, niaD and nirA genes have been introduced into Aspergillus. Both the instability tests, (section 8.2.3) and the genomic Southerns indicate that autonomously replicating plasmids, containing these genes, are present.

However, the plasmid rescues, (section 8.2.4), failed to isolate plasmid that contained the genes of

interest; plasmids were isolated but these plasmids did not complement the Aspergillus mutations when used in transformation experiments.

Various factors influence plasmid rescues. As plasmid size increases, the transformation frequency in E.coli decreases. The presence of chromosomal DNA also reduces the transformation frequency; the chromosomal DNA competes with the plasmid DNA for DNA binding sites and subsequent uptake into the host cell, (Gems 1990). The structure of the plasmid determines whether or not it is viable in an E.coli host; rearrangements in both the amp^R gene or bacterial plasmid origin can lead to a plasmid being "unrescuable". The level of competence of the host cell also affects plasmid rescue.

Failure of the Instant Gene Bank plasmid rescues can be explained by any one of the above possibilities e.g. only 5kb plasmids were rescued, the pHELP1/gene cointegrates are at least 10kb in size, if small plasmids are favoured over larger plasmids then it is less likely that the pHELP1/gene cointegrates will be rescued.

One way of increasing the frequency of plasmid rescues is to alter the ratio of chromosomal DNA to plasmid DNA. I attempted to alter the ratio by pretreatment with phenol which I hoped would enrich the amount of plasmid DNA in the samples, (G.Griffith, personal communication). The results show that this was not particularly successful.

Using highly competent and expensive host cells did not lead to any noticable increase in rescue frequency.

There is also another way of altering the chromosomal/plasmid DNA ratio in favour of the plasmid DNA. It is possible to isolate all the free autonomously replicating plasmid from fungal transformants without using E.coli hosts. In Chapter 5, I described how ARp1 was purified from fungal transformants by using EtBr/CsCl gradients and ultracentrifugation. Using this method it should be possible to isolate the pHELP1/gene cointegrates plus any other free plasmid present. Once this free plasmid DNA was prepared, aliquots could then be used to transform E.coli hosts, which could be screened for the presence of the pHELP1/gene cointegrate. Transformation of auxotrophic fungal mutants, followed by selection, would check that the pHELP1/gene cointegrate had been isolated and that this plasmid was fully functional. There is one drawback with this approach. One litre of culture yielded only 1µg of ARp1, so scale-up for isolation of useful amounts of pHELP1/gene cointegrates is a problem.

The Instant Gene Bank is useful in that the technique answers certain questions quickly. Is the gene of interest present? Will genes from one species be functional/expressed in a different species? Various workers have demonstrated that it

is possible for genes from one fungal species to be expressed in a different fungal species: Ballance and Turner (1985) transformed an A.nidulans mutant with the N.crassa pyr4 gene; Whitehead et al (1989) transformed a Penicillium chrysogenum mutant with both the A.niger and A.nidulans niaD genes; Beri and Turner transformed P.chrysogenum with the A.nidulans amdS gene. It is also possible to isolate genes for which there may not be any identified, homologous genes that could be used as probes to screen conventional libraries.

There are also certain drawbacks to the technique. The total time taken to isolate genes, assuming the plasmid rescues work, is much longer than isolating genes from a plasmid or phage library. Another limitation of the technique, which is the same for a normal gene bank, is the requirement for specific mutant strains in which to do the initial complementation/selection.

If the problems with plasmid rescues can be solved then the Instant Gene Bank could be a powerful technique for isolation of fungal genes. Indeed, the Instant Gene Bank technique has been used successfully to clone the trpC gene from Penicillium canescens, (Aleksenko, personal communication.)

Chapter 9.

Concluding remarks.

9.1 Amal: a summary.

The amal sequence consists of a composite of Aspergillus chromosome-derived DNA and pUC-like DNA, (see Chapters 3 and 6). The amal sequence is composed of two similar 3kb arms, separated by a 345bp unique region, and contains internal, inverted repeats.

The subclone results described in Chapter 4 suggest that no specific region of amal can be identified as being critical for autonomous replication. The same results do confirm that some component(s) of amal play a central role in autonomous replication.

The role of pUC8-like DNA in amal function has not been clearly determined, but this topic has been discussed in detail previously, (see Chapters 3 and 4). The results of cotransformations with pY184 and its rearranged, rescued derivative (Chapter 4), strongly suggests that such DNA or rather some component of this DNA, does appear to play some part in autonomous plasmid replication.

The amal sequence promotes autonomous plasmid replication in both Aspergillus and also to a limited extent in Penicillium. Amal-like sequences isolated from Penicillium promote autonomous replication in both Penicillium and Aspergillus, (see Chapter 7).

9.2.1 Plasmid recombination and ARp1.

Plasmid recombination is of some interest when considering both the behaviour and replication mechanism of ARp1. Plasmid recombination can be divided into three broad areas: (1) recombination leading to integration/excision of a plasmid, Moore and Simon (1987), Orr-Weaver and Szostak (1983), Szostak *et al* (1983); (2) recombination between homologous regions within a plasmid resulting in rearrangements, Gems (1990); (3) recombination between plasmid molecules to produce a cointegrate plasmid, Puchta and Hohn (1991), Maryon and Carroll (1991) and Lyznik *et al* (1991).

The three types of integration event: Type I, Type II and Type III, as identified by Hinnen (1978); were described in detail in Chapter 1. Integration and excision via recombination are probably not relevant when considering the behaviour of ARp1 and derived subclones, with the possible exception of pILJ20, (see below). Recombination between plasmids is much more frequent than recombination between plasmid and chromosome.

Recombination leading to rearrangements is of some importance. As described in the results Chapters, ARp1 and most of the subclones contain pUC-like DNA within the *ama1* sequence. Recombination between these regions and the pUC8 DNA could lead to a variety of different rearrangement products e.g.

the rearranged pILJ25 derivatives, (see Chapter 4). That homologous recombination could lead to plasmid rearrangements is only speculation because the rearrangement mechanism is not known, but recombination cannot be discounted.

Recombination events leading to the formation of selectable cointegrate plasmids was the basis for much of the work described in Chapter 8. The mechanism of cointegrate formation has been recently described in Xenopus laevis by Maryon and Carrol (1991) and in plants by Puchta and Hohn (1991) and Lyznik et al (1991). Recombination between plasmids can be described by two different models; either the double strand break repair model or the single strand annealing model. Both models attempt to explain how double strand breaks within a DNA duplex enhance the rate of recombination, see Orr-Weaver and Szostak (1983) for a review.

9.2.2. The double strand break repair model.

In this model, recombination is initiated by a double strand break in one of the two DNA molecules. The double strand break is enlarged by nucleases and the 3′ end of one of the digested strands (the donor), invades the other duplex (the recipient), at a homologous site, producing a heteroduplex with a single Holiday junction. The double strand break is repaired by DNA synthesis using the unbroken DNA

strand as a template; this process produces a double crossover intermediate consisting of two Holiday junctions. Resolution of the double crossover structure can proceed in two ways; either flanking markers remain on the same DNA molecule (non-recombinant) or markers are exchanged (recombinant). The probability of either event occurring should be equal. The double strand break repair mechanism is conservative because both recombining molecules are repaired/restored.

9.2.3 The single strand annealing model.

In this model, recombination is initiated by a double strand break in each of the two DNA molecules at different positions. The double strand breaks act as substrates for single strand exonucleases, which digest away one of the DNA strands in each DNA molecule. Alternatively, helicase can generate single stranded DNA by unwinding a double stranded DNA substrate. In either case, the generated single strand ends are complementary and the two undigested strands from the two different DNA molecules basepair. The intermediate structure is repaired using the double stranded intermediate as a primer. This process is not conservative because the free ends of the non-recombining strands in both DNA molecules are degraded, so only recombinant molecules survive.

9.2.4 ARp1 and the recombination models.

The results from Chapter 8, in which digested pHELP1/digested genomic DNA (two double strand breaks) and undigested pHELP1/digested genomic DNA (single double strand break) were used in cotransformations, could be of help in identifying which of the above mechanisms is functional in plasmid recombination in Aspergillus. However, the results appear to be inconclusive since both sets of cotransformations produce the same number of transformants; in Yeast, two double strand breaks enhance recombination rates 3000-fold, Szostak et al (1983). The mechanism by which ARp1 and associated plasmids recombine is unclear at this time.

9.2.5 Does ARp1 recombine with the chromosome to replicate?

How does ARp1 replicate autonomously? This question has a number of possible answers; ama1 could promote autonomous replication; ama1 might inhibit stable integration i.e. ARp1 might recombine with the chromosomal DNA, replicate then be excised.

The mechanism by which ama1 might promote autonomous replication has been described in detail in previous Chapters. Ama1 might inhibit stable integration by promoting recombination, which would lead to plasmid excision. The integration of ARp1

into the genome must be short-lived since no stable ARp1 fungal transformants were ever isolated. There is no convincing evidence that ARp1 does integrate into the genome.

However, the results with pILJ20, an ARp1 subclone, suggest that this plasmid is not markedly different from pILJ16. As described in section 4.3.6, further investigation suggests that pILJ20 has not integrated but that the plasmid has been rearranged.

It is known that excision of rescuable plasmid DNA does occur in Aspergillus. Johnstone et al (1985), transformed an argB2 brlA42 mutant of A.nidulans with a wild-type A.nidulans gene bank and rescued out a plasmid carrying the chromosomal copy of the mutant brlA42 allele. The excision event was the result of homologus recombination, (Type III integration), between a plasmid carrying the wild-type brlA42 gene and the chromosomal mutant brlA42 gene.

Clues as to how the ARp-derived ama1 sequence "works" could be found by comparing and contrasting the ama1 sequence with the sequences of the ama1-related, cosmid-borne Aspergillus genomic sequences (Chapter 6), and the Penicillium pam sequences, (Chapter 7). Such comparisons may also illustrate how the genomic ama1 precursor DNA was rearranged to produce the ARp1-borne ama1 sequence.

It is clear that there is still a lot of

research left in this field because there are many unanswered questions regarding *ama1* and it's related sequences e.g. how did the *ama1* composite structure come about? Have the ARp1-borne pUC8 and *argB* sequences been rearranged? Does ARp1 replicate via a Double Rolling Circle mechanism? What is the extent of DNA similarity between *ama1* and both the cosmid-borne sequences described in Chapter 6 and the *Penicillium*-derived sequences described in Chapter 7?

It is likely that autonomously replicating plasmids such as ARp1 and pFOLT4, (Powell and Kistler 1991), will become increasingly important as research tools.

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