Transformation and Development of *Aspergillus nidulans*.

A thesis submitted for the Degree of
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by

David Herbert Gems

Institute of Genetics
Church Street
Glasgow

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Dedicated to
William Olaf Stapledon
(1881-1950)

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

Contents

Abbreviations

1. Chemicals.

Ac - acetate
ATP - adenosine triphosphate
BSA - bovine serum albumen
Cit - citrate
DNA - deoxyribonucleic acid
DNase - deoxyribonuclease
DTT - dithiothreitol
EDTA - disodium ethylenediamine tetra-acetate
EtBr - ethidium bromide
EtOH - ethanol
mRNA - messenger RNA
MOPS - 3-N-morpholinopropanesulphonic acid
RNA - ribonucleic acid
RNase - ribonuclease
SDS - sodium dodecylsulphate
Tris - tris (hydroxymethyl) aminoethane

2. Measurements

Ci - curie(s)
mA - milliamps (10^{-3}A)
bp - base pair(s)
kb - kilobase pair(s) or 1,000bp
kda - kilodalton(s) or 1,000 daltons
^{o}C - degrees centigrade
g - centrifugal force equivalent to gravitational acceleration
g - gramme(s)
mg - milligramme(s) (10^{-3}g)
ug - microgramme(s) (10^{-6}g)
ng - ananogramme(s) (10^{-9}g)
l - litre(s)
ml - millilitre(s) (10^{-3}l)
ul - microlitre(s) (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)]
min - minute(s)
V - volts
W - Watts

3. Miscellaneous

AGE - agarose gel electrophoresis
AIp - integrating Aspergillus/E.coli shuttle vector
ARp - autonomously replicating Aspergillus/E.coli shuttle vector
dH_2O - distilled water
log - logarithm
mt - mitochondrial
ori - origin of DNA replication
PAGE - polyacrylamide gel electrophoresis
RER - rough endoplasmic reticulum
UV - ultraviolet
% - percentage

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Summary

The main aim of the study of the molecular genetics of development in Aspergillus nidulans is to understand how master genes orchestrate the expression of large numbers of genes which determine development. brlA has been shown to be one master gene in the development of the head of the conidiophore (asexual reproductive apparatus) (Clutterbuck 1969; 1977; Boylan et al., 1987; Adams et al., 1988).

A number of genes affecting conidiophore morphology and pigmentation have been cloned and shown to be regulated by brlA (O Hara et al, 1988, Birse and Clutterbuck, 1991). In this thesis the cloning of a gene, ygA (yellow-green), which affects conidial pigmentation, is described.

However, prior to this, with the aim of improving the standard A.nidulans gene cloning methods, a plasmid, ARp1, was characterised. This plasmid, which transforms Aspergillus with a very high frequency, was previously isolated in this lab by I.L. Johnstone. By a combination of genetic and Southern analysis it was shown that this plasmid replicates autonomously, not only in A.nidulans, but also in A.oryzae and A.niger.

It was then found that ARp1 recombines at high frequency with cotransforming integrative (AIp) plasmids. This increases the transformation frequency of AIp plasmids by two orders of magnitude as they become part of autonomously replicating plasmid cointegrates. A "helper" plasmid, pHELP3, was constructed, consisting of a sequence conferring autonomous replication in Aspergillus inserted into pUC8. The gene adD was then cloned by cotransformation of pHELP3 with a gene bank constructed in the AIp vector pILJ16, of an adD3 strain.

The adD gene was used to probe an A.nidulans gene library in the lambda replacement vector EMBL3, constructed by Gareth Griffith. Homologous lambda clones were isolated which contained the gene ygA, which maps 0.1 map units from adD. A third gene in this cluster, adC, was also cloned.

It was also found that cotransformation between another helper

plasmid, pHELP1, and non-homologous linear DNA occurred at high frequency. On the basis of this, sonicated A.nidulans DNA and pHELP1 were used in cotransformations to clone the gene trpB. The technique was dubbed the "instant gene bank" method.

Finally a family of enhancer mutations of the developmental mutation medusa (medA$^-$) were isolated. Designated sthenyo, (Medusa's sister; Riley, 1851), a number of alleles were mapped to two loci, sthA and sthB. These mutations have no detectable phenotypes in the absence of medA$^-$ allele. It was shown that sthA1 in combination with another mutation without a phenotype - the temperature sensitive morphological mutation brlA42 grown at the permissive temperature - shows severe abnormalities in conidiophore morphology. It is proposed that the small number of conidiophore specific mutations (Martinelli and Clutterbuck, 1971) relative to the number of different conidiophore specific poly(A)$^+$mRNAs (Timberlake 1980) may be due partly to the existence of families of polygenes, or partially redundant genes, exemplified by sthA, sthB and medA.

CHAPTER 1

Introduction

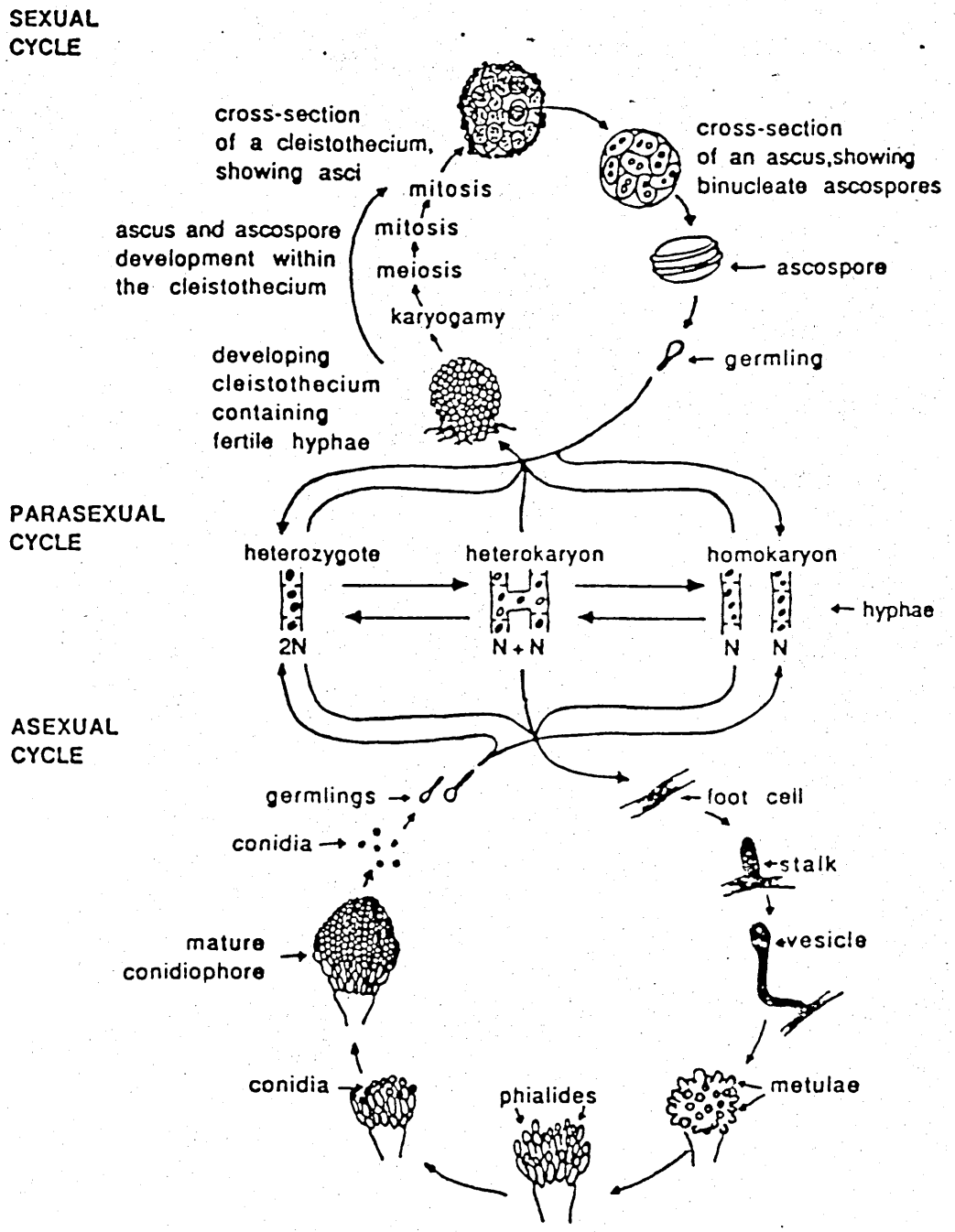

Figure 1.1: The life cycle of Aspergillus nidulans. (Courtesy of M.A. Marshall).

Introduction

Apart from its use as the subject of genetic studies the Euascomycete filamentous fungus Aspergillus nidulans is of little importance. It is not a serious pathogen of man or agriculture; it is of little use industrially or commercially; nor does it possess any unusual features of biological interest. On the other hand, many features of A.nidulans make it a convenient organism for genetic and molecular genetic studies: it has a short life-cycle (Fig.1), replicates sexually and asexually, and is haploid, but may be propagated as a diploid (Pontecorvo et al, 1953); it can be used for gene manipulation studies involving e.g. transformation (Ballance et al, 1983; Tilburn et al, 1984), gene cloning (Johnstone et al, 1985), and targeted gene disruption or replacement (Miller et al, 1985a); its genome is relatively small (31 mb) with 8 chromosomes ranging in size from 2.9 to 5.0 mb, which can be separated by pulse field electrophoresis (Brody and Carbon, 1989).

As a multicellular lower eukaryote which undergoes relatively simple developmental and morphogenetic processes its very ordinariness make it an appropriate organism for the study of the universal in the particular. In other words, A.nidulans can be used as a model organism to investigate fundamental questions of biology, particularly those concerning the genetic determination of development.

The work described in this thesis was devised to contribute to the answering, in the long term, of some of the following questions:
a) How is a complex succession of spatially and temporally regulated changes of gene expression determined?
b) How do the genes involved in development encode morphologically complex, muticellular, three dimensional structures? What kinds of structural components of the cell give rise to particular morphological features?
c) How are gene regulatory, and morphogenetic changes coordinated so that appropriate genes are activated and deactivated in the appropriate morphological structures? (c.f. Stragier et al, 1988).

These investigations were directed specifically to the development of the organ of asexual reproduction of A.nidulans: the conidiophore. In recent years conidiogenesis has been the focus of intensive genetic (Clutterbuck, 1969; 1990a; 1990c) and molecular genetic (Timberlake, 1980; Timberlake and Barnard, 1981; Boylan et al , 1987; Adams et al,

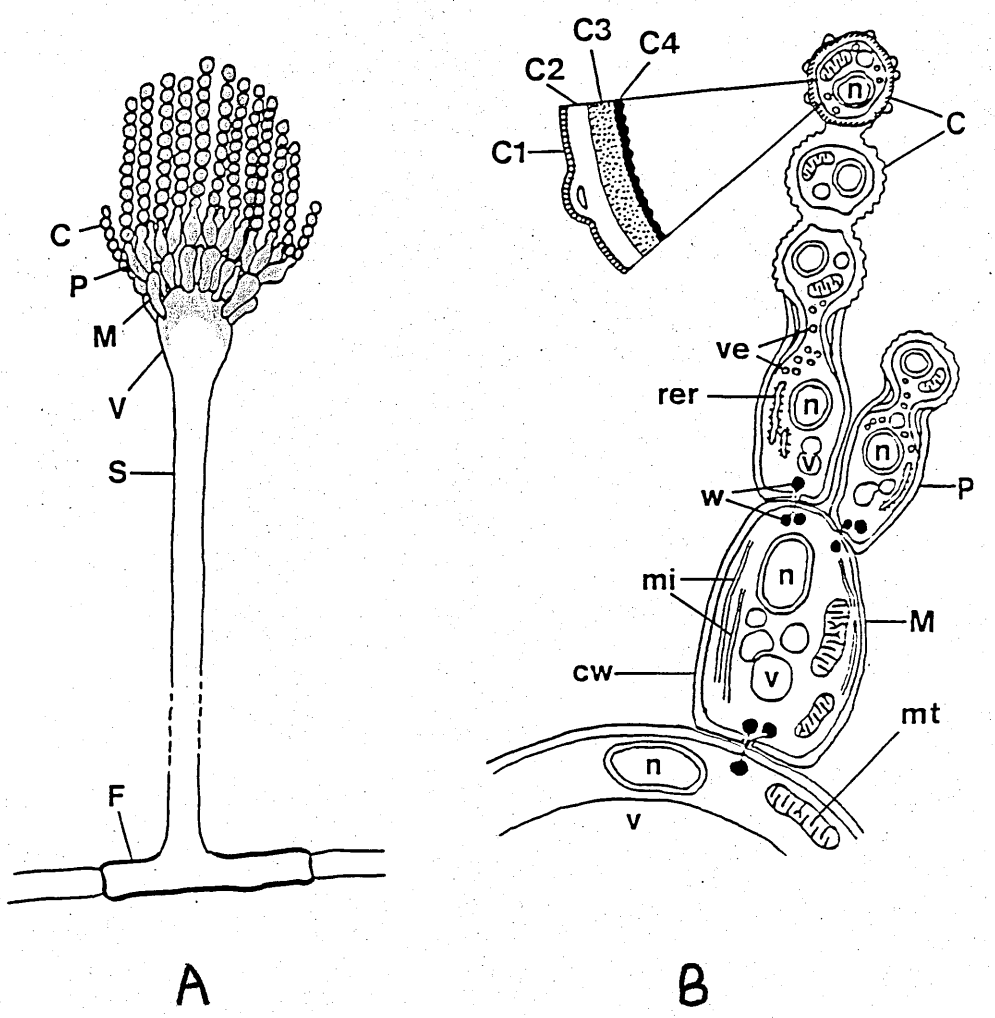

Figure 1.2: A, **mature wild-type <u>Aspergillus</u> conidiophore.** C, conidium ; F, foot cell; M, metula; P, phialide; S, stalk; V, vesicle. B, **diagrammatic cross-section of metula, phialide and conidia.** (Based on Timberlake, 1990). C1-C4, spore wall layers; cw, cell wall; mt, microtubules; mi, mitochondrion; n, nucleus; rer, rough endoplasmic reticulum; v, vacuole; ve, vesicle; w, Woronin body.

1988; Mirabito et al , 1989; Marshall and Timberlake, 1990) analysis.

In addition, this thesis deals with the properties of plasmids which replicate autonomously in Aspergillus, and the events forming the basis of cotransformation.

DEVELOPMENTAL GENETICS

1.1.1 Conidiophore development in A.nidulans.

Conidophore development has been studied by light microscopy (Clutterbuck, 1969), transmission electron microscopy (TEM), using chemical fixation (Oliver, 1972) and freeze substitution, as well as scanning electron microscopy (SEM) (Mims et al, 1988; Sewall et al, 1990a; 1990b).

Conidiogenesis begins 20 hours after germination at 37^{o}C with the formation of a hyphal compartment with a thickened wall, or foot cell (Fig 1.1A). From the foot cell a thick walled, aerial hypha, or conidiophore stalk develops. How the orientation of the stalk is determined is not clear, but geotropism is not involved. The tip grows by means of the fusion with the plasma membrane at the apex of large numbers of apical vesicles containing cell wall components and polymerising enzymes, which are produced by Golgi bodies following the growing tip. Nuclei remain further back down the stalk. Invariably, at a length of approximately 100uM apical growth ceases and the apex swells to form the vesicle (10uM diameter); this accumulates grey-brown conidiophore melanin in the cell wall.

Now the number of apical vesicles declines, although a few are seen round the vesicle periphery, vacuoles become prominent, and nuclei move up from the stalk into the vesicle. At this point unknown determinants give rise to a complex pattern formation event: apical vesicles form into a regular clustered array under the plasma membrane, and up to 60 evenly spaced buds emerge simultaneously. These develop into support cells or metulae (aka primary sterigmata), and into these migrate mitochondria, RER, and other organelles. 60-odd nuclei are then positioned one directly subtending each metula, and then divide simultaneously, along with all the other nuclei in the vesicle and stalk. One daughter nucleus then enters each metula initial, after which a centripetally developing septum forms, separating vesicle and metula, except for a central pore; at each side of this pore are seen dark staining spherical organelles of

uncertain function, the Woronin bodies (Fig. 1.2B).

Next, sporogenous phialide cells (aka secondary sterigmata) form from the tips of the metulae, and similar process of directed nuclear division, septum formation and the appearance of the Woronin bodies, occurs. Phialide cells have a distinctive morphology: bottle-(or phial-) shaped with a distal, elongated neck region (Fig 1.2). After septation, a second phialide often forms from the side of the metula, just under the first one (Fig. 1.2B). Metulae and phialides are both 5-7uM in length. Meanwhile, a large vacuole develops in the vesicle, occupying the bulk of its volume.

The conidium initial then forms as a protrusion from the end of the phialide neck. The single phialide nucleus enters the cell neck, divides, and the distal daughter nucleus enters the conidium. A septum forms by radial invagination of the plasma membrane and wall deposition.

The conidial wall comprises four layers, C1-C4 (Fig. 1.1B). The outer two layers are formed by the phialide, and the inner two layers by the conidium itself. The outer spore wall is highly hydrophobic, and contains a polyphenolic, dark-green pigment. While the conidium enters dormancy, further conidia are generated by the phialide, until a long chain of conidia is formed.

1.1.2 Genetic analysis of conidiogenesis

Most of the genetic and molecular genetic investigation has focussed on development from vesicle formation onwards. However, it is known that at 20 hours after germination, the oldest cells in a colony attain developmental competence (Axelrod et al, 1973). Mutations resulting in precocious competence have been identified (Axelrod et al, 1973; Kurtz and Champe, 1979). Studies of mutants unable to attain competence defined three loci, acoA, acoB and acoC, which are also blocked in sexual development (Butnick et al, 1984a; 1984b).

In cells which are competent to develop, conidiation will only occur at an air interface (Clutterbuck, 1977), although under certain conditions (e.g. C and N starvation) a low level of conidiation will occur in vigorously shaken submerged culture (Saxena and Sinha, 1973; Martinelli, 1976). Also required for induction of conidiation is red light (Mooney and Yager, 1990); exposure to far-red light leads to a

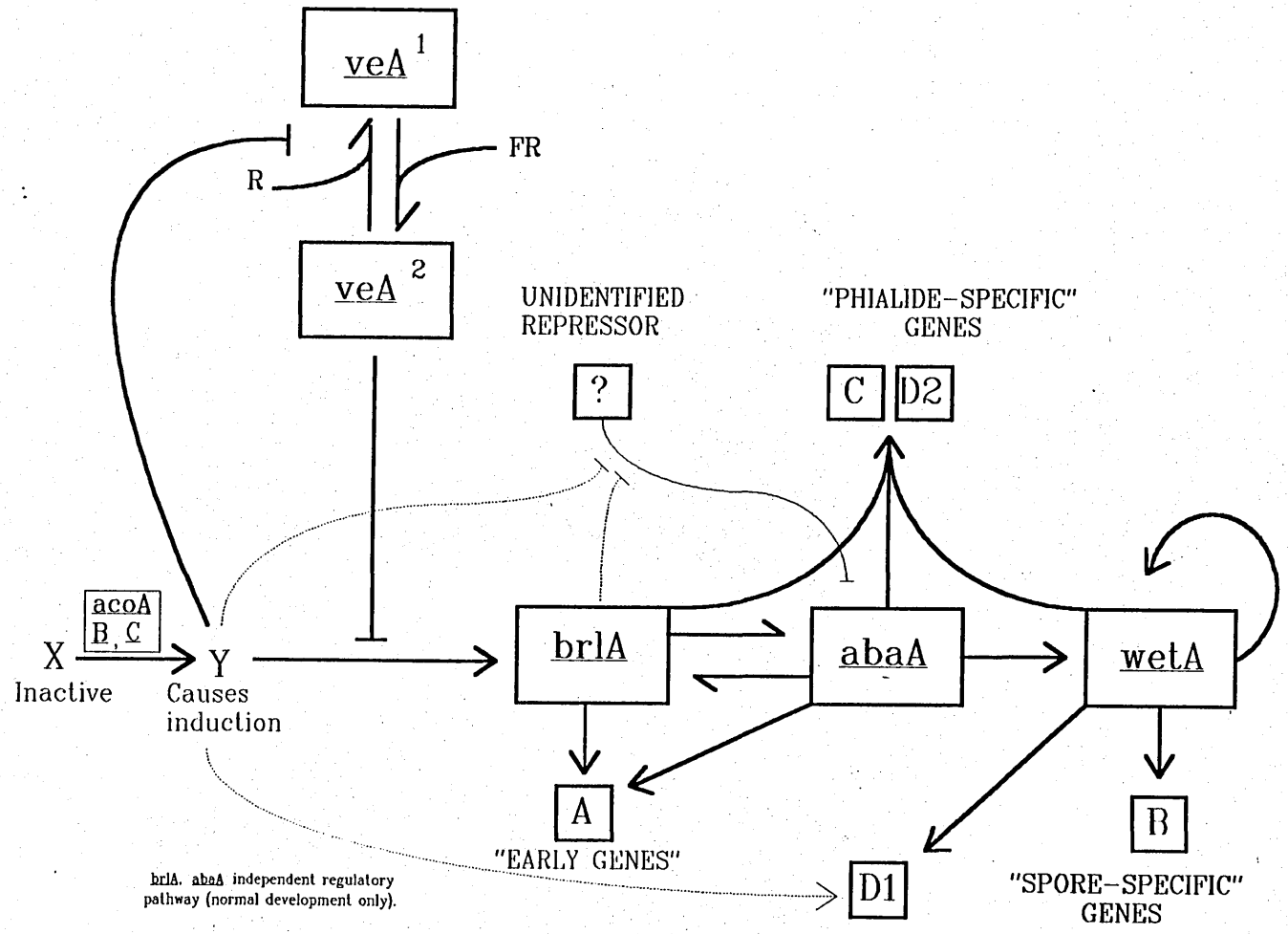

Figure 1.3: **Model of genetic control of conidiation in A.nidulans.** Left to right represents the direction of time. Arrows and bars represent positive and negative regulation respectively. (Adapted from Timberlake, 1990).

partial reversal of such induction, indicating that a growth-affecting phytochrome-like pigment may be present. However, most laboratory strains contain mutations in the velvet (veA) gene (Kafer, 1965), rendering conidiation light independent. Many aco mutants are found to be partially suppressed by the veA1 mutation (Butnick et al, 1984b).

Timberlake has suggested a model for the control of competence and induction of conidiation by veA and the aco genes (Fig. 1.3). Here the aco genes encode enzymes catalysing the conversion of a metabolite, X, which is inhibitory to conidiation, to Y, which is not. In aco$^-$ mutants, X accumulates, inhibiting conidiation. Since veA1 is a partial suppressor of the aco phenotype, it is suggested that the formation of the form of the veA gene product which does not inhibit conidiation is inhibited by X (Fig. 1.3). Thus, in veA1 strains one form of repression of conidiation by X is lifted, which reduces the effect of elevated concentrations of X.

In a screen for mutants affected in conidiophore development in the stages after stalk development, a number of classes of mutation were described (Clutterbuck, 1969; 1990c). 1) Those with modified conidia. 2) Oligosporogenous: where conidiophore structure was altered without blocking conidiation. 3) Aconidial. 4) With modified conidiophore pigmentation. Mutations affecting both vegetative and reproductive hyphae were ignored, as were many which simply resulted in rather sick-looking conidiophores; only those in which clearly definable alterations had occurred were studied further.

Conidial modification mutants include ones with changes from the wild-type dark green pigmentation, e.g. yellow (yA, yA), yellow-green (ygA), white (wA, wB), and fawn (fwA); and in conidial wall structure, e.g. wet-white (wetA), and dark (drkA). Only one type of conidiophore colour mutant, ivory, was identified, determined by 3 loci: ivoA, ivoB and ygA. Conidial and conidiophore pigmentation is described in depth in chapter 7 of this thesis.

Phenotypes of oligosporogenous and aconidial mutants are shown in Fig 1.4. Oligosporogenous: medusa, (medA) produces supernumerary layers of metulae at 37^{o}C, but has an aconidial phenotype at 30^{o}C: this latter consists of elongated metulae with no phialides or conidia. The extent of the morphological phenotype also varies with pH. medusa mutants are also deficient in conidiophore pigmentation at both temperatures. stunted (stuA) produces tiny, thin walled conidiophores.

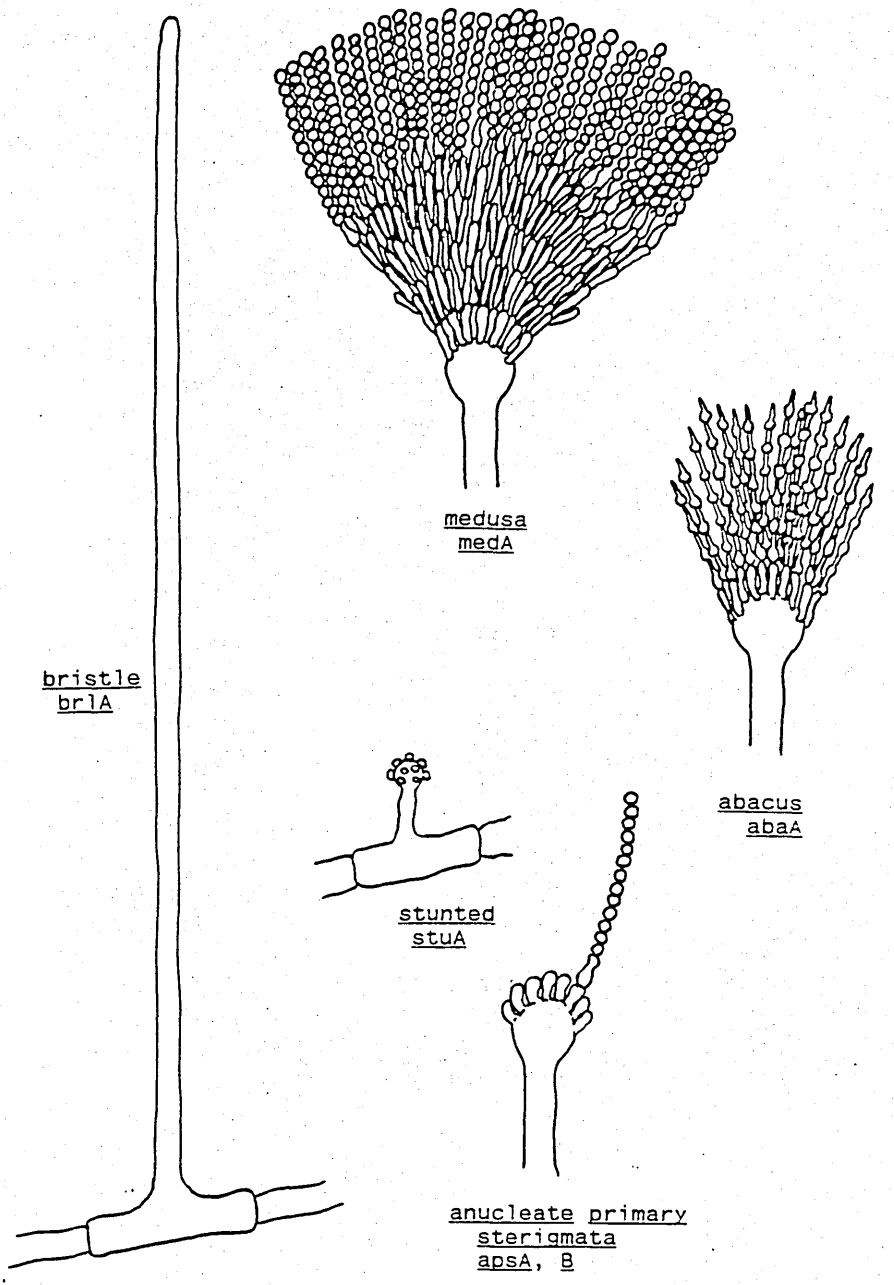

Figure 1.4: **Morphological mutants of *A.nidulans*.**

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In anucleate primary sterigmata (apsA, apsB) mutants, nuclei fail in most cases to migrate into the metula initials. Occasionally a nucleus will enter a metula by accident, resulting in development of apparently normal metula, phialide and conidia (Fig 1.4). Distribution of nuclei is also abnormal in vegetative hyphae. **Aconidial:** bristle (brlA) fails to produce a vesicle, instead developing an elongated stalk, and abacus (abaA) produces supernumary layers of what appear to be immature phialides.

Studies of double conidiation mutants showed the epistatic relationship brlA>abaA>wetA (Martinelli, 1979). Complex interactions were generally seen between the oligosporogenous mutations. However, stuA, apsA and apsB were found to be epistatic to medA, although the medA$^-$ deficiency in conidiophore pigmentation was evident in all three cases.

1.1.3 Molecular genetic analysis of conidiogenesis

The genes brlA, abaA, wetA, fwA, yA, ivoB, wA, stuA, veA, and ivoA have been cloned (Johnstone et al, 1985; Boylan et al, 1987; Turner et al, 1985; O'Hara and Timberlake, 1989; Birse and Clutterbuck, 1991; Mayorga and Timberlake, 1990; Miller, pers. comm.; Yager and Mooney, pers. comm.; Griffith and Clutterbuck, pers. comm.), by means of transformation of A.nidulans mutant strains to wild type by complementation of mutations with A.nidulans DNA libraries. These were constructed in plasmid (Johnstone et al, 1985; Ballance and Turner, 1986) or cosmid (Yelton et al, 1985) vectors. A much larger number of other Aspergillus genes were cloned as cDNAs prepared from poly(A)$^+$RNAs extracted from developing cultures, from which transcripts produced by vegetative hyphae had been removed by subtractive hybridisation (Zimmermann et al, 1980).

A 38-kb sequence of A.nidulans DNA, SpoC1, coding for 19-20 transcripts, at least 14 of which were developmentally regulated and spore specific, was identified using these cDNAs (Timberlake and Barnard, 1981; Gwynne et al, 1984). These transcripts comprise >2% of the mass of the conidial poly(A)$^+$RNA and represent 7% of the genes expressed specifically in the spore. Interestingly, deletion of the entire cluster does not result in any detectable phenotype whatsoever (Aramayo et al, 1989). This is discussed in chapter 8 of this thesis.

With DNA sequence analysis, and the results of gene expression

Table 1.1: Conidiation mutants of A.nidulans.

Gene	Phenotype	Peak expression time (hrs)[1]	Gene function/ putative protein
	Aconidial		
brlA	bristle aconidial	15	Zn finger DNA binding protein
abaA	abacus aconidial	18	leucine zipper DNA binding protein
	Conidiophore morphology		
stuA	stunted conidiophore	<15	?
medA	medusa morphology	?	?
veA	velvet morphology	?	biosynthesis of phyto-chrome-like pigment?
	Conidial morphology		
wetA	wet-white conidia	25	DNA binding protein, regulating cell cycle?
	Conidiophore pigmentation		
ivoA	ivory conidiophores	20	tryptophan hydroxylase?
ivoB	" "	18	phenol oxidase
ygA	" "	?	Cu^{++} uptake/distribution?
brlA	as above	15	as above
medA	" "	?	" "
	Conidial pigmentation[2]		
wA	white conidia	30	?
wetA	as above	25	as above
yA	yellow conidia	20.5	Conidial laccase I
ygA	yellow-green conidia	?	as above

[1]Hours after germination at 37°C; the time at which peak expression levels are first reached is shown.

[2]A full list of conidial pigmentation mutants including those uncharacterised is shown in Table 7.1.

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studies of the brlA, abaA, wetA, yA, wA, ivoA, ivoB and stuA genes, along with various cDNAs corresponding to developmentally regulated transcripts, a complex network of gene interactions has been elucidated (Fig. 1.3).

The putative proteins BrlA, AbaA and WetA: These all bear similarities to DNA binding proteins. BrlA possesses a sequence motif similar to the cys_2-his_2 Zn(II) coordination sites ("Zinc-fingers") first identified in Xenopus laevis transcription factor IIIA (TFIIIA) (Adams et al, 1988; Miller et al, 1985b). If, in either Zn-finger of brlA, a cysteine is changed to a serine (-SH to -OH) by site directed mutagenesis, there is a complete loss of brlA activity, confirming that the Zinc finger motifs are essential to brlA function (Adams et al, 1990).

The putative AbaA protein contains a potential "leucine zipper" sequence (Mirabito et al, 1989), yet lack the "basic region" consensus sequence often associated with DNA binding proteins (Vinson et al, 1989). The least that can be said is that the leucine zipper allows AbaA dimerisation. The putative WetA protein (Marshall and Timberlake, 1990) is basic (+6) and the putative amino acid sequence shows 27% sequence identity with the wee1 encoded polypeptide of S.pombe, which regulates mitosis (Russel and Nurse, 1987). It also has a number of $p32^{cdc2}$ phosphorylation sites. However, unlike the wee1 encoded protein it lacks a consensus ATP binding site or a protein kinase catalytic domain. Descriptions of conidiation mutants, and data on expression and putative products is summarised in Table 1.1.

Temporal regulation of expression. Results of Northern analyses of RNA prepared from synchronised Aspergillus cultures at different intervals after induction of conidiation mostly correspond to known epistatic relationships: in $brlA^-$ strains abaA, wetA, ivoB, yA and wA transcripts do not accumulate; in $abaA^-$ strains, wA and wetA transcripts do not accumulate; and in $wetA^-$ strains only wA transcripts do not accumulate (Boylan et al, 1987; O'Hara and Timberlake, 1989; Birse and Clutterbuck, 1990b; Mayorga and Timberlake, 1990). Only the stuA gene is transcribed before brlA, transcripts appearing at the same time as the onset of developmental competence (Miller, pers. comm.).

The question of which gene switches on which was further elucidated by the use of fusions of the A.nidulans alcA gene promoter (p) with

the coding regions of the brlA, abaA and wetA genes (Adams et al, 1988; Mirabito et al, 1989; Marshall and Timberlake, 1990). alcA encodes catabolic alcohol dehydrogenase. This gene is repressed during growth on glucose as a carbon source, and strongly induced when threonine is a carbon source. Induction of alcA(p)::brlA in submerged Aspergillus culture caused the formation of vesicles, phialides and conidia, and the abaA, wetA, yA and ivoB genes were expressed. Induction of alcA(p)::abaA led to cessation of vegetative growth and vacuolation, but not formation of conidia; this was despite the fact that not only the wetA, but also the brlA gene was activated. It was inferred that the correct order of gene expression is essential for morphogenesis (Mirabito et al, 1989). Induction of alcA(p)::wetA caused a reduction of vegetative growth, but did not activate brlA or abaA. From a reduction in wetA mRNA levels in wetA$^-$ strains it was inferred that the wetA gene is positively autoregulated.

When the effects of the brlA$^-$, abaA$^-$ and wetA$^-$ mutations, and expression of the three alcA(p) constructs on the expression of genes defined by the developmental cDNAs, it was found possible to subdivide them into 5 classes, A, B, C, D1, and D2, each with a distinct pattern of regulation (Fig 1.3). A, or "early genes", require brlA$^+$ and/or abaA$^+$, are not expressed in conidia, and probably include ivoA and ivoB. Class B, or "spore specific" genes are switched on by wetA. Class C, or phialide genes, require brlA, abaA and wetA expression, and accumulate in the spores. Expression of class D genes is slightly more complex: Class D1 genes are like class B genes, and class D2 like class C genes. The only difference is that both are also switched on during normal development in brlA$^-$ strains. Thus, they are subject to regulation by a second regulatory system. The network of gene interactions outlined here is summarised in Fig. 1.3.

As well as activating developmental genes, misscheduled expression of brlA and abaA has been shown to repress expression of alcohol dehydrogenase (alcA) and aldehyde dehydrogenase (aldA) genes (Adams and Timberlake, 1990). A general reuduction in levels of mRNA and protein synthesis is also observed which is believed to reflect a generalised metabolic shutdown caused by brlA expression.

When brlA and abaA upstream regulatory sequences were fused to E.coli lacZ gene sequences expression in Aspergillus led to colonies where only the conidiophores stained blue with IPTG-X-gal. This demonstrates that the brlA and abaA genes are expressed only in the conidiophore (Aguirre et al, 1990).

1.2.1 Transformation of Aspergillus

Transformation strategies

Aspergillus species, like S.cerevisiae, are usually transformed by incubation of protoplasts (sphaeroplasts) with DNA in the presence of $CaCl_2$; polyethylene glycol (PEG) is then added, which results in protoplast fusion and entry of the DNA into the cells. The protoplasts are then suspended in an osmotically stablising medium which selects for growth only of protoplasts in which the marker gene on the transforming DNA is expressed.

Recently several other DNA mediated transformation strategies have been used. Similar transformation frequencies to those found using PEG were obtained when protoplasts from A.niger and A.awamori were electroporated in the presence of DNA (Ward et al, 1989). N.crassa has recently been transformed by blasting hyphae with DNA-coated tungsten microparticles fired from a particle gun (Armaleo et al, 1990). This technique is described as "biolistic" transformation.

RNA mediated transformation, or retrotransformation, of Aspergillus has also been reported (Zucchi et al, 1989). This utilises a PEG induced protoplast fusion protocol, and is believed to be mediated by reverse transcriptase activity in Aspergillus (hence "retrotransformation".)

Integrative transformation of A.nidulans

The first DNA mediated transformation methods for A.nidulans (Ballance et al, 1983; Tilburn et al, 1984) were based on those used for S.cerevisiae (Hinnen et al,1978). The cell walls of hyphae grown in liquid culture is digested away with a complex mixture of enzymes extracted from Trichoderma harzianum (Peberdy, 1979), now marketed as NovoZym 234. This includes alpha-1,3-glucanase, B-1,3-, B-1,6-glucanase and chitinase, as well as proteases, DNase and RNase; hyphal wall digestion results in the release of protoplasts. In the Tilburn et al system, use of $MgSO_4$ as an osmotic stabiliser causes vacuolation of the protoplasts. The resulting reduction in density allows them to be separated from mycelial debris by centrifugation.

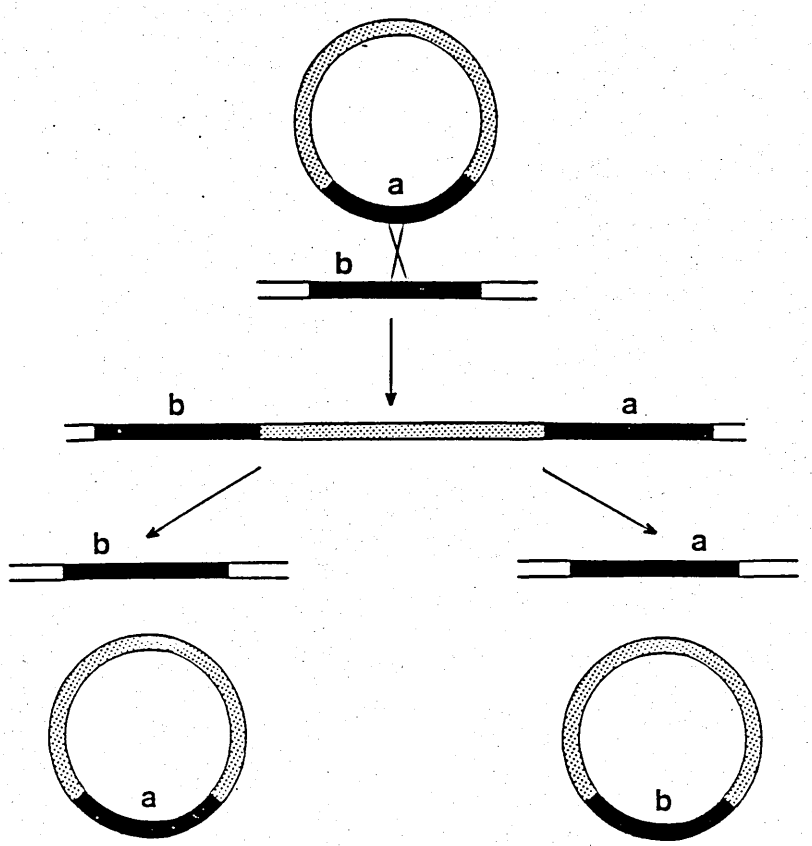

Figure 1.5. **Integration and excision of plasmid sequences by homologous recombination.**

Black boxes represent homologous Aspergillus gene sequences (alleles **a** and **b**); shaded boxes: E.coli vector sequences. Homologous recombination between plasmid borne allele **a** and chromosomal allele **b** results in plasmid integration. In the resulting direct repeat, integrated E.coli vector sequences are flanked by two copies of the gene. Homologous recombination between duplicate alleles may result in excision of allele **a** or **b**. Excision of **b** constitutes two-step gene replacement.

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After serial washing in an iso-osmotic sorbitol solution (1.2M) to remove the NovoZyme 234, and transformation, protoplasts are embedded in solid osmotically-stabilising selective medium (1M sucrose), and left to regenerate at 37°C. This takes approximately 3 days.

The transformation frequency depends largely on vector sequences, but also to an extent on the selectable marker used, and the length of the DNA insert carrying it. DNA mediated transformation of filamentous fungi was first achieved when N.crassa was transformed to inositol prototrophy using uncut chromosomal DNA (Mishra and Tatum, 1973). More recently when the amdS (acetamidase) and pyr4 (orotidine-5'-phosphate decarboxylase) genes were cloned in the E.coli pBR322 plasmid, maximum transformation frequencies of 25 and 10 transformants per ug DNA respectively were obtained (Tilburn et al, 1984; Ballance et al, 1983). In a detailed study 33 $argB^+$ transformants of A.nidulans with a plasmid consisting of the argB gene in the E.coli plasmid pUC19 were characterised (Upshall, 1986). 17/33 transformants showed integration of the argB plasmid at the argB locus. This was the result of homologous recombination between host and transforming DNA sequences, and resulted in the formation of a tandem repeat flanking the bacterial vector sequence (Fig. 1.5). In 7/33 cases gene conversion, or double crossover had occurred, resulting in regeneration of $argB^+$ (Fig. 1.5). The remaining 5/33 showed complex patterns of integration.

The use of certain bacterial plasmid components greatly enhances the transformation frequency of Aspergillus shuttle vectors. Johnstone (1985a) found that transferring the argB gene from the yeast shuttle vector pBB29 to pUC8 (to form pILJ16) resulted in an increase in transformation frequency from 1-5 to 250-500 transformants per ug DNA. The pUC plasmids (Vieira and Messing, 1982) may have a limited ability to replicate autonomously in A.nidulans. This is suggested a) by the enhanced transformation frequency; b) by the fact that using uncut DNA prepared from an initial Aspergillus transformant it is sometimes possible to reisolate the plasmid by transformation of E.coli (Johnstone, 1985a); and c), that in some initial transformants with such plasmids a degree of mitotic instability is observed (Upshall, 1986). Thus the enhancement of transformation may occur due to limited replication of the transforming plasmid upon entry into the host nucleus, which increases the probability of the occurrence of

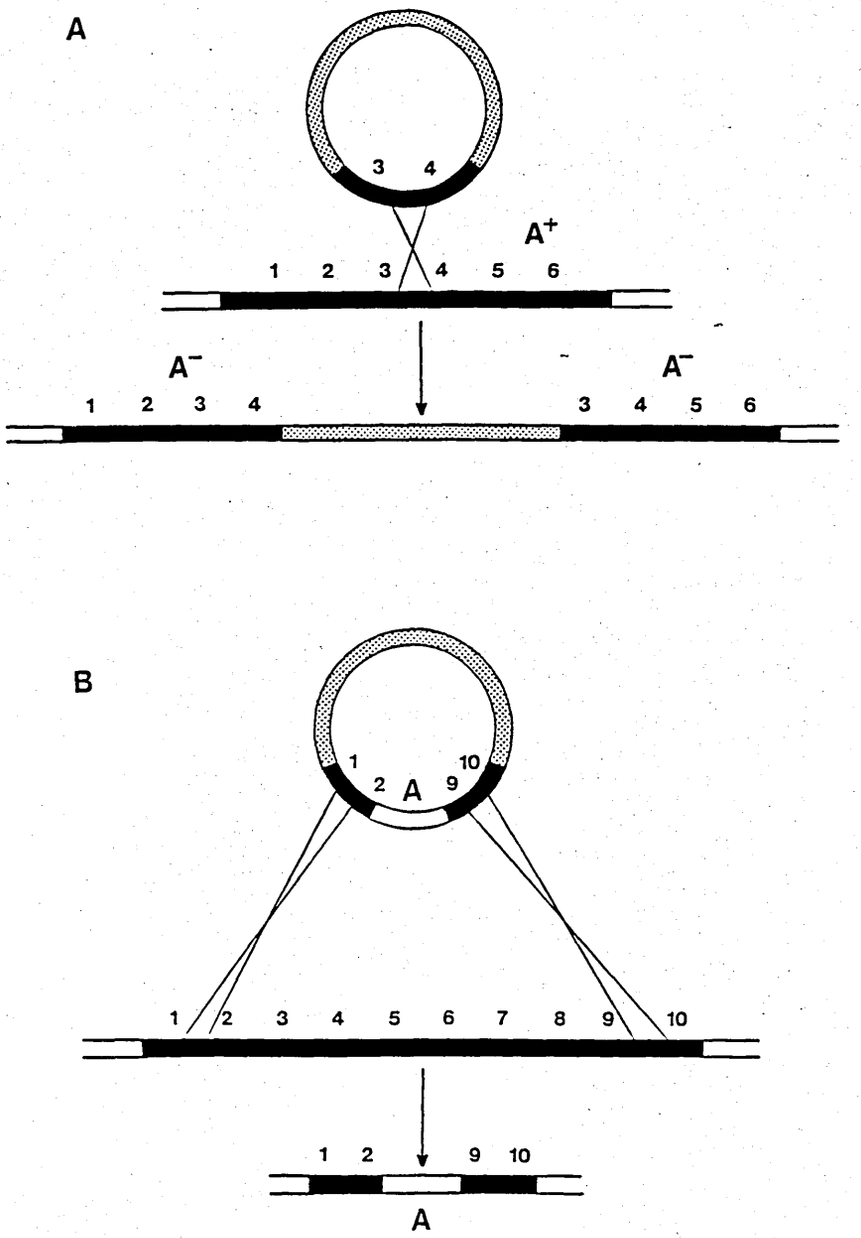

Figure 1.6. **Targeted gene disruption and replacement.**
A: Targeted gene disruption. The black box represents gene A; the shaded box, E.coli vector sequences. The numbers 1-6 represent the extent of the gene. The transforming plasmid contains an internal fragment of gene A. Homologous recombination as shown results in formation of two incomplete, non-functional copies of gene A.

B: Targeted deletion. Black boxes, DNA sequence to be deleted; shaded boxes, as above; open box, selectable marker gene A. Among A^+ transformants will be some which are the result of double homologous recombination events as shown, which result in replacement of DNA sequence 2-8 with gene A (after Aramayo et al, 1989).

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stable integration.

Integrative transformation has served a wide variety of purposes in molecular biological research.

Shotgun cloning of genes in Aspergillus

Integrative transformation can be used to clone genes. Aspergillus DNA libraries have been constructed in plasmid shuttle vectors (Johnstone et al, 1985; Ballance and Turner, 1986) and cosmids (Yelton et al, 1985). These were then used to transform mutant Aspergillus strains with selection for wild-type colonies. From these the plasmid containing the complementing DNA insert was reisolated by plasmid rescue into E.coli; or, in the case of cosmids, it was packaged into a lambda bacteriophage, and transfected into E.coli.

Gene manipulation and the study of gene function and expression in A.nidulans

As well as gene cloning, integrative transformation has been used to introduce genes which have been altered in vitro, such as the alcA(p) and lacZ fusion constructs described in section 1.1.3. Altered gene sequences can be used to replace resident, wild-type copies by means of two-step gene replacement: after homologous integration resulting in tandem repeat formation, passage through the sexual cycle stimulates a second homologous recombination event resulting in plasmid excision Fig. 1.5A (Miller et al, 1985a). This may result in loss of either the transforming, or the resident gene sequence. One step gene replacement may also occur as the result of a double crossover event (Fig. 1.5B).

Targeted gene disruption can be achieved by homologous integration of a DNA sequence containing part of a gene (Fig.1.6A). Targeted deletions can be carried out by replacing the centre of a large sequence with a selectable marker, and isolation of transformants where one step gene replacement has occurred by recombination between the chromosome and each end of the deleted sequence (Fig.1.6B, after Aramayo et al, 1989.)

The boundaries of a gene on a cloned DNA fragment can be defined by forcing ectopic integration of a given gene sequence, and then testing for wild-type levels of expression. This has been done by transforming with a vector carrying part of a selectable marker

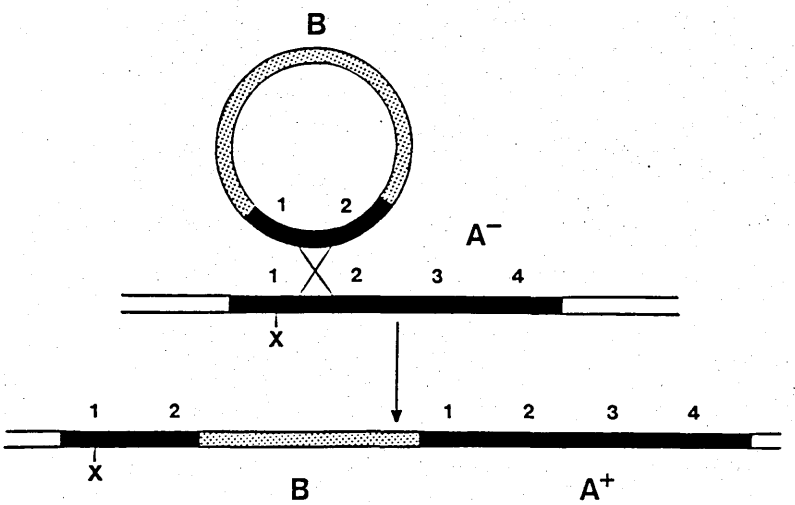

Figure 1.7. **Forced integration of gene B at locus A.**
Transformation with a cloned DNA fragment (shaded box) complements a mutation in gene B. To ensure that the entire B gene is present integration is forced at locus A (black box). This is achieved by using a plasmid containing a part of gene A, which corresponds to the end of the gene containing the lesion. Thus transformation to A^+ may only occur through regeneration of gene A^+ by the recombination event shown.

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corresponding to that part of the resident mutant allele containing the gene lesion (Fig. 1.7). In this case transformation can only occur by homologous recombination between partial transformation marker and resident mutant allele to form a wild type gene (Hamer and Timberlake, 1987; Birse 1989).

The availability of so many ways to manipulate genes using integrative transformation make A.nidulans a versatile system for the study of gene function and expression.

Autonomously replicating plasmid vectors

Up until recently no plasmid vectors which are maintained extrachromosomally by autonomous replication in Aspergillus have been available. Five major advantages to the use of such vectors are 1) increased frequency of transformation. 2) By virtue of 1), facilitated shotgun cloning, and 3) easier plasmid rescue. 4) Easy definition of gene boundaries. 5) Increases in gene copy number resulting in an overall increase of gene expression may be useful for preparation of gene products (e.g. in industry). Autonomously replicating plasmids are discussed in detail in chapter 3 of this thesis.

Cotransformation

A typical transformation of Aspergillus using 10^7 protoplasts and 3ug of the integrating plasmid pILJ16 yields approximately 1,000 ARG^+ transformants (Johnstone, 1985a). One in 10^4 protoplasts are transformed. This suggests that if transformation of a double mutant to wild type with two genes on separate plasmid were carried out, the frequency of double transformation would be one in $(10^4)^2$ = one in 10^8 protoplasts. From this it could be predicted that if transformation by one plasmid were selected for, 0.01% of transformants would also be transformed by the second plasmid. In actual experiments, however, it is found that up to 95% of transformants also contain the unselected gene (Wernars et al, 1987).

When this phenomenon, known as cotransformation, was first observed in Aspergillus (Kelly and Hynes, 1985) it was argued that it reflected the existence of a subpopulation of 1 in 10^4 protoplasts with a very high degree of competence. The rest were presumably not competent at all. This interpretation was derived from that of Hicks et al (1978),

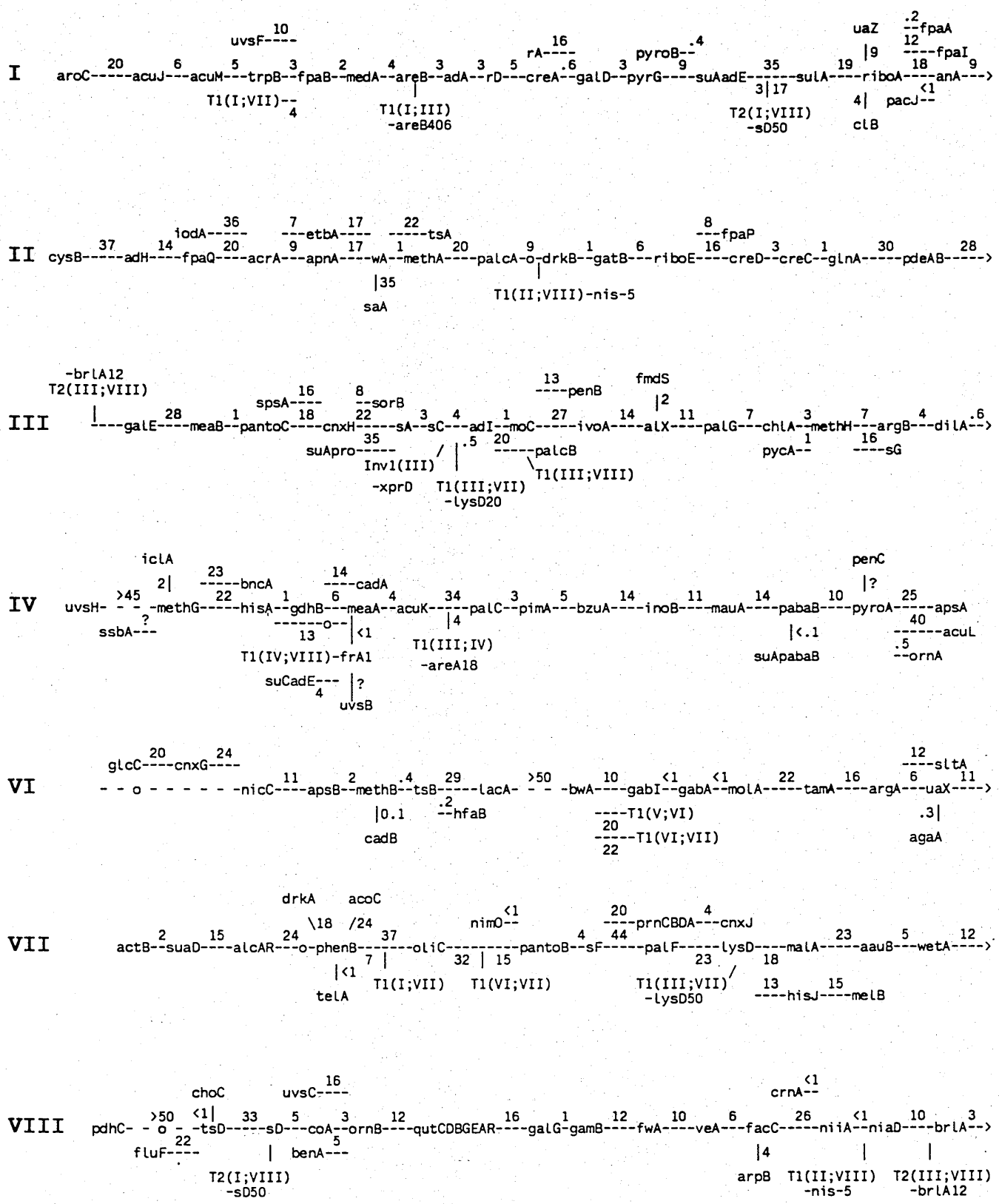
who first observed cotransformation in S.cerevisiae, also using integrative plasmids, and a similar transformation methodology using protoplasts and PEG. This in turn was based on the argument of Kretschmer et al (1975) who observed 50-85% cotransformation in E.coli, using pairs of autonomously replicating plasmids, and cells made competent for transformation by treatment with $CaCl_2$.

Chapter 4 of this thesis contains a detailed discussion of the evidence for this interpretation of cotransformation.

CHAPTER 2

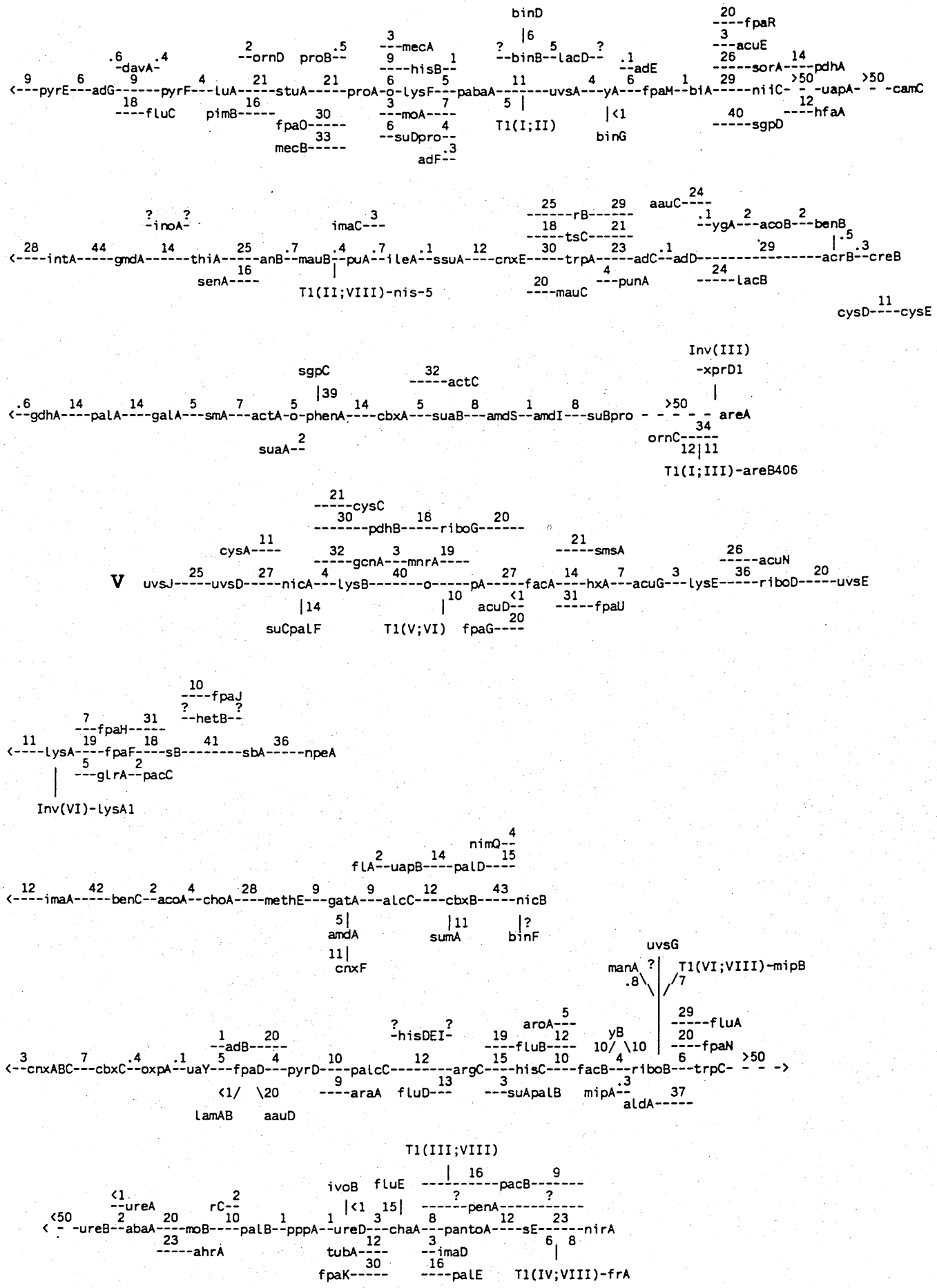
Materials and Methods

Linkage map of



Revised June 1989

ASPERGILLUS NIDULANS



2.1 List of Materials

Material	Source
General chemicals and organic solvents.	B.D.H., Hopkins & Williams, Koch-Light Laboratories, May and Baker.
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.	Amersham.
Geneclean kit.	Amersham.
Prepagene kit.	Bio-Rad.
Frozen competent E.coli cells.	Gibco BRL

All enzymes were obtained from Gibco BRL except the following:

Lysozyme.	Sigma.
NovoZym 234.	Novo Biolabs.

2.2 Bacterial Strains

All those used were derivatives of *E. coli* K-12.

Name	Genotype	Source
DH1	F^-, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, (r^-,m^+), *supE*44.	Hanahan, 1983.
DH5	(As DH1)	Hanahan, 1983.
DS941	*recF*143, *proA*7, *Str*31, *thr*1, *leu*6, *tsx*33, *mtL*1, *his*4, *argE*3, *lacY*1, *galK*2, *ara*14, lambda$^-$, *lacI*^{q}, *lacZ M*15, *lacY*$^+$.	Horii and Clarke, 1973. (Derived from JC9239).
NM538	*supF*, *hsdR*.	Frischauf et al, 1983.
NM539	*supF*, *hsdR*, (P2*cox*3).	Frischauf et al, 1983.
SURE	(*hsdRMS*), *mcrA*, *mcrB*, *mrr*, *endA*1, *supE*44, *thi*-1, -, *gyrA*96, *relA*1, *lac*-, *rebB*, *recJ*, *sbcC*, *umuC*, *uvrC*, [F', *proAB*, *lacIqz M*15, Tn10, (*tetr*)].	Stratagene catalogue

2.3 Plasmids and Bacteriophage

This list includes those used whose construction is not described in this thesis:

Plasmid	Description	Selectable marker	Reference
pUC8, pUC18	Derived from pBR322	*amp*^{R}	Vieira and Messing, 1982.
pIC20-R	Derived from pUC18	*amp*^{R}	
pACYC184	Derived from P15-A cryptic miniplasmid	*cam*^{R}, *tet*^{R}	Chang and Cohen, 1978.
pBLUESCRIPT II KS+	M13-based sequencing vector	*amp*^{R}	Stratagene catalogue pp.104-105, 1988.
pILJ16	AIp	*argB*	Johnstone et al, 1985.
pILJ16 library	Gene bank of *A.nidulans* DNA	*argB*	Johnstone, 1985a.
ARp1	ARp	*argB*	Johnstone, 1985a.
pILJ20, 23, and 25	Subclones of ARp1	*argB*	Johnstone, 1985a.
pTA11	pIC20-R based AIp	*trpC*	Mullaney et al, 1985; Tom Adams, pers. comm.
pMS1	Site-directed AIp	*argB*	Moira Stark, pers.comm.
pCEB218	AIp	*argB*, *ivoB*	Birse, 1989.

Bacteriophage	Description	Reference
EMBL3	Lambda replacement vector	Frischauf et al, 1983.

2.4 Aspergillus strains

The strains of A.nidulans used in this work were from Glasgow stocks (Clutterbuck, 1974), and from A.J. Clutterbuck's personal collection or were constructed for the work described in this thesis. Strains of A.oryzae were provided by Dr. C.A. Batt, and A.niger by Dr. F.P.Buxton of Allelix Inc.

Strain	Genotype
A.nidulans	
G089	fpaB37 riboA1 biA1
G0112	yA2;pyroA4;methB3
G12	fpaB37 adF17 pabaA1 yA2
G34	yA2; methH2 argB2
G35	yA2;methH2 phenA2
G52	adG14 yA2; lysB5
G53	ygA; nicA2 pA3 facA303 riboD5; methB3
G115	riboA1 adG14 luA1 yA2
G122	riboA1 proA1 yA2 adE20; pyroA4
G135	luA1 yA2; adD3
G141	proA1 adF9 pabaA1 biA1
G151	adF33 pabaA1 yA2; wA3
G175	adF9 pabaA1 yA2
G195	suA1adE20 yA2 adE20; acrA1;galA1; pyroA4; facA303; sB3;nicB8; riboB2
G196	trpB403 pabaA1 yA2
G197	suA1adE20 lysF88 pabaA1 yA2 adE20
G225	yA2 pyroA4; adC1
G523	proA1 pabaA1 yA2; cysA1
G712	yA2; palD8 nicB8 wetA6 malA1
AJC9.4	pabaA1; argB2;brlA42 ivoB63
AJC9.41	argB2: uvsB314; ribo A/B
AJC9.45	biA1; argB2; uvsD153; fwA2;
AJC9.43	biA1 pabaA1; argB2; uvsC114

(cont.)

Strain	Genotype
DHG001	yA2; acrA1;galA1 argB2; pyroA4; facA303; sB3; nicB8; riboB2 niaD17
DHG019	yA2 pabaA1; trpC801; argB2; brlA42; methH2
DHG042	medA11^{ts} pabaA1; brlA42
DHG050	medA11^{ts} pabaA1 yA2
DHG054	medA13 adF17 pabaA1
DHG056	medA17 adF17
DHG060	medA13 adF17 pabaA1;sthB1
DHG065	medA14 yA2 biA1; sth-65
DHG066	medA13 biA1; sth-66
DHG067	medA13 biA1; sth-67
DHG068	medA13 biA1; sth-68
DHG069	medA13 biA1; sth-69
DHG070	medA13 biA1; sth-70
DHG072	medA17 adF17 sthA1
DHG076	medA17 sthA1 yA2; argB2; methB3
DHG080	yA2; sthB1; methB3
DHG081	yA2 adF17; sthB1
DHG085	medA14 biA1; pyroA4; sthB1
DHG087	medA17 sthA1 adF17
DHG088	medA13 adF17; sthB1
DHG090	medA17 sthA1 biA1; pyroA4
DHG124	ygA6; riboE6, thiA4; argB2;methB3
DHG135	biA1 methH2 argB2; trpC801
DHG151	medA17 yA2 pabaA1 sthA1
A.oryzae	
YTH-13	argB$^-$
A.niger	
350.25	argB52

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. Solid L-agar: as LB-broth with the addition of 12g $N^{O}.3$ Oxoid agar. For phage work a supplement of 10mM $MgSO_4$ was added to LB-Broth.

BBL Agar: 10g Trypticase peptone (BBL11921), 5g NaCl, made up to 1 litre in distilled water, adjusted to pH7.2 with NaOH and solidified with the addition of 10g Taiyo agar.

BBL Agarose Overlay: As for BBL agar with the addition of 2.5g $MgSO_4.6H_2O$ before solidification with 6.5g Agarose (type 1 low EEO A6013).

2.6 Aspergillus Culture Media

Liquid Minimal Medium (LMM): 10g glucose, 2g $NaNO_3$, 10mls -CN solution, 1ml Trace Elements Solution, adjust volume to 1 litre with distilled water. **Solid minimal medium:** as liquid, with the addition of 12g Taiyo agar.

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

Complete Medium (CM): As for minimal medium plus 2g peptone, 1g Yeastrel, 1.5g Casein Hydrolysate, 1ml Vitamin Solution.

Nitrogen Free Sucrose Osmotically Stablised Medium (SOS-N): 342g sucrose, 10mls -CN Solution, 1ml Trace Element Solution, 20g Difco Agar, volume brought up to 1 litre with distilled water.

-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 $CuSO_4$, 800mg $FePO_4$, 800mg $MnSO_4.4H_2O$, 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 Supplements

Only those required by strains commonly used in this study are listed.

Supplement	Final Concentration
adenine	50mM
aneurine	0.2mg/ml
biotin	0.04ug/ml
L-arginine	1.0mM
L-leucine	4mM
L-lysine	1.0mM
L-methionine	1.0mM
L-proline	4mM
L-tryptophan	4mM
nicotinamide	2ug/ml
p-amino benzoic acid	5.0uM
pyrodoxine HCl	0.05ug/ml
riboflavin	5mM

2.7 Sterilisation

All growth media were sterilised by autoclaving at 120^{o}C for 15 minutes, with some supplements and buffers autoclaved at 108^{o}C for 10 minutes. L-tryptophan, which breaks down at 120^{o}C, was sterilised by filtration through a 0.22um filter.

2.8 Buffer Solutions

Electrophoresis

DNA

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

10x TAE Buffer: 48.4g Tris, 3.6g NaAc, 3.6g $Na_2EDTA.2H_2O$, made up to 1 litre with distilled water, pH adjusted 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.

RNA

10x MOPS: 41.8g Morpholinopropanesulphonic acid, 4.1g NaAc, 1.86g Na_2EDTA, adjust pH to 7.0 with NaOH then make up to one litre with RNase free (R/F) distilled water.

MMF: 500ul Formamide, 162ul 37% formaldehyde, 100ul MOPS, 338ul H_2O.

Formaldehyde Gel Loading Buffer: 50% glycerol, 1mM Na_2EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol, made up in R/F distilled water.

DNA Manipulation

Restriction and Ligation Buffers: Obtained form Gibco BRL

ATP Stock Solution (100mM): Dissolve 60mg ATP in 0.8ml distilled water, adjust pH to 7.0 with 0.1M NaOH, make up to 1ml with water; store at $-20^{o}C$.

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

10x Klenow Buffer: 0.5M Tris, 0.1M $MgSO_4$, 1mM dithiothreitol, 500ug/ml bovine serum albumen; store at $-20^{o}C$.

DNA and RNA Hybridisation

Nick Translation Buffer: as 10x Klenow buffer.

10x Kinase Buffer: 100mM KCl, 70mM Tris, 10mM $MgCl_2$, pH to 7.6, 5mM DTT (added after autoclaving.)

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

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

Denaturing Solution: 1.5M NaCl, 0.5M NaOH.

Denhardt's Solution: 0.2mg/ml BSA, 0.2mg/ml Ficoll-400, 0.2mg/ml polyvinyl pyrolidone.

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

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

DNA and RNA 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 phage storage and dilution:- 5.8g NaCl, 2g $MgSO_4$, 2% gelatin, 1 mM Tris, pH to 7.5 then made up to 1 litre with 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, made up fresh.

Birnboim Doly Buffer III: 5M KAc, pH4.8, mix equal volumes of 3M CH_3COOK, and CH_3COOH, pH should be 4.8.

STET Buffer: 8% sucrose, 5% Triton-X100, 50mM EDTA, 50mM Tris, pH to 8.0.

A.nidulans **DNA Extraction Buffer:** 200mM Tris, 300mM NaCl, 25 mM EDTA, 0.5% SDS, pH to 8.5.

A.nidulans **RNA Extraction.** All glassware used for work involving RNA was heat baked at 250°C overnight to render RNase inactive. Plasticware was soaked in 1% DEPC overnight. All solutions termed "RNase free" (except those containing amine groups), were treated with 0.1% DEPC overnight followed by autoclaving at 120°C for 15 minutes. Compounds containing amine groups (e.g. Tris, EDTA) were taken from previously unopened bottles and added to RNase free water. Gloves were always worn when working with RNA.

5x RNB: 1.0M Tris, 1.25M NaCl, 0.25M EGTA, pH to 8.5 with NaOH.

RNA Extraction Mix: 4.8g p-amino salicylic acid (PAS) and 0.8g of tri-isopropylnaphthalene sulphonic acid are dissolved separately in 40mls of distilled R/F water. The PAS is added to the TNS with constant stirring and 20ml of 5x RNB is added to the PAS-TNS to produce the extraction buffer. This mixture can be stored on ice for up to 6 hours.

Transformation

A.nidulans

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 phoshate buffer, pH to 5.6.

E.coli

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

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

Antibiotics: **ampicillin:** Added to both liquid and plates to a final concentration of 50ug/ml. Stock solution: 100mg/ml in sterile distilled water, stored at 4°C for up to a month. **Chloramphenicol:** Added to both liquid and plates to a final concentration of 10ug/ml. Stock solution: 34mg/ml in 100% EtOH, stored at -20°C for up to a year.

In all cases medium was cooled to <55°C before adding the antibiotic.

2.9 E.coli Growth Conditions: Liquid cultures for transformation or plasmid or phage DNA preparations were routinely grown in L-broth at 37°C with vigorous shaking. Plate cultures were grown on L-agar with antibiotics added as required. When plating bacteriophage, phage particles were mixed with plating cells and incubated at 37°C for 20 minutes to allow the phage to adsorb to the bacteria. This suspension was then added to 3.0mls of cooled (47°C) BBL Agarose Overlay, then poured onto a hardened BBL agar plate. After setting, the plates were inverted and incubated at 37°C. Plaques were counted and picked after 12-16 hours of incubation.

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

2.10 Aspergillus Growth Conditions: Strains were kept and subcultured on solid medium, complete medium being used for all strains with the exception of transformants which were cultured on minimal medium to maintain selection for the transformed phenotype. Incubation was normally at 37°C, healthy strains conidiating after 2-3 days.
Liquid medium with the appropriate supplements was used for the production of mycelia for transformation experiments and for DNA preparations.

2.11 In vivo Techniques : E. coli

Standard methods as described in Maniatis et al, 1982.

Transformation of E.coli

Competent Cells

A single colony was picked and grown in 5mls of L-broth at 37°C with shaking to $O.D._{550}$ = 0.3 (2 hours). This culture was used to inoculate 100mls LB-broth prewarmed to 37°C. Growth was allowed to continue until a cell density of approximately $1x10^8$ cells/ ml (mid-log phase: $O.D._{550}$ = 0.48 for DH1) had been reached. Cells were chilled on ice for 10 minutes, transferred to 40ml tubes and then harvested by centrifugation at 1,000g for 5 minutes at 0°C. The pellet was resuspended in 30mls of ice-cold TfbI. Cells were then spun down immediately, as above, and very gently resuspended in 4mls TfbII by gently drawing them through a cut-tipped 1ml pipette. The cell suspension was then incubated at 0°C for 15 minutes. 200ul aliquots were dispensed into pre-cooled Eppendorf tubes, then snap-frozen by dropping them into liquid nitrogen. Cells produced in this manner and stored at -70°C remained competent for at least 5 years.

Transformation

Cells were thawed at room temperature until just liquid, before incubation at 4°C for 5-10 minutes (60 minutes maximum.) The transforming DNA was then added to the cells and then mixed by gentle stirring. The DNA should be added in a volume equal to or less than one quarter of the volume of the competent cells and at a concentration of less than 0.1ug/200ul cells. The cells were left on ice for 20 minutes before heat-shocked at 42°C for 45 seconds. The cells were then put back on ice for 1-2 minutes to quench the heat shock. 4 volumes of LB-broth were then added and the cells incubated at 37°C for 40-60 minutes to allow expression of the antibiotic resistence gene. Transformed cells were then poured onto antibiotic supplemented L-agar plates which had previously been air dried for several hours.

2.12. In vivo Techniques : Aspergillus

Classical Techniques: All methods were as described in Pontecorvo et al, 1953 and Clutterbuck, 1974.

Meiotic Crosses: Conidia of the two strains to be crossed (both carrying different auxotrophic markers) were mixed in a small volume of CM and plated in the centre of a thick plate of MM. The plate was incubated for 24 hours at 37°C, then sealed with tape and incubated for a further 10 days to 3 weeks at 37°C. Mature cleistothecia were picked and freed from mycelial debris and Hulle cells by rolling them with a dissecting needle over the surface of an agar plate. They were then crushed and suspended in distilled water and appropriate dilutions plated out onto CM; the presence or absence of markers form both parents among progeny showed whether a cleistothecium was hybrid or selfed. Master plates each containing an ordered array of 24 progeny from recombinant cleistothecia were constructed on CM, and replica plated onto MM with appropriate supplements in order to determine the genotype of the recombinant progeny.

Parasexual Analysis

Formation of heterokaryons: Conidia from two strains with different nutrient requirements and different spore colour phenotypes were streaked in parallel lines approximately 4-5 mm apart on CM and incubated at 37°C for 2 days. When the edges of the two colonies had grown together, a sterile scalpel blade was used to cut out $2mm^3$ cubes of CM and fungus from the junction region. These were plated on MM. Under these conditions only heterokaryotic mycelium can grow. After 3-4 days growth at 37°C the heterokaryotic colony fills the plate. $2mm^3$ cubes taken from the colony periphery were subcultured on MM, and then after another 3-4 days, subcultured again.

Selection for diploids: After 3-4 days a suspension of conidia from the heterokaryon was prepared; this was filtered through cotton wool to remove heterokaryotic mycelial debris. Conidia were added to hand-hot molten MM to different concentrations and plates poured. When set, these were incubated at 37°C for 2-3 days after which bright green diploid colonies were seen. To check that these were diploids, sizes of conidia were compared microscopically with conidia from a haploid

strain, using an eyepiece graticule. Chains of 3 conidia were examined. [length of diploid chain]^3 was roughly [length of haploid chain]^3 x 2.

Haploidisation: Diploids were then stab inoculated onto CM plus 2mg/ml benlate (benomyl) which stimulates mitotic haploidisation (McCully and Forbes, 1965; Hastie, 1970). Haploids appeared as fast growing sectors, which were used to inoculate master plates. Genotypes of haploid segregants were then ascertained by standard means.

Molecular Techniques

Transformation of Aspergillus: The method of transformation used was that described by Tilburn et al, 1984 and modified by Johnstone, 1985a.

Culture of mycelium: Mycelium was grown in liquid MM with appropriate supplements for auxotrophic strains. 1 litre flasks containing 200mls of medium were incubated for 12-16 hours at 37^{o}C with vigorous shaking. Normally conidia from fresh growth on solid medium was used for inoculation: about 10^8 conidia / 200ml culture medium. The mycelium was harvested using sterile conditions.

Protoplast Production: The mycelium was suspended in APB (about 1g wet weight/5mls, which should give a pea-soup consistency) in a 20ml universal container to which B-mercaptoethanol and BSA were added to final concentrations of 5mM and 2mg/ml respectively. This step increased protoplast viability after transformation 10-fold (Johnstone, 1985a). NovoZym 234 was then added to a final concentration of 4mg/ml. This was incubated from 90-180 minutes in an orbital shaker (100 rpm) at 30^{o}C. The protoplasted mix was transferred to 30ml Corex centifuge tubes and an overlay of 1ml of 0.5x ATB was gently added to the surface of the contents of each tube. The protoplasts were separated from debris by centrifugation at 5,000g for 10 minutes at 4^{o}C in a swing out rotor; the protoplasts formed a sharp band at the interface between APB and 0.5x ATB.

The protoplasts were gently drawn off using a cut-tipped pipette and resuspended in three volumes of ATB relative to the protoplast mix, and pelleted by centrifugation at 4,000g for five minutes. The

protoplasts were washed twice in the same volume of fresh ATB and finally resuspended in ATB at a density of $5 \times 10^7 - 2 \times 10^8$ protoplasts/ml.

Transformation: 200-300 ng transforming DNA was added per 10^6 protoplasts. DNA was in a volume of 10ul TE to a 100ul volume of protoplast suspension. This was incubated at room temperature for 15 minutes; 10 volumes of 60% PEG MW 8,000 (in ATB) was then added, followed by a further 15 minutes incubation. The PEG was then diluted by the addition of 5 volumes of ATB.

Regeneration: Protoplast suspensions were gently pipetted onto petri plates. Luke warm molten SOS-N agar, with appropriate supplements, was then poured into the plates and mixed with the protoplasts by agitating the plates by hand. After setting plates were incubated at 37^oC . Transformed colonies appeared after 2 days in the case of ARp plasmids, and 3 days with AIp plasmids.

2.13 In Vitro Techniques

The standard methods were as described in Maniatis et al, 1982.

Plasmid Preparation

The plasmid content of *E. coli* transformants was routinely screened by a combination of Single Colony Gel Electrophoresis, and restriction mapping following plasmid isolation.

Single Colony Gel Electrophoresis: This technique enables the plasmid content of an *E. coli* transformant to be observed. A single colony is patched out ($1cm^2$) on a selective plate and grown overnight. Using a toothpick a blob of cells is collected and resuspended in 100ul of Single Colony Gel Buffer. 25ul of the suspension containing the plasmid is then loaded onto an agarose gel.

A more detailed screening of plasmid content involved firstly purification of plasmid DNA followed by restriction analysis.

Boiling / STET Preparation

The boiling or STET plasmid miniprep (Holmes and Quigley, 1981) allows isolation of a small amount of restrictable DNA from a large number of E. coli transformants in a relatively short time.

Cells from a 1.5ml overnight culture were pelleted by spinning in a microfuge for 20 seconds. The pellet was resuspended in 350ul of STET buffer and 25ul of lysozyme (10mg/ml) was added. Lysis having been achieved through incubation in a boiling water bath for 40 seconds, the coprecipitated cellular debris and denatured chromosomal DNA was pelleted by centrifugation for 15 minutes and removed with a toothpick. The plasmid DNA was then precipitated by adding 40ul 3M NaAc and 400ul cold isopropanol. The precipitated DNA was pelleted by spinning for 7 minutes, and washed with 70% EtOH. The excess alcohol was drained off and the Eppendorf tubes used left inverted for 15 minutes to dry. The pellet was then resuspended in 50ul TE.

Birnboim-Doly large scale preparation of plasmid DNA

A modification of the Alkaline-SDS extraction of Birnboim and Doly (1979) was used for large-scale preparation of plasmid DNA.

100ml of plasmid containing cells were pelleted by centrifugation at 3,840g for 5 minutes. The pellet was resuspended in 4mls of Birnboim/Doly solution I and incubated at room temperature for 5 minutes. Cells were then lysed by the addition of 8mls of freshly prepared Birnboim/Doly solution II, with mixing by gentle inversion; the mixture was then left on ice for 5 minutes. 6mls of cooled Birnboim/Doly solution III was then added, and again gently mixed until viscosity was reduced. The white, flocular precipitate was spun down by centrifugation at 35,000g at $4^{o}C$ for 10-20 minutes and the supernatent poured into a tube containing 12mls of isopropanol. After mixing the plasmid DNA was left at room temperature for 15 minutes to precipitate before being centrifuged at 35,000g for 10 minutes at room temperature. The pellet was washed carefully with 70% ethanol, dried and resuspended in TE.

The plasmid DNA was purified by CsCl/EtBr density gradient ultracentrifugation: 4.5g CsCl was dissolved in 4.6mls of cleared lysate. 240ul EtBr (10mg/ml) was added and this solution transferred to a 6ml polypropylene ultracentrifuge tube and spun at 55K (267,000g) at 20°C for at least 4 hours (Vti65 rotor). The plasmid band was removed with a hypodermic syringe. After removal of EtBr by repeated extraction with water-saturated butanol, CsCl was removed by 3 successive dialyses against a 1,000X volume of TE. The first two dialyses were for 20 minutes, and the final one, 60 minutes.

Bacteriophage DNA Preparation

Preparation of plating bacteria: 50mls of LB-broth containing 0.2% maltose in a 250ml flask was inoculated with a single colony of E.coli (e.g. non-permissive strain NM539) and incubated at 37°C overnight with shaking. The cells were then pelleted by spinning at 4,000g for 10 minutes at room temperature, and resuspended in 100mls 10mM $MgSO_4$; this should give a cell density of about 1.5 x 10^9/ml (OD_{600} = 2). Plating bacteria prepared in this way stored at 4°C remain viable for up to 3 weeks.

Purification of Bacteriophage: A single viral plaque was picked from a plate (>12 hours old, at 37°C) with a toothpick and added to 1ml of SM buffer, and vortexed. 100ul of the resulting phage suspension was added to 250ul of plating bacteria, plated, and incubated overnight at 37°C. This results in confluent lysis. 10mls of SM buffer was then added to the plate, which was then incubated for 2 hours at room temperature on a rocking platform. 5mls of the resulting suspension was then added to 400ml of cultured E.coli cells (e.g. permissive strain NM538), OD_{600} = 0.3, grown in LB-broth supplemented with 10mM $MgSO_4$, in a baffled 2 litre flask. This was then incubated at 37°C until lysis had occurred (4-6 hours).

10mls of chloroform was then added and incubation allowed to continue for a further 10 minutes. Cellular debris was removed by centrifugation at 14K (15,000g) for 20 minutes. The supernatant was transferred to a new vessel and DNase and RNase (2ug/ml final concentration) were added and incubated for 20 minutes at room temperature. Next, NaCl was added to a final concentration of 2% w/v and left for one hour on ice to precipitate unwanted proteins. These

were pelleted by centrifugation for 5 minutes at 15,000g at 4°C. The phage particles were then precipitated from the supernatent by the addition of PEG 6,000 to a final concentration of 8% w/v. After incubation at 4°C for 4 hours the phage were pelleted by a 5 minute spin at 15,000g at 4°C, and the supernatent decanted. The centrifuge vessels were left inverted for 10 minutes to drain off all of the supernatent. The precipitated phage were then resuspended in 12mls SM, then purified by density gradient ultracentrifugation.

Following the addition of 0.71g CsCl to each 1ml of SM, a phosphate precipitate formed which was removed by centrifugation for 10 minutes at 12,000g, 20°C. The CsCl/phage containing supernatent was transferred into a polypropylene tube and spun to equilibrium (270,000g, 4 hours, Vti65 rotor). The band of phage, which against a black background appears a pale bluish grey, was removed using hypodermic syringe; the CsCl was then removed by serial dialyses against SM.

DNA Extraction: Removal of DNA from the bacteriophage suspension was carried out after incubation with EDTA (20mM), proteinase K (50ug/ml) and SDS (0.5%) for a period of 60 minutes at 37°C. The DNA was then extracted twice against phenol/chloroform and then once against chloroform with residual chloroform being removed by dialysis against a 1,000x volume of TE, twice for 20 minutes and then once for one hour.

Preparation of High MW DNA from Aspergillus: based on the method of Raeder and Broda (1985).

Mycelium grown for 14-20 hours at 37°C was harvested by filtration, then immersed in liquid nitrogen and ground to a fine powder using a pestle and mortar. 10mls of extraction buffer was quickly added to each 1g of the freeze dried powder and this was mixed by inversion. A 70% volume of buffered phenol was added and mixed in, followed by a 30% volume of chloroform. After 30 seconds of mixing by gentle inversion the suspension was centrifuged for 60 minutes at 13,000g and the aqueous upper layer was removed. To this was added RNase to a final concentration of 250ug/ml; this was left to incubate for 30 minutes at room temperature on a tube roller. Next, the solution was extracted with 1 volume of chloroform and centrifuged for 10 minutes

at 13,000g. The upper, aqueous phase was transferred to a new tube and 0.51 volumes of isopropanol added to precipitate DNA. After 15 minutes the DNA was pelleted by centifugation for 10 minutes at 13,000g. The supernatent was discarded, and the pellet gently washed with 70% ethanol, dried, and then dissolved in TE.

Precipitation of DNA:

DNA was precipitated either using EtOH or isopropanol. In each case DNA solutions were made 0.3M with respect to NaAc by the addition of 1/9 volume of 3M NaAc pH 5.3. For ethanol precipitation 2.5 volumes of EtOH (absolute alcohol) were added and the precipitation was carried out at $4^{o}C$ for 30 minutes, or 10 minutes at $-20^{o}C$. Isopropanol precipitation was performed by adding 0.6 volumes of isopropanol to the DNA solution. This was then left for 30 minutes at room temperature.
Precipitated DNA was pelleted by centrifugation at 12,000g in a microfuge for 15 minutes or Beckman JA2-21 JA20 rotor for 20 minutes at 18K (40,000g). Temperature was $0^{o}C$ in the case of EtOH precipitation, and $20^{o}C$ in the case of isopropanol precipitation. After decanting the supernatent, traces of salt were removed from the pellet by washing with 70% EtOH. The pellet was then dried under a vacuum.

Restriction of DNA:

DNA was routinely restricted in 0.4ml Eppendorf tubes. Reactions were usually performed in a total volume of 20ul containing 0.1-1.0 ug of DNA, 2ul 10x restriction buffer and 1.0 to 10.0 units of enzyme, the volume being made up with dH_2O. The reactions were incubated at the appropriate temperature, as recommended by the suppliers (usually $37^{o}C$) for 1 hour after which time the digestion was usually complete. Restriction was arrested by either the addition of final sample buffer or if serial treatments were required phenol extraction and EtOH precipitation. Restrictions involving Lambda DNA for size markers were always heated to $65^{o}C$ for 5 minutes to melt the cohesive ends prior to loading on a gel.

Ligation of DNA:

T4 ligase catalyses the formation of covalently joined hybrid DNA molecules from "sticky" or blunt ended molecules. The concentration of fragment "ends" to vector "ends" was adjusted to approximately 2 to 1, and the total concentration of DNA was adjusted to 5-20 ug/ml. A typical 20ul reaction mix comprised about 100ng of DNA (vector and fragment), 4ul 5x BRL ligase buffer, and 0.1 unit of T4 DNA ligase for "sticky ended" ligations, or 1 unit for "blunt ended" ligations, the volume being made up with distilled water. For "blunt-ended" ligations the reaction was allowed to proceed for more than 12 hours at 16^{o}C, whilst "sticky-ended" reactions were normally carried out at room temperature for about 4 hours.

Phosphatase Treatment:

Self-ligation of cloning vector molecules dramatically reduces the efficiency of DNA cloning. By removing the 5' terminal phosphate groups on linearised vector DNA prior to mixing with target fragment, self-ligation is minimised because to work T4 DNA ligase requires a 5' terminal phosphate group on one precursor molecule. As calf intestinal phosphatase (CIP) functions in most restriction buffers, it was usually added (0.1 unit) to a vector DNA restriction digest. To dephosphorylate protruding 5' termini, incubation was allowed to proceed for 30 minutes after restriction at 37^{o}C before a second aliquot (0.1 unit) was added and incubated for a further 30 minute period. When dephosphorylating DNA with blunt ends or recessed 5' termini, incubation took place at 37^{o}C for 15 minutes, followed by 56^{o}C for 15 minutes. Following the addition of another 0.1 unit of CIP incubation at both of these temperatures was repeated.

To remove CIP the "phosphatased" system was heated to 68^{o}C for 15 minutes in the presence of 2% SDS and extracted twice with phenol/chloroform. DNA was recovered by either precipitation or the use of the Gene-clean or Prepagene systems.

Extraction of DNA from Agarose Gels Using Gene-Clean and Prepagene:

These methods were carried out according to the manufacturers' instructions. Both processes were used for the purification of bands from agarose gels either for cloning, radiolabelling for use as a hybridisation probe, or for transformation of Aspergillus.

DNA is first run on a gel (in the case of Gene-clean this has to be a TAE gel). The desired band is cut out, weighed in an Eppendorf tube and then chopped into small pieces. 2.5 volumes of NaI are added and the Eppendorf placed in a 55^{o}C water bath for 10 minutes, or until the agarose gel has dissolved. Alternatively, the agarose block is placed in a small Eppendorf tube with a pin-hole in the bottom overlayed with a glass wool plug. This is then placed in a large Eppendorf tube, and spun in a microfuge for 2 minutes. The DNA in solution is then withdrawn from the large Eppendorf tube. 5ul of glass milk (a suspension of powdered glass in dH_2O) is then added and the tube vortexed briefly; the suspension is incubated for 5 minutes on the bench (or on ice in the case of Gene-Clean) and then spun in a microfuge for 5 seconds. The supernatant is discarded and the pellet resuspended in 600ul NEW (Gene-Clean) or wash (Prepagene) buffer. It is then pelleted as before and washed again twice before DNA is eluted by resuspension in TE.

Extraction of RNA: - modified from Timberlake, 1980.

Mycelial mats were peeled from agar-filled petri dishes and immediately submerged in liquid nitrogen. The mycelium was ground to a fine powder with a pestle and mortar, which was added quickly to a cooled mixture of 8ml of RNA extraction buffer and 4mls of buffered phenol per mycelial mat. The mixture was vortexed for 10 minutes intermittently: 30 seconds of vortexing followed by 30 seconds on ice. 4 mls of chloroform was added and then the mixture was centrifuged for 10 minutes at 12,000g and 4^{o}C. The aqueous phase was re-extracted twice with phenol/chloroform. LiCl (10M) was added slowly to a final concentration of 2M and RNA allowed to precipitate overnight at 4^{o}C. The RNA was pelleted at 8,000g for 15 minutes at 4^{o}C, then washed with 70%EtOH.

2.14 : Nucleic Acid Hybridisation

Probe Radiolabelling

Nick Translation: The enzyme E.coli DNA polymerase I was used in conjunction with DNase I to prepare ^{32}P-labelled DNA suitable for use as a hybridisation probe. DNA polymerase I has a 3' to 5' polymerase, and a 3' to 5' and a 5' to 3' exonucleolytic activity, facilitating the replacement of pre-existing nucleotides with radioactive ones. The enzyme requires a nicked double strand DNA template, the nicks being produced by the activity of the enzyme DNase I.

A typical nick translation consisted of 0.2 to 1.0ug of DNA (usually plasmid DNA or a gel purified restriction fragment of a plasmid) , 2ul of a 1mM solution of each of the 3 unlabelled dNTPs, 20uCi [-^{32}P]dATP 1,000Ci/mMole, 5ul 10x Nick Translation Buffer, the volume being made up to 50ul with sterile distilled water and chilled on ice. Nicks were introduced by the addition of 0.5ul DNase I (0.1ug/ml) prior to the addition of 5 units of DNA polymerase I. This was incubated for at least 2 hours at 16^{o}C. 2ul 0.5M EDTA was then added to stop the reaction and the unincorporated dNDPs and dNTPs separated from labelled DNA by passage through a Sephadex G-50 column with the marker dyes phenol red and dextran blue. The dNDPs, dNTPs and phenol red enter the matrix and are retarded; DNA and phenol blue are excluded from the matrix and is eluted first.

Random priming: In this reaction DNA to be radiolabelled is first linearised by restriction (if it isn't linear already) and then denatured by heating to 95-100^{o}C for 2 minutes, then chilled on ice. Random sequence hexanucleotides can be used to prime DNA synthesis on a single stranded DNA template by the DNA polymerase I Klenow fragment (Feinberg and Vogelstein, 1983). Using this technique, up to 80% incorporation of radiolabelled dNTPs added can be achieved; using random priming the probe specific activity obtained is usually considerably higher than that using nick translation.

Random priming reactions were usually carried out in a volume of 50ul. This contained 10-50ng linear DNA, 5ul 10x reaction buffer, 5ul hexanucleotide primer solution, 4ul 1mM solutions of each of the 3 unlabelled dNTPs, and 20uCi (-^{32}P)dNTP 1,000 Ci/mMole, (usually dATP or dCTP), and 20 units of DNA polymerase I Klenow fragment. The volume was made up to 50ul with dH_2O. This was incubated at 37^{o}C for 30

minutes. Separation of radiolablled DNA was as described for nick translation.

Southern Blotting:

DNA was transferred from agarose gels to Hybond-N (Amersham) nylon membrane, using the procedure of Southern (1975), and those modifications suggested by the manufacturers of the membrane.

After electrophoresis, when Aspergillus chromosomal DNA was present, the DNA was partially depurinated by soaking the gel in 0.25M HCl for 30 minutes. The gel was then soaked in denaturing and then neutralising solution for 30 minutes with gentle agitation, e.g using a rocking platform. A glass plate spanning two reservoirs of 20x SSC was covered with a sheet of filter paper (Whatmann 3MM), the filter paper being submerged in 20x SSC on both sides. The gel was placed on top of the paper and a piece of nylon membrane, cut to size, was placed on the gel, with care to exclude any air-bubbles. The membrane was then covered with two sheets of filter paper and then a thick stack of absorbant paper towels, a sheet of glass, and finally a weight (about 1kg). The transfer of DNA then took 12-16 hours after which the membrane was removed, washed for a few seconds in 2x SSC, air dried for 15 minutes, and then exposed to U.V. radiation in a Stratalinker U.V. oven to crosslink the DNA to the filter.

Northern Blotting:

The presence of formaldehyde in RNA gels meant that no pre-treatment of the gel to denature the nucleic acid was necessary, and so after electrophoresis the agarose gel was simply inverted, covered with Hybond-N, filter paper and paper towels. RNA was then transferred onto the nylon membrane through the capillary action of 20x SSc being absorbed into the paper towels. After blotting the RNA was UV-fixed to the Hybond-N before hybridisation.

Nucleic Acid Hybridisation

Radiolabelled probe (high stringency): The methodology is modified from that recommended by the manufacturer of Hybond-N membrane. The membrane was prehybridised in sealed bags in 5ml 5x SSPE, 0.2% SDS per 100 cm^2. The membrane was incubated for one hour at 65^oC, with gentle agitation. Hybridisation with the radiolabelled probe was performed in fresh prehybridisation buffer. The probe was denatured by boiling for 2 minutes, chilled on ice, then added to the bag. This was incubated for 65^oC. Incubation time varied with the abundance of DNA target sequences. In the case of lambda plaques, or digests of plasmid DNA, 4-8 hours is sufficient. In the case of single sequences in digests of genomic DNA >12 hours is necessary. The bag was then opened and the radioactive probe discarded. The membrane was washed three times in Pall Blot-wash for 20 minutes at room temperature and then wrapped in Saran-Wrap. The intensity of the radioactive signal on the filter was then checked with a Geiger counter. This was then placed in an autoradiography cassette with a sheet of Kodak Xomat S1 and an intensifying screen, and incubated at -70^oC for from 1 hour to 2 weeks, depending on the intensity of the radioactive signal on the filter.

Low stringency hybridisation: This was as high stringency except that a different hybridisation buffer was used, and incubation was at a lower temperature. The buffer used (from DeFeo-Jones et al, 1983) was 5x SSPE, 0.1% SDS, %x Denhardt's solution, 40% deionised formamide and 5% (w/v) dextran sulphate. 5mg/ml denatured sonicated salmon sperm was added to the prehybridisation buffer. Hybridisation was carried out at 42^oC for >18 hours. Filters were washed serially, for 20 minutes in conditions of decreasing stringency: 2x SSC, 0.1% SDS, then 1x SSC, 0.1% SDS, and then 0.5x SSC, 0.1% SDS at 22^oC, and then the same 3 washes at 42^oC (DeFeo-Jones et al, 1983).

Enhanced chemiluminescence probe (ECL): This system involves the electrostatic binding of horseradish peroxidase (HRP), complexed with a cationic polymer, to anionic single stranded DNA. Addition of glutaraldehyde then results in covalent cross-linkage of enzyme to probe. This is then hybridised to DNA previously Southern blotted onto a nylon membrane. Probe detection involves incubation with hydrogen peroxide, coupled to an oxidation reaction involving the cyclic

hydrazide luminol, which produces blue light. Light production by this reaction is enhanced by addition of a synthetic component of the firefly bioluminescent system, D-luciferin (Whitehead et al, 1983), and can be detected using Xomat film with exposure times of 2-60 minutes.

Reactions were carried out according to the instructions of the manufacturer of the ECL kit (Amersham). Added to 200-500ng of single stranded linear DNA in 20ul of dH_2O was 20ul of HRP solution, and then 20ul glutaraldehyde. Labelled probe may be stored in 50% glycerol at -20°C for long periods. Hybridisation is then carried out at 42°C in a buffer supplied by Amersham. For hybridisation time, see radiolabelled probe. Filters were then washed twice, for 20 minutes at 42°C in primary wash buffer (urea, 360g; SDS, 4g; 20x SSC, 25ml, made up to 1 litre), and then twice in secondary wash buffer (2 x SSC). Filters were then incubated for 1 minute in the luminol/D-luciferin detection solution (Amersham), drained, wrapped in SaranWrap and placed in a film cassette with a sheet of Xomat film. A 5 minute test exposure was carried out, then, based on the results of that, a second exposure, usually between 10 and 60 minutes.

2.15 Electrophoresis Through Gels

DNA: Agarose gels of 1%, 0.8% and 0.4% were used here for the separation of DNA molecules. DNA was made visible by ultraviolet fluorescence after staining with EtBr. The EtBr was either added to the agarose at a final concentration of 0.2ug/ml, or after running, the gel was soaked in 1x running buffer plus 0.6ug ul/ml EtBr. Agarose powder was dissolved at 100°C in 1x running buffer (either 1x TAE or 1x TBE). The molten agarose was cooled to 55°C before being poured into a horizontal gel former using an appropriate teflon well-former, or comb. After setting the gel was transferred to a gel running tank, and submerged with running buffer. After loading the samples the gel was run at 40-150 volts giving a running time of 3-12 hours.

Gels were photographed with UV transillumination (wavelength: 240nm) using a Polaroid camera loaded with Polaroid 4x5 Land Film (no. 57) or a Pentax 35mm SLR loaded with Ilford HP5 film; both were fitted with a Kodak Wratten Filter No.9 (red).

RNA: RNA, stored as a suspension of a precipitate at -20^{o}C, was aliquoted into Eppendorf tubes and pelleted by centrifuation in a microfuge for 15 minutes. The pellets were then dried by vac uum dessication for 10 minutes. The pellets were then resuspended in 25ul of MMF buffer and incubated at 60^{o}C for 15 minutes. After 10ul of Formaldehyde Gel Loading Buffer had been added, the samples were ready to be loaded.

Formaldehyde gels were prepared by melting 4.5g agarose in 300mls 1x MOPS buffer. Upon cooling to 60^{o}C, 48.6mls of the agarose solution was removed and replaced with an equal volume of formaldehyde. The solution was then poured into the gel former and a comb put in place. After setting (20 minutes), the gel was covered with running buffer (1x MOPS), the comb removed and the samples loaded. The gel was run at 100-150 volts for 6-8 hours, the running buffer being circulated through the system by means of a peristaltic pump.

CHAPTER 3

Autonomous replication of plasmids in *Aspergillus*

Introduction

The search for autonomously replicating sequences (ARSes)

At present molecular genetic techniques involving transformation of Ascomycete filamentous fungi utilise plasmid shuttle vectors which transform by integrating into the chromosome of the recipient fungal strain. This is true for A.nidulans (Ballance et al, 1983; Tilburn et al, 1984), A.niger (Buxton et al, 1985; Kelly et al, 1985) A.oryzae (Hahm and Batt, 1987), Neurospora crassa (Case et al, 1979), Podospora anserina (Debuchy and Brygoo, 1984), Penicillium chrysogenum (Bull et al, 1988) and Cephalosporium acremonium (Queener et al, 1985).

Of the various attempts to develop autonomously replicating plasmid vectors for the filamentous fungi the common strategy has been to try to define a sequence of DNA which, spliced into an integrative plasmid vector, would confer upon it the ability to replicate autonomously within the fungus. It was expected that such an autonomously replicating sequence (ARS) would function as an ori, but also perhaps possess secondary functions e.g. affecting plasmid partitioning during nuclear division, or stimulating recombination, or copy number amplification.

There are a number of potential sources of ARSes: chromosomal DNA, mt genomic DNA, mt plasmid DNA, indigenous nuclear plasmid DNA or viral DNA. In each case the source species could be the same or different to the species to be transformed. An additional approach is the use of other chromosomal components: e.g. centromeric and telomeric DNA sequences.

ARSes from indigenous nuclear plasmids

The autonomously replicating yeast episomal plasmid (YEp) shuttle vectors are based on the naturally occurring 2uM circle plasmid of Saccharomyces cerevisiae. The first such plasmid vector was constructed by Beggs (1978) who cloned a bacterial plasmid, pMB9, and the yeast leu2 gene, into the 2uM circle. 2uM circle-like nuclear plasmids have been found in other yeasts e.g. Schizosaccharomyces pombe (Guidice et al, 1979), Saccharomyces rouxii (Toh-e et al, 1982), Kluyveromyces drosophilarum (Chen et al, 1986), and the dimorphic yeast Candida albicans (Kurtz et al, 1987), but in none of the filamentous fungi. No ARS activity of yeast 2uM DNA in filamentous

fungi has been reported.

ARSes from chromosomal DNA

A fragment of yeast chromosomal DNA was isolated by Stinchcomb et al (1979) as an insert which conferred a 100x increase in transformation frequency over the parent integrative vector, YIp5. This fragment was believed to carry a yeast chromosomal origin of DNA replication and was designated ars1. Sequences acting as ARSes in yeast were also isolated from DNA of five other eukaryotes, including Drosophila melanogaster (Stinchcomb et al, 1980). Whether these correspond to origins of replication in the source species is unknown.

The results of Ballance and Turner (1985) suggest otherwise: over 100 A.nidulans sequences were isolated which acted as ARSes in S.cerevisiae. None of these were found to do so in A.nidulans. This indicates that the DNA sequences recognised as replication origins by the two species may be different. It also indicates that ARS activity should be tested in the species in which it is hoped ultimately to utilise the ARS bearing plasmid. This was the strategy used by Buxton and Radford (1984), and by Johnstone (1985a, see below).

Although none of the A.nidulans DNA sequences isolated by Ballance and Turner which acted as ARSes in yeast did so in A.nidulans, one 3.5-kb A.nidulans DNA fragment was found to bring about a 50 - 100-fold increase in transformation frequencies. Designated ans1, it was found to be mitotically stable in transformants without selective pressure being maintained, suggesting integration of transforming DNA. This was confirmed by Southern blot analysis of unrestricted DNA of Aspergillus transformants with ans1, where no band which might correspond to plasmid running separately from chromosomal DNA was observed (Ballance and Turner, unpublished observations). ans1 was also found to have multiple regions of homology in the A.nidulans genome, but it has been shown that enhanced transformation efficiency is not the result of integration at these sites (Cullen et al, 1987b). It remains unclear whether the properties of ans1 reflect a limited ability to replicate autonomously which could stimulate integrative transformation by allowing increased time for it to happen, or whether it contains a sequence which promotes recombination and integration. ans1 shows similar enhancement of the transformation frequency of Penicillium chrysogenum (Cantoral et al, 1987).

It has been calculated that ARSes are present on chromosomal DNA every 32 kb in S.cerevisiae (Chan and Tye, 1980) and that sequences acting as ARSes in S.cerevisiae are present every 19 kb of S.pombe DNA (Maundrell et al, 1985), and every 15 kb of D.melanogaster DNA (Stinchcomb et al, 1980). It was a little surprising then, that when Buxton and Radford (1984) screened 700 cloned N.crassa DNA sequences of between 1.2 and 6.8 kb (1,400 kb in all) for sequences acting as ARSes in N.crassa, none were found. Buxton and Radford argued that presence of an ori on a plasmid might not be sufficient for ARS activity in a filamentous fungus. It was observed that at each mitotic division the probability of loss of an ARS-containing plasmid in yeast is 30-60% per division under selective conditions. Thus it is only powerful selection against the cells that have lost the plasmid that maintains the level of plasmid containing cells in the population. It was argued that in the multinucleate hyphal compartments cross-feeding of untransformed by transformed nuclei would effectively reduce selective pressure against the former. This combined with plasmid instability would result in continual reduction in the proportion of transformed nuclei, until eventually the transformant colony would cease to grow. Thus, transformation of any coenocytic organism such as Neurospora with an unstably maintained ARS plasmid is an impossibility. Alternatively, Neurospora ARS sequences may be longer than 6 kb, or may not be cloneable in E.coli due to sequence peculiarities.

The argument of Buxton and Radford was thrown into doubt by the subsequent fortuitous discoveries of ARSes active in various other fungi. In the zygomycete Mucor circinelloides an ARS was discovered when cloning the M.circinelloides gene leu (van Heeswijck et al, 1982; Roncero et al, 1989). The 4.4-kb PstI fragment containing the gene also contained an ARS. Similar accidents led to the discovery of ARSes in the basidiomycetes Ustilago maydis (Tsukuda et al, 1988) and Phanerochaete chrysosporium (Randall and Reddy, 1991).

A different hypothesis to explain why an ori might not act as an ARS in some species of fungi was put forward by Begueret (1989). It was found that addition of telomeres from Tetrahymena thermophila to the ends of a plasmid which normally transformed Podospora anserina by integration, resulted in an autonomously replicating linear plasmid (Perrot et al, 1987). Begueret suggested that linear, but not circular plasmids may be able to replicate in Podospora, due to the

lack of an enzyme (e.g. a topoisomerase) necessary for replication of circular DNA.

ARSes from mitochondrial DNA

Hyman et al (1983) has shown that sequences occur every 1.7-kb around the yeast mt genome which, when cloned into YIp5 act as ARSes in S.cerevisiae. Parts of the A.nidulans mt genome have been cloned into A.nidulans vectors and do not show ARS behaviour in A.nidulans (Turner and Ballance, 1985b).

It has been reported that sequences from a mt plasmid from Podospora anserina act as ARSes in that species (Stahl et al, 1982), and likewise, that sequences from a mt plasmid from Neurospora intermedia did so in N.crassa (Stohl and Lambowitz, 1983; Stohl et al, 1984). However, these results have not been confirmed, and are now widely regarded as erroneous.

ARSes from other sources

It has been shown that integration of the S.cerevisiae centromere CEN11 into the A.nidulans chromosome does not disrupt normal chromosome separation during mitosis (Boylan et al, 1986). This indicates that CEN11 does not function as a centromere in A.nidulans, and that the DNA sequences recognised as centromeres by the two species differ. It has subsequently been shown that the centromeres of S.pombe differ widely from those of S.cerevisiae (Clarke, 1990).

Although the existence of dsDNA mycoviruses of some species of Aspergillus has been known for some time, to date no attempt to isolate ARSes from viral DNA has been reported.

Isolation of plasmid ARp1 from an A.nidulans transformant

Johnstone (1985a) employed a strategy similar to that of Buxton and Radford (described above) to isolate an A.nidulans ARS. In the process of cloning the A.nidulans gene brlA by transformation of a brlA42 mutant strain with an A.nidulans gene library (constructed in the integrative plasmid vector pILJ16) it was observed that a small proportion of transformed colonies grew more slowly than the others, and had a ragged , heterokaryon-like morphology. Johnstone argued that transformation with an autonomously replicating plasmid could result

in a reduction of transformant stability compared with that of integrative transformants which might be reflected in colony morphology. It was also argued that such plasmids would transform with a higher frequency than integrative ones, and hence transformants containing ARS plasmids would be over-represented among the population. Finally, given that such transformants contain unintegrated plasmid, it would be possible to reisolate the plasmid into E.coli by transformation with uncut Aspergillus transformant DNA.

That this was the cause of the morphological abnormalities observed was confirmed by the reisolation of an 11.5 kb plasmid from one of three slow growing colonies picked from among a total of $2x10^4$ transformants. The properties of this plasmid indicated that it might contain an ARS.

Characterisation of ARp1

A preliminary characterisation of this plasmid by Johnstone (1985a; 1985b, referred to there as pUATB1) yielded the following results:

a) It transformed A.nidulans with a frequency of 3,000-5,000 transformants/ug DNA, an approximately 10-fold increase in transformation efficiency over the parent plasmid pILJ16.

b) Transformants had a somewhat ragged, heterokaryon-like appearance which was taken to be the result of plasmid instability - hence the original designation, pUATB1: Unstable Appearance of Transformant. The plasmid was subsequently redesignated ARp1 (Aspergillus Replicative plasmid, Gems et al, 1991).

c) ARp1 was mitotically unstable: 52-88% of conidial progeny of ARG^+ ARp1 transformants were ARG^-.

d) Southern analysis of uncut ARp1 transformant DNA clearly showed the presence of free plasmid, but whether or not some plasmid sequence had integrated into the chromosome was unclear.

e) Restriction mapping of the DNA insert in the parent plasmid moiety of ARp1 showed that it consisted of two inverted repeats of approximately 3 kb each, with a unique central region (Fig. 3.1). The insert was designated AMA1 (Autonomous Maintenance in Aspergillus). One strand of the central 470bp of the insert was sequenced and found to consist of a 376 bp central unique region and two distal 47 bp inverted repeats.

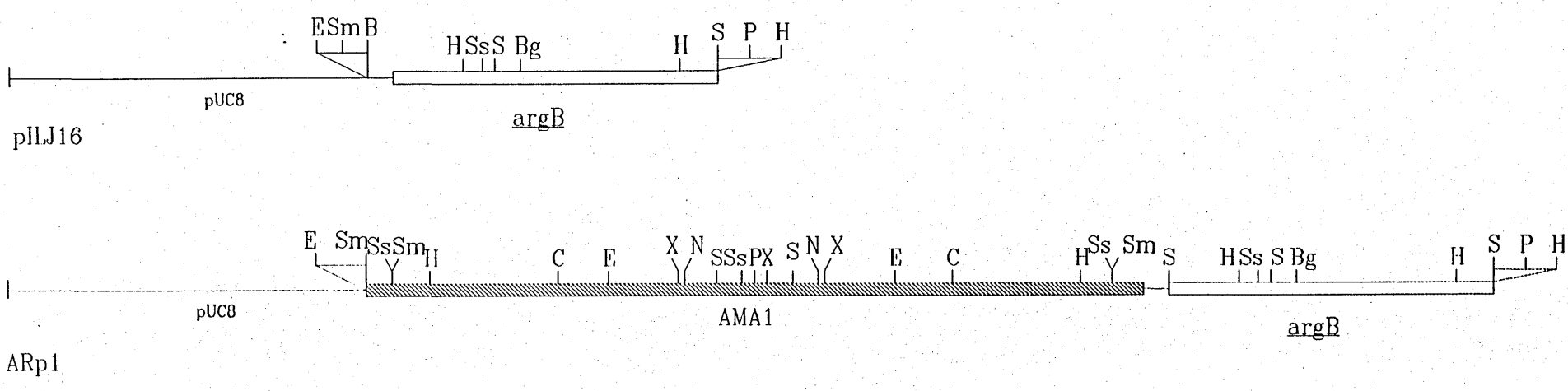
f) Southern analysis of restricted A.nidulans DNA probing with sequences from AMA1 suggest that the inverted repeat structure was

also present in the chromosome as part of a larger repeat - perhaps as large as 10 kb. In addition it was thought that a further copy of one arm of the repeat was present.

From these results Johnstone was not able to distinguish whether a) ARp1 contained an ARS and was replicating autonomously in the transformants, or b), it contained a recombinogenic sequence similar to ans1 which stimulated integration, but also gene amplification and excision. The presence of the latter would equally well explain the instability of the transformant phenotype and the unintegrated copies of ARp1 seen on Southern blots.

Research aims

The basic aims of the work to be described in this section were a) to discern whether AMA1 contains an ARS or a recombinogenic sequence. b) If there is an ARS, to locate it within AMA1 and c) to construct a plasmid vector suitable for gene bank construction by subcloning a fragment of AMA1 containing the ARS. Finally, d) to ascertain whether AMA1 shows similar activity in species related to A.nidulans such as A.oryzae and A.niger. Both these species have long been used for food production, e.g. of soy sauce, koji and citric acid (Wood, 1977; Berry et al, 1977), and recently have been used for heterologous expression of genes with saleable products, e.g. bovine chymosin, Rhizomucor derived aspartic proteinase, and somatostatin (Cullen et al, 1988; Christensen et al, 1988; R.W. Davies, pers. comm.).



facing page 48a

Figure 3.1. Linear restriction maps of plasmids pILJ16 and ARp1.

The open box represents the cloned Aspergillus argB sequences; the cross-hatched box, the AMA1 sequence; the single line, E.coli plasmid sequences (pUC8); the triangles, pUC8 polylinker sequences.

Abbreviations for restriction enzyme sites are: B, BamHI; Bg, BglII; C, ClaI; E, EcoRI; H, HindIII; N, NruI; P, PstI; S, SalI; Sm, SmaI; Ss, SstI; X, XhoI.

ARp1 contains no restriction sites for the following enzymes: AflII, KpnI and MluI.

All restriction sites mapped by DHG are highlighted in yellow.

Table 3.1. **A summary of ARp1 restriction digest products as derived from the gel presented in Fig. 3.2.**

Sizes were estimated using lambda HindIII/ EcoRI size standards, and are given in kb.

Restriction enzyme(s)	Estimated fragment sizes	sum of frags.
ClaI	8.5, 3.1	11.6
SmaI	5.7, 5.7, 0.14	11.54
NruI	10.5, 1.1	11.6
XhoI	10.2, 0.7, 0.47	11.37
SalI	4.7, 2.9, 2.4, 1.25	10.95
XhoI/SstI	3.9, 2.1, 2.1, 0.5, 0.47, 0.29	10.51

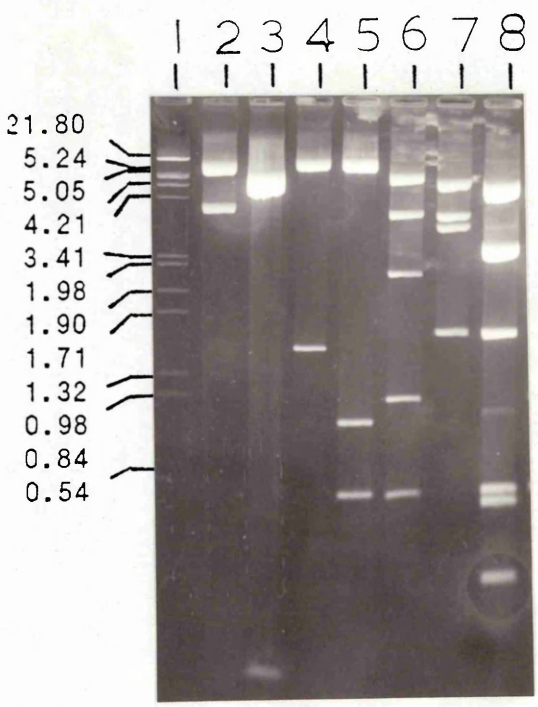


Figure 3.2. **Restriction digests of plasmid ARp1.**
1% agarose gel. Sizes of lambda HindIII/EcoRI markers are given in kb.

Digests are as follows:
Lane 1) Lambda HindIII/ EcoRI, 2) - 8) are ARp1 digests: 2) ClaI, 3) SmaI, 4) NruI, 5) XhoI, 6) SalI, 7) Sst1, 8) XhoI/SstI.

Table 3.2. A summary of the transformation frequencies of 22 transformations of A.nidulans with ARp1.

Approximately 1ug of plasmid DNA was used per 10^6 protoplasts.

Strain	Number of transformants/ 10^6 protoplasts ARp1	pILJ16	pTA11	ARp/AIp
G34	30,000	-	-	-
G34	20,000	-	-	-
G34	65,000	-	-	-
G34	11,000	1.6	-	6,875
G34	200	1.4	-	143
G34	250	6	-	42
G124	5,000	-	-	-
DHG135	100	-	1	100
DHG135	140	-	2.1	67
DHG135	1,080	-	0	>1,080
AJC9.4	110	-	-	-
AJC9.4	220	-	-	-
AJC9.4	2,400	10	-	240
DHG019	16,000	-	50	320
DHG019	2,000	-	-	-
DHG019	700	-	138	5.1
DHG019	200	-	-	-

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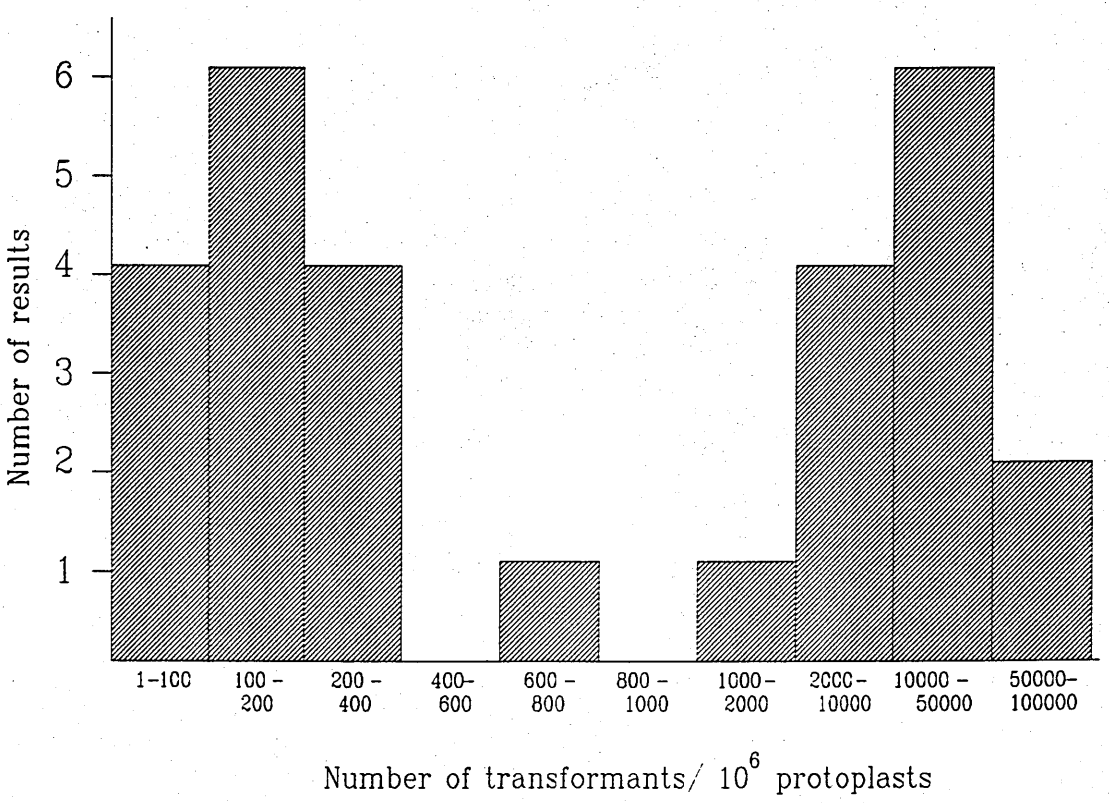

Figure 3.3. Histogram showing variation in frequency of transformation of A.nidulans by ARp1.

The right hand group of results are taken as reflecting transformations carried out under optimum conditions. The average of these latter is about 20,000 transformants per 10^6 protoplasts.

3.1 Restriction analysis of ARp1

Restriction mapping of ARp1 was initially carried out by Johnstone (1985a). Further restriction mapping was carried out in order to identify restriction enzymes cleaving AMA1 but not pILJ16 sequences. Plasmids pILJ16 and ARp1 (Fig. 3.2) were incubated with a variety of 6-cutter restriction endonucleases, and then subjected to AGE. Those enzymes which cut only within the AMA1 region were used for further restriction mapping. The results are presented in Fig. 3.1 and 3.2, and Table 3.1. All restriction sites not mentioned in the text, but shown in Fig. 3.1 were mapped by Johnstone.

KpnI, MluI and AflII did not cut ARp1. ClaI cuts ARp1 twice, producing an 8.4-kb and a 3.1-kb fragment. NruI likewise cuts twice, producing a 10.5-kb and a 1.0-kb fragment. Since AMA1 is palindromic (Johnstone, 1985a) these patterns must correspond to pairs of restriction sites at identical locations on each arm of the inverted repeat, equidistant from the middle of AMA1. XhoI digestion produces 3 fragments of size 10.4 kb, 0.7 kb, and 0.4 kb, suggesting one pair of restriction sites 0.6 kb from the centre of AMA1 and a third site within the insert, about 0.1 kb off centre, towards the argB end of AMA1. This is confirmed by the presence of an XhoI site in the sequence of the central unique 376bp (Johnstone 1985a), 117-123bp from the centre. SmaI digestions produced three fragments of 5.7 kb, 5.6 kb, and 0.2 kb. There is a SmaI site in the pUC8 polylinker. The other two sites must be located 2.9 kb from the centre of AMA1, at about the same position as the pair of SstI sites located 0.2 kb from the distal ends of AMA1.

3.2.1 Efficiency of transformation with ARp1

Johnstone (1985a) showed that ARp1 transformed A.nidulans strain G324 (yA2; wA3; ivoA63 sC12 methH2 argB2 galA) at a frequency of 3,000-5,000 transformants per ug ARp1 DNA. In 22 transformations carried out here with a variety of strains of A.nidulans a wide variety of frequencies were observed (Table 3.2). Fig. 3.3 shows a histogram showing the variation in frequencies. There are two peaks. The right

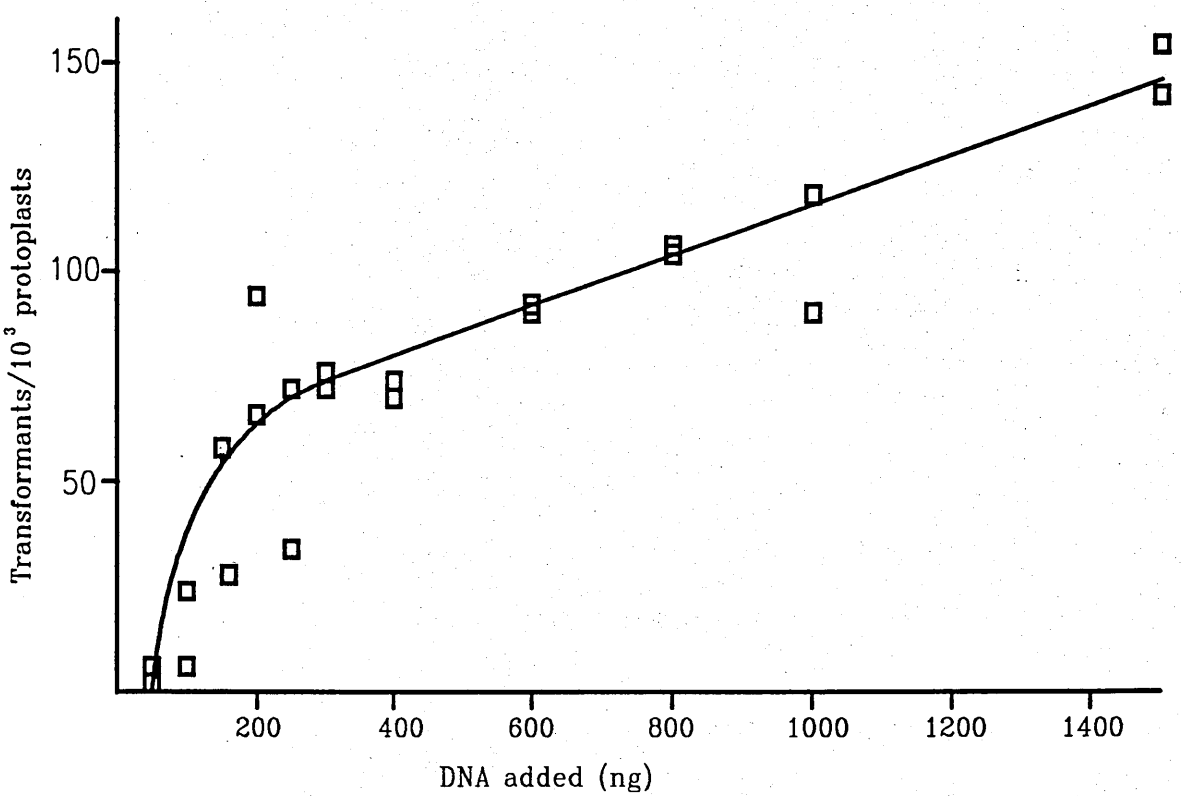

Figure 3.4. **Variation of frequency of transformation of Aspergillus with mass of ARp1 added.**

Strains G34 was transformed from *argB*$^-$ to *argB*$^+$ with ARp1. Approximately 10^6 protoplasts were used to determine each point. All transforming DNAs were made up to 30ul with TE before being added to protoplasts. Note that the transforming frequencies obtained in this experiment were somewhat higher than usual.

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hand peak, which presumably represents transformations which occurred under optimal conditions, taken by itself corresponds to an average transformation frequency of about 20,000 transformants per 10^6 protoplasts. The left hand peak presumably corresponds to non-optimal conditions. The causes of the reduced transformation frequencies are unclear, but possible explanations are failure to completely remove NovoZym (which contains DNase) from protoplast preparations, or contamination of glassware with detergent, or of plasmid DNA with EtBr, both of which could result in protoplast death.

In one experiment, frequency of transformation per 10^6 protoplasts was plotted against the quantity of plasmid DNA used per transformation (Fig. 3.4). Transformation rose sharply up to 200-300 ng of plasmid DNA per 10^6 protoplasts, then increased more gradually at a rate of about 60 transformants per ng of transforming DNA. The results of two identical transformations performed at the same time were averaged to give each point on the graph.

In transformations Johnstone added about 3 ug plasmid DNA to about $3x10^7$ protoplasts. This corresponds to about 100 ng DNA per 10^6 protoplasts: near to the saturating level of ARp1. If it is assumed that the saturation point for AIp and ARp plasmids are similar, then it can be said that since pILJ16 gives a frequency of 80 transformants per 10^6 protoplasts, ARp1 transformation occurs with a 20,000/80 = 250-fold greater efficiency than pILJ16.

20,000 transformants per 10^6 protoplasts using 200 ng of DNA corresponds to 100,000 transformants per 10^6 protoplasts per ug DNA. In order to realistically reflect the numbers of transformants seen the convention of expressing transformation frequency per ug of DNA has been replaced by expression of the frequency at near saturation levels of DNA per 10^6 protoplasts, where "near saturation levels of DNA" is defined as approx. 300ng DNA.

.. Given that only 10-15% of protoplasts are capable of regenerating (Johnstone 1985a) 20,000 transformants per 10^6 protoplasts represents a transformation frequency of 13-20% of all viable protoplasts. This figure may be even higher if PEG induced protoplast fusion is taken into account.

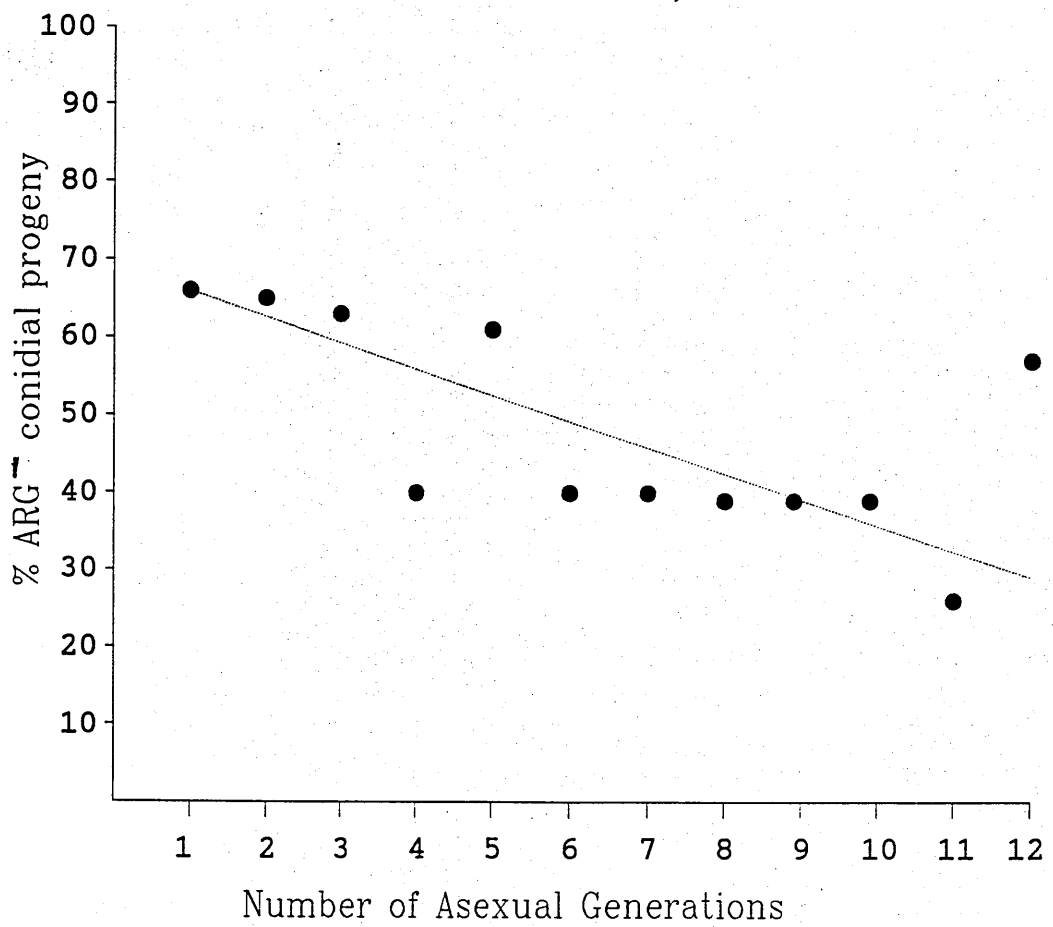

Figure 3.5. **Reduction in mitotic instability of ARp1 transformants of <u>Aspergillus</u> resulting from subculture.**

An ARG^+ ARp1 transformant of strain G34 was subcultured through 12 cycles of asexual reproduction and the proportion of ARG^+ progeny counted at each generation. Conidia were plated on CM (on which ARG^+ and ARG^- colonies may be distinguished) at a density of about 50 colonies per plate. ARG^+ colonies were used to inoculate fresh plates, and so on. Note the increase in stability which occurs between generations 3 and 6.

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3.2.2 Stability of transformants with ARp1: asexual reproduction

Johnstone (1985a) showed that ARp1 transformed colonies grown under conditions selective for ARG^+ gave conidia 52-88% of which were ARG^-. Here an ARG^+ ARp1 transformant of A.nidulans strain G34 was serially subcultured, and the proportion of ARG^+ conidial progeny measured at each generation, through 12 generations (Fig. 3.5). CM was used, which contains a suboptimal concentration of arginine, such that ARG^- strains may grow but do not conidiate. Thus ARG^+ and ARG^- strains are easily distinguishable. (CM was used both for subculture and screening). It was found that the ARG^+ phenotype remained unstable throughout. The proportion of the ARG^+ progeny began at 35%, but after six generations increased to 53% (Fig. 3.5). This might reflect an alteration of the plasmid structure resulting in increased stability. The sustained instability of the transfomant phenotype in asexual progeny suggests that integration of ARp1 does not readily occur.

DNA was prepared from one ARG^- colony from among progeny of a ARp1 transformant. Southern blot analysis of this DNA showed that no pUC8 sequences were present, implying that ARp1 had been lost (data not shown).

3.3 Transmission of ARp1 through the sexual cycle

Two cleistothecia were picked from a cross between A.nidulans strains AJC9.4 (pabaA1; argB2; brlA42 ivoB63) and an ARG^+ ARp1 transformant of strain G34 (yA2;argB2 methH2). Ascospores were plated on CM, on which ARG^+ and ARG^- colonies can be easily distinguished (see Section 3.3). Plates were incubated at 30°C to allow conidiation of brlA42 strains. Of 220 ascospores from the first hybrid cleistothecium, 29.5% gave rise to ARG^+ colonies, and of 375 from the second, 61.3% gave rise to ARG^+ colonies. This indicates that ARp1 can be transmitted through the sexual cycle.

3.4 Linkage analysis of *argB*$^+$ in ARp1 transformants

From one of the master strains of McCully and Forbes (1965) an *argB2* derivative, DHG001, was constructed. Diploids were formed from four ARG$^+$ ARp1 transformants of *A.nidulans* strain AJC9.4 and DHG001, and the products of mitotic haploidisation were characterised. Because any ARG$^-$ haploidisation products could have resulted from plasmid instability, only ARG$^+$ segregants were considered. If ARp1 had integrated into one of the chromosomes the *argB*$^+$ allele should segregate with the wild type allele of the marker gene, identifying the linkage group corresponding to that chromosome.

In three out of the four cases the *argB*$^+$ allele behaved as unlinked to all eight linkage groups (Table 3.3). In one case, 3), the *argB*$^+$ allele appeared linked to group IV, segregating with the *pyroA*$^+$ allele, suggesting that in transformant 3, ARp1 was integrated into the chromosome. However, when the stability in asexual reproduction of the ARG$^+$ phenotype of the four transformants was compared with that of ARG$^+$ segregants from the four haploidisations it was found that in all four initial transformants and haploid segregants from diploids 1, 2, and 4, the ARG$^+$ phenotype was unstable, whereas all ARG$^+$ segregants from haploidisation of transformant 3 were stable.

These results suggest that ARp1 did not integrate, at least not stably, in the case of transformants 1,2 and 4. The segregants from transformant 3 suggest that ARp1 did integrate into linkage group IV; however, the analysis of stability before and after passage through the parasexual cycle suggests that integration occurred during the cycle, and may not reflect the condition of the transforming plasmid in the initial transformant.

Segregation of the *pabaA1* allele from AJC9.4 and the *yA2* marker gene from DHG001 can be taken as a positive control for the haploidisation mapping process (Table 3.4). Exceptions in segregants from transformant 3 may be due to inaccurate replica plating.

Table 3.3 **Haploidisation mapping of *argB*$^+$ in 4 ARp1 transformants**

This table shows ARG$^+$ segregants from the haploidisation of diploids formed between master strain DHG001 (*yA*2; *acr*1; *galA*1 *argB*2;*pyroA*4; *facA*303; *sB*3; *nicB*8; *riboB*2 *niaD*17) and four ARG$^+$ transformants with ARp1 of strain AJC9.4 (*pabaA*1; *argB*2 *brlA*42 *ivoB*63), named 1, 2, 3 and 4. Only ARG$^+$ segregants are analysed since ARG$^-$ segregants may be the result of plasmid instability. If ARp1 integrates into one chromosome of the transformant strain, then the *argB*$^+$ allele should segregate with the wild type allele only of the marker gene identifying the linkage group corresponding to that chromosome.

Linkage Group:	I		II		III		IV		V		VI		VII		VIII	
	yA		*acrA*		*galA*		*pyroA*		*facA*		*sB*		*nicB*		*riboB*	
ARG$^+$ progeny	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
1)	8	11	3	16	16	3	7	12	8	11	14	5	13	6	0	19
2)	9	21	12	18	29	2	24	6	12	18	26	4	25	5	3	27
3)	13	19	12	20	14	18	32	0	20	12	26	6	22	10	3	29
4)	16	32	20	28	41	7	26	22	29	19	40	8	43	5	4	44

The *argB*$^+$ allele only shows any linkage in transformant 3), to linkage group IV.

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Redo

Table 3.4 **Segregation of yA2 and pabaA1 in haploidisation analysis of four ARp1 transformants.**

This table shows that the yA2 allele from DHG001 and the pabaA1 allele from AJC9.4 do not segregate together. This can be taken as a positive control for the haploidisation mapping the results of which are displayed in Table 3.3.

		Linkage group I	
		yA2	
T'f'nt	Progeny	+	−
1)	$PABA^+$	0	5
	$PABA^-$	10	0
2)	$PABA^+$	0	6
	$PABA^-$	11	0
3)	$PABA^+$	1	9
	$PABA^-$	9	3
4)	$PABA^+$	0	11
	$PABA^-$	12	0

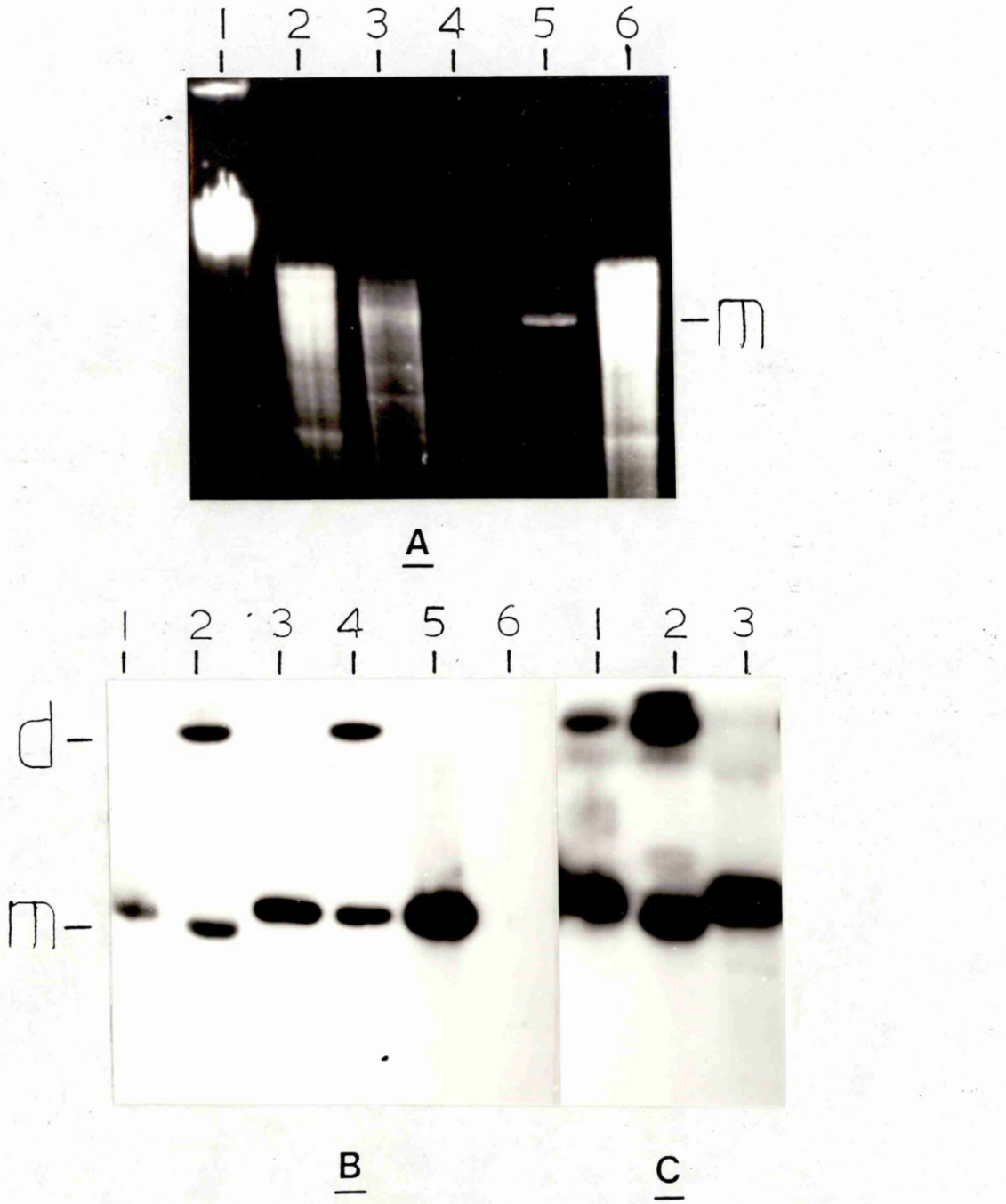

Figure 3.6. **Southern blot of DNA from A.nidulans transformants with ARp1.**

Panel A shows DNA from one argB$^+$ ARp1 transformant of A.nidulans strain G34, separated on a 0.8% agarose gel. Lanes: 1, uncut; 2, cut with BamHI; 3, cut with BglII; 4, ARp1 DNA prepared from E.coli uncut, and 5, cut with BamHI. Probe: radiolabelled pUC8. Lane 6: untransformed A.nidulans DNA.
Panel B shows the autoradiograph prepared from the gel in panel A.
Panel C shows a longer exposure of lanes 1, 2 and 3.
m, monomer; d, dimer.

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3.5 Southern blot analysis of ARp1 transformants

Southern blot analysis by Johnstone (1985a) of ARp1 transformant DNA appeared to show ARp1 both integrated into, and running separately from the chromosome. However, in Southern blot analysis of uncut ARp1 transformant DNA it is not easy to distinguish whether hybridisation of probe to the chromosomal DNA band is due to a) plasmid integration into the chromosome, b) ARp1 dimer running at the same position as the chromosomal DNA, or c) plasmid DNA in some way entangled with the chromosomal DNA. To distinguish between these cases DNA extracted from ARG^+ ARp1 transformants was run on an agarose gel i) uncut, ii) cut with BamH1, (which does not cleave ARp1), and iii) cut with BglII, (which cuts ARp1 once), yielding the linear plasmid monomer. These were run alongside ARp1 extracted fron E.coli (Fig. 3.6A). Southern blots of these gels were probed with radiolabelled pUC8 (Fig. 3.6B).

The probe did not hybridise with the uncut chromosomal DNA but to bands corresponding in size to ARp1 cccDNA monomer and dimer, running below and slightly above the chromosomal DNA respectively. In a longer exposure of the same autoradiograph (Fig. 3.6C) the position of intact chromosomal DNA between monomeric and dimeric ARp1 is revealed by non-specific hybridisation. In the track containing transformant DNA cut with BglII, a single band is seen, corresponding in size to linear ARp1 monomer (11.5 kb).

No bands are seen which might correspond to junction fragments between ARp1 and chromosomal DNA, which would be expected if ARp1 had integrated into the chromosome. An autoradiograph from a similar experiment is shown in Fig. 3.7.

When a large amount of BamH1 digested DNA from an ARp1 transformant was separated on an agarose gel the high running dimeric form was clearly seen (Fig. 3.8).

These results strongly suggest that ARp1 is not generally integrated into the chromosome in Aspergillus transformants. If this is the case it must, to be maintained, bear the capacity to replicate autonomously within the Aspergillus transformant. If so, ARp1 represents the first plasmid found which can do so within Aspergillus, or indeed, any filamentous ascomycete. Thus two classes of Aspergillus transforming vectors now exist: ARps (Aspergillus Replicative plasmids), which may be contrasted with Aspergillus Integrative plasmids (AIps).

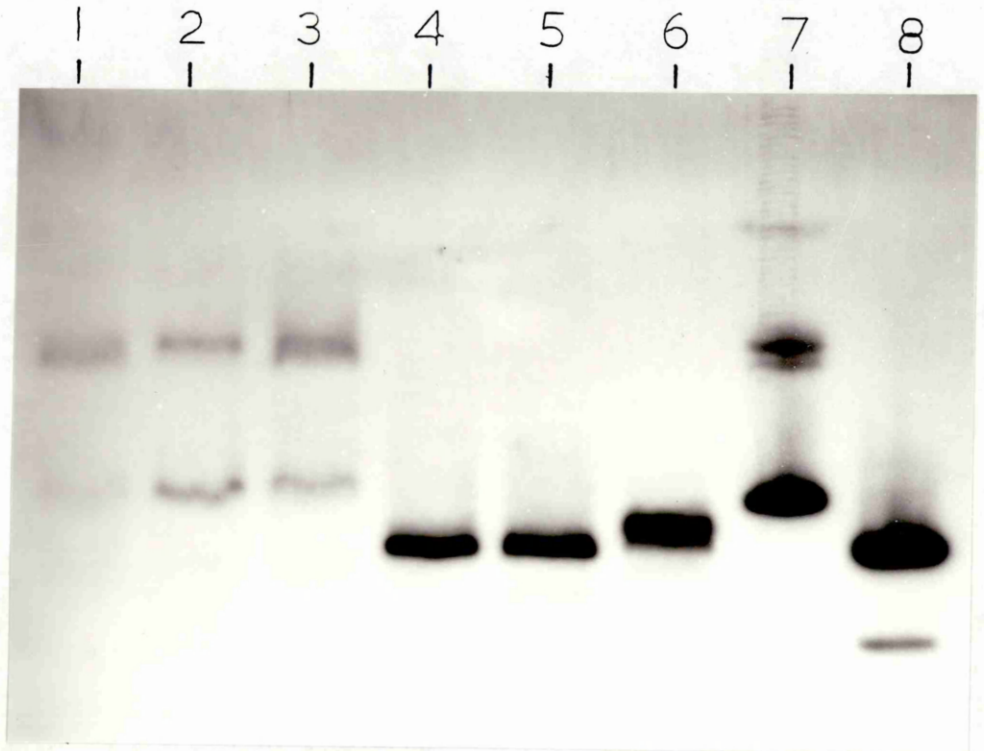

Figure 3.7. **Southern blot analysis of DNA from 3 A.nidulans transformants with ARp1.**

Probe: radiolabelled pUC8.

As Fig. 3.6. Lanes 1, 2, 3, DNA from 3 ARp1 transformants of Aspergillus cut with BamHI; 4, 5, 6, cut with BglII; 7, ARp1 from E.coli, uncut, and 8, cut with BglII.

This blot shows particularly clearly the presence of only unintegrated ARp1 in Aspergillus.

facing page 52b

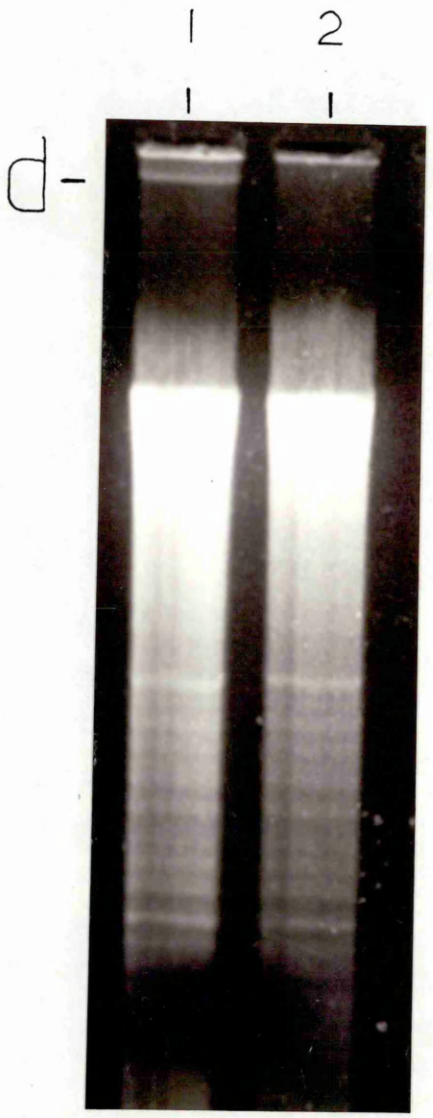

Figure 3.8. **Restriction digests of DNA from an ARp1 transformant of A.nidulans.**

Digested DNA was separated on a EtBr stained 0.8% agarose gel. Lane 1, DNA from A.nidulans strain G34 transformed with ARp1; 2, untransformed. In both cases DNA was digested with BamHI, which does not cut ARp1. The ARp1 dimer is clearly visible in the track containing the transformant DNA. d, dimer.

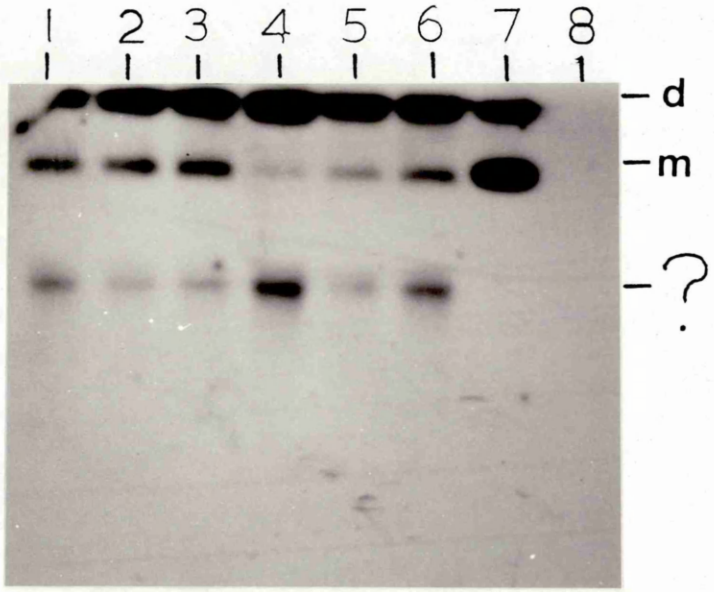

Figure 3.10. **Southern blot of A.nidulans transformants with monomeric and dimeric plasmid ARp1 DNA.**

The gel was 0.8% agarose; the Southern blot was probed with radiolabelled pUC18; all Aspergillus DNAs were digested with BamHI. Lanes 1, 2, 3, three A.nidulans strain G34 transformants with ARp1 monomer; 4, 5, 6, three A.nidulans transformants with ARp1 dimer; 7, ARp1 DNA extracted from E.coli (monomer and dimer); 8, untransformed strain G34. M, monomer; D, dimer; ?, unidentified band, possibly linear monomer.

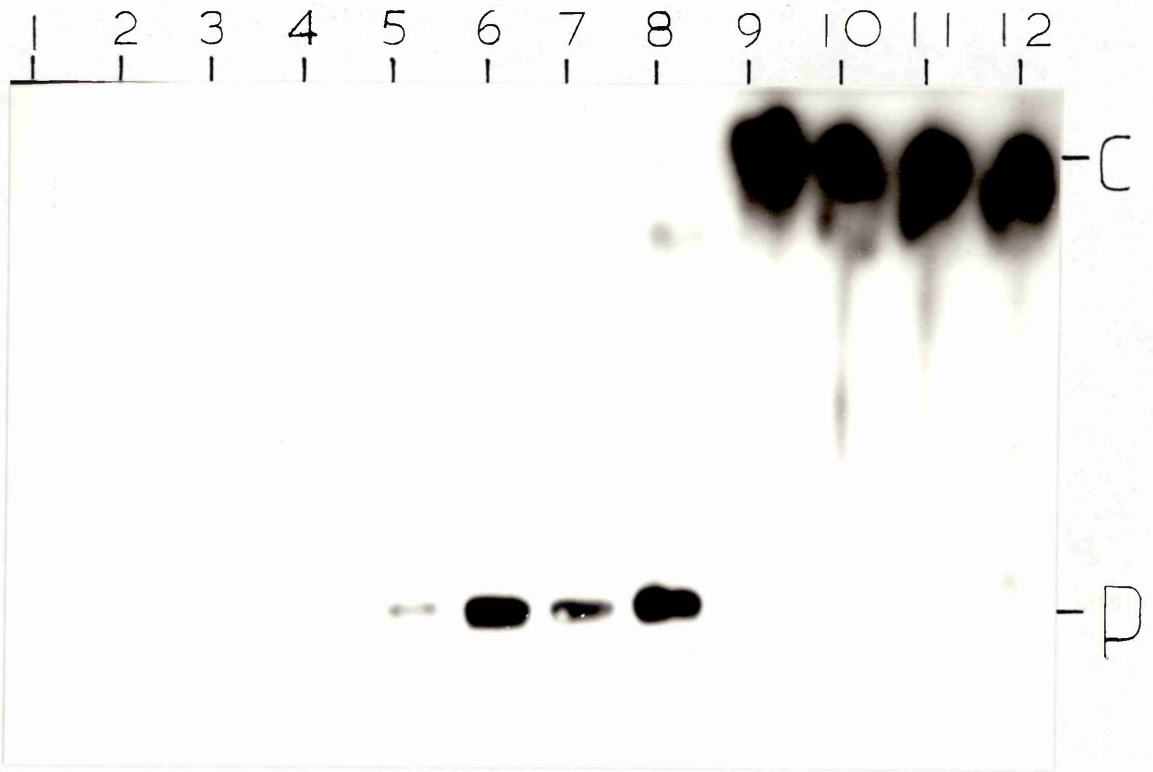

Figure 3.11. **Southern blot of A.nidulans DNA from an ARp1 transformant after density gradient separation.**

About 25ug of DNA was subjected to CsCl/ EtBr density gradient ultracentrifugation in a volume of 6 mls. This was fractionated into 24 250-ul aliquots, numbered from the bottom of the gradient. DNA was extracted from each fraction, and fractions 5-16 subjected to electrophoresis on a 0.8% agarose gel. A Southern blot prepared from the gel was probed with radiolabelled pUC8. Lanes 1-12 correspond to fractions 5-16. C, chromosomal DNA; P, plasmid DNA.

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3.6 Interconversion of monomeric and dimeric ARp1 in Aspergillus

By comparison of ARp1 DNA on agarose gels with a range of other plasmids of known size, it was shown that the two forms of ARp1 most commonly seen correspond to supercoiled cccDNA monomer and dimer (Fig. 3.9). Since both forms are seen in both E.coli and A.nidulans transformants it was asked whether the presence of both forms in the fungus was the result of transformation with a mixture of the two forms extracted from E.coli. Alternatively, interconversion between monomer and dimer could occur in Aspergillus.

ARp1 was separated on an agarose gel and the monomer and dimer separately gel purified. DNA from ARG^+ A.nidulans transformants with each form was subjected to Southern blot analysis (Fig. 3.10). It was observed that where monomeric transforming plasmid had been used most of the plasmid in the transformant was in dimeric form (Fig. 3.10, lanes 1, 2 and 3). Furthermore, where dimeric transforming ARp1 was used, only a very small proportion of the plasmid was present as monomer (Fig. 3.10, lanes 4, 5 and 6.). This suggests that a), ARp1 monomers are readily converted into dimers in Aspergillus, perhaps by recombination between monomers (yet not appreciably into higher multimers); b) that resolution of dimers to form monomers does occur, but less frequently; and c) that the presence of near equimolar amounts of monomer and dimer seen in Aspergillus transformants (e.g. in Fig. 3.6B and 3.7) does result in part from transformation by both forms extracted from E.coli.

3.7 Separation of ARp1 from A.nidulans transformant DNA by density gradient ultracentrifugation

When ARp1 transformant DNA was subjected to CsCl density gradient ultracentrifugation no EtBr stained plasmid band was observed. However, when the gradient was fractionated, and DNA extracted from serial fractions was subjected to AGE and Southern blotting, radiolabelled pUC8 hybridised strongly to DNA migrating faster than the chromosome in the three fractions from immediately beneath the band of chromosomal DNA in the gradient (Fig. 3.11). This suggests that chromosomal and supercoiled plasmid DNA had been separated. (The hybridisation of probe to the chromosomal DNA may be due to open circle plasmid DNA entangled with the chromosome). This indicates the future possibility, especially should higher copy number derivatives

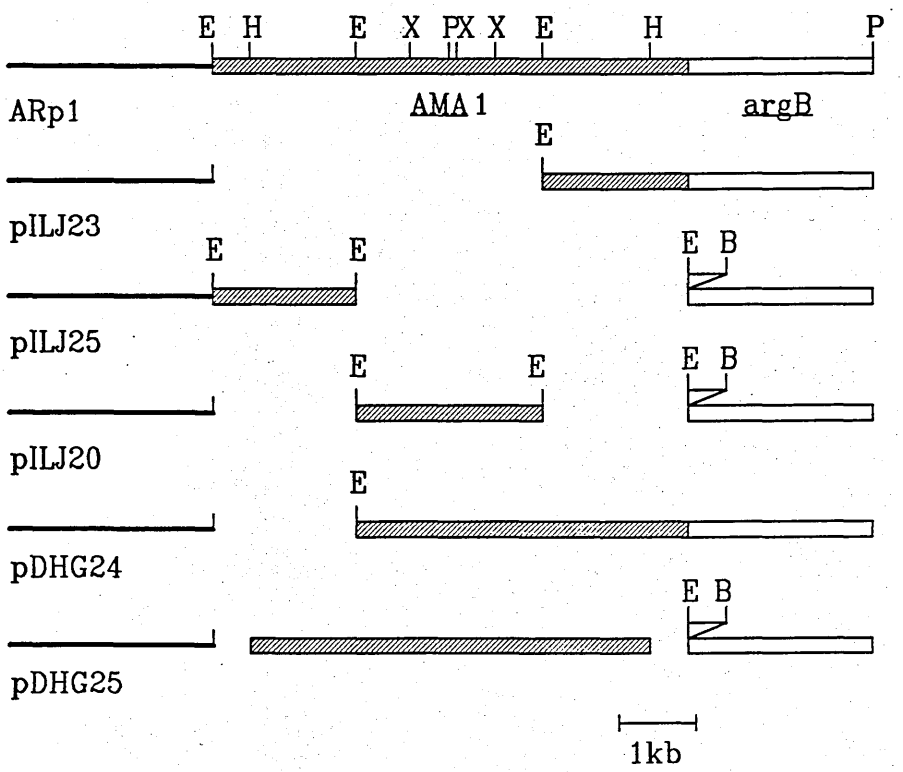

Figure 3.12. **Linear restriction maps of the subclones of ARp1.**
The open boxes repesent A.nidulans argB sequences. The cross-hatched boxes represent AMA1 and AMA1-derived sequences. The single lines represent pUC8 sequences, and the triangles, pUC8 polylinker sequences. For construction of ARp1, pILJ20, pILJ23 and pILJ25 see Johnstone, 1985a. pDHG24 was constructed by partial digestion of ARp1 with EcoRI, followed by self-ligation. pDHG25 was constructed by blunt end ligating the 5.1-kb HindIII fragment of AMA1, with Klenow-filled ends, into the SmaI site of pILJ16 (Fig. 3.1).
Abbreviations for restriction enzyme sites: B, BamHI; E, EcoRI;, HindIII; P, PstI; X, XhoI.

facing page 54a

of ARp1 be developed, of extracting plasmid directly from Aspergillus transformants without retransforming E.coli.

3.8.1 Construction of subclones of ARp1

The construction of three subclones of ARp1: pILJ20, pILJ23 and pILJ25 has been described by Johnstone (1985a; Fig. 3.12). Two further subclones were constructed. A partial digest of ARp1 with EcoRI was carried out and the 9.4-kb EcoRI partial fragment was gel purified. This was then self-ligated and transformed into E.coli strain DH5, selecting for ampicillin resistance to form pDHG24. ARp1 was then restricted with HindIII and the 5.1-kb fragment of AMA1. The 4 bp 5' ssDNA overhangs were then filled in by the action of the Klenow fragment of DNA polymerase I. The resulting DNA was then ligated with SmaI-restricted and phosphatased (calf intestinal phosphatase) pILJ16, to form pDHG25. The structure of pDHG24 and pDHG25 was confirmed by EcoRI digestion (data not shown).

3.8.2 Transformation of A.nidulans with ARp1 subclones

It was hoped that the region conferring free replication could be localised within AMA1 by observing the behaviour of subclones of ARp1 in transformations. pILJ20, pILJ23, pILJ25, pDHG24, pDHG25 and for comparison, pILJ16 and ARp1, were transformed into A.nidulans strain G34, with selection for ARG^+ colonies. The results (Table 3.5) classify the plasmids into three types: firstly ARp1, pDHG24 and pDHG25 showed no greater variation in transformation frequency than is often encountered between batches of the same plasmid DNA (Table 3.5). Secondly, pILJ20 behaved like an AIp, with a transformation frequency only slightly higher than that of pILJ16. However, heterokaryon-like colonies were occasionally seen. Thirdly, pILJ23 and pILJ25 had intermediate properties: a transformation frequency 10 - 15-fold greater than pILJ16, but less than a tenth that of ARp1, pDHG24 and pDHG25. In addition, a large number of weakly growing pILJ23 and pILJ25 transformant colonies were seen which did not grow if subcultured (abortive transformants).

3.8.3 Stability of transformants with ARp1 subclones

Conidia from ARG^+ transformants were plated on CM and the percentage

Table 3.5. **Frequency of transformation of Aspergillus with subclones of ARp1.**

Strains used were G34, G034, AJC9.4, AJC541.20, AJC12.6, and AJC12.7. There was no apparent significant variation of strain with transformation frequency.
(Data printed on the same line do not represent transformations carried out at the same time or of the same strain.)

Number of transformants/ 10^6 protoplasts						
ARp1	pILJ16	pILJ20	pILJ23[1]	pILJ25[1]	pDHG24	pDHG25
30,000	6	1	80	100	240	1,600
20,000	10	3.8	600	400	4,400	530
65,000	23	300	60	35	5,300	3,400
10,000	55	1			40,000	10,000
200	1.6					5,400
250						
5,000						
100						
140						
1,080						
110						
220						
2,400						
16,000						
2,000						
700						
200						
8,176	16.1	76.5	246	246	12,485	4186

[1]Estimates: strong growing true transformant colonies as against abortive transformants.

facing page 54b

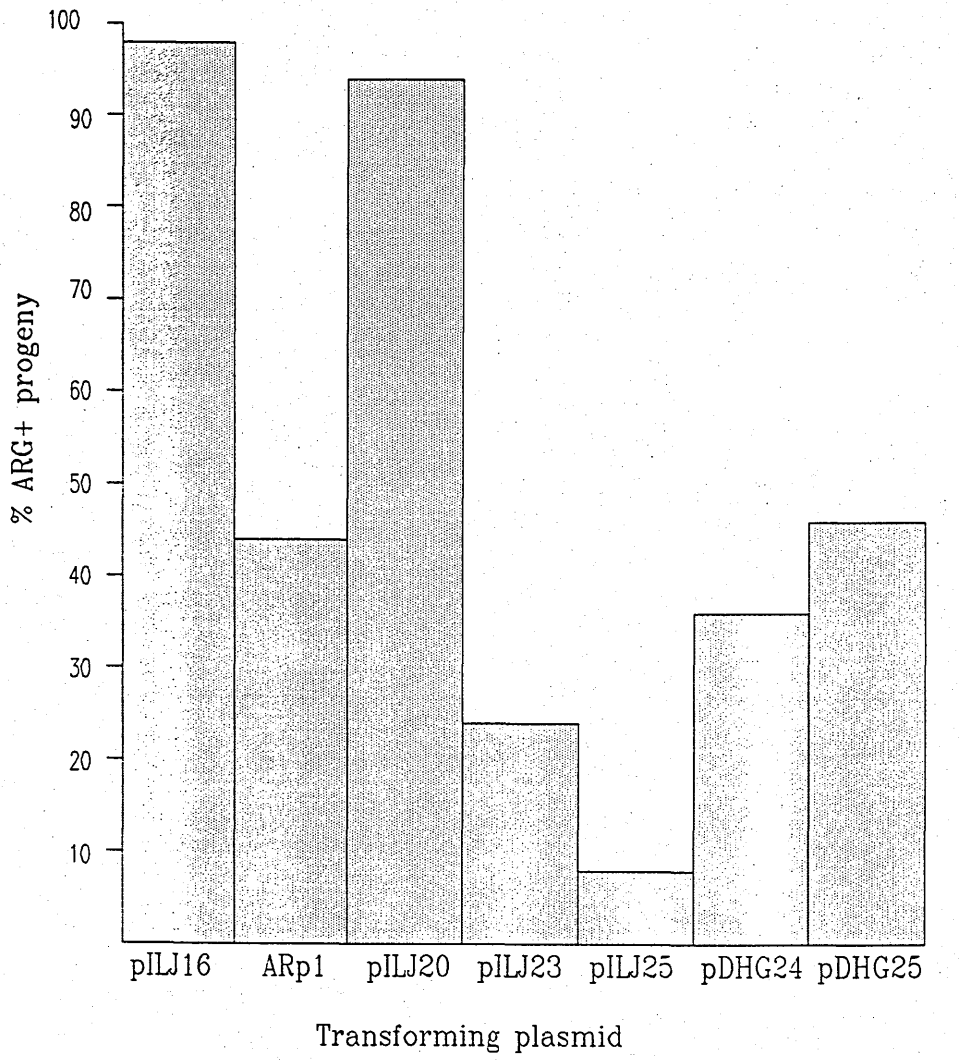

Figure 3.13. **Histogram showing instability of transformants with ARp1 and subclones.**

This figure shows the proportion of asexual progeny of transformants with ARp1 and subclones which have retained the transformant phenotype (ARG^+). This was measured by plating conidia from transformants onto CM, on which ARG^+ and ARG^+ colonies are easily distinguished (the latter do not conidiate.)

Table 3.6. **Stability of ARG$^+$ transformants of A.nidulans strain G34 with pILJ16, ARp1 and subclones.**

ARG$^+$ and ARG$^-$ colonies were distinguished by plating conidia from transformants onto CM on which only the ARG$^+$ conidiate.

% ARG$^+$ colonies (bracketed: sample size)						
pILJ16	ARp1	pILJ20	pILJ23	pILJ25	pDHG24	pDHG25
95(21)	15(39)	100(80)	59(29)	[90(21)]*	28(154)	33(118)
100(21)	36(56)	100(27)	15(34)	0(23)	35(251)	46(48)
100(20)	37(38)	100(37)	6(31)	31(16)	41(249)	31(70)
100(23)	55(42)	73(72)	[88(25)]*	0(19)	46(201)	76(106)
100(38)	50(44)	100(94)	11(27)	0(6)	-	26(54)
[24(28)][1]	61(49)	100(11)	-	-	-	59(81)

*These are stabilised transformants, probably resulting from integration of transforming DNA into the chromosome, and have been excluded from the average.

[1]This datum was excluded from calculation of the average since *typically* pILJ16 transformants are 100% stable.

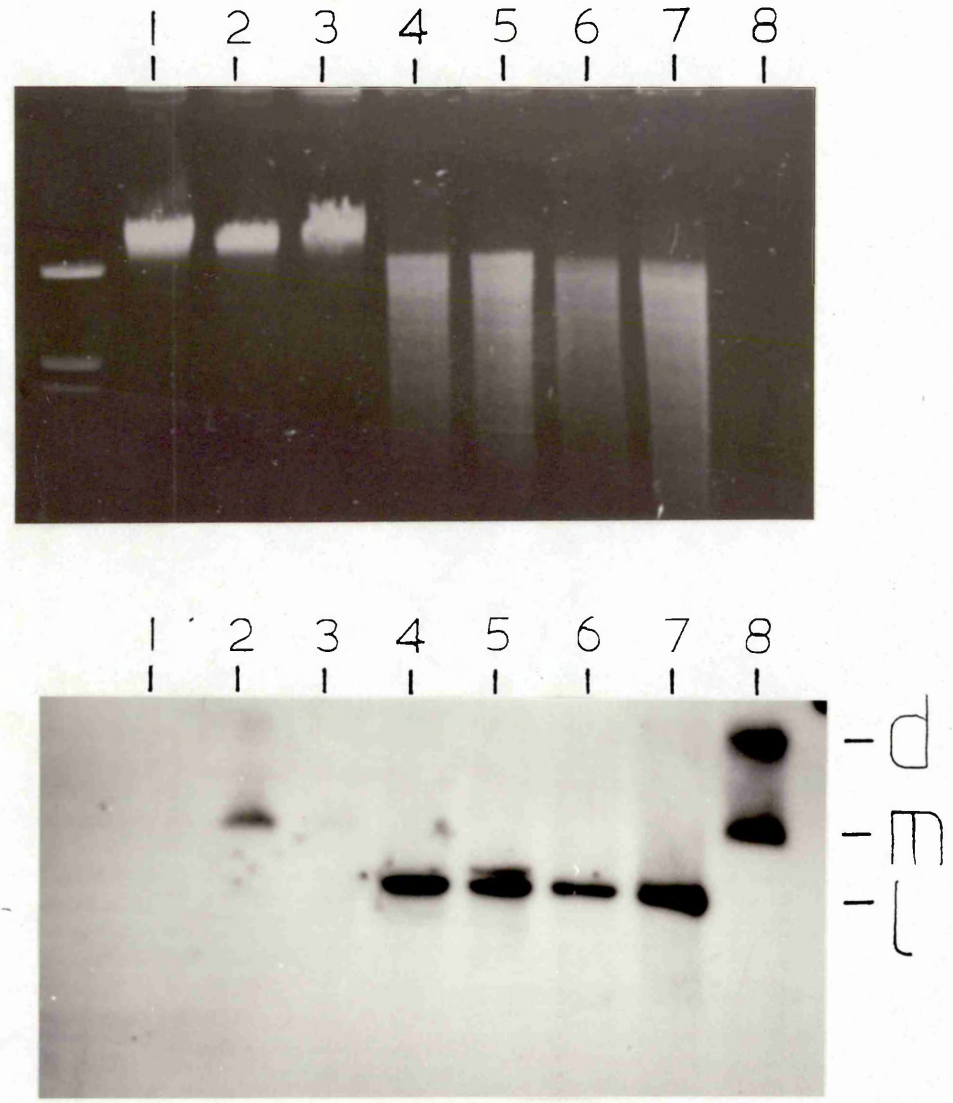

Figure 3.14. **Southern blot of A.nidulans transformants with pDHG25.**
The blot was probed with radiolabelled pUC18. DNAs from 2 transformants, T25.1 and T25.2, of strain G34 with pDHG25 were examined.

Lane 1, DNA from untransformed strain G34; 2, 3, T25.1 and T25.2, uncut; 4, 5, cut with BamHI; 6, 7, cut with BglII; 8, pDHG25 DNA extracted from E.coli, uncut.
m, supercoiled cccDNA monomer; d, supercoiled cccDNA dimer; l, linear plasmid monomer.

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of ARG$^+$ colonies among the progeny was scored. It was found that the proportion of ARG$^+$ progeny of transformants of pDHG24 and pDHG25 were similar to that of ARp1 transformants (Table 3.6). Of six pILJ20 transformants, five were found to be stable; in one, 83% of progeny were ARG$^+$. This is similar to the result obtained from the pILJ16 transformants: of six, four were stable, and two unstable, having 95% and 24% ARG$^+$ progeny respectively. Occasional unstable pILJ16 Aspergillus transformants were observed by Upshall (1986) who found that the ARG$^+$ became stable if the unstable transformants were subcultured.

The ARG$^+$ phenotype of most pILJ23 and pILJ25 transformants was found to be more unstable. If the two more stable colonies are excluded 23% of progeny of pILJ23 and 8% of progeny of pILJ25 transformants are ARG$^+$. The data on transformant stability is summarised in a histogram in Fig. 3.13.

3.8.4 Southern analysis of A.nidulans transformants with pDHG25

DNAs from transformants of A.nidulans strain G34 with pDHG25, T25.1 and T25.2, grown under selective conditions, were subjected to Southern analysis using a radiolabelled pUC8 DNA probe (Fig. 3.14). Two bands were visible in the tracks containing uncut transformant DNA (tracks 2 and 3), which correspond in position to monomer and dimer of pDHG25 (see track 8). Restriction of transformant DNAs with BamHI (tracks 4 and 5) and BglII (tracks 6 and 7) which cut pDHG25 once, gave a single band in the case of T25.1 (tracks 4 and 6). A second band, running slightly above the main band , was visible in the case of T25.2 (tracks 5 and 7), suggesting that a rearrangement of the plasmid had occurred resulting in a slight increase in size. These results, taken with the properties of pDHG25 described in sections 3.8.2 and 3.8.3 suggest that pDHG25 is maintained within transformants as an unintegrated plasmid (ARp) without undergoing major structural rearrangements.

3.9 Transformation of uvs mutants of A.nidulans with ARp1

It has been suggested that the inverted repeats present in the yeast 2uM circle are necessary for plasmid copy number amplification (Futcher, 1986). It was argued that recombination between inverted

Table 3.7. Transformation of 3 *uvs* mutants of *A.nidulans* with pILJ16 and ARp1.

Strain	N° transformants/10^6 protoplasts pILJ16	ARp1	ARp1/pILJ16
G34 (*uvs*$^+$)	1,600	6,000	3.75
AJC9.41 (*uvsB*314)	320	300	0.9
AJC9.43 (*uvsC*114)	500	1,050	2.1
AJC9.45 (*uvsD*153)	300	850	2.1

Note: in this experiment the transformation frequencies with pILJ16 were unusually high, and those with ARp1 unusually low, unless there is something wrong with the G34 control transformations.

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wrong way around

repeats after one repeat only had replicated would theoretically result in reversal of the direction of one replication form relative to the other (see discussion); this would result in double rolling circle replication and copy number amplification.

If recombination between inverted repeats is required for maintenance of ARp1, then it might be expected that in strains in which there was depression or enhancement of DNA recombination rates, there would be a concommitant change in the transformation frequency with ARp1, or of the stability of the plasmid in transformants.

A number of mutations have been characterised which affect mitotic recombination in A.nidulans. Mutations in the genes uvsB and uvsC cause an increase in sensitivity to UV radiation (Jansen, 1970a). It was found that in uvsC14/uvsC14 diploids the rate of mitotic recombination was 10-100x lower than that in $uvsC^+/uvsC^+$ strains. On the other hand there was a 5-10x increase in mitotic recombination in uvsB10/uvsB10 over $uvsB^+/uvsB^+$ diploids (Jansen, 1970b). It is believed that the mutant uvsC14 is defective in recombination repair, whereas uvsB10 is defective in excision repair of UV damaged DNA.. In a further mutant, uvsD53, uvsD53/uvsD53 diploids were found to have a 25x higher mitotic recombination rate than $uvsD^+/uvsD^+$ strains (Fortuin, 1970). This mutant was found to be defective in enzymatic photoreactivation of UV damage (Fortuin, 1971).

Four $argB^-$ strains of A.nidulans were transformed with pILJ16 and ARp1. These were G34 (uvs^+), AJC9.41 (uvsB314), AJC9.43 (uvsC114), and AJC9.45 (uvsD153). It was argued that in mutants with enhanced mitotic recombination rates, pILJ16 should transform at a higher frequency, since the frequency of homologous integration should be enhanced. Similarly, if recombination between inverted repeats of ARp1 is important for its stable maintenance, transformation of enhanced mitotic recombination mutants might also give enhanced rates of transformation. However, if Futcher-type copy number amplification does not occur, then an increased frequency of transformation of pIILJ16 relative to ARp1 might be expected. Similarly, transformation of mutants with suppressed mitotic recombination rates might or might not increase the transformation frequency of ARp1 relative to pILJ16 depending on whether or not Futcher-type copy number amplification was occurring.

The results of one set of transformations (Table 3.7) show a general drop in transformation frequency into all three uvs strains relative to the uvs^+ strain. This may reflect increased protoplast mortality

due to failure of DNA repair. Between uvs strains there is little variation in transformation frequencies. The only possible significant variation is an increase in the relative frequency of transformation of pILJ16 over ARp1 in the case of uvsB. This hints that ARp1 transformation is recombination independent - at least uvsB mediated recombination. However, a much more extensive, thorough study would be necessary to be certain.

3.10 Transformation of Aspergillus with linear ARp DNA

The transformation frequency of A.nidulans with ARp1 cut with BglII, and pDHG25 DNAs cut with BglII, BamHI and SmaI were ascertained, and compared with those of the uncut plasmid incubated in REact buffers alone. The effect of incubation with BamHI on ARp1 (which contains no BamHI sites) and SmaI on pDHG25 (which contains no SmaI sites) were also checked. That DNAs were digested to completion was checked by examining the DNA digests run on EtBr stained agarose minigels. The whole restriction solution was then used for the transformation, without prior purification of DNA. The results are displayed as a histogram in Fig. 3.15. Surprisingly it was found that ARp1 cut with BglII transformed Aspergillus with the same frequency as uncut DNA incubated with BamHI (both incubations were carried out with REact 3 restriction buffer). A similar result was obtained comparing pDHG25 cut with BamHI or BglII with plasmid incubated with SmaI.

In S.cerevisiae there are two ways in which homologous recombination between transforming plasmid and chromosome give rise to plasmid integration: a single recombination event may result in the integration of the entire plasmid (type-I integration). Alternatively, two recombination events (double crossover, or integration followed by gene conversion) may result in transformation by gene replacement (type-III integration). It has been observed in S.cerevisiae that the frequency of type I integration over type III could be increased 50-fold by cutting the transforming DNA within the coding region of the selectable marker gene (Orr-Weaver and Szostak, 1983). This effect is not so marked in Aspergillus. Clutterbuck (unpublished) has shown that type I integration is enhanced just 2-fold by cutting within the transforming gene regions (also see Yelton et al, 1985), and similar results were obtained in Neurospora (Kim and Marzluff, 1988). The results obtained with the two ARps suggests that the reason for the difference between the behaviour of linear

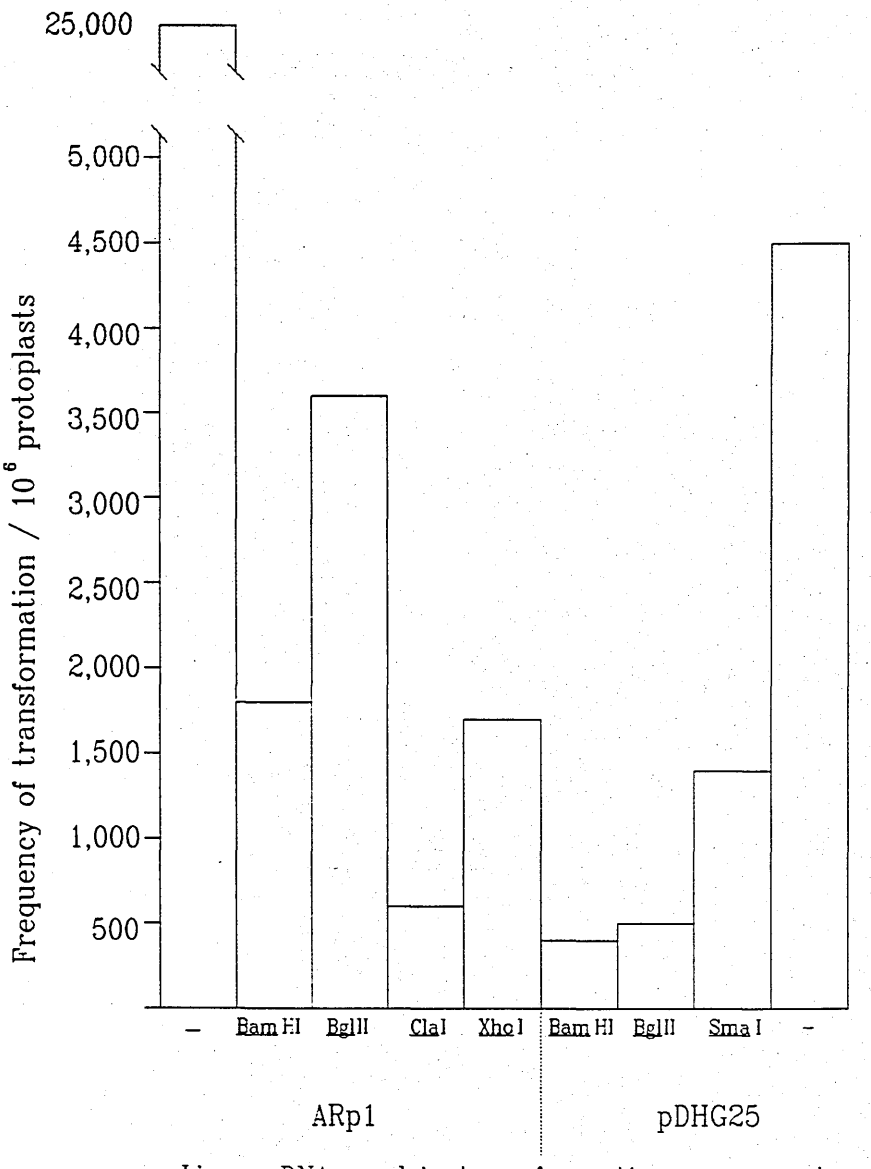

Figure 3.15. **Histogram showing the effect of linearising transforming plasmids upon frequency of transformation of Aspergillus.**
Restriction digests were added directly to transformations without any purification steps. That restrictions had occurred to completion was checked by AGE.

For ARp1 and pDHG25 restriction sites see Figs. 3.1 and 3.12. BamHI does not cut ARp1, but cuts pDHG25 once. BglII cuts ARp1 and pDHG25 once. SmaI cleaves pDHG25 once. ARp1 is cleaved by ClaI and XhoI twice and three times respectively.

facing page 57b

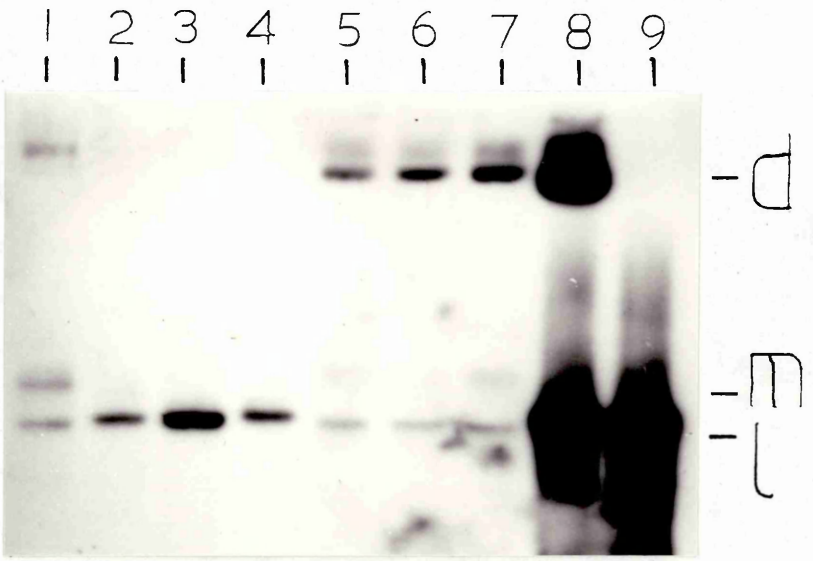

Figure 3.16. **Southern blot analysis of Aspergillus transformants with linear plasmid pDHG25 DNA.**

The blot was probed with radiolabelled pUC18.

Lanes 1, 2, DNA from 2 transformants of strain G34 with pDHG25 linearised with BamHI; the transformant DNA was also cut with BamHI. 3, 4, DNA from 2 transformants with pDHG25 linearised with BglII. The transformant DNA was cut with BglII. 5, 6, 7, DNA from 3 transformants with pDHG25 linearised with SmaI; the transformant DNA was also cut with SmaI; 8, pDHG25 DNA extracted from E.coli, uncut and 9, cut with BamHI; 10, DNA from untransformed Aspergillus strain G34.

d, supercoiled cccDNA dimer; m, supercoiled cccDNA monomer; l, linear monomer.

Note that recircularisation of linear DNA in the transformant has led to regeneration of the restriction site in the two transformants with pDHG25 cut with BglII and one with BamHI. In the case of all 3 transformants with SmaI-linearised pDHG25 loss of the SmaI site has occurred.

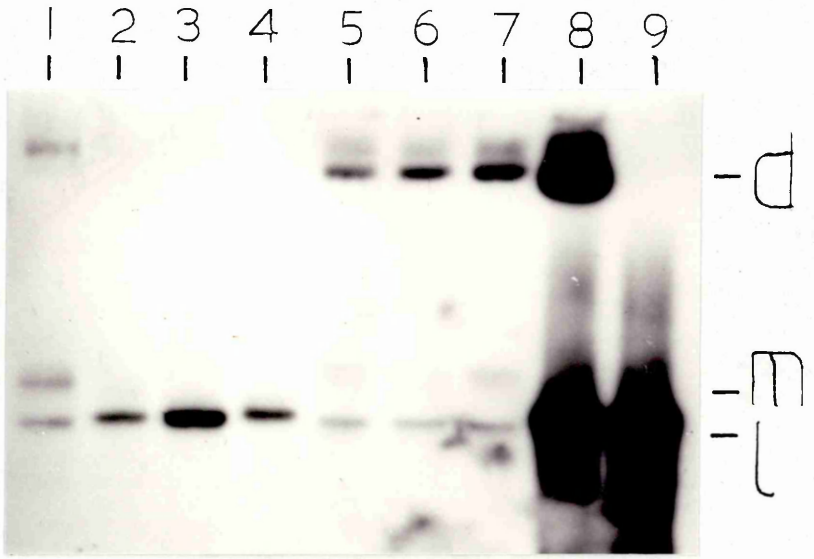

Figure 3.16. **Southern blot analysis of Aspergillus transformants with linear plasmid pDHG25 DNA.**

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Lanes 1, 2, DNA from 2 transformants of strain G34 with pDHG25 linearised with BamHI; the transformant DNA was also cut with BamHI. 3, 4, DNA from 2 transformants with pDHG25 linearised with BglII. The transformant DNA was cut with BglII. 5, 6, 7, DNA from 3 transformants with pDHG25 linearised with SmaI; the transformant DNA was also cut with SmaI; 8, pDHG25 DNA extracted from E.coli, uncut and 9, cut with BamHI; 10, DNA from untransformed Aspergillus strain G34.

d, supercoiled cccDNA dimer; m, supercoiled cccDNA monomer; l, linear monomer.

Note that recircularisation of linear DNA in the transformant has led to regeneration of the restriction site in the two transformants with pDHG25 cut with BglII and one with BamHI. In the case of all 3 transformants with SmaI-linearised pDHG25 loss of the SmaI site has occurred.

transforming DNA between yeast and Aspergillus (and perhaps Neurospora as well) is that in the filamentous fungi linear DNA is recircularised with high efficiency upon entry into the protoplast. Possibly a DNA ligase does this.

Since the BglII site in ARp1 and pDHG25 lies in the selected argB marker gene, the ligation of linear plasmid DNA ends must occur very precisely. To confirm this DNA was prepared from transformants of A.nidulans strain G34, two with pDHG25 linearised with BamHI, two with BglII, and three incubated with SmaI (control). These were then restricted again with the same enzyme, and subjected to Southern blot analysis (probe: pUC18) to see whether the restriction sites had been regenerated (Fig. 3.16). It was found that while one of the BamHI restricted plasmids was relinearised (track 2) the other was not (track 1). However, plasmids derived from BglII digested DNAs both relinearised (tracks 3 and 4). This suggests that ligation in transformants of linear plasmid ends may occur precisely or small sequence changes may occur.

Transformations were also carried out with ARp1 restricted with XhoI. Recircularisation of the largest XhoI fragment would result in the presence of plasmid ARp1 with the central XhoI fragments deleted. It was observed that XhoI restricted ARp1 transformed A.nidulans with a similar frequency as ARp1 linearised with BglII (Fig. 3.15). When the stability of XhoI deleted ARp1 transformants was examined, it was found to be the same as that of ARp1 transformants. This indicates that a) the central unique region of AMA1 contained within the two central XhoI fragments is not required for autonomous maintenance of ARp1; and b) a perfect palindromic structure functions as well as a pair of inverted repeats separated from each other.

3.11 Estimation of plasmid copy number in ARp1 transformants

DNA was prepared from two transformants of A.nidulans strain G34 with ARp1 grown under selective conditions, and five aliquots taken from each prep containing 5 ug, 2.5 ug, 1.2 ug and 0.6 ug respectively of DNA. These were restricted with EcoRI and subjected to Southern blot analysis using as a probe a radiolabelled 1.6-kb HindIII fragment containing most of the A.nidulans argB gene. The intensity of bands corresponding to the 7.4-kb EcoRI fragment of ARp1 (containing the $argB^+$ allele), and the approximately 9-kb fragment of chromosomal DNA containing the argB2 allele were compared (Fig. 3.17). The upper (9-

Table 3.8. Analysis of asexual progeny of 3 heterokaryons between ARG^+ ARp1 transformants of Aspergillus strain G034 (biA1; argB2), and strains G34 (yA2; argB2 methH2).

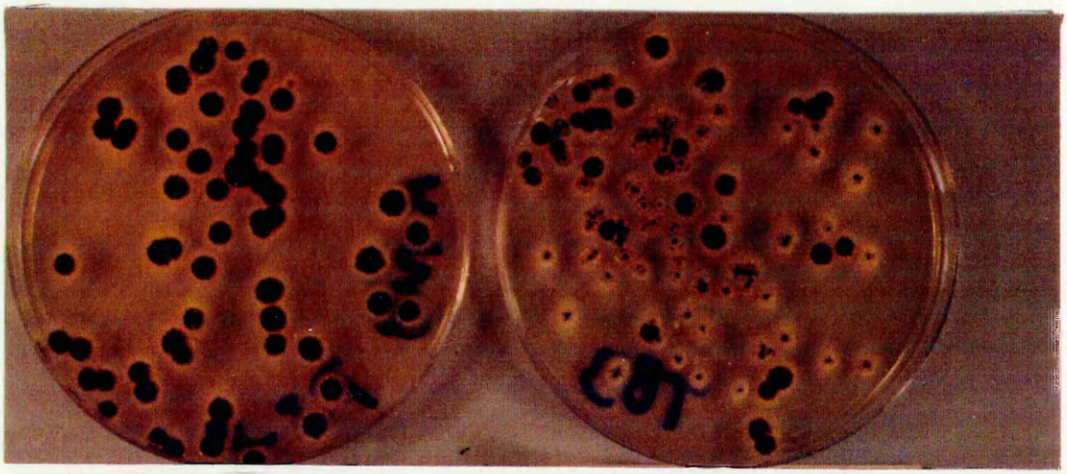
Parent	Growth supplements	%yA^+	Sample size
T1	meth, bi	100	32
T2	meth, bi	100	44
T3	meth, bi	100	22
T1	arg, meth, bi	20	210
T2	arg, meth, bi	49	144
T3	arg, meth, bi	46	138

kb) bands in tracks 5 and 9 respectively are slightly less intense than the lower (7.4-kb) bands in tracks 2 and 6 respectively. From this it can be deduced that the 7.4-kb band is approximately 10x more intense than the 9-kb band. Thus the plasmid copy number of ARp1 is about 10 per haploid genome.

However, it is possible that the overall mean copy number may not reflect the actual copy number in an individual transformed nucleus. The proportion of ARG^+ conidial progeny of ARp1 transformants (35%) may reflect the proportion of nuclei within the mycelium containing ARp1. This would parallel the situation in budding yeast where it has been shown that ARS plasmids are only present in 5-20% of selectively growing cells (Stinchcomb et al, 1979). If this is so, the true mean copy number per transformed nucleus would be 10/0.35 = 29 per nucleus. This compares with a copy number of 50-100 per cell for the yeast 2uM-circle episome and 5-20 per chromosome for the plasmid ColE1 of E.coli.

3.12 Test for cytoduction of ARp1 between nuclei in A.nidulans

Heterokaryons were prepared between 3 ARG^+ transformants of A.nidulans strain GO34 (biA1; argB2) with ARp1 and strain G34 (yA2; argB2 methH2). Standard methods were used (Pontecorvo et al, 1953; Clutterbuck 1974). Once established, the heterokaryons were allowed to grow to fill a 9cm petri plate. $1mm^3$ cubes of mycelium taken from the periphery were then used to inoculate new plates. When colony peripheries had reached the edge of these new plates, conidia were harvested from the periphery, and plated a) on MM supplemented with biotin and methionine, and b) on MM plus biotin, methionine and arginine (control). The results are summarised in Table 3.8. On MM plus biotin and methionine no yA^- colonies were seen. On the control plates an average of 62% of colonies were yA^-. Approximately 2,000 conidial progeny from each heterokaryon were then screened at high density on MM plus biotin and methionine for yA^- colonies. None were observed. The lack of any evidence for transfer of ARp1 between nuclei (cytoduction) suggests that ARp1 is strictly confined within the transformant nucleus. This conforms with the observation that the nuclear membrane does not break down during mitosis in A.nidulans. (Robinow and Caten, 1969).



Progeny of pILJ16 transformant

Progeny of ARp1 transformant

Figure 3.18. **Progeny of ARG$^+$ transformants of A.niger with pILJ16 and ARp1.**

These have been grown on CM at 37^{o}C for about two days. Notice that in the case of the ARp1 transformant progeny two distinct colony sizes are seen. The large colonies are ARG$^+$ and the small ones are ARG$^-$.

facing page 60a

3.13.1 Transformation of Aspergillus oryzae and Aspergillus niger with ARp1

Transformations of A.oryzae strain YTH-13 (argB$^-$) and A.niger strain 350.52 (argB52) were carried out under the same conditions used for A.nidulans with one exception. The hyphae of A.oryzae and A.niger are larger and the hyphal walls thicker than those of A.nidulans, and more resistant to digestion with NovoZym 234. Digestions of mycelial walls to release protoplasts utilised a NovoZym concentration of 16mg per ml - a four-fold higher concentration than is required in A.nidulans transformation.

Aspergillus species in submerged shake-flask cultures tend to form pellets (spherical mycelial aggregates) and it was thought that these might reduce hyphal wall digestion by NovoZym 234. Trinci (1983) found that pelleting was prevented by addition to the submerged culture of the anionic polymer Junlon-110. Here, with respect to A.oryzae, it was found that although Junlon-110 increased the yield of mycelium dramatically, and completely prevented pelleting, the overall yield of protoplasts was unchanged (data not shown). Addition of the enzyme B-glucuronidase was also found not to increase protoplast yield.

The results of four transformations of A.oryzae and A.niger with ARp1 and pILJ16 are shown in Table 3.9. ARp1 transforms A.oryzae and A.niger with a 30x and up to an 80x higher frequency than pILJ16 respectively. pDHG25 was found to transform A.oryzae at a similar frequency to ARp1 (not tested in A.niger).

3.13.2 Stability of A.oryzae and A.niger ARp1 transformants

Conidia from ARp1 transformants of A.oryzae strain YTH-13 were plated on CM. It was found that due to the rather wispy and irregular colony morphology and asynchronous conidiation, ARG$^+$ and ARG$^-$ colonies were not distinguishable. However, when Triton-X100 was added to the media (final concentration: 0.0025%) smaller, more distinct colonies grew and more strongly conidiating ARG$^+$ colonies were distinguishable from weakly conidiating ARG$^-$ ones. This interpretation of colony appearance was confirmed by stab-inoculating a number of colonies of each type onto MM and MM plus arginine. Conidial progeny of five ARp1 transformants were examined (Table 3.10). On average 48.6% of progeny were ARG$^+$ (sample size: 584).

Progeny of A.niger ARp1 transformants grown on CM showed two

Table 3.9 Transformation of A.oryzae and A.niger with ARp1 and pILJ16.

	Number of transformants/10^6 protoplasts	
A.oryzae	ARp1	pILJ16
1	100	0
2	6,000	220
3	400	0
4	700	0
A.niger		
1	660	66
2	40	64
3	1,200	15
4	approx. 40	0

facing page 60b

Table 3.10. **Stability of transformants of A.oryzae and A.niger with ARp1.**

ARG^+ and ARG^- progeny from A.oryzae transformants plated on CM plus Triton-X100 could be distinguished on the basis of strength of conidiation. ARG^+ and ARG^- progeny of A.niger transformants could be distinguished on the basis of colony size.

A.oryzae	Transforming plasmid	Sample size	%ARG^+ colonies
T'formant 1	ARp1	112	30
" 2	"	116	31
" 3	"	95	63
" 4	"	147	76
" 5	"	114	43
A.niger			
T'formant 1	ARp1	58	23
" 2	"	84	18
" 3	"	89	26
" 4	"	76	20
" 1	pILJ16	62	98
" 2	"	71	100
" 3	"	153	100
" 4	"	44	100

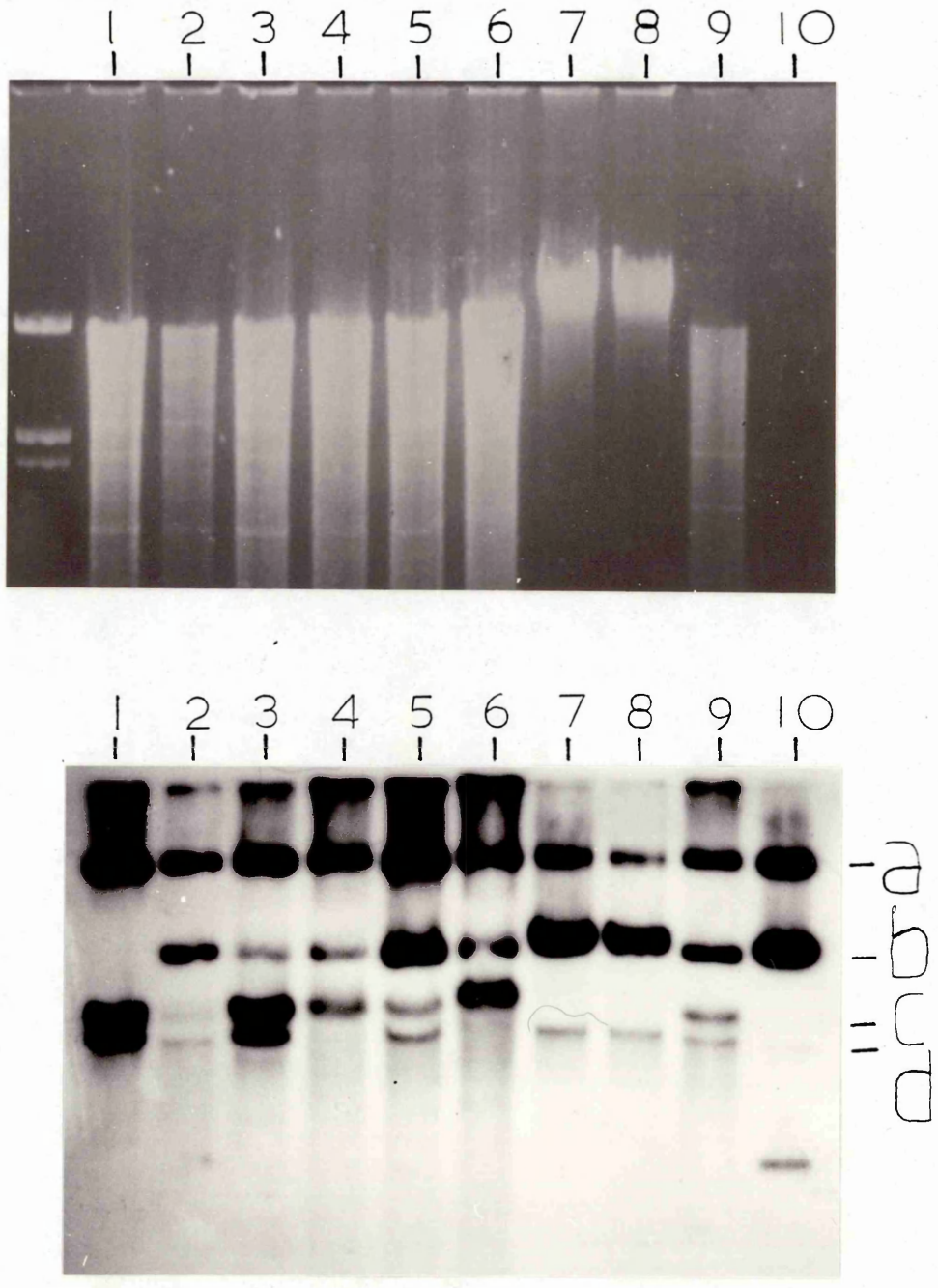


Figure 3.19. **Southern blot analysis of ARp1 transformants of A.nidulans and A.oryzae.**

The blot was probed with radiolabelled pUC8. All transformant DNAs were restricted with BamHI, which does not cut ARp1.

Lanes 1-3, DNA from 3 ARp1 transformants of A.nidulans strain AJC9.4; 4-6, 3 ARp1 transformants of A.nidulans strain G34; 7-9, 3 ARp1 transformants with A.oryzae strain YTH-13. 10, uncut ARp1 DNA extracted from E.coli. Note: DNA in tracks 7 and 8 did not restrict.

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distinct sizes of colony (Fig. 3.18). That the large colonies were ARG^+ and the small colonies ARG^- was confirmed by subculturing onto selective and non-selective medium. Conidial progeny of four ARp1 transformants were examined (Table 3.10). An average of 21% of progeny were ARG^+ (sample size: 307). By comparison, among the progeny of four pILJ16 transformants of A.niger 99.6% were ARG^+ (sample size: 330).

3.13.3 Southern analysis comparing ARp1 transformants of A.nidulans and A.oryzae

DNA from three transformants of A.oryzae strain YTH-13 with ARp1 grown under conditions selecting for maintenance of the plasmid was restricted with BamHI and subjected to Southern blot analysis using a radiolabelled pUC8 probe. For comparison, BamHI digested DNA from three transformants of A.nidulans strains AJC9.4 and G34 with ARp1, and ARp1 prepared from E.coli was run in adjacent tracks (Fig. 3.19). Four bands are seen designated A, B, C and D. In tracks 1-10 the two top bands, A and B, corresponding to ARp1 monomer and dimer can be seen. Unintegrated plasmid is thus present in the three A.oryzae transformants, as well as the six A.nidulans transformants. It can also be seen that no change in plasmid size has occurred. In track 1 the band corresponding to monomeric ARp1 (band B) is absent. In addition, two lower bands, C and D are seen in most of the 9 tracks containing transformant DNA. Examination of results of a number of Southern analyses of BamHI digested ARp1 transformant DNAs suggests that bands C and D may correspond to linear ARp1 dimer (C) and monomer (D). These may be the products of BamHI* activity on a single BamHI* site in ARp1, rather than non-specific nicking by contaminating DNase, which would result in the formation of open circle monomer and dimer, and linear dimer in addition to linear monomer.

There are a number of reasons for making this interpretation, rather than that bands C and D represent junction fragments resulting from integration of ARp1. Firstly, these two extra bands are sometimes seen in BamHI digests but never BglII digests of ARp1 transformant DNA. Secondly, these two lower bands are often not seen in Southern analyses of BamHI digested ARp1 transformant DNA. Thirdly, the same two lower bands are seen in nine ARp1 transformants, six for A.nidulans and three from A.oryzae: bands produced by integration of ARp1 would be unlikely to be the same in both species.

Discussion and Future Prospects

The properties of ARp1 described strongly suggest that it is maintained in transformants by means of autonomous replication, rather than by integration into and replication with the chromosome. Given that this is so, the question arises why previous approaches to finding Aspergillus and Neurospora ARS plasmids have not succeeded. A plausable explanation rests with the Buxton and Radford hypothesis (1984) that an ori alone will not act as an ARS in these species. This suggests that a true Aspergillus ARS (e.g. AMA1) must contain, as well as an ori, some other element which enhances stability.

Possible roles for this stability factor are a) a sequence stimulating active partitioning of the plasmid, analogous to the STB region of the yeast 2uM circle (Kikuchi, 1983), b) a recombinogenic site facilitating resolution of plasmid multimers, analogous to the cer site of the E.coli plasmid ColE1 (Summers and Sherratt, 1984), or c) an element promoting plasmid copy number amplification, a role suggested for the inverted repeats in the yeast 2uM circle (Futcher, 1986).

Sequences stimulating plasmid partitioning: The stability of plasmids in a number of species has been found to be enhanced by the presence of a sequence of DNA which interacts with the cytoskeleton or nuclear membrane during division. The STB site, in conjunction with the trans-acting products of two plasmid coding regions, REP1 and REP2, promote the equipartitioning of the yeast 2uM circle (Kikuchi, 1983). Similarly, the par region of bacterial plasmids R1 and pSC101 have been shown to interact with the bacterial membrane, a process which brings about active plasmid partitioning (Gustafsson et al, 1983). However, in Aspergillus the only known source of chromosomal sequences which might have such properties in ARp1 is the centromere.

Recombinogenic sites: There exists some evidence to support the possibility that the stabilising factor is a recombinogenic site. Results in section 3.6 suggest that recombination between ARp1 monomers to form dimers occurs at high frequency, and that resolution of dimers to form monomers also occurs. The possible existence of a recombination hot-spot is indicated by results shown in chapter 5. It has been shown for the E.coli plasmid ColE1 that plasmid stability is correlated inversely with degree of plasmid multimerisation, and that

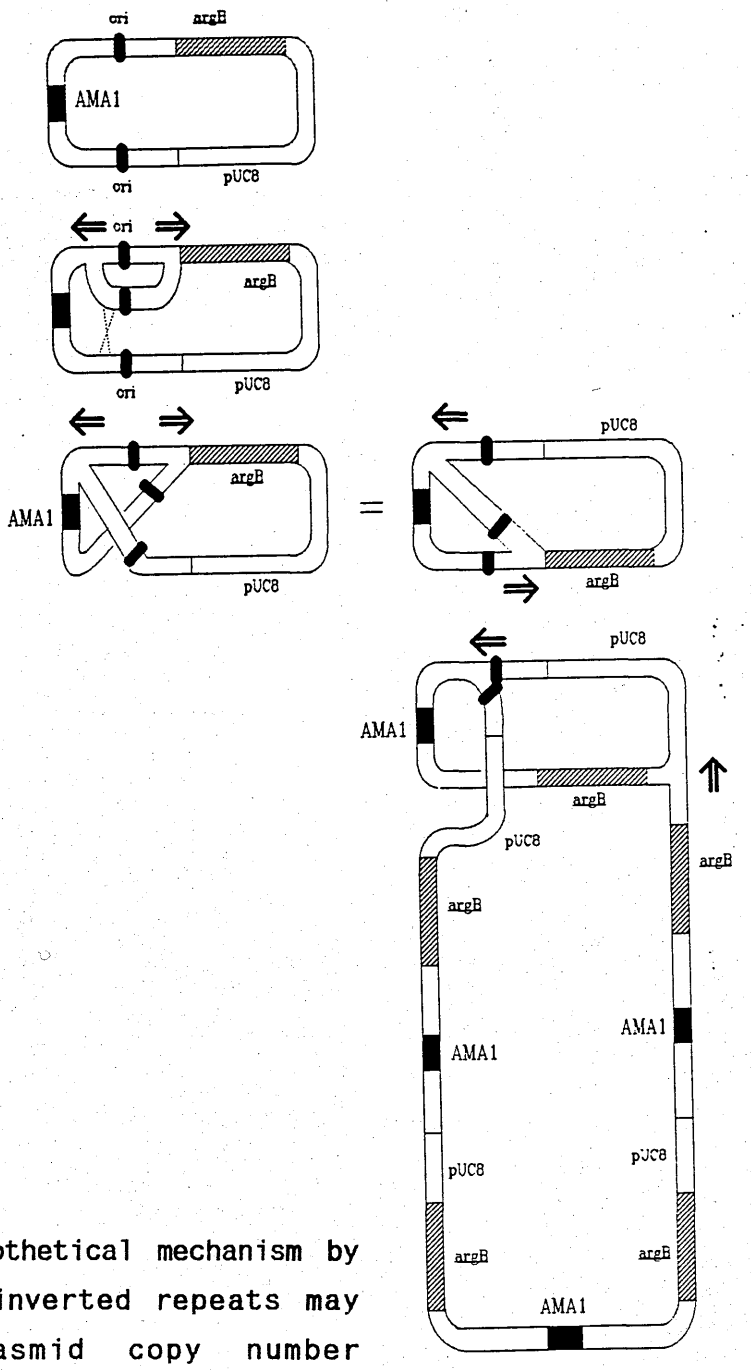

Figure 3.20. Hypothetical mechanism by which the AMA1 inverted repeats may result in plasmid copy number amplification (based on Futcher, 1986).

Recombination between inverted repeats when only one has undergone replication would theoretically result in a reversal of direction of one DNA replication fork relative to the other (as shown) resulting in double rolling circle replication. In chapter 6 of this thesis it is demonstrated that recombination *does* occur between the AMA1 inverted repeats.

stabilisation of this plasmid is effected by multimer resolution to form monomers. This is mediated by a sequence, cer, which stimulates site specific recombination (Summers and Sherratt, 1985). Conceivably ARp1 multimers are resolved through a recombination hot-spot in AMA1. However, this would imply the existence of some kind of ori counting mechanism for maintaining plasmid copy number, as is the case for ColE1 and E.coli. The existence of such a mechanism could be investigated a) by looking for reduced plasmid stability in uvs recombination mutants and determining whether there was a concommitant increase in multimerisation and b), constructing ARp1 derivatives with mutiple origins, which should have reduced stability if an origin counting mechanism is active.

A precedent exists for studies of fungal recombination mutants as an extension of the much more thoroughly investigated DNA recombination systems in E.coli: the rec1 gene of the basidiomycete Ustilago maydis has been found to encode a protein which is functionally homologous to the E.coli RecA protein (Kmiec and Holloman, 1982)

Copy number amplification and inverted repeats: That the inverted repeat structure of AMA1 may be important for maintainance of the plasmid is suggested by the structure of the 2uM-circle episome of S.cerevisiae. This also contains two inverted repeats, of 599 bp. Futcher (1986) has suggested a role for these in plasmid copy number amplification. He observed that if recombination between inverted repeats occurred when only one repeat had undergone DNA replication, the direction of one DNA replication fork would be reversed relative to the other. This would result in double rolling circle replication of the plasmid and copy number amplification. The formation of inverted duplications is also known to be a common first step in gene amplification in mammalian cells - e.g. the oncogenic polyoma virus middle T-antigen in transformed rat cells (Passananti et al, 1987). A diagram showing how this process could occur with ARp1 is shown in Fig. 3.20. Note that such recombination would generate new sequences (the bottom of the concatemer as shown in Fig. 20). Southern blots of BglII digests of ARp1 Aspergillus transformants, using the argB as a probe, did not detect the appearance of such predicted novel sequences (data not shown).

It remains unclear whether the inverted repeat is important as a repeat, or because of its inverted structure, or both (or neither). In future, the clearest answer to this question will come from looking

at the behaviour of ARp1 derivatives in which the orientation of one of the AMA1 repeats in relation to the other has been inverted generating a direct rather than an indirect repeat. This would leave the number of copies unchanged, but destroy the inverted repeat structure.

The probable presence in AMA1 of a stabilising element raises the possibility that the ori on ARp1 might lie outside the AMA1 sequence. Johnstone (1985b) found that the plasmid pILJ16 transforms A.nidulans with a 25-fold higher frequency than the plasmid from which the argB gene was derived, pSAL43. It is possible that the reason for this difference is that pUC8 sequences in pILJ16 contain a sequence recognised as an ori by Aspergillus. Thus, transient plasmid replication may occur which enhances the integrative transformation frequency. That this may be the case is supported by work involving ans1. ans1 is found to enhance transformation efficiency of the plasmid pJDB1 (pyrG), based on the bacterial vector pBR325, 50 - 100-fold. However, when inserted into the pUC18-based plasmid pA1 (argB$^+$) ans1 only conferred a 4-fold increase in transformation efficiency. pUC18 differs from pUC8 only in its polylinker sequence. (Alternatively, this difference could be due to the different mode of integration of the markers.)

Differences between Neurospora and Aspergillus: An entirely different explanation for being able to isolate a sequence conferring replication of an autonomous plasmid in Aspergillus but not Neurospora lies in the differences between the cellular stuctures of the mycelia in these two fungi. Although in Neurosopora mycelium is interrupted by septa, nuclei move freely between compartments, and anastomotic connections commonly form between hyphae (Atwood and Pittenger, 1955). On the other hand, in Aspergillus nuclei do not move between compartments, and anastomoses are rarely seen (Clutterbuck and Roper, 1966). This is thought to be the basis of differences between the two genera in the properties of heterokaryons. In the case of Neurospora it has been found that the ratio of two types of nuclei in a heterokaryon reflects the ratio of the two types of conidia used in heterokaryon formation, and this ratio remains constant during growth (Pittenger and Atwood, 1955). This was so even where one nuclear type was much more abundant than the other, resulting in a suboptimal growth rate. In other words Neurospora heterokaryons do not show

adaptive change. In contrast, Aspergillus heterokaryons do show adaptive change of nuclear ratios in response to environmental change (Warr and Roper, 1964). This appears to be the result of selection between hyphae, rather than individual nuclei (Clutterbuck and Roper, 1966).

It is possible, then, that ARp1 contains only an ori, and no second sequence conferring stable plasmid maintenance, and that the plasmid, though poorly maintained in individual nuclei, is maintained within the mycelium by strong selection between the hyphae. The heterokaryon-like appearance of ARp1 transformants colonies conforms with this hypothesis. If this view is correct, it should also be possible to isolate autonomously replicating plasmids for Penicillium since adaptive change in nuclear ratios in this genus has also been demonstrate (Jinks, 1952), and in other colonial fungi, but not Neurospora.

Results shown in section 3.10 suggest that deletion of the central two XhoI fragments in AMA1 does not reduce transformation frequency below that of ARp1. Taken together with data set out in sections 3.8.2 and 3.8.3, this suggests the following: the region of AMA1 conferring the power to replicate freely lies in each arm of the inverted repeat between the HindIII and XhoI sites. That it does not lie within the unique central region is suggested by the almost complete lack of replicon behaviour by pILJ20, and by the replicon behaviour of XhoI deleted ARp1 (section 3.10). The properties of pILJ23 and pILJ25: intermediate transformation frequency, large number of abortive transformants and extreme instability of transformants, may reflect a reduction of the plasmids' ability to replicate and lends itself to two basic interpretations. Firstly, that the regions conferring free replication are bissected by the EcoRI sites in AMA1, such that pILJ23 and pILJ25 have diminished replicon behaviour and pILJ20 almost none. Secondly, that the relevant sequence is present in pILJ23 and pILJ25, but that two copies of it, or the inverted repeat structure, or both are required for full replicon activity.

In section 3.10 it is also suggested that ligation of ends of linear transforming DNA in filamentous fungi but not in yeast is the reason why only in yeast does the use of linear transforming DNA stimulate gene replacement. An alternative explanation lies with the properties of the polymerase-nuclease believed to initiate DNA recombination by

asymmetric strand transfer (Meselson and Radding, 1975). This, it has been suggested, has a higher affinity for its substrate in yeast than in other fungi such as Neurospora. Thus, in yeast, the ends of linear DNA, by being effective substrates for the strand transfer reaction, may stimulate homologous recombination more strongly.

As described in section 3.11, although the mean ARp1 copy number per haploid genome is approximately 10, the mean copy number per transformed nucleus is likely to be approximately 30. This would be the case, it was argued, if the proportion of ARG^+ conidial transformant progeny was a reflection of the proportion of transformed nuclei. However, the possibility exists that plasmid instability may be a property of conidiogenesis, occurring only during the rapid nuclear divisions which occur in the phialide to generate the conidial nuclei. ARS plasmids in budding yeast have been found to show a strong bias to segregate to the mother cell (Murray and Szostak, 1984). It has been observed that the similarity of yeast budding and conidial development from phialides may reflect the common ancestry in evolution of these two Ascomycetes. However, the slightly heterokaryon-like appearance of ARp1-transformed colonies argues against ARp1 instability being confined to conidiogenesis.

The sequence AMA1 shows clear potential for use in the development of plasmid cloning vectors for Aspergillus species. The plasmid pDHG25 may serve as such a vector. It has the same high frequency of transformation and relative structural stability as ARp1, but also is 1 kb smaller, and contains a unique BamH1 site into which pseudorandom SauIIIA digested chromosomal DNA could be inserted to form a gene bank.

CHAPTER 4

Cotransformation of autonomous and integrative plasmids

Introduction

Possible mechanisms of cotransformation

The initial discovery of high levels of cotransformation of unselected DNA during transformations of bacteria (Kretschmer et al, 1975) and yeast (Hicks et al, 1978) was interpreted as follows: only a small subpopulation of those cells, or protoplasts used in a transformation were truly competent to be transformed. This subpopulation gets multiply transformed, either by one or more transforming DNA species. Subsequent to its discovery cotransformation has been used in a variety of organisms, including unicellular, dimorphic, filamentous and other fungi, for a number of processes. These include introduction of unselected DNA, one step gene replacement, locus directed mutagenesis and localisation of genes within large stretches of cloned DNA. During the course of some of this work it has become clear that in addition to the existence of a small subpopulation of competent protoplasts, a second factor underlies cotransformation, namely: recombination between transforming plasmids upon entry into the protoplast.

The simplest indication of the occurence of such plasmid recombination would be the resulting genetic linkage of independently introduced genes. Suppose that cotransformation results from the integration into the chromosome of plasmids previously recombined into a cointegrate structure: the two plasmids would then be integrated at the same locus. In the case of an autonomously-maintained plasmid cotransformed with an integrative one, recombination would result in the formation of an autonomously replicating cointegrate. Conversely, if no recombination occurs, two integrating plasmids would be expected to integrate at different loci; the basis of maintenance of cotransforming autonomous and integrating plasmids would remain unchanged, remaining unintegrated and integrated respectively.

The occurrence of recombination between plasmids during recombination suggests an alternative explanation of recombination to the competent protoplast subpopulation model. It is possible that DNA uptake into all protoplasts occurs quite efficiently, and that the rate limiting step is integration into the chromosome. That transformation is limited by homologous recombination has been shown in yeast, where increasing available homology increases transformation

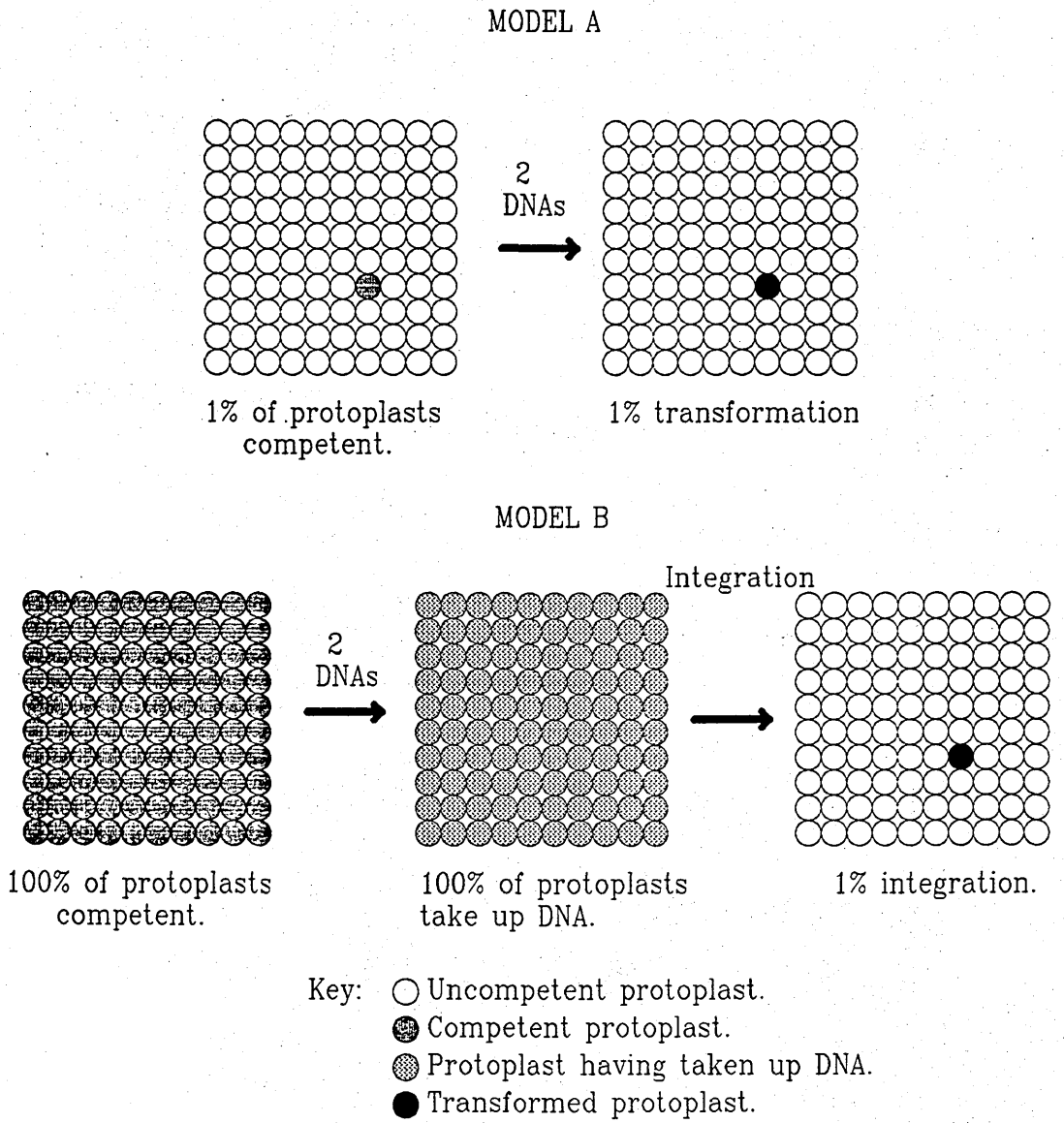

Figure 4.1. **Schematic representation of two models of events underlying cotransformation in fungi.**
These models describe a hypothetical situation where 1% of protoplasts are transformed, and 100% of transformants cotransformed.

A, Competent protoplast model: Here only one of 100 protoplasts is competent; DNA uptake into this one protoplast occurs efficiently, followed by efficient stable integration into the chromosome.

B, Recombination model: Here all the protoplasts are equally competent. DNA uptake occurs efficiently, followed by a high frequency of recombination between transforming DNAs. However, the probability of stable integration occurring in any protoplast is only 1%.

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frequency (Szostak and Wu, 1979). If the frequency of recombination between plasmids was much higher than this limiting rate then a situation would exist in which cotransformation might occur at the frequencies seen without the existence of any subpopulation of competent protoplasts, since rare homologous recombination events between plasmid and chromosome would be likely to result in integration of plasmid heterodimers. Thus the high frequency transformation of unselected DNA seen in cotransformation depends on plasmid recombination. The two models of cotransformation are schematically represented in Fig. 4.1.

Alternatively, plasmid recombination may be purely incidental to, and not essential for cotransformation; if this is so, the competent protoplast subpopulation model of fungal cotransformation may be correct.

A review of the behaviour of cotransforming plasmids observed in a range of organisms gives some idea to what extent recombination is a causal factor.

Cotransformation of the yeasts

High frequency of cotransformation in Saccharomyces cerevisiae was first reported by Hicks et al (1978) using two integrative plasmid vectors. This was interpreted as reflecting the presence of a subpopulation of competent cells. The authors speculated that a cell's position in the cell cycle might determine its competence.

When an integrating plasmid carrying the E.coli gene conferring resistance to the antibiotic G418 on Tn601(903) was added to transformations with an autonomous plasmid carrying the LEU2 gene, 8% cotransformation to G418 resistance was observed (Jimenez and Davies, 1980). It was found that under non-selective conditions the G418 resistance and LEU2 genes were lost at the same frequency. This was interpreted as meaning that cotransformation was the result of recombination of the two plasmids. This was confirmed by Southern blot analysis, and by reisolation into E.coli of the plasmid cointegrates. The report of this work does not state whether homologous or non-homologous recombination had occurred. In subsequent work, however, *in vivo* homologous recombination of episomal and (normally) integrating plasmids was used for the purposes of plasmid construction (Valinger et al, 1989).

A different type of cotransformation occurred when an autonomously replicating plasmid carrying the gene TRP1 was cotransformed with linear DNA fragments carrying the yeast MAT gene (Siliciano and Tatchell, 1984). In 1% of TRP^+ transformants integration of the MAT gene into the chromosome had occurred. In another study this type of cotransformation was found to occur at a frequency of 4% (Rudolph et al, 1985).

In the fission yeast Schizosaccharomyces pombe homologous recombination between autonomously replicating plasmids has been shown to occur at high frequency to form multimers (or polymers) of 10 or more plasmid copies (Sakaguchi and Yamamoto, 1983). It was also found that if a leu1 ura1 strain of S.pombe was transformed with the non-replicating YIp32 plasmid carrying the S.cerevisiae gene LEU2, plus an autonomously replicating plasmid carrying the ura1 gene, a 250 - 280-fold in the frequency of transformation to LEU^+ over that using YIp32 alone, occurred (Sakai et al, 1984). 20 such LEU^+ colonies when tested were all found to be also URA^+. Both phenotypes were found to be lost under non-selective conditions. Southern analysis suggested that homologous recombination between plasmids had led to the production of large heteropolymers containing a 10 to 1 ratio of ura1 to LEU2 plasmid.

Homologous recombination between autonomously replicating plasmids has been described in another yeast, Kluyveromyces lactis (Bianchi et al, 1989). The plasmids involved were the resident K.lactis yeast 2uM plasmid homologue, pKD1, and transforming derivatives of it. Parallel studies in S.cerevisiae with S.cerevisiae plasmids yielded similar results (Thomas and James, 1980).

Cotransformation of dimorphic fungi

When the dimorphic fungus Yarrowia lipolytica was transformed with a plasmid gene library carrying the LYS2 gene, tandem arrays of 2-10 copies of incoming DNA were found to be integrated into the genome (Gaillardin et al, 1985). When two LYS^+ transformants with 5 and 10 integrated copies were subjected to Southern analysis it was found that each integrated plasmid copy in the tandem array had a different DNA library insert. It was inferred that homologous recombination between plasmids had led to the formation of plasmid multimers which had subsequently integrated into the chromosome. In a separate experiment 2/12 LYS^+ transformants were found to be cotransformed with

an unselected DNA sequence (Gaillardin et al, 1985).

More complex cotransformation events were observed in studies involving the pathogenic, diploid, dimorphic fungus Candida albicans. An autonomous ARS plasmid of this species was found to recombine to form large plasmid oligomers, consisting of head to tail tandem repeats (Kurtz et al, 1987). A derivative of this plasmid carrying URA3 was used to cotransform a LEU^+ ura3 strain, with a plasmid carrying a leu2 gene disrupted by an insert of phage lambda DNA (Kelly et al, 1988). Colony hybridisation was carried out using a lambda DNA probe. 30% of colonies were found to contain lambda DNA, and 5 strongly hybridising strains were picked and cured of the URA3 ARS plasmid. Southern analysis of the cured strains showed that one copy of the LEU2 gene had been disrupted in 3 out of 5 cases. However, in the other 2 the lambda DNA was lost with the ARS plasmid, implying that it had recombined with it.

Cotransformation of basidiomycete fungi

Wang et al (1989) used cotransformation to identify a DNA fragment carrying a ferrichrome biosynthesis gene in the corn smut fungus, the basidiomycete Ustilago maydis. Transformations were carried out of a Fec^- strain with the linear DNA fragments produced by restriction with HindIII of a cosmid with a hygromycin B gene in the vector sequence, and an uncharacterised 46 kb insert containing Fec^+. 28% of the resulting HYG^+ transformants were found to be Fec^+. In a further experiment, one HindIII fragment of the cosmid insert subcloned into pUC18 was found to cotransform with a HygB plasmid to Fec^+ at a frequency of 48%. Thus, in this case cotransformation of a HygB plasmid with non-homologous linear DNA and homologous cccDNA both result in high levels of cotransformation.

High levels of cotransformation have also been observed in the basidiomycete fungus Coprinus cinereus. Mellon et al (1987) found 48% of TRP^+ transformants were cotransformed with the unselected acu-7 gene. Integration of the latter gene had occurred non-homologously and multiply, at different sites around the genome. Higher levels of cotransformation of this species with unselected DNA was reported by Casselton and Herce (1989).

Cotransformation of ascomycete filamentous fungi

Cotransformation of Neurospora crassa was first reported by Vollmer and Yanofsky (1986); Austin and Tyler (1990), in studies using the ga-2 and Bml^R (benomyl resistence) genes observed a maximum of 80-90% cotransformation. All transformations involved plasmid integration.

A clearer study of the mechanism of cotransformation itself involved the barley leaf spot and glume blot disease fungus, Septoria nodorum (Cooley et al, 1990). Three different DNAs were found to have cotransformed transformants with HygB at a frequency of 50%, including one plasmid with the coding region of the E.coli LacZ gene fused to the expression signals of the A.nidulans gdp gene. In each case a large number of "abortive " colonies were seen. In cotransformations involving the HygB and lacZ fusion genes 112 "abortives" were picked and grown on medium containing Xgal (the chromogenic substrate for B-galactosidase), in the absence of hygromycin. None showed the presence of B-galactosidase. It was inferred that the protoplasts producing "abortives" are deficient in an essential step in transformation and are not an intermediate stage in which the final step, integration, occurs only at a low frequency. Cooley et al concluded that their results suggested the existence of a small subpopulation of competent protoplasts.

Cotransformation has been observed in a number of Aspergillus species observed including A.oryzae (Russ et al, 1990), A.nidulans (Wernars et al, 1985) and A.niger (Kelly and Hynes, 1985). In one study of A.niger (Goosen et al, 1989) a $pyrA^+trpC^+$ strain was transformed with a construct consisting of the A.niger trpC gene disrupted by insertion of a lacZ gene in frame into the coding sequence. 20% of PYR^+ transformants were found to express lacZ, but none were $trpC^-$. Where instead of the trpC-lacZ plasmid, a linear DNA fragment consisting of the lacZ gene flanked by short sequences of the trpC gene, 0.15% of PYR^+ transformants were found to be cotransformed to LAC^+TRP^-. Southern analysis of these cotransformants showed that a one step gene replacement event involving the lacZ-trpC DNA fragment had occurred at the resident $trpC^+$ allele causing mutagenesis. In addition, multiple integration of $pyrA^+$ sequences at the pyrA locus had occurred. The incidence of $PYR^+LAC^+TRP^+$ cotransformants was 0.016%.

Aspergillus nidulans. In most organisms lack of homology between transforming plasmid and the genome of the recipient organism depresses frequency of integrative transformation. This was not found to be the case in transformations involving the amdS (acetamidase) gene and the amdS$^-$ deletion mutants of A.nidulans (Wernars et al, 1985). In addition, Southern analysis of AMD$^+$ transformant DNA showed the presence of up to 21 integrated copies of the vector sequences in a tandem array. A considerable degree of vector rearrangement was also observed in these arrays, as was a high degree of recipient strain variation with respect to incidence of multiple integrations. To explain these findings it was proposed that upon entry into the protoplast recombination between plasmids occurred, mediated by a non-homologous recombination system, generating "scrambled" cointegrates which subsequently integrated. The differences between strains would reflect genetic variation in this recombination system.

In transformations involving the amdS gene 95% cotransformation with the trpC gene was found to occur (Wernars et al, 1987). In a complex experiment an amdS$^-$ TRP$^+$ strain was transformed to AMD$^+$ and cotransformed with a non-functional trpC gene containing an in-frame lacZ insertion. 10 AMD$^+$LAC$^+$TRP$^-$ transformants were identified. Two of these, when transformed to TRP$^+$ with the trpC$^+$ gene, yielded a low frequency of transformants which were LAC$^-$AMD$^-$. This implies that integration of the amdS gene had occurred, with the trpC-lacZ sequence, at the resident trpC locus. Thus, gene replacement by the trpC$^+$ gene had resulted in loss of both amdS$^+$ and lacZ sequences. This was confirmed by Southern analysis of transformant DNA and interpreted as meaning that recombination of trpC-lacZ and amdS sequences had occurred prior to integration into the chromosome.

Cotransformation of linear DNA with transforming cccDNA in A.nidulans is also known to occur (Timberlake et al, 1985). This was used to locate cloned genes within cosmid inserts: cotransformations were first carried out using restriction digests of the cosmids with a variety of enzymes. Then, when those enzymes which did not cut within the gene of interest had been identified, cotransformations with gel purified individual restriction fragments generated by those enzymes were carried out. These were found to cotransform ARG$^+$ or TRP$^+$ transformants with a frequency of about 10%. Thus the restriction fragments carrying the genes of interest were identified without the need to construct a large number of subclones from the cosmid inserts.

Cotransformation of other eukaryotes

Protozoa: In the slime mold Dictyostelium discoideum cotransformation with integrating plasmids has been demonstrated, involving about 100 copies of each plasmid (Nellen and Firtel, 1985). These are present as large, head to tail tandem arrays of one or the other plasmid, rather than arrays containing both plasmids. Whether both tandem arrays integrate at the same site is not clear. In a further study carried out under different conditions, cotransformation was found to involve homologous recombination between the two plasmids (Katz and Ratner, 1988). However, when no homology existed between plasmids, non-homologous recombination occurred, resulting in similar levels of cotransformation.

Plants: Cotransformation can occur in most plant transformation systems with varying frequencies, e.g. 10-15% in Chlamydomonas reinhardtii (Kindle, 1990). This may involve the integration of large plasmid concatemers, e.g. in transformation by electroporation of Nicotinia tabacum (tobacco) (Riggs and Bates, 1986). On the other hand, cotransformation of N.rustica via transfection by Agrobacterium rhizogenes with T-DNA plasmids involves the integration of low copy numbers of the two cotransforming plasmids at separate chromosomal sites (Hamill et al, 1987).

Nematodes: Transformation of the nematode worm Caenorhabditis elegans has been achieved by microinjection with plasmid cccDNA (Stinchcomb et al, 1985). In this case integration into the chromosome does not occur; rather, transforming DNA is maintained extrachromosomally in the form of large, head to tail concatemers containing 80-300 plasmid sequences, presumably the result of homologous recombination between plasmids. If linear plasmid DNA is used, concatemers containing both head to head, and head to tail arrangements of plasmids occur. This was interpreted as the result of the ligation of linear plasmid ends.

Cultured insect and mammalian cells: Cotransformation of the fruit fly Drosophila melanogaster heat shock gene with a selectable marker was demonstrated in a D.hydei cultured cell line (Sinclair et al, 1985). Transformed cells were found to contain tandem arrays of 50-100 copies of the cotransforming plasmid, arranged head to tail, integrated into the chromosome. Whether or not integration of the two plasmid species

had occurred at the same site was not investigated.

Cotransformation of mammalian cells occurs at high frequency. Wigler et al (1979) found that 15 out of 16 tk^+ (thymidine kinase) transformants of cultured mouse cell line Ltk contained unselected cotransforming plasmid sequences. Again, these were present as tandem repeats of up to 100 copies, arranged in a head to tail tandem array, integrated into the chromosome; and again, whether or not cointegration of the two plasmid species at a single site had occurred was not investigated.

That homologous recombination does occur between DNAs transforming mammalian cells was demonstrated when plasmids containing the same polyoma virus gene sequences with different gene lesions were transfected together into Rat-1 cells (Pomerantz et al, 1983). This resulted in formation of the wild-type gene at high frequency, as evidenced by high levels of cellular transformation.

General description of cotransformation

Overall, similar processes appear to give rise to cotransformation in a variety of eukaryotic species. The exception to this is the yeast S.cerevisiae, where linear DNA ends stimulate DNA integration. In many other species examined, e.g. D.discoideum, A.nidulans and C.elegans transformation of linear DNA results in the ligation of linear DNA ends, to form plasmid monomers or concatemers, and no marked increase in plasmid integration into the chromosome. However, a 10-fold enhancement of integration by linearising DNA has recently been reported in A.nidulans (Mayorga and Timberlake, 1990). These exceptions having been noted, certain loose rules can be adduced from the results described in this review to describe how DNA species will behave in cotransformations.

I) Cotransformation usually involves recombination between cotransforming DNAs to form plasmid cointegrates.

II) Where homology exists between two cotransforming DNA species, cointegration will occur by homologous recombination; where it does not, non-homologous recombination will result in cointegration at a lower frequency.

III) Cotransformation, as opposed to simple transformation, involving integration into the chromosome occurs more readily where there is an impediment to homologous integration into the chromosome. This could be due to lack of homology between transforming DNA and chromosome

(e.g. transformation of an amdS deletion mutant of A.nidulans with the amdS gene; Wernars et al, 1985); or to large genome size.
IV) Cotransformation with an unselected linear DNA plus a selected cccDNA will usually occur by recombination between the two DNAs. Exceptions to this are a) cotransformations of yeast, where linear DNA is more likely to recombine with the chromosome, and b) situations where integration of the linear DNA species by a double crossover event is specifically selected for, e.g. as described in the case of A.niger (Goosen et al, 1989). Here the transforming and cotransforming DNAs integrated at different sites, and such cotransformation occurred at a frequency of only 0.16% of all transformants.

The results described in chapters 4, 5, and 6 of this thesis allow a more precise gauge of the extent to which cotransformation of A.nidulans conforms with these loose rules.

Table 4.1 **Stability of ARG^+ and IVO^+ phenotypes in ARp1-pCEB218 cotransformants**

Conidia from progeny of ARG^+IVO^+ cotransformants were plated on CM and on CM plus arginine. On CM the proportion of ARG^+IVO^+ (grey-brown) and ARG^+IVO^- (ivory), and ARG^- (no conidiophores) colonies were scored. On CM plus arginine the proportion of IVO^+ and IVO^- colonies were scored. The IVO^+ phenotype clearly shows instability, and the overall proportion of IVO^+ colonies which could be scored in each case is the same - even though most of the colonies on CM are ARG^- and could not be scored with respect to IVO.

	Number of colonies on CM				
ARG^+IVO^+ strain	ARG^+IVO^+	ARG^+IVO^-	ARG^-	$\%ARG^+$	$\%IVO^+$[1]
3.1	2	7	81	10	2.2
3.2	26	4	74	29	25.0
3.3	4	0	40	9	9.1
6.1	13	0	44	23	22.8
6.2	8	3	22	33	24.2
7.1	3	0	28	10	9.6
7.2	4	17	27	44	8.3
7.3	0	15	78	16	0
				Average:	12.7

	Number of colonies on CM plus arginine		
ARG^+IVO^+ strain	IVO^+	IVO^-	$\%IVO^+$
3.1	0	86	0
3.2	30	110	21
3.3	2	42	4
6.1	7	36	16
6.2	12	27	31
7.1	3	24	11
7.2	8	70	10
7.3	4	87	4
		Average:	12.1

[1] As a percentage of both ARG^+ and ARG^- colonies; in the latter the phenotype with respect to IVO is not known.

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Results

4.1 Stability of ARG^+ IVO^+ ARp1-pCEB218 cotransformants

A.nidulans strain AJC9.4 (pabaA1; argB2; brlA42 ivoB63) was simultaneously transformed with approximately equal amounts of ARp1 and pCEB218. pCEB218 consists of the bacterial plasmid vector pUC8 and a 5.0-kb SalI fragment insert containing the conidiophore melanin gene ivoB. Transformants were grown at 37°C at which temperature brlA42 mutants do not conidiate, and the IVO phenotype is visible. Approximately 10% of ARG^+ ARp1 transformants had the grey-brown IVO^+ phenotype, indicating that they had been cotransformed with pCEB218. ARG^+ IVO^+ cotransformants 3, 6 and 7 were streaked onto MM supplemented with PABA to obtain pure cotransformed strains. Several such progeny of each of the three cotransformants were then subcultured at 30°C, at which temperature brlA42 mutants conidiate. Conidia were then plated on CM, and on CM supplemented with arginine. On CM the percentage of ARG^+ progeny was scored, and the percentage of the ARG^+ colonies which were IVO^+ was also scored. Since ARG^- colonies do not conidiate on CM their phenotype with respect to IVO could not be scored. On CM plus arginine the percentage of IVO^+ progeny was scored. The results are summarised in Table 1.

It was found that on CM 21.7% of progeny were ARG^+, and on CM plus arginine 12.1% were IVO^+. It was also found that 63% of the ARG^+ colonies scored on CM were also IVO^+. In addition, if the proportion of IVO^+ colonies which could be scored on CM were calculated, and the ARG^- colonies were included in this calculation, this gave a figure of 12%. This strongly implies that the ARG^- colonies seen on CM were all IVO^-. If this is so, then the proportions of progeny phenotypes were as follows: ARG^+IVO^+ : 12%, ARG^+IVO^- : 9.2%, ARG^-IVO^+ : 0%, ARG^-IVO^- : 78.8%.

These results may be interpreted as follows: firstly, the high frequency of loss of the IVO^+ phenotype implies that the plasmid pCEB218 did not integrate into the chromosome of cotransformants. That pCEB218 a) normally transforms by integration into the chromosome, and b) in this case shows a level of instability similar to that of ARp1 strongly implies that ARp1 and pCEB218 are interacting in some way. The simplest hypothesis to account for this is the occurrence of recombination between pCEB218 and ARp1. This would result in the

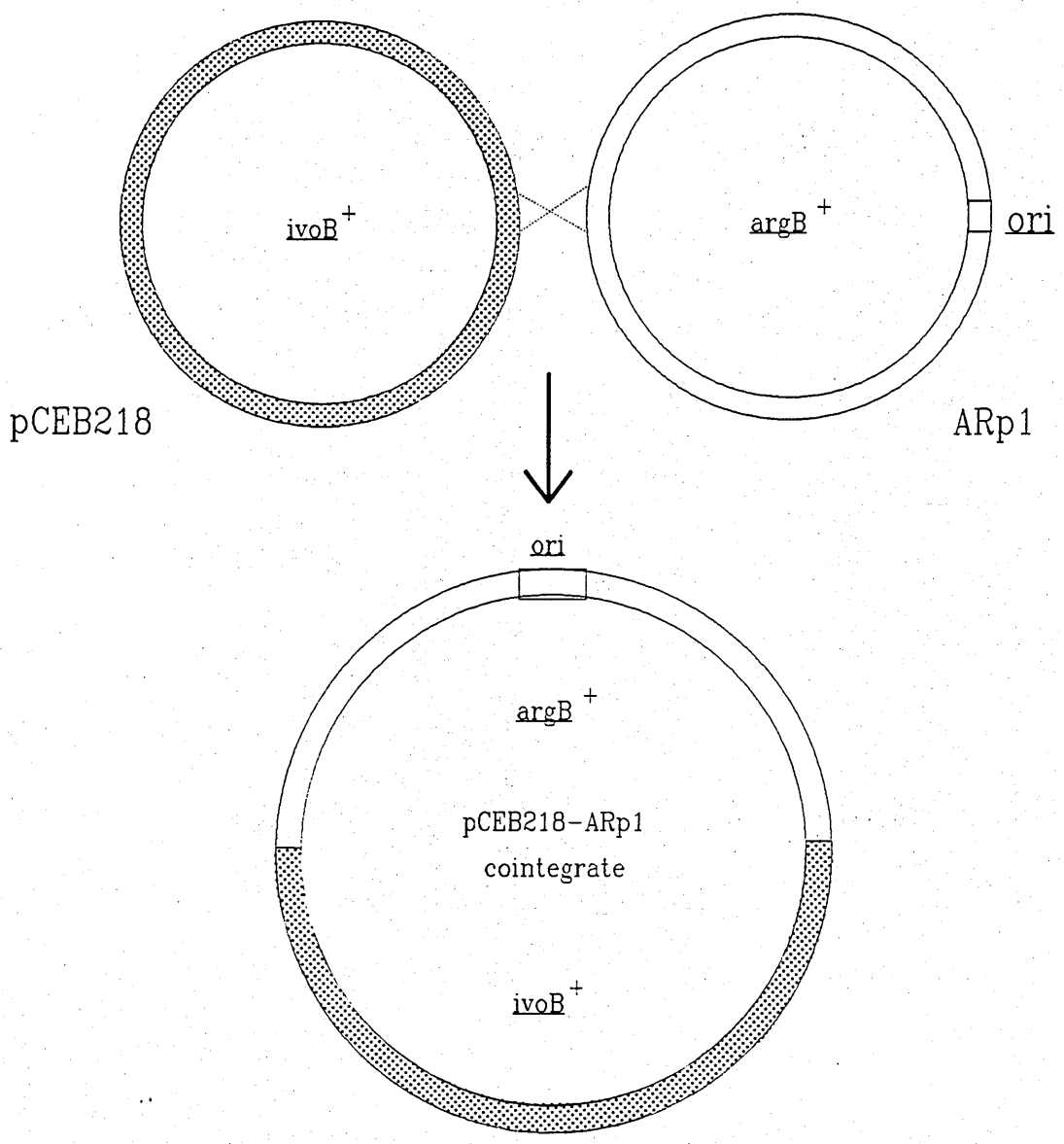

Figure 4.2. Recombination between ARp and AIp plasmids results in formation of an ARp cointegrate.

facing page 78a

formation of a cointegrate structure (Fig.4.2), which would retain properties of ARp1, notably autonomous replication and mitotic instability. This idea is further supported by the fact that the *ivoB*$^+$ and *argB*$^+$ alleles in cotransformants behave as if linked, being more often retained or lost together than separately. It was decided that the testing of this hypothesis should be carried out by means of an investigation of cotransformations involving two directly selectable markers.

4.2 Construction of *A.nidulans* strains DHG019 and DHG135

It was decided to investigate cotransformation between ARp1 and the integrative plasmid pTA11 (kindly supplied by Dr. T.H. Adams), which contains the *A.nidulans* *trpC* gene (Fig. 4.3). In order to do this two *trpC*$^-$*argB*$^-$ strains were constructed. From progeny of a cross between *A.nidulans* strain G34 (*yA2*; *argB2* *methH2*) and AJC12.7 (*pabaA1*;*brlA42* *trpC801*), strain DHG019 (*yA2*; *argB2* *methH2*; *brlA42* *trpC801*) was isolated. Then from a cross between DHG19 and G0141 (*pabaA1* *biA1*), strain DHG135 (*biA1*; *argB2* *methH2*;*trpC801*) was isolated.

Both these strains were found to grow very poorly on CM supplemented with arginine and tryptophan. In order to test the possibility that repression of amino acid uptake by NO_3 or NH_4 was occurring strain DHG135 was inoculated onto MM plus the various supplements required, with or without NO_3 or NH_4. Diameters of three colonies in each case were measured after 3 days at 37^{o}C. It was found that addition of NO_3 and NH_4 caused a 24.0% and a 25.1% decrease respectively in colony diameter. It was concluded that repression of amino acid uptake by NO_3 and NH_4 had been occurring. Most subsequent work with these strains was carried out using arginine or tryptophan as a nitrogen source.

4.3 Construction of pDHG29

A new plasmid was constructed containing AMA1, *argB* and *trpC*, for use in transformations of *argB2* *trpC801* strains in order to compare with the behaviour of pTA11 and ARp1 in cotransformations. pTA11 was restricted with *Xho*I and the 4.4-kb *Xho* fragment containing the *trpC* gene was gel purified. ARp1 was then restricted with *Xho*I and treated with CIP (calf intestinal phosphatase) in order to remove 3'-phosphate groups and prevent self-ligation. The 4.4-kb *Xho*I DNA fragment was then ligated into ARp1, replacing the unique region between the

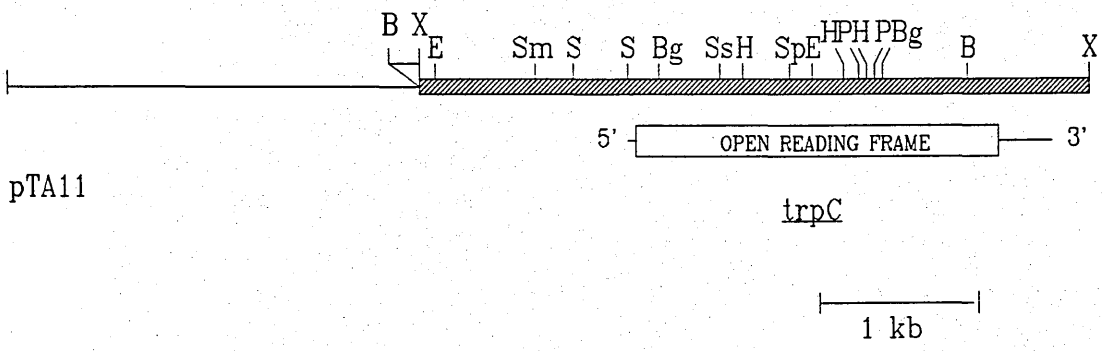

Figure 4.3. **Linear restriction digest map of the plasmid pTA11.**
The single line in the upper figure represents pIC20-R sequences, and the cross-hatched box the trpC sequences. In the lower figure the single line and open box represent untranslated and translated regions of the trpC mRNA respectively (courtesy of Mullaney et al, 1985).
Abbreviations for restriction enzyme sites are B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; P, PstI; S, SalI; Sm, SmaI; Ss, SstI; X, XhoI.

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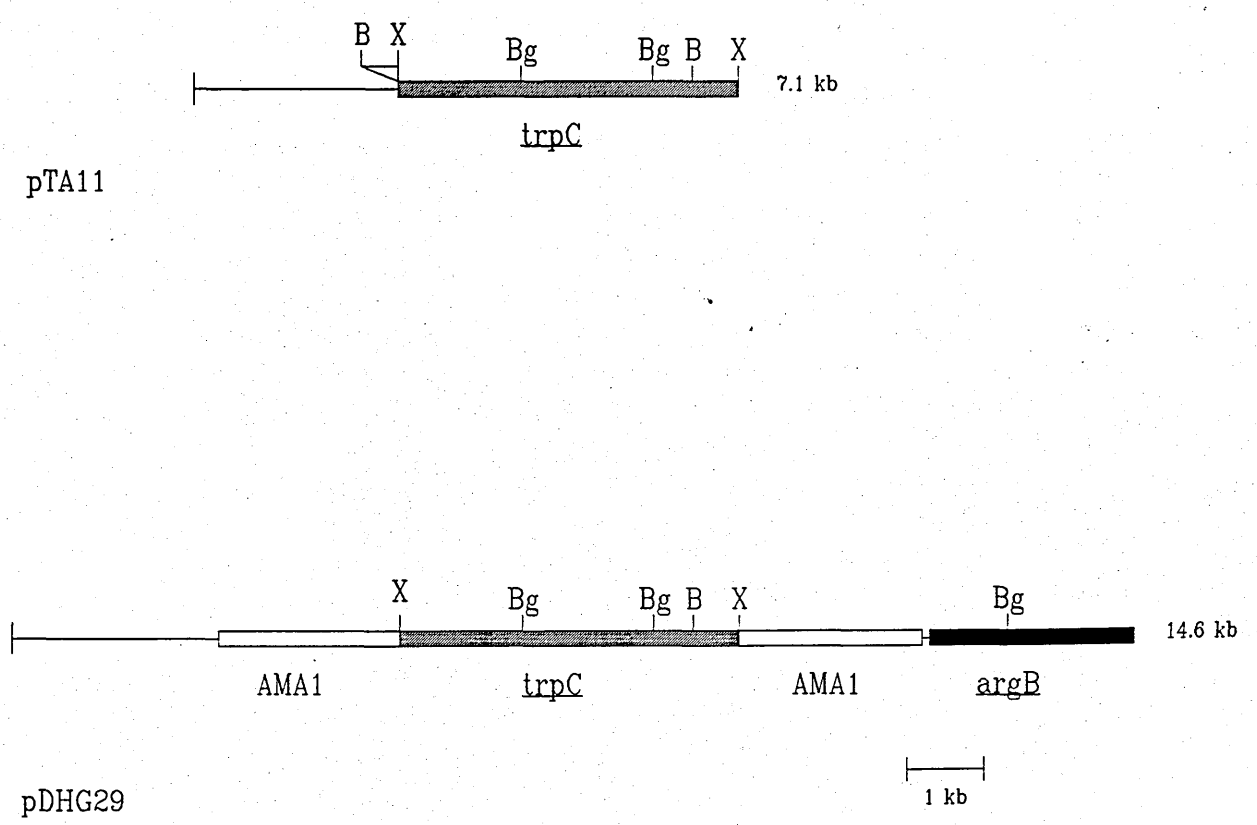

Figure 4.4. Linear restriction digest maps of plasmids pTA11 and pDHG29.

The single lines represent E.coli vector sequences: pIC20-R in the case of pTA11; pUC8 in the case of pDHG29. The open boxes represent AMA1 sequences, the cross-hatched boxes, trpC sequences, and the black box, argB sequences. Plasmid pDHG29 was constructed by ligating the 4.4-kb XhoI fragment of pTA11 between the XhoI sites of the CIP-treated, XhoI digested ARp1 (see Fig. 3.1).

Abbreviations for restriction enzymes: B, BamHI; Bg, BglII; X, XhoI.

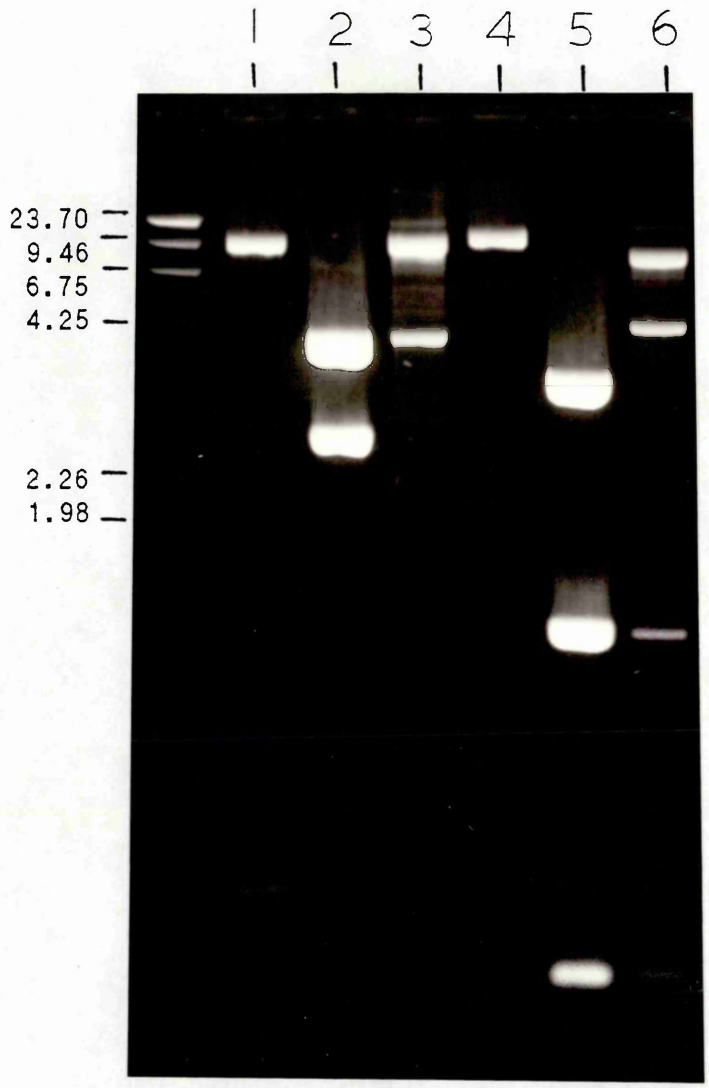

Figure 4.5. **Restriction digests to confirm the identity of pDHG29.** Lambda HindIII size standards are given in kb.

Single and double digests are as follows:
Lane 1, ARp1, XhoI; 2, pTA11, XhoI; 3, pDHG29, XhoI; 4, ARp1, BamHI/BglII; 5, pTA11 BamHI/BglII; 6, pDHG29, BamHI/BglII.

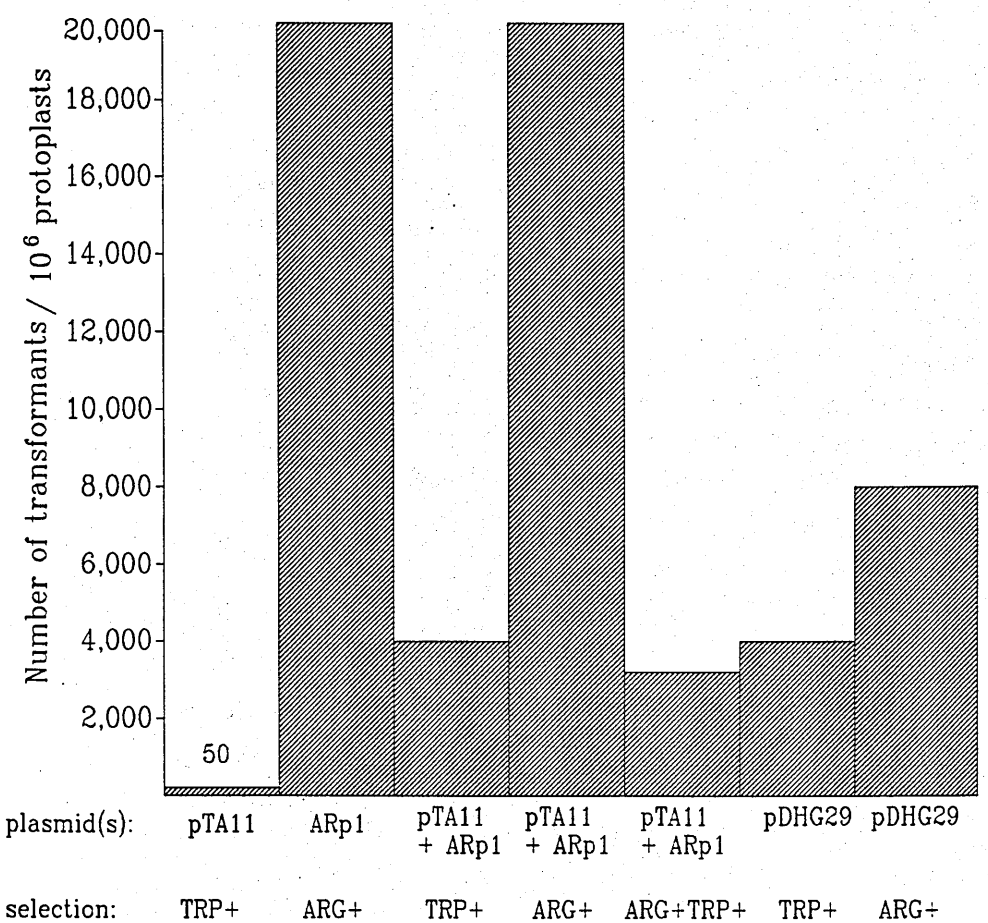

Figure 4.6. **Histogram showing transformation frequencies obtained using pTA11, ARp1 and pDHG29.**

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inverted repeats of AMA1 (Fig. 4.4). The identity of the plasmid, designated pDHG29, was checked by restriction analysis (Fig. 4.5). The orientation of the insert was ascertained by the restriction pattern resulting from a BamHI/BglII double digest.

4.4.1 Cotransformation of pTA11 with ARp1

Five transformations of strain DHG019 using pTA11 and ARp1, and selecting for TRP^+, gave on average approximately 4,000 transformants per 10^6 protoplasts. This compared with an average transformation efficiency with pTA11 alone of 50 transformants per 10^6 protoplasts. Thus, transformation efficiency of a $trpC^-$ strain to TRP^+ with pTA11 is enhanced 80-fold by the presence of ARp1. Transformations using pTA11 and ARp1 together were also carried out selecting for ARG^+. Transformation efficiencies were characteristic of ARp1 alone. One transformation of DHG019 using pTA11 and ARp1 and selecting for ARG^+ and TRP^+ gave 3,300 ARG^+TRP^+ colonies per 10^6 protoplasts.

Transformation of strain DHG019 with pDHG29 selecting for TRP^+ gave an average frequency of approximately 4,000 transformants per 10^6 protoplasts. However, the same transformation selecting for ARG^+ gave an average of 8,000 transformants per 10^6 protoplasts. This compares with a frequency of 20,000 transformants per 10^6 protoplasts using ARp1 alone. Transformation efficiencies are compared in a histogram in Fig. 4.6.

The 80-fold increase in transformation efficiency found upon addition of ARp1 to transformations of $trpC^-$ strains to TRP^+ with pTA11 conforms with the hypothesis that an interaction between pTA11 and ARp1 is occurring.

4.4.2 Characterisation of cotransformants

If the unstable TRP^+ transformants produced by transformation of $trpC^-$ $argB^-$ strains with pTA11 and ARp1 contain pTA11-ARp1 cointegrates, then the TRP^+ colonies, when tested, should turn out to be ARG^+ as well, since the cointegrate should contain the $argB^+$ gene (unless it has been disrupted in the recombination process). 13 TRP^+ colonies produced in this way were examined. All had a somewhat unstable appearance - i.e. ragged colony edges and uneven growth. 11 out of the 13 colonies were found to be ARG^+ (i.e. were able to grow on MM

Table 4.2 Stability of the ARG^+ and TRP^+ phenotypes in pTA11-ARp1 cotranformants of A.nidulans strain DHG019.

24 progeny each of 8 ARG^+TRP^+ transformants were examined. (All were $METH^-$).

	Phenotypes of progeny			
Transformant	%ARG^+TRP^+	%ARG^+TRP^-	%ARG^-TRP^+	%ARG^-TRP^-
1	100	0	0	0
2	32	0	0	68
3	5	10	0	84
4	17	0	0	82
5	100	0	0	0
6	18	6	12	65
7	31	0	5	63
8	87	4	0	9

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supplemented with methionine alone). None of the colonies grew on MM alone, suggesting that none were contaminants.

In a complementary experiment, 16 ARG^+ transformants of strain DHG019 with pTA11 and ARp1 were similarly examined. Only one out of 16 proved to be TRP^+. None grew on MM alone. This compares to the 10% of ARG^+ ARp1 transformants of A.nidulans strain AJC9.4 found to be cotransformed to IVO^+ with pCEB218 described in section 4.1.

In a control experiment 24 colonies each of four ARG^+ ARp1 transformants of strain DHG019 were tested for TRP^+. No TRP^+ colonies were found. Similarly, 24 progeny each of four TRP^+ transformants of strain DHG019 with pTA11 were tested for ARG^+. No ARG^+ colonies were found.

The stability of eight ARG^+TRP^+ pTA11-ARp1 cotransformants was examined. Conidia were plated on non-selective conditions (CM supplemented with arginine and tryptophan), and progeny used to stab inoculate master plates (non-selective conditions.) The phenotypes of the resultant colonies were then tested. Sample size was 24 progeny tested per original transformant. Five transformants were found to be highly unstable. On average 72% of their progeny were ARG^-TRP^-, and only 21% were ARG^+TRP^+. ARG^+TRP^- and ARG^-TRP^+ were even rarer: 3% of each. Two of the transformants were stable: all progeny were ARG^+ TRP^+. One was slightly unstable: 9% of progeny were ARG^-TRP^-. Full results are shown in Table 4.2.

These results are congruent with the two cotransforming plasmids having recombined to form a cointegrate, or heterodimeric structure. Thus when the cointegrate is lost during mitosis both $\underline{argB}^+$ and $\underline{trpC}^+$ are lost. Otherwise both are retained. The rare ARG^+TRP^- progeny could result from intramolecular recombination resulting in resolution of the cointegrate and loss of the pTA11 component. It has been shown that this sort of recombination happens in the case of the ARp1 dimer (section 3.6). The stable transformants may result from the integration of the cointegrate into the chromosome. The ARG^-TRP^+ progeny might occur by integration of resolved pTA11 molecules followed by loss of ARp1 through mitotic instability.

The most probable type of recombination event that might occur between the two cotransforming plasmids is homologous recombination between the common bacterial regions. In A.nidulans most integrative transformation events occur by homologous recombination between the

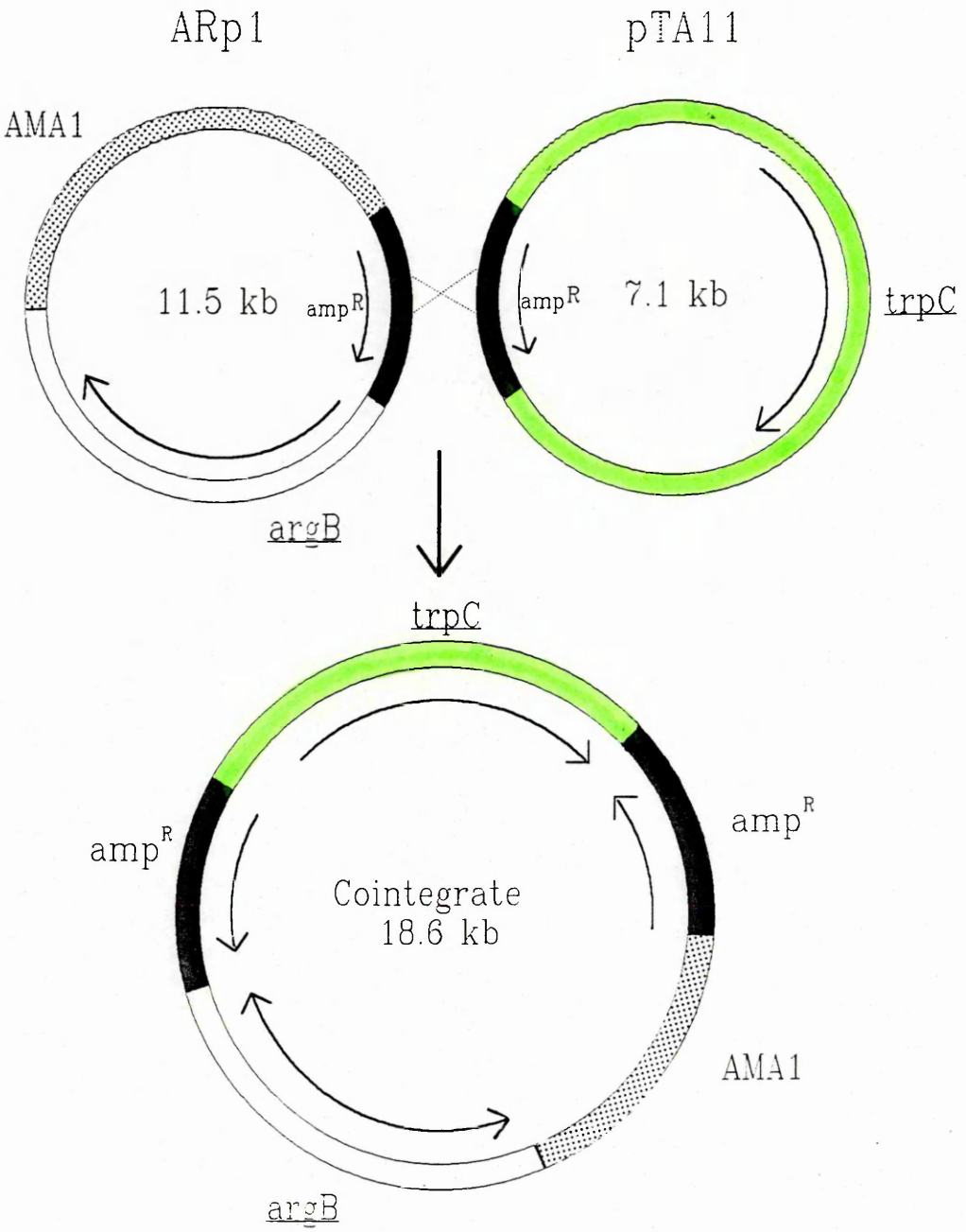


Figure 4.7. Schematic representation of homologous recombination between ARp1 and pTA11 and the structure of the cointegrate that results.

The open boxes represent argB sequences, the green boxes trpC sequences, the shaded boxes AMA1 sequences, and the black boxes E.coli vector sequences. The arrows show orientation of gene transcripts, and position of gene coding regions. The crossed dotted lines represent a recombination event. (Not to scale).

transforming wild type and the resident mutant alleles (Yelton et al, 1984; Upshall 1986). The cointegrate structure that would result from such recombination between ARp1 and pTA11 is shown in Fig. 4.7.

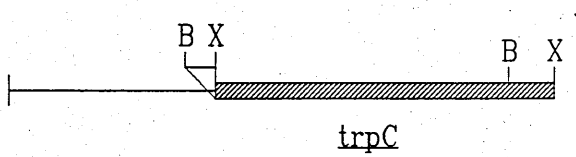
One feature of such a hypothetical cointegrate is that the direction of transcription of the *argB*$^+$ and *trpC*$^+$ genes would both point towards an *ori* located in the AMA1 region. In studies of the *E.coli* plasmid pBR322 Liu and Wang (1987) have shown that gene transcription may lead to the formation of positive supercoils in the DNA template in advance of the direction of movement of the RNA polymerase, and negative supercoils behind it. Thus, simultaneous transcription of *argB* and *trpC* might cause positive supercoiling of the AMA1 region, which might reduce initiation of DNA replication. If this were so, reversal of the orientation of the *trpC* gene might increase frequency of cotransformation and/or mitotic stability of the plasmid cointegrate.

4.5 Construction of pTA11D

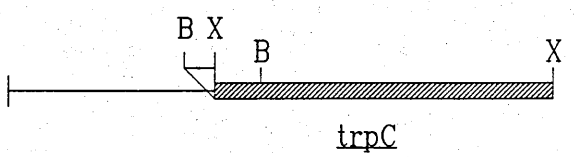
For this purpose a derivative of pTA11 was constructed with the *trpC* gene in the opposite orientation with respect to pUC8. pTA11 DNA was digested with *Xho*I, phenol and chloroform extracted and then ethanol precipitated. This DNA was then used as a substrate for a ligation reaction, which was subsequently transformed into *E.coli*. Transformant colonies patched on LB-agar plus ampicillin were subjected to single colony gel analysis and colonies containing plasmids with DNA inserts were identified. Restriction analysis of STET plasmid minipreps of these using *Bam*HI were then carried out. A clone was chosen which yielded 6.5-kb and 0.6-kb *Bam*HI fragments, denoting a reversal of insert orientation, (Fig. 4.8) and designated pTA11D.

4.6 Cotransformation of pTA11D with ARp1

Transformation of strain DHG019 to TRP$^+$ with pTA11D and ARp1 occurred with a similar frequency to that found using pTA11 and ARp1. A similar level of mitotic instability of the TRP$^+$ phenotype of pTA11-ARp1 cotransformants to that of pTA11-ARp1 cotransformants was also observed. In a control experiment pTA11D was found to transform strain DHG019 to TRP$^+$ with a similar efficiency to that using pTA11. These results suggest that DNA supercoiling resulting from gene transcription is not an important factor in the determination of plasmid stability in *A.nidulans*.



pTA11



pTA11D

Figure 4.8. Linear restriction digest maps of plasmids pTA11 and pTA11D.

Single lines represent E.coli vector sequences (pIC20-R), triangles, pIC20-R polylinker sequences; cross-hatched box, trpC sequences.

Abbreviations for restriction enzyme sites:

B, BamHI; X, XhoI.

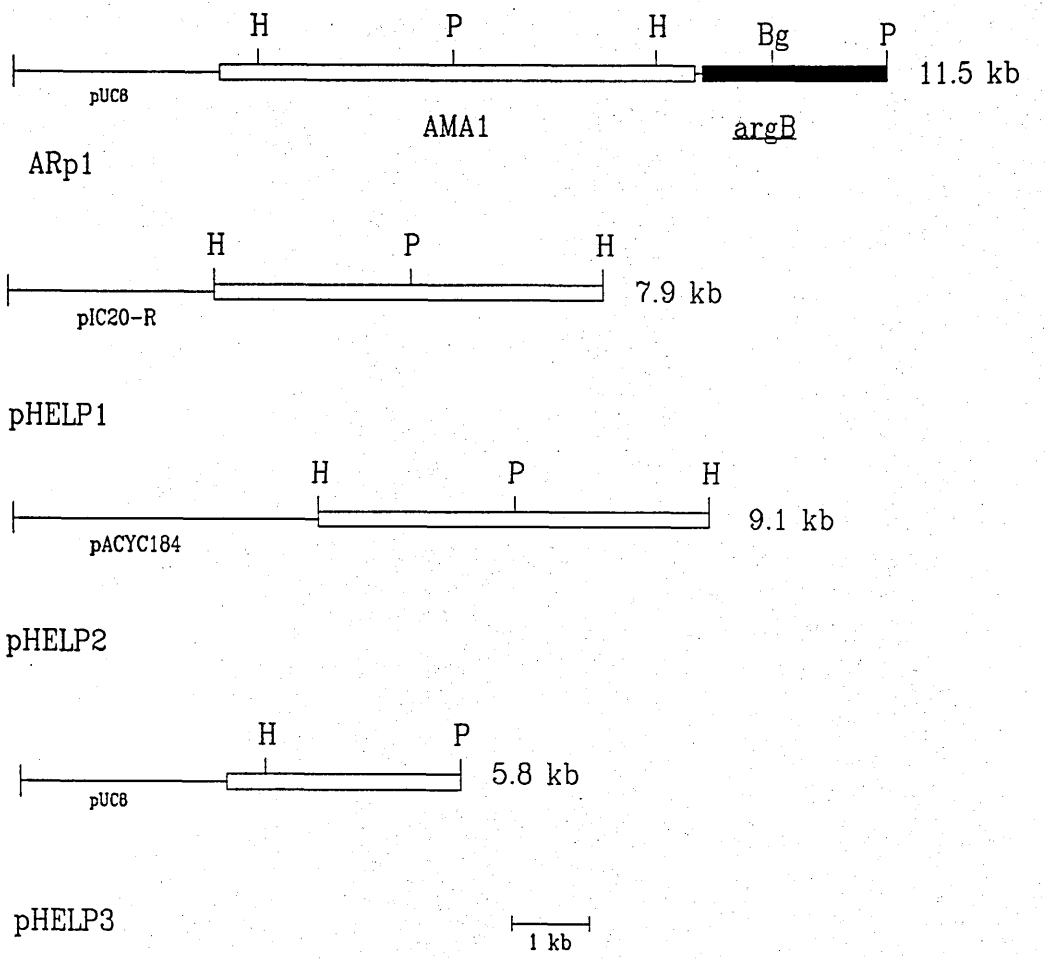


Figure 4.9. Linear restriction digest maps of plasmids ARp1, pHELP1, pHELP2 and pHELP3.

Single lines represent E.coli vector sequences. In the cases of ARp1 and pHELP3 this is pUC8; in the case of pHELP1 it is pIC20-R; in the case of pHELP2 it is pACYC184. Open boxes represent AMA1 and AMA1-derived sequences. The black box represents argB sequences.

pHELP1 was constructed by ligating the 5.1-kb HindIII fragment of ARp1 into the polylinker of the plasmid pIC20-R. pHELP2 was constructed by inserting the same DNA fragment into the P15-A-based plasmid pACYC184. pHELP3 was made by digesting ARp1 with PstI, and then self-ligating it.

Abbreviations for restriction enzyme sites: Bg, BglII; H, HindIII; P, PstI.

4.7 Construction of helper plasmids pHELP1, pHELP2 and pHELP3

It was thought that the easiest way to show whether cotransformation of ARp plasmids with integrative ones occurs by means of the formation of plasmid heterodimers would be to reisolate the latter into E.coli. Repeated attempts to do this using DNA from ARG^+TRP^+ pTA11-ARp1 cotransformants failed. It was argued that the large size of such a cointegrate (18.6 kb) would reduce the efficiency of plasmid rescue. Cotransformation with subclones of ARp1 would theoretically result in the formation of smaller cointegrates which would be more easily rescued.

The 5.1-kb HindIII fragment of AMA1, which is sufficient to confer ARp properties in pDHG25, was ligated into the HindIII site of the E.coli plasmid pIC20-R, which contains the amp^R gene. The resulting 7.9-kb plasmid was designated pHELP1 (Fig. 4.9). The same HindIII fragment was also ligated into the HindIII site of another E.coli plasmid, pACYC184, which contains the cam^R (chloramphenicol resistance) and tet^R (tetracycline resistance) genes. The resulting 9.1-kb plasmid was designated pHELP2 (Fig. 4.9). A third plasmid derivative of ARp1 was constructed by restricting it with PstI, and then religating it. This effectively deleted the right hand repeat of AMA1 and the $argB^+$ gene from ARp1. The resulting 5.8-kb plasmid was designated pHELP3 (Fig. 4.9). The identity of each plasmid was confirmed by restriction analysis (data not shown).

The term "helper plasmid" is a convenient description of a plasmid which may be used in cotransformations with an integrative plasmid in order to convert the latter into a replicon through in vivo recombination of the two plasmids. For this reason the pHELP designation was chosen.

4.8.1 Cotransformation of pILJ16 with helper plasmids

A number of transformations of strain G34 to ARG^+ using pILJ16, alone or in the presence of pHELP1, pHELP2 or pHELP3 were carried out. All three helper plasmids were found to stimulate transformation frequency. Where pILJ16 alone gave on average 60 transformants per 10^6 protoplasts over four transformations, pILJ16 plus pHELP1 generated an average of 12,000 transformants per 10^6 protoplasts (3 transformations), a 200-fold increase in transformation efficiency. pILJ16 plus pHELP2 transformed strain G34 to ARG^+ at somewhat lower

Table 4.3 Frequency of transformation of strain G34 to ARG^+ using pILJ16 and helper plasmids pHELP1, pHELP2 and pHELP3.

	Number of transformants per 10^6 protoplasts			
Plasmid added to pILJ16:	none	pHELP1	pHELP2	pHELP3
T'formation 1	66	-	-	6,500
2	30	3,000	100	5,000
3	100	20,000	300	-
4	40	13,000	2,000	-

frequency: 800 transformants per 10^6 protoplasts (average of 3 transformations); this constitutes a 13-fold increase in transformation efficiency over pILJ16 alone. pILJ16 plus pHELP3 yielded 6,000 transformants per 10^6 protoplasts (average of 2 transformations), a 100-fold increase of pILJ16 alone. Full transformation results are shown in Table 4.3.

In control transformations using pHELP1, pHELP2 or pHELP3 alone no ARG^+ transformants were seen.

The transformation frequency using pILJ16 plus pHELP1 is similar to that of pDHG25 (around 10,000 ARG^+ transformants per 10^6 protoplasts). This strongly implies that recombination between the two plasmids occurs at a similar or higher frequency to that of the entry of transforming DNA into the protoplasts. The lower transformation frequency in the case of pILJ16 and pHELP2 may reflect the greatly reduced homology between the two plasmids: whereas pILJ16 and pHELP1 have 2.7-kb of bacterial sequences in common, pILJ16 and pHELP2 contain different bacterial sequences. This supports the hypothesis that predominantly homologous recombination causes cointegrate formation. The relatively high frequency of transformation using pILJ16 and pHELP3 implies that the inverted repeat is not essential for autonomous plasmid maintenance. However, it cannot be ruled out that during transformation recombination regenerates an inverted repeat structure.

4.8.2 Stability of pILJ16-helper plasmid transformants

Conidia were harvested and suspended in water, where the conidial concentrations were measured using a haemocytometer. They were then plated on CM at a density of approximately 50 per plate. On CM ARG^+ and ARG^- colonies were readily distinguished and the degree of instability of the ARG^+ phenotype gauged. Progeny of 13 ARG^+ transformants of strain G34 with pILJ16 and pHELP1 were examined. The number of ARG^+ colonies was on average 20.8%, and numbers varied between extremes of 11% and 47%. Overall sample size was 1,384. Of progeny of 6 ARG^+ transformants with pILJ16 and pHELP2, 36.7% were ARG^+, with variation between 27% and 49%. Overall sample size was 210. Among progeny of 8 ARG^+ transformants with pILJ16 and pHELP3, 15.5% were ARG^+ with numbers varying between 5% and 31%. However, a further two transformants had 86% and 72% ARG^+ progeny, and if these are included the overall proportion of ARG^+ progeny is 28.2%. Total sample

Table 4.4 Mitotic instability of ARG^+ phenotype in pILJ16-helper plasmid cotransformants.

Helper plasmid added to pILJ16					
pHELP1		pHELP2		pHELP3	
sample size	%Arg+	sample size	%ARG+	sample size	%ARG+
82	17	31	44	64	9
103	25	29	31	49	31
74	44	35	49	55	12
54	11	26	38	25	8
39	31	49	31	80	5
180	47	40	27	67	22
150	21			31	16
113	17	Total: 210	36.7[1]	59	86
165	12			83	72
68	31			68	21
123	46			Total: 581	28.2[1]
171	22				
62	44				
1,384	20.8[1]				

[1]Average

size was 581. Full results are shown in Table 4.4.

The instability of the ARG^+ phenotype observed can only be accounted for by recombination of pILJ16 with helper plasmids. Since addition of the latter results in a 13 - 200-fold enhancement of transformation efficiency, it is not surprising that all transformants examined were unstable. A much larger number of transformants would have to be screened to rule out the occurrence of a low frequency of integrative transformation.

The higher degree of instability of the pILJ16-pHELP3 cotransformants suggests that the second copy of the repeat found in AMA1, or the inverted repeat structure does, after all, affect plasmid maintenance. The increase in stability observed in the cases of two of these cotransformants may be due to a) initial formation of a different cointegrate structure, b) formation of the usual cointegrate followed by a rearrangement, or c) to a subpopulation of the nuclei within the transformant having become stably transformed by plasmid integration into the chromosome.

4.8.3 Plasmid rescue from a pILJ16-pHELP2 cotransformant.

DNA was prepared from an unstable ARG^+ transformant of strain G34 with pILJ16 and pHELP2 grown under selective conditions. Competent cells of E.coli strain DH5 were transformed with the transformant DNA and one ampicillin resistant colony was obtained.

Repeated attempts at plasmid rescue using DNA from unstable ARG^+ pILJ16-pHELP1 and pILJ16-pHELP3 cotransformants were not successful.

4.8.4 Restriction mapping of pILJ16-pHELP2 plasmid cointegrate

Results of the restriction mapping of the plasmid isolated from the pILJ16/pHELP2 cotransformant, designated pCOT2, are presented in Figs. 4.10, 4.11 and Table 4.5. The restriction patterns seen conform with the formation of pCOT2 by the recombination of pILJ16 and pHELP2. The 3.0-kb and 3.8-kb EcoRI fragments of pHELP2 comprising the pACYC184 sequence and the distal 1.4-kb of the AMA1 sequence are present (Fig. 4.10, gel C, lanes 3 and 4). A single 1.2-kb EcoRI/PstI fragment only is present, however, implying that the point of recombination with pILJ16 lies in the other 1.2-kb EcoRI/PstI fragment of AMA1. The central 1.1-kb NruI, and 0.6-kb SalI fragments of AMA1 are also

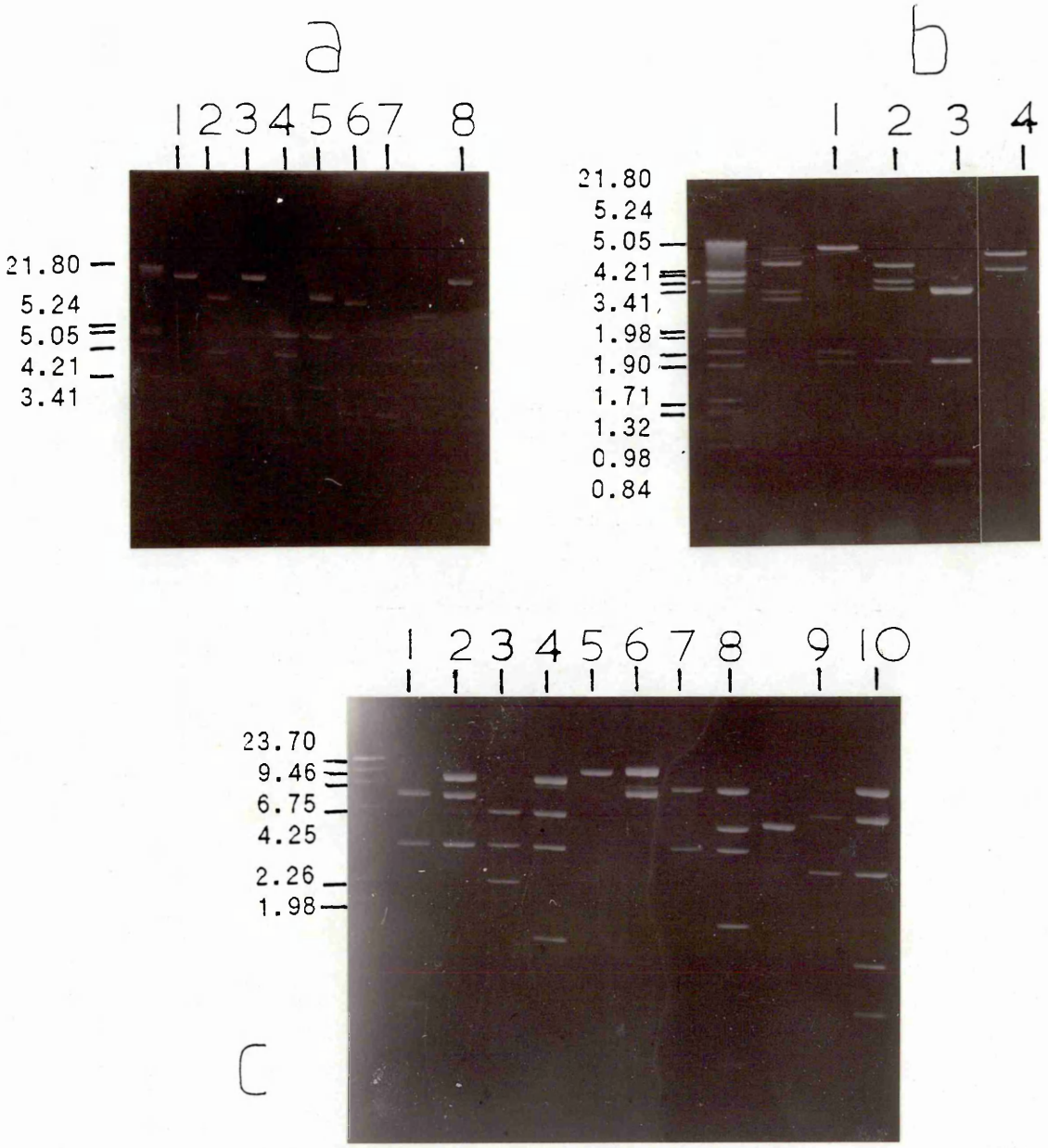


Figure 4.10. **Restriction digests of pCOT2.**

All DNAs are pCOT2 unless otherwise stated. Only significant digests are labelled; lambda HindIII/EcoRI size standards are given in kb. Single and double digests are as follows:

Gel A: Lane 1) BamHI, 2) EcoRI, 3) PstI, 4) EcoRI/BglII, 5) BamHI/BglII, 6) EcoRI/BamHI, 7) EcoRI/PstI, 8) BglII.

Gel B: Lane 1) Pst/BglHII, 2) HindIII, 3) pILJ16, HindIII, 4) SstI.

Gel C: Lane 1) pHELP2, NruI, 2) NruI, 3) pHELP2, EcoRI, 4) EcoRI, 5) pHELP2, Sst1, 6) Sst1, 7) pHELP2, Sal1, 8) SalI, 9) pHELP2, PstI/HindIII, 10) PstI/HindIII.

Table 4.5 DNA fragment sizes from the restriction of pCOT2 measured from the gels presented in Figure 4.10.

Restriction enzymes	Estimated size of fragments (kb)	Sum of fragments
BamHI	12.0, 3.5	15.3
EcoRI	6.0, 3.8, 3.0, 1.6	14.7
PstI	12.5, 2.9,	15.4
HindIII	6.0, 4.1, 3.6, 1.7, 0.4	15.8
BglII	15.0	15.0
SstI	10.0. 5.8	15.8
ClaI	10.5, 5.2, 1.0	16.7
NruI	7.6, 5.0, 2.9	14.1
SalI	5.5, 3.4, 2.8, 1.7, 0.8, 0.55	14.8
EcoRI/BglII	5.0, 4.2, 2.9, 2.4, 1.6	16.1
BglII/BamHI	6.9, 4.9, 3.2	15.9
EcoRI/BamHI	7.3, 2.8, 2.2, 1.8, 1.5	15.6
PstI/EcoRI	3.9, 3.1, 2.7, 2.7, 1.5, 1.2	14.9
PstI/BglII	13.0, 1.6, 1.4	16.0
PstI/HindIII	5.6, 3.7, 2.35, 1.4, 1.0, 0.4	14.5

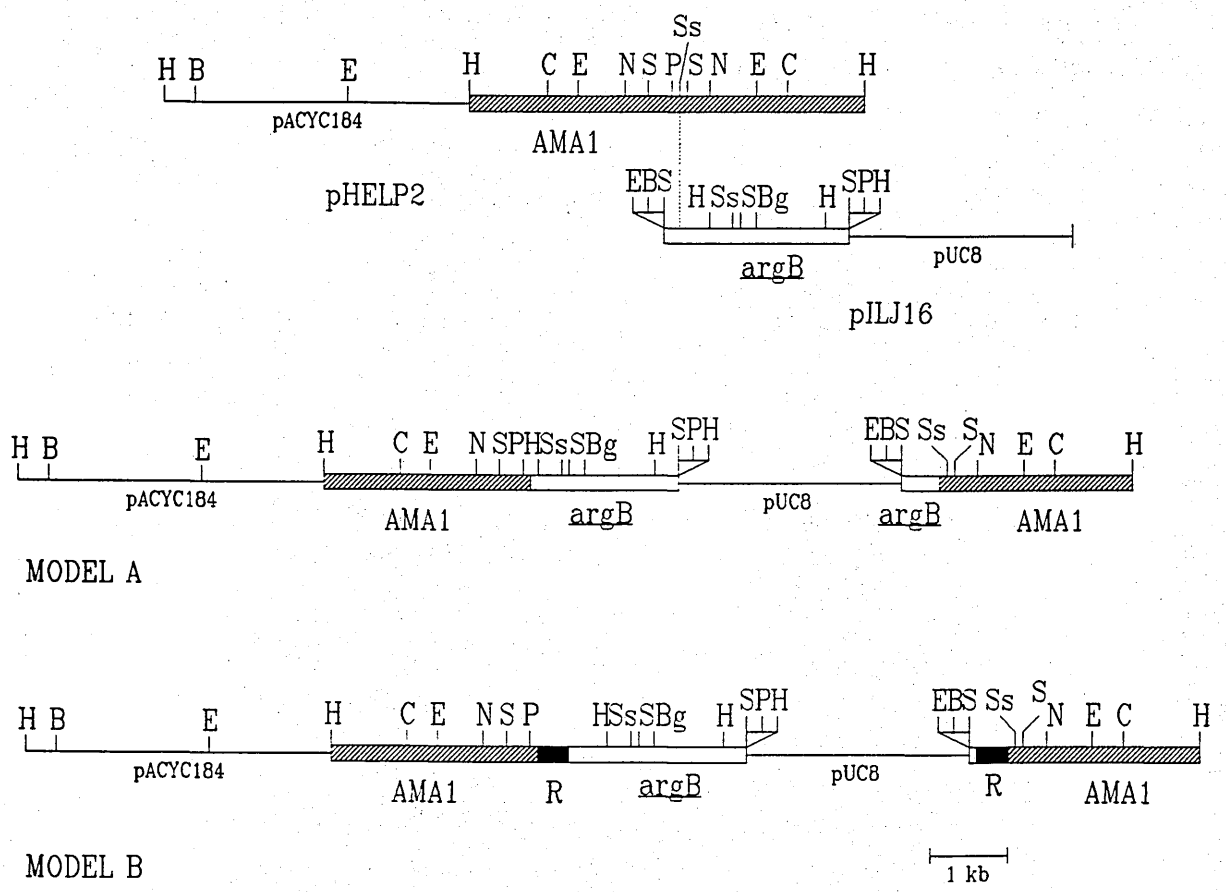


Figure 4.11. **Linear restriction maps of pCOT2.**
pCOT2 is the product of apparently non-homologous recombination between plasmids pILJ16 and pHELP2. Maps of pHELP2 and pILJ16 are shown for comparison.

Model A: the first map constructed from the data shown in Fig. 4.10 and Table 4.5.
Model B: represents the final map.
The single line represents E.coli vector sequences (pACYC184 and pUC8 as indicated); the open boxes represent argB sequences, and the cross-hatched boxes AMA1-derived sequences; the black boxes represent duplicated sequences.
Abbreviations for restriction enzymes are: B, BamHI; Bg, BglII; C, ClaI; E, EcoRI; H, HindIII; N, NruI; P, PstI; S, SalI; Ss, SstI.

absent, suggesting that the point of recombination lies between the central PstI site and one of the flanking SalI sites (Fig. 4.10, gel C, lanes 1, 2, 7 and 8).

With respect to pILJ16: the 1.7-kb HindIII fragment containing most of the argB gene is present (see Fig. 4.10, gel B, lanes 2 and 3). The 2.7-kb EcoRI/PstI fragment corresponding to the pUC8 residue is present. The 1.6-kb BglII/PstI fragment comprising the right hand side of the argB gene as shown in Fig. 4.11 is present (Fig. 4.10, gel B, lane 1). Thus the point of recombination with pHELP2 must lie in the right hand 0.5-kb BamHI/HindIII fragment of the argB gene.

Four hypothetical plasmids can be drawn which would result from the recombination of pILJ16 and pHELP1 in the regions defined: where recombination occurs in the left or right hand repeats of AMA1, with the two DNAs in either orientation with respect to each other. The only hypothetical cointegrate which conforms roughly with the restriction patterns observed is Model A, shown in Fig. 4.11. To conform with the SstI restriction pattern seen (Fig. 4.10, gel B, lane 4), recombination must have taken place between the central PstI site of AMA1, and the SstI site located 0.1 kb to the right of it as shown. However, comparing Model A with the restriction data, one discrepancy is clear: the sum of the sizes of pILJ16 and pHELP1 is 14.5 kb. The average size of the cointegrate given by the restriction digests is 15.4 kb. It is also seen that the sizes of the small fragments corresponding to the junction between the two plasmids in Model A are consistently underestimated - e.g. in the EcoRI/BglII, HindIII, PstI/BglII digests, and most notably in the PstI/HindIII digest (Fig. 4.10, gel C, lane 10) where the map predicts a 0.2-kb junction fragment, and a 1.0-kb fragment is seen. Thus it would appear that a 0.8-kb increase in size has occurred during recombination, presumably the result of a sequence duplication (Fig. 4.11, Model B).

4.9 Supertransformation of pDHG25 transformants with pTA11

One question raised by these results is why recombination between plasmids occurs so much more readily than between plasmids and chromosomes. Possible explanations include i) the relatively large number of plasmids in the cell increases the probability of the association of homologous DNA; ii) the supercoiled nature of the plasmids stimulates recombination; iii) recombination is stimulated by a sequence on AMA1, which might be the ori. Other possible

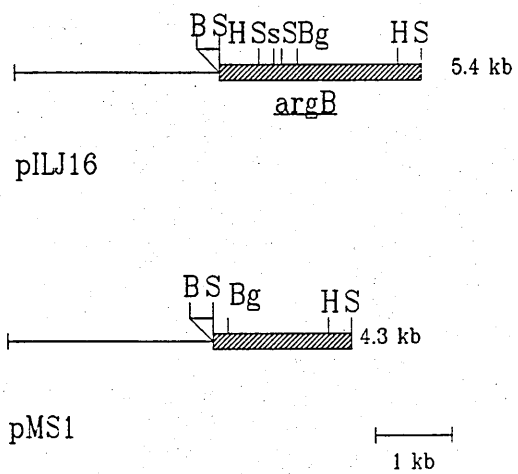


Figure 4.12. **Linear restriction map of pMS1 with pILJ16 for comparison.**

Single lines represent pUC8 sequences and cross-hatched boxes argB sequences.

Abbreviations for restriction enzyme sites are: B, BamHI; Bg, BglII; H, HindIII; S, SalI; Ss, SstI.

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explanations see such recombination as stimulated by the transformation process itself, e.g. i) as a result of the way the cell reacts to exogenous DNA; ii) the cellular location of the transforming DNA immediately after entry into the protoplast or iii), the nakedness (lack of DNA binding proteins, e.g. histones) of the transforming DNAs.

In order to test these latter hypotheses protoplasts were prepared from an ARG^+ pDHG25 transformant of A.nidulans strain DHG135 grown under conditions selecting for maintenance of pDHG25. These protoplasts were then transformed to TRP^+ with the plasmid pTA11. A single experiment gave a frequency of approximately 2,000 ARG^+TRP^+ transformants per 10^6 protoplasts. In two transformations of untransformed strain DHG135 carried out simultaneously, pTA11 alone gave approximately 600 transformants per 10^6 protoplasts, and pTA11 plus pDHG25 gave 3,400 transformants per 10^6 protoplasts.

This result suggests that plasmid recombination does not occur as the result of the manner of cellular processing of exogenous DNA or naked DNA, nor is it the result of any unusual cellular location of transforming DNA immediately after entry into the cell, but that resident plasmid will recombine with incoming DNA as readily as a newly added plasmid.

4.10 Cotransformation of helper plasmids with plasmid pMS1

Plasmid pMS1 is a derivative of pILJ16 constructed in this laboratory by Moira Stark. The 0.8-kb SalI fragment containing part of the argB gene has been deleted. This leaves only a part of the argB gene which corresponds to the region containing the lesion in the argB2 mutant allele (Fig. 4.12). This plasmid can only transform by integrating at the argB locus thus reconstituting an $argB^+$ allele. pMS1 transforms argB2 strains of A.nidulans at low frequency: 2 transformants per 10^6 protoplasts (Birse, 1990). As a control experiment the effect of the addition of pHELP1 and pHELP3 to transformations of argB2 strain G34 with pMS1 was examined. Over three transformations pMS1 alone gave 0.7 transformants per 10^6 protoplasts. pMS1 plus pHELP1 gave 0.4 ARG^+ transformants per 10^6 protoplasts, and pMS1 plus pHELP3 gave 0.1 ARG^+ transformants per 10^6 protoplasts. Control transformations with pHELP1 and pHELP3 alone gave no transformants.

The failure of the helper plasmids to enhance pMS1 mediated transformation confirms that the enhancement of transformation

efficiency by helper plasmids does not involve interaction with the chromosomal allele of the transformation marker.

4.11 Stability of ARG^+ pMS1 transformants

Progeny of four ARG^+ transformants with pMS1 were examined. No ARG^- colonies were seen, implying that stable integration of pMS had occurred (total sample size: 972). The progeny of 25 ARG^+ colonies resulting from transformation with pMS1 in the presence of pHELP1 were also examined. In the case of 22 transformants, no ARG^- progeny were seen (total sample size: approximately 2,000). However, in three cases, MSH5, MSH10 and MSH12, 84%, 100%, and 72% respectively of progeny were ARG^-. The stability of 6 each of the ARG^+ progeny of MSH5 and MSH12 was ascertained. On average 75% and 77% of progeny were ARG^+.

There are two possible explanations for this result: firstly, experimental error - i.e. contamination of pMS1 DNA with a very small amount of pILJ16; secondly, that recombination between pMS1 and pHELP1 to form a plasmid cointegrate occurred. This cointegrate then integrated at the argB locus regenerating a functional argB allele. The argB-pHELP1 sequence then excised by means of an unusual non-homologous recombination event, generating an autonomously replicating plasmid carrying the argB gene plus flanking sequences from the chromosome.

Discussion and prospects

The results described in this chapter show that, as in the cases of S.cerevisiae (Jimenez and Davies, 1980), and S.pombe (Sakai et al, 1984) cotransformation of normally integrating with autonomous plasmids results in recombination to form autonomously replicating plasmid cointegrates. As in the case of S.pombe this was found to result in an increase in transformation efficiency of the normally integrative plasmid of two orders of magnitude. It was also shown that the frequency of such cotransformation is likely to be dependent on the existence of sequence homology between cotransforming plasmids: the substitution of the bacterial sequence pIC20-R of pHELP1, which has extensive homology with the pUC8 sequence of pILJ16, with the non-homologous P15-A - based pACYC184 sequence of pHELP2, results in a 94%

decrease in transformation efficiency. Whether the recombination event giving rise to the cointegrate pCOT2 resulted from a small region of homology between the two plasmids, or to a completely random recombination event is not clear. To determine this it would be necessary to sequence the DNA of the junction region between the two plasmids and compare it with the sequence of pACYC184 and AMA1 (Scott Robertson, work in progress).

These results conform in two ways with the rules adduced from the review in the chapter introduction: cotransformation involves plasmid recombination to form heterodimers, and homologous recombination appears to be favoured over non-homologous (see also chapter 5). The occurrence of plasmid recombination as demonstrated does not, of course, prove that it is the basis of cotransformation, rather than incidental to it. On the other hand, it does offer a simpler, more plausable, and more complete explanation of the phenomenon.

Since the work described in this chapter was carried out similar results have been described using ARp1 in cotransformations of A.oryzae (Russ et al, 1990). In transformations with an integrative plasmid and ARp1, cotransformation occurred at a high frequency, and Southern analysis revealed that cointegrates between the two plasmids had formed. In a further experiment transformations of ARp1 and a non-homologous integrative plasmid carrying the gene encoding the artificial sweetener thaumatin, one cotransformant was found which showed a 1,000-fold enhancement of thaumatin production (Hahm and Batt, 1990). Since the plasmid copy number in this transformant was not significantly higher than normal it was inferred that an increase in gene expression had occurred, perhaps as a result of non-homologous recombination between cotransforming plasmids.

CHAPTER 5

Use of cotransformation to clone the gene *adD*

Introduction

The use of ARp1 based plasmids in gene cloning

As described in the main introduction, autonomously replicating plasmid vectors are in most respects superior to integrating ones for the purpose of gene cloning by complementation of mutant alleles with a gene library. It is expected that pDHG25 will serve as an effective vector for library construction and gene cloning. Jim Kinghorn (pers. comm.) is presently constructing a gene library of A.niger DNA in this vector. However, in this project a different approach was taken to using ARp1 based plasmids to improve gene cloning methodology which doesn't necessitate library construction.

It was shown in chapter 4 that addition of helper plasmids carrying the AMA1 sequence to transformations with normally integrating plasmids results in a) greatly enhanced efficiency of transformation and b) maintenance of the normally integrating plasmid as part of a freely replicating cointegrate. This implies that rather than having to construct a gene library in an autonomously maintained vector de novo, it should be possible to convert integrative vectors used for gene bank construction to autonomous vectors in vivo simply by cotransforming them with a helper plasmid. Construction of a gene library of A.nidulans DNA in the integrative vector pILJ16 has been described by Johnstone (1985a).

Cloning by cotransformation

The single clear disadvantage to such a method is that the large size of cointegrates resulting from recombination between helper and pILJ16 gene library plasmids might hinder their reisolation into E.coli. An examination of factors affecting the efficiency of plasmid rescue is described in the first part of the results section of this chapter.

A further consideration, which would equally apply to the use of a pDHG25 gene library, concerns the number of potential transformants that it would be necessary to generate in order to have a given probability of cloning a given DNA sequence. Clarke and Carbon (1976) devised a formula to relate the number of gene bank transformants to the probability of complementation of a mutation in the transformed strain:

$$N = \ln(1-P)/\ln(1-1/n)$$

where N = number of (potential) transformants, P = probability of the occurrence of complementation and n = size of genome/size of the library DNA insert. However, one assumption of this formula is that each transformant has been transformed by a single plasmid. Since, as has been shown, transformation with ARp1 based plasmids usually occurs by uptake of more than one transforming sequence, and also, given that only a proportion of plasmid vectors contain library inserts, the real value of n:

$$n' = n/xy$$

where x = the average number of plasmids taken up per protoplast and y = the fraction of vectors containing library inserts. Thus:

$$N' = \ln(1-P)/\ln(1-1/n')$$

or:

$$N' = N/xy$$

Clearly, in the case of ARp1 based gene libraries, $N' < N$, suggesting that complementation should occur that much more frequently. Theoretically, a very approximate value of x could be calculated if a small number of transformants are obtained, N' and y are known, and P is supposed to be between 0.5 and 0.9. With respect to the pILJ16 gene library, average insert size = 5 kb, y = 0.5 and the A.nidulans genome size is 31,000 kb (Brody and Carbon, 1989). x is unknown, but if it were 5 then:

$$N = 14,275$$
$$N' = 5,710$$

for an 90% probability of a single occurrence of complementation.

The genes adC and adD both encode the adenine biosynthetic enzyme AIR carboxylase (Pontecorvo et al, 1953; Foley et al, 1965). The adD gene was picked for cloning because of its proximity to the gene ygA which affects production both of conidiophore melanin and the dark-green conidial pigment, rather than any interest in adD itself. ygA is discussed in detail in chapter 7.

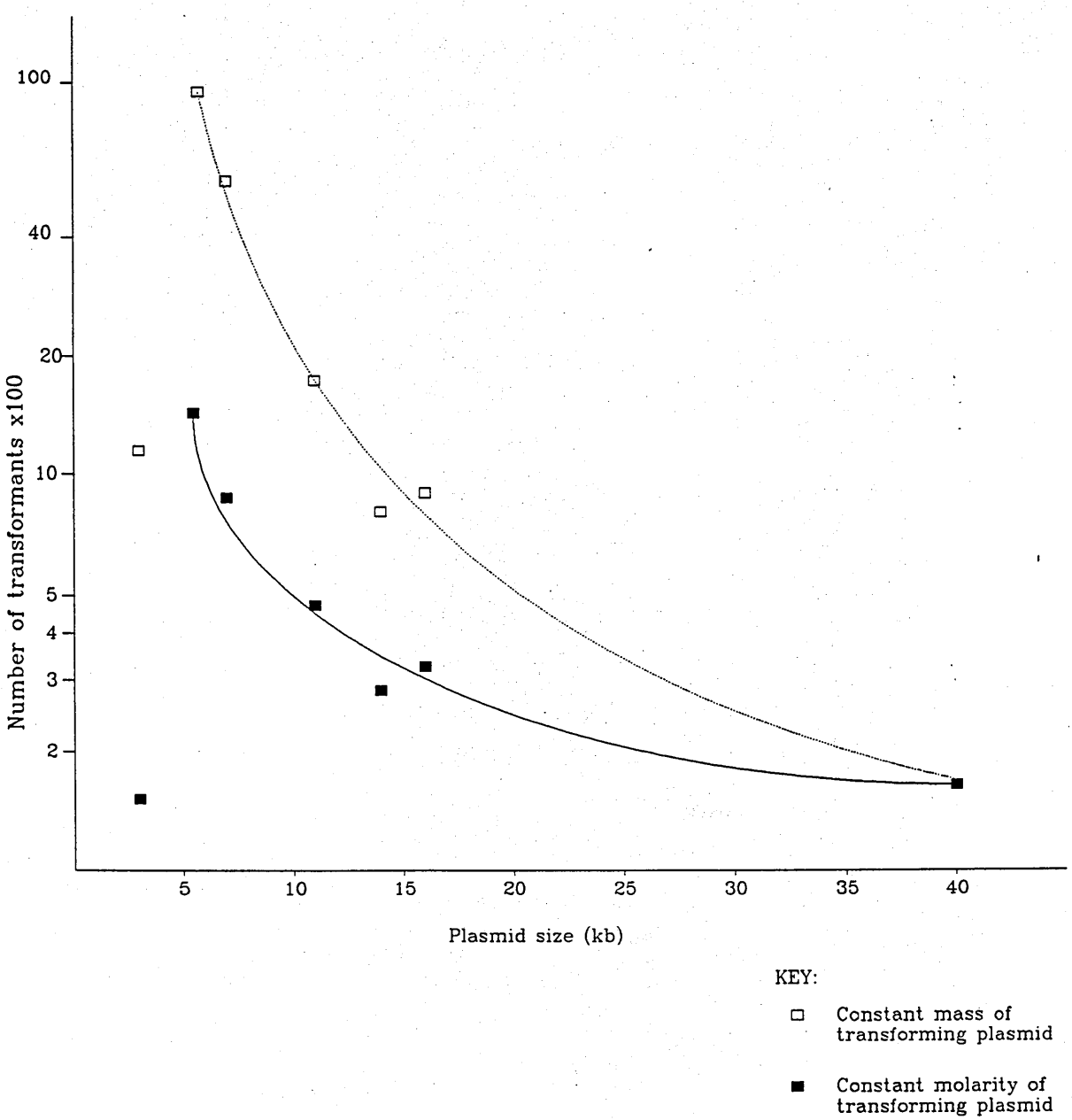

Figure 5.1. **Graph showing variation of transformation frequency with size of transforming plasmid in E.coli.**

These results were obtained from a single experiment. Equal masses of plasmid DNA was used for each transformation; resulting data are represented as open boxes. Data were then divided by the quotient of the size of the largest plasmid (40 kb) and the size of the transforming plasmid, in order to show transformation frequencies at a constant molarity. Resulting data are represented as black boxes.

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Results

5.1 Investigation of factors affecting efficiency of plasmid rescue

Firstly, the effect of plasmid size on efficiency of transformation of E.coli was examined. Equal amounts of 7 plasmids carrying the amp^R gene ranging in size from 2.7 kb up to 40 kb were used to transform competent cells of E.coli strain DH1. The resulting numbers of transformants were plotted against plasmid size (Fig. 5.1). The transformation frequencies divided by their molarity relative to the largest plasmid were also plotted. A clear negative correlation of plasmid size with transformation efficiency was seen, as expected. It is believed that the low frequency found using the 2.7-kb plasmid (pUC18) was the result of experimental error. From this graph it can be calculated that plasmid rescue of pDHG25 with a 5-kb library insert (15.5 kb) would occur 70% more efficiently than that of a cointegrate of pILJ16 with a 5-kb insert and pHELP1 (18.3 kb). To maximise plasmid rescue efficiency it was decided to use pHELP3 (5.8 kb) which would form 16.2-kb cointegrates with pILJ16 with a library insert, although the enhancement of transformation efficiency with pILJ16 by pHELP3 is only 50% of that using pHELP1.

The effect of the presence of chromosomal DNA on efficiency of transformation of E.coli with plasmids was examined. In transformations using a 5.4-kb and a 14.0-kb plasmid varying amounts of Aspergillus chromosomal DNA was added and the effect on transformation efficiency noted (Fig. 5.2). It can be seen that a decrease in plasmid to chromosomal (p:c) DNA ratio of 1:20 to 1:160 causes a 100-fold drop on transformation efficiency. Given an mean copy number of 30 plasmids per haploid genome, a 16-kb plasmid would be present at a p:c ratio of 480 kb : 31,000 kb = 1 : 65.

The Holmes and Quigley (1981) boiling plasmid miniprep involves a step which increases p:c ratio. When lysed cells are boiled in STET buffer, denatured chromosomal DNA and protein form an insoluble aggregate which can be spun down, while plasmid DNA rapidly renatures and remains in solution in the supernatent. In order to reproduce this effect varying amounts of BSA (bovine serum albumen) was added to STET buffer containing 0.5 ug/ul DNA from an A.nidulans ARG^+ transformant with ARp1. A boiling prep procedure was then followed, and the

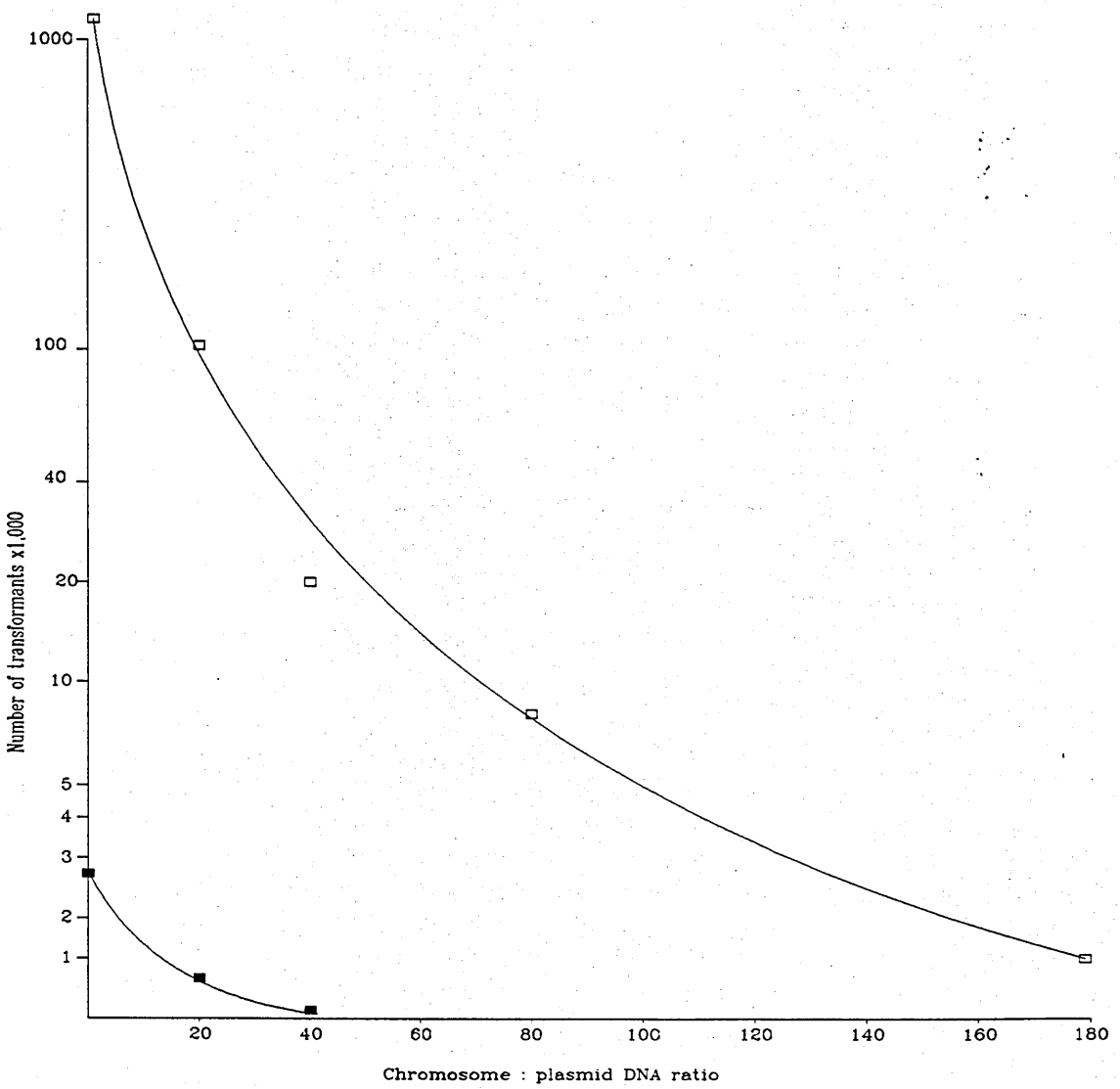

Figure 5.2. Variation of frequency of transformation of E.coli with plasmid DNA, with the ratio of chromosomal to plasmid DNA.
Open boxes: 5.4-kb plasmid; black boxes, 14.0-kb plasmid.

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resulting DNA precipitated and resuspended in TE. DNA concentration was then assayed visually on an EtBr stained minigel. The results are displayed in Table 5.1. A reduction in the amount of chromosomal DNA with increasing amounts of BSA is evident. 700 ng of each of the resulting DNAs were then used to transform competent cells of *E.coli* strain DH1 with selection for *amp*^{R} (competence: 1.2 x 10^6 transformants/ ug DNA). The results are shown in Table 5.1. Plasmid rescue was only successful in one case which used a DNA in which the amount of chromosomal DNA had been reduced. This may or may not be due to an increase in the p:c ratio as a result of the precipitation of the chromosomal DNA.

5.2 Cotransformation of an *adD*3 strain of *A.nidulans*

30 transformations of strain G135 (*yA*2;*adD*3;*leuA*1) were carried out selecting for AD$^+$. 1 ug each of pHELP3 and the pILJ16-based gene library and 1x10^7 protoplasts were used in each transformation. The amounts of DNA used were considerably below saturating levels in order to maximise the number of transformants per unit DNA. Two AD$^+$*yA*$^-$ colonies, both with unstable, heterokaryon-like appearance, were obtained.

5.3 Characterisation of two AD$^+$ transformants

Conidia from the two transformants, TAD1 and TAD2, were stab-inoculated onto MM, and MM supplemented with leucine. It was found in both cases that growth occurred on MM plus leucine, but not on MM alone, suggesting that these colonies were not the result of contamination.

Conidia from TAD1 and TAD2 were plated on CM supplemented with adenine (non-selective conditions) and progeny used to stab inoculate 24 colony array master plates (also on CM plus adenine). 8 master plates of each were then tested for AD$^+$. In the case of TAD1 3/192 (1.5%) were AD$^+$. And of TAD2 progeny 13/192 (6.8%) were AD$^+$. Thus, the transforming *adD*$^+$ sequence is highly mitotically unstable.

It was also found that when conidia from TAD1 and TAD2 were plated onto CM, 80-90% of progeny in each case failed to conidiate. The rest grew and conidiated normally. When tested, non-conidiating progeny were found to be AD$^-$ and conidiating progeny AD$^+$.

Table 5.1 **Use of boiling procedure to increase plasmid to chromosomal (p : c) DNA ratio**

DNA of an ARG^+ transformant of A.nidulans with ARp1 was treated in the presence of various concentrations of BSA to coprecipitate with chromosomal DNA. 700ng of each DNA was then used to transform E.coli strain DH1 selecting for ampicillin resistence.

Concentration of BSA (ug/ul)	DNA concentration after treatment (ug/ul)	Number of amp^r transformants
-*	(0.4)	0
0	0.4	0
2.5	0.4	0
12.5	0.2	1
63	0.1	0

* Not subjected to the boiling prep.

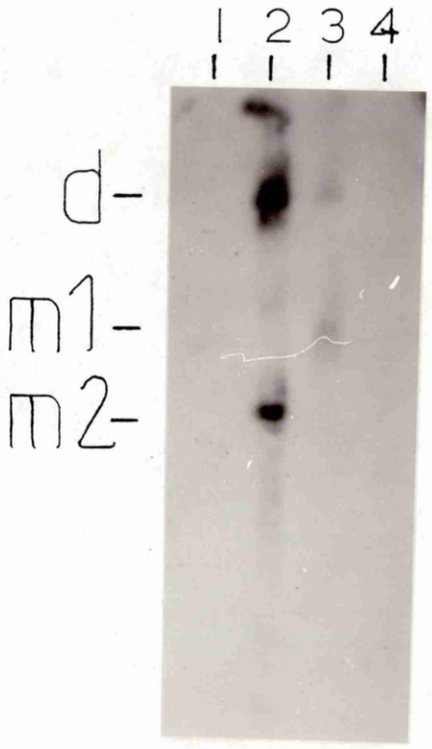

Figure 5.3. **Southern blot analysis of DNA from TAD1 and TAD2.**
Uncut chromosomal DNA was run on a 0.8% agarose gel. The blot prepared from this gel was probed with radiolabelled pUC18.

Lane 1). DNA from untransformed A.nidulans strain G34.
Lane 2) ARp1 transformant DNA of strain G34.
Lane 3) TAD1.
Lane 4) TAD2.
d, dimeric cccDNA; m1, adD^{+} plasmid cccDNA monomer; m2, ARp1 cccDNA monomer.

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5.4 Southern analysis of TAD1 and TAD2

DNA was prepared from transformant strains TAD1 and TAD2 grown under selective conditions. This was then subjected, uncut, to Southern analysis using a radiolabelled pUC18 probe. Uncut DNA from an ARG^+ ARp1 transformant was run alongside for comparison. In the TAD1 track pUC8 hybridised to DNA running at approximately the same position as the dimeric ARp1 (23 kb) and also to approximately the position of the chromosomal DNA (Fig. 5.3). Whether this latter represents pUC sequences integrated into the chromosome, or a smaller, free plasmid running at the same position is unclear. In the TAD2 track no hybridisation was detected. This may be due to a lower abundance of transforming plasmid; the instability of TAD2 characteristic of transformation by an ARp plasmids, described in section 5.3, suggests that free plasmid *is* present. Alternatively, pUC8 derived sequences may have been lost from the transformant.

5.5 Plasmid rescue from transformant TAD1

Whole DNA from TAD1 and TAD2 were used to transform competent cells of E.coli strain DS941. 14 AMP^R colonies were obtained using TAD1 DNA, and none were obtained using TAD2 DNA. Single colony gel analysis showed that plasmids from different colonies were not identical (data not shown). Large scale preparations were carried out of three apparently different plasmids.

5.6 Transformation of A.nidulans with rescued plasmids

These plasmids, designated padD3, padD7 and padD11 were used to transform A.nidulans strain G135, selecting for AD^+. padD3 and padD11 did not give AD^+ transformants, and restriction analysis showed these to be pILJ16 and pHELP3 respectively. padD7 gave a low frequency of transformation to AD^+: 1-5 transformants per 10^6 protoplasts over eight transformations.

The stability of the AD^+ phenotype in four AD^+ transformants was tested by plating conidia onto CM and observing the proportion of conidiating AD^+ progeny. 86-93% of progeny had lost the adD^+ gene. Initial restriction analysis suggested that padD7 consisted of two plasmids which were designated padD7a and padD7b.

Figure 5.4. Single colony gel analysis of ampicillin resistant E.coli colonies transformed with unrestricted DNA from subcultured A.nidulans transformant strain TAD1.

Lane 1) pILJ16.

Lane 2) ARp1.

Lane 3) - 15) Plasmids rescued from subcultured TAD1 DNA.

Lanes 5 and 6 correspond to pDHG7 and pDHG8.

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5.7 Separation of padD7a and padD7b

padD7 was used to transform E.coli strain DS941 to AMP^R. Single colony gel analysis was used to identify colonies containing one or other of the two plasmids. Plasmid DNA was prepared in each case and used to transform A.nidulans strain G135. No AD^+ colonies were seen. Restriction analysis of padD7a and padD7b was carried out. padD7a was a 7.9-kb plasmid the restriction pattern of which bore no resemblance to either pILJ16 or pHELP3. padD7b was pILJ16. It was concluded that neither of these could contain adD^+, therefore a third plasmid was present in the padD7 plasmid mixture at a very low abundance. Various attempts to isolate the third plasmid from the mixture were not successful.

5.8 Plasmid rescue from subcultured AD^+ transformants

In order to circumvent the problem of reisolating the wrong plasmid it was decided to subculture AD^+ transformants under selective conditions in order to try to cure them of unselected plasmids. Progeny of four AD^+ transformants using padD7 were serially subcultured under selective conditions through 3 asexual generations. At each generation some conidia were plated on CM and the same high level of instability of the AD^+ phenotype was seen. DNA was then prepared and used, uncut, to transform competent cells of E.coli strain DH5. From one of these transformations 13 AMP^R colonies were obtained. Single colony gel analysis revealed that all 13 colonies contained a single plasmid species greater than 12 kb in size (Fig. 5.4).

5.9 Transformation of A.nidulans with rescued plasmids

Plasmid DNA was prepared from two AMP^R colonies and used to transform A.nidulans strain G135 (adD3) selecting for AD^+. Over three transformations the two plasmid preps, designated pDHG7 and pDHG8, gave an average of 18,000 and 17,000 AD^+ transformants respectively.

Located 0.1 map units proximal to the adD locus on linkage group II is the adC locus. In order to test the possibility that the neighbouring adC had been cloned in the same DNA fragment containing adD, A.nidulans strain G225 (yA2;adC1;pyroA4) was transformed with pDHG7 and pDHG8, selecting for AD^+. Transformation occurred at a frequency of 0.5 and 1.1 AD^+ transformants respectively per 10^6

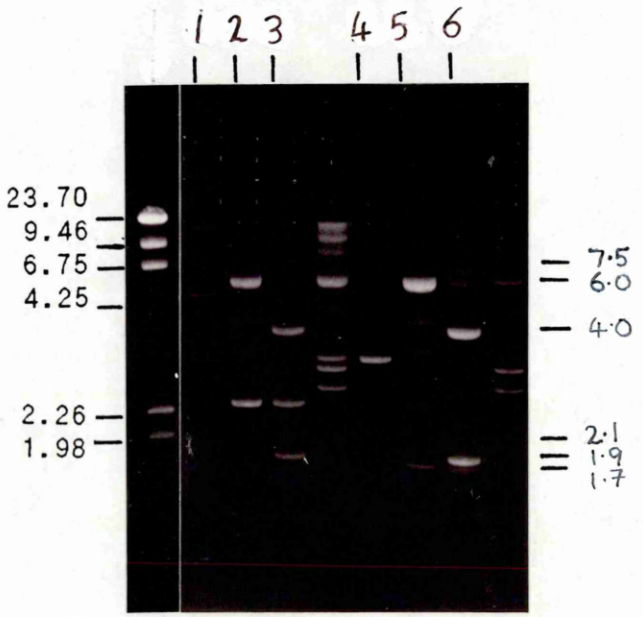

Figure 5.5. **Restriction digests of pDHG7 and pDHG8.**
Only significant digests are labelled; lambda HindIII size standards are given in kb on the left.
Digests are as follows:

Lane 1) pDHG7, BamHI, 2) pDHG7, SalI, 3) pDHG7, EcoRI, 4) pDHG8, BamHI, 5) pDHG8, SalI, 6) pDHG8, EcoRI.
Estimated sizes of EcoRI fragments of pDHG8 are given on the right.

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protoplasts, and transformant colony growth was weak. Growth of eight adC$^+$ colonies was tested on MM, and MM plus pyrodoxine. All were found to grow on MM plus pyrodoxine, but not on MM alone, suggesting that these were transformants, and not contaminants. This result may be interpreted as signifying that part of the adC gene only has been cloned.

5.10 Characterisation of AD$^+$ transformants with adD$^+$

adD$^+$ transformants had a slightly uneven colony morphology, similar to that seen in ARG$^+$ transformants with ARp1. All were yA$^-$, LEU$^-$.

Transformants were unstable: conidia from four transformants with each of pDHG7 and pDHG8 were plated on CM. On average 13.7% and 22.7% respectively of progeny were AD$^+$ (total sample size: 577).

5.11 Characterisation of pDHG7 and pDHG8

When pDHG7 and pDHG8 were restricted with EcoRI, bands of two different intensities were seen, suggesting that both plasmid preps contained two plasmid species of differing abundance (Fig. 5.5, Table 5.2). The sum of the sizes of the high and low abundance plasmids were calculated. Assuming that the plasmids did not have bands in common, in pDHG7 they were 8.3 kb and 17.8 kb respectively; in pDHG8 they were 5.9 kb and 17.8 kb respectively. The restriction patterns with SalI and EcoRI strongly suggested that the smaller plasmid in pDHG8 was pHELP3, and in pDHG7, a slightly enlarged derivative of pHELP3. It was surmised that the larger plasmid in each preparation was likely to be a cointegrate formed from pHELP3 and pILJ16 with a library insert. The large plasmid in each case was designated pDHG7a and pDHG8a, and the small one pDHG7b and pDHG8b.

5.12 Attempted separation of pDHG8a and pHELP3

In order to purify the putative cointegrate competent cells of E.coli strain DH1 were transformed to AMP^R with pDHG8. Single colony gel analyses of 68 transformants were carried out. All contained pHELP3.

In a different approach pDHG8 was restricted with a range of enzymes in order to find one which linearised pDHG8a. The linear pDHG8a could then be gel purified, ligated to itself and transformed into E.coli. ClaI was found to linearise pDHG8a (Fig. 5.6), and the plasmid was

Table 5.2. **Restriction analysis of pDHG7 and pDHG8**
When pDHG7 and pDHG8 were restricted with EcoRI (Fig. 5.5) and subjected to AGE, bands of two different intensities were seen in each, indicating that each contained a mixture of two plasmids.

Plasmids	Fragment sizes resulting from restriction with EcoRI	Sum of fragment sizes (kb)
pDHG7a	7.5, 6.0, 2.1, 1.7, 1.0	17.8
pDHG7b	4.0, 2.4, 1.9	8.3
pDHG8a	7.5, 6.0, 2.1, 1.7, 1.0	17.8
pDHG8b	4.0, 1.9	5.9

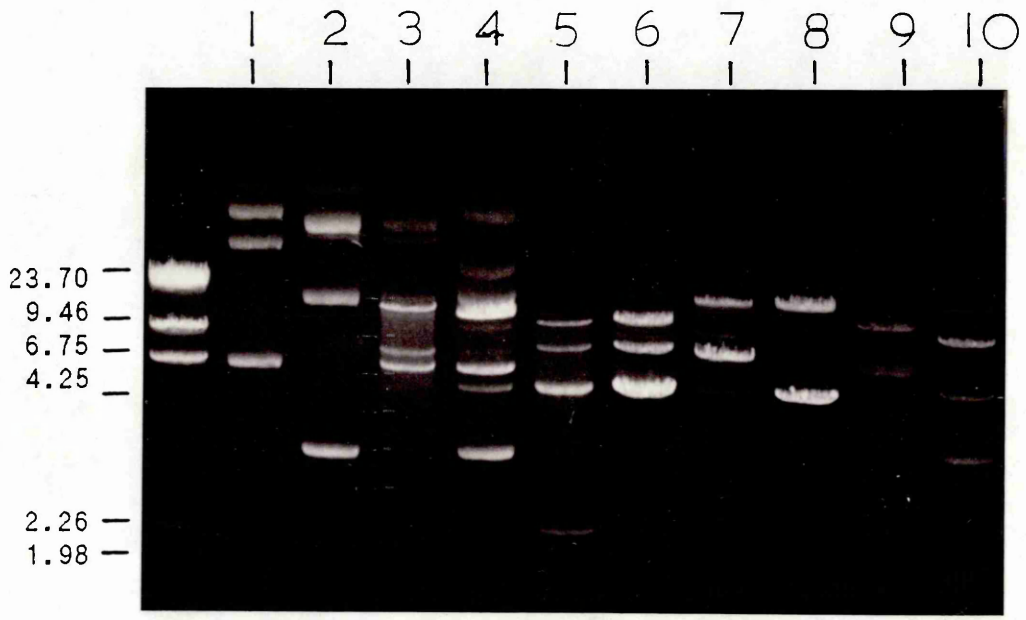

Figure 5.6. **Restriction digests of plasmid mixtures pDHG7 and pDHG8.** Lambda HindIII size standards are given in kb, on the right. Digests are as follows:

Lane 1) pDHG7, uncut, 2) pDHG8 uncut, 3) pDHG7, BamHI, 4) pDHG8, BamHI, 5) pDHG7, XhoI, 6) pDHG8, XhoI, 7) pDHG7, ClaI, 8) pDHG8, Cla1, 9) pDHG7, BglII, 10) pDHG8, BglII.
Estimated sizes of ClaI restriction fragments of pDHG8 are given on the left.

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isolated as described and transformed into E.coli strain DH1. Boiling preps of plasmid DNA from three resulting AMP^R colonies were carried out. The plasmid yields were found to be unusually low. These were restricted with ClaI and run on a gel next to ClaI restricted pDHG8 for comparison. Each of the plasmid minipreps yielded a single linear ClaI fragment corresponding in size to pDHG8a (Fig. 5.7). However when a large scale prep of one of these plasmids, designated pDHG15, was carried out it proved to contain both pDHG8a and pHELP3 (Fig. 5.8). The same proved to be true when large scale preps of the other plasmids were carried out.

These results were interpreted as follows: the pIL16-library/pHELP3 cointegrate for some reason is very poorly maintained by E.coli, hence the low yield of pDHG8a from the boiling preps. Thus when resolution occurs to yield the two plasmid components, cells containing pHELP3 grow rapidly. The pILJ16-library component, however, is disabled and cannot replicate at all. The selective advantage for the resolved pHELP3 is so great that resolution is seen even in the recA strains of E.coli used. This is not seen when plasmid from boiling preps using a 1.5ml E.coli culture is used. However, in the large scale plasmid prep, using 200ml culture volumes where many more cell divisions occur, sufficient time is given for the occurrence of rare cointegrate resolutions. The products of these lead to the cells containing them rapidly outgrowing those containing the intact cointegrate; on the other hand B-lactamase produced by the amp^R gene in the former cells may allow enhanced growth of the latter. Thus, separation of pDHG8 and pHELP3 is not possible.

5.12 Restriction mapping of pDHG8a

Since preparation of pDHG8a in the absence of pDHGb was not possible restriction mapping was carried out as follows: pDHG8b was taken to be pHELP3, and it was assumed that the cointegrate contained a pHELP3 component. Restriction fragments seen on gels which were both a) of higher abundance, implying that they came from the more abundant pDHG8b, and b) would be expected to be generated by restriction of unrecombined pHELP3, but not pHELP3 recombined through the pUC8 component, were ignored. Six restriction digests were carried out of pDHG8, and these were separated on an agarose gel. DNA fragment sizes were calculated, and then Southern blot analysis was carried out, using a radiolabelled pUC8 probe. The restriction mapping gel, and the

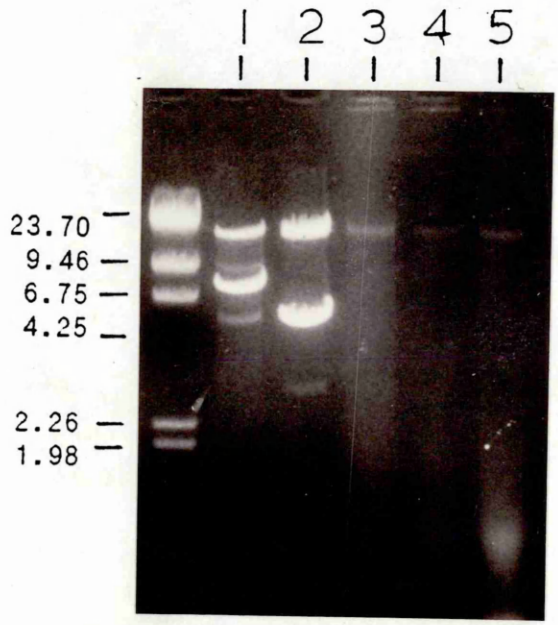

Figure 5.7. **Restriction digests of DNA minipreps of pDHG8a.**
Lambda HindIII size standards are given on the right.
Digests are as follows:

Lane 1) pDHG7, ClaI.
Lane 2) pDHG8, ClaI.
Lane 3) - 5) pDHG8a, ClaI.

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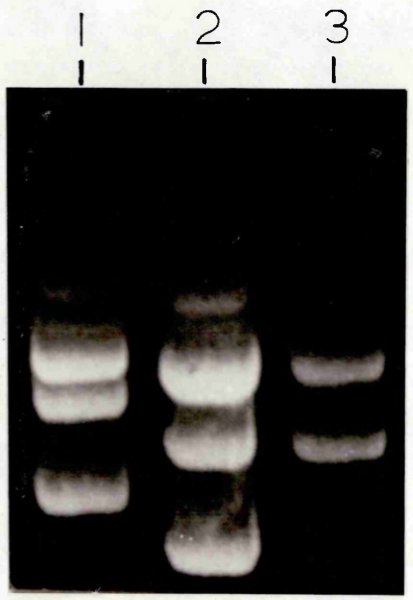

Figure 5.8. Agarose gel electrophoresis of plasmids pDHG7, pDHG8 and pDHG15 prepared from 200 ml cultures of E.coli.

Lane 1) pDHG7.

Lane 2) pDHG8.

Lane 3) pDHG15.

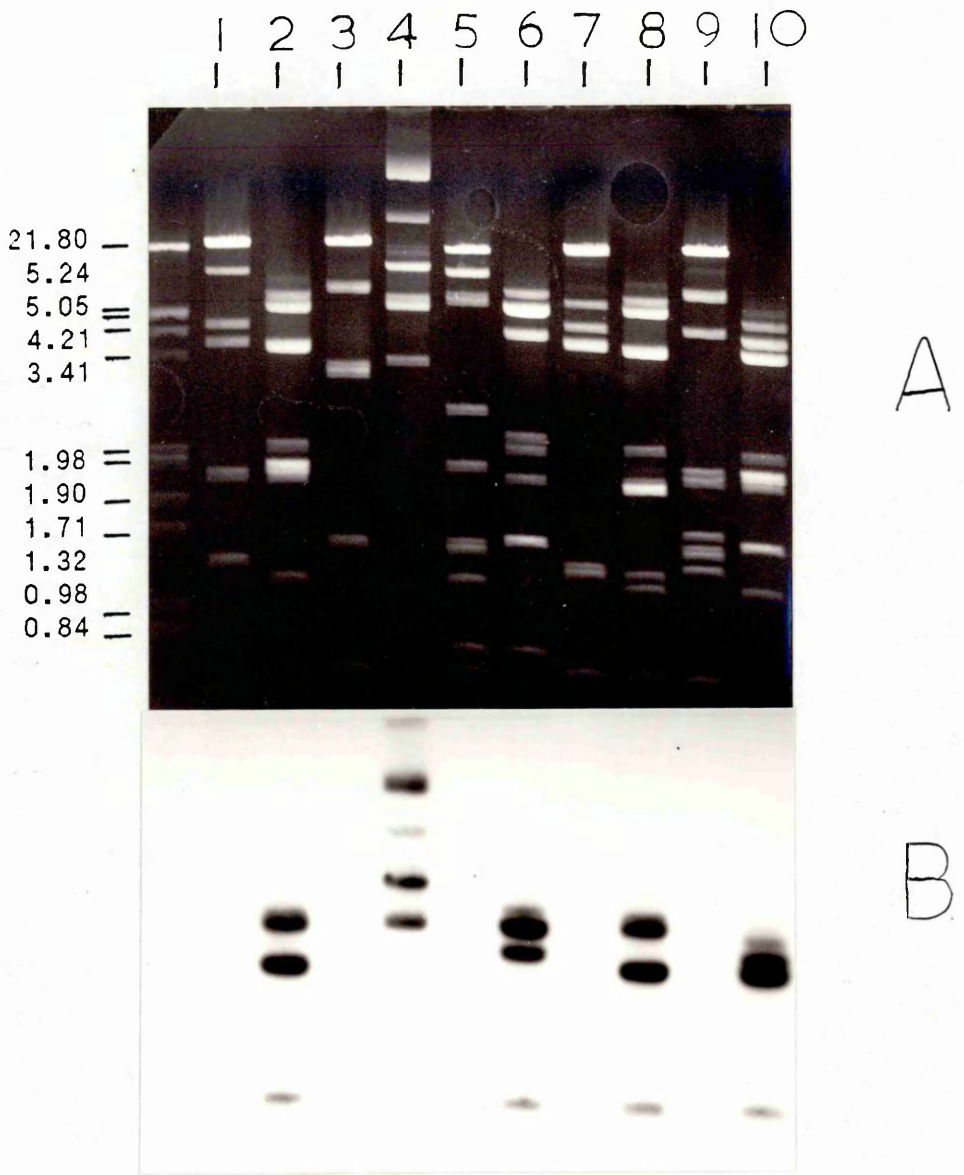

Figure 5.9. **Restriction digests and Southern blot analysis of pDHG8.**

A: gel. Only significant digests are labelled; lambda HindIII/EcoRI size standards are given in kb. LadD11 is an EMBL3 lambda vector with an A.nidulans DNA insert containing the entire adD region (see chapter 7).
Single and double digests are as follows:

Lane 1) LadD11, EcoRI, 2) pDHG8, EcoRI, 3) LadD11, BglII, 4) pDHG8, BglII, 5) LadD11, BglII/SmaI, 6) pDHG8, BglII SmaI, 7) LadD11, EcoRI/SmaI, 8) pDHG8, EcoRI SmaI, 9) LadD11, EcoRI BglII, 10) pDHG8, EcoRI BglII.

B: blot, prepared form gel above. Probed with radiolabelled pUC18.

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Table 5.3 Restriction and Southern blot analysis of pDHG8

DNA fragments believed to have come form pDHG8b alone are bracketted, and not included in the sum of restriction fragment sizes. Fragments hybridising with radiolabelled pUC8 and underlined.

Enzymes(s)	Restriction fragment sizes (kb)	Sum of fragment sizes (kb)
EcoRI	6, 5.6, (3.6), 2, 1.8, 1.75, 1.1	18.25
BglII	10, 5.6, 3.3[1]	18.9
BglII/SmaI	6.4, (5.2), 4.1, 2.1, 2, 1.7, 1.3, 0.82	18.42
EcoRI/SmaI	6, 5.6, (3.4), 2, 1.7, 1.1, 1.05, 0.72	18.7
EcoRI/BglII	4.6, 3.8, (3.4), 1.9, 1.9, 1.7, 1.6, 1.3, 1.1	17.95
PstI/ClaI	[6.0, 5.0, 4.1,][2] 2, 1.5	18.6

Average sum: 18.4

[1] Other bands seen on gel correspond to uncut plasmid pDHG8b.

[2] These values were calculated from a separate gel and Southern blot (not shown). The nature of hybridisation to the upper 3 bands was not clear.

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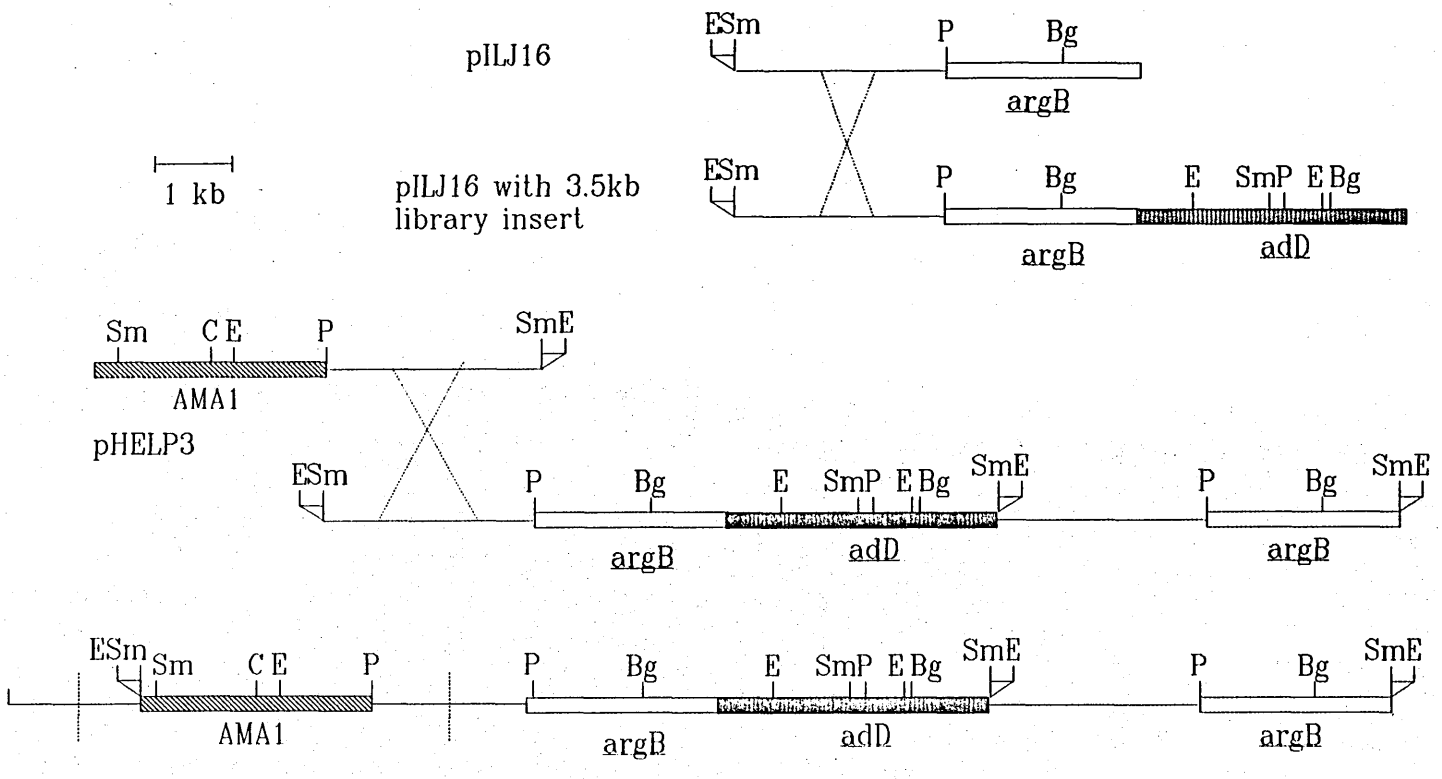

Figure 5.10. Linear restriction map of pDHG8a, and scheme describing how the cointegrate could have been generated by recombination between 3 plasmids.

In this scheme pILJ16 recombines homologously with pILJ16 containing an adD gene insert.This dimer then recombines non-homologously with pHELP3.

The single lines represent pUC8 sequences, the open boxes, argB sequences, the cross-hatched boxes, AMA1-derived sequences, and the grey boxes adD gene sequences. The triangles represent pUC8 polylinker sequences. The crossed dotted lines represent recombination events.

Abbreviations for restriction enzymes sites: Bg, BglII; C, ClaI; E, EcoRI; P, PstI; S, SalI; Sm, SmaI.

autoradiograph derived from it are shown in Fig. 5.9. DNA fragment sizes are shown in Table 5.3, with fragments derived from the free DHG8b only (which were ignored) in brackets. DNA fragments with which radiolabelled pUC8 hybridised are underlined.

EcoRI and SmaI, but not BglII, cut at a single site in the pUC8 polylinker. None of these enzymes cut elsewhere in pUC8. Nonetheless, all three enzymes gave three DNA fragments of pDHG8a which hybridised with pUC8. Thus, pDHG8a appears to contain three copies of pUC8 derived sequences, which in turn implies that it is the product of recombination between three plasmids, i.e. a trimer. The presence of just one ClaI site in pDHG8a suggests that only one of these is pHELP3. The second plasmid is likely to be derived from pILJ16 with a library insert of approximately 5 kb.

Southern analysis also showed the presence of a 2.0-kb PstI fragment, and 1.7-kb EcoRI, SmaI and/or EcoRI/SmaI fragments hybridising to pUC8 (Fig. 5.9, lanes 2 and 8). Since pUC8 sequences are 2.7 kb in size, and have a PstI site at one end, and EcoRI and SmaI sites at the other, this suggests that in the formation of pDHG8a a non-homologous recombination event has occurred between pUC8 sequences in opposite orientations relative to each other. It also implies that in this process 1.7 kb of pUC8 DNA was deleted.

The average size of pDHG8a calculated from the sums of restriction fragment sizes is 18.4 kb. Given that pILJ16 plus approximately 5-kb library insert, plus pHELP3 are likely to constitute 16.2 kb of the cointegrate; and given that a 1.7-kb deletion of pUC8 sequences has occurred, a remainder of 3.5 kb is left to account for the third plasmid. When the assumption was made that this third plasmid was pILJ16 (5.4 kb) and that the DNA library insert containing adD^+ was 3.6 kb in size a model was generated which corresponded to the restriction and Southern hybridisation patterns seen. In this model, pILJ16 with a 3.5-kb library insert has recombined homologously with a molecule of pILJ16 without an insert, and non-homologously, as described, with pHELP3 (Fig. 5.10).

In chapter 7 the isolation and subcloning of adD sequences from an A.nidulans DNA library in a lambda bacteriophage vector is described. The detailed restriction map of a 3.1-kb BglII fragment containing the adC and adD genes was found to correspond to that of the 3.5-kb insert in pDHG8a. This is shown in Fig. 5.11 which compares pDHG8a with pYG12, a plasmid containing the AdD gene on a 3.1-kb BglII fragment inserted into pBLUESCRIPTII KS+. In the gel shown in Fig. 5.9

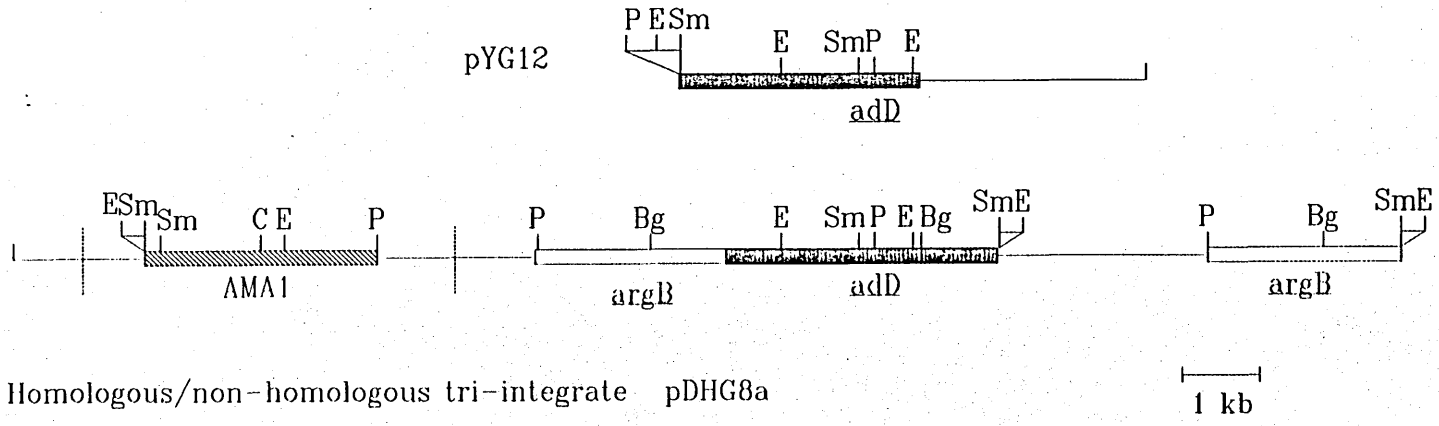

Figure 5.11. Linear restriction maps of plasmids pYG12 and pDHG8a.

The single lines represent E.coli vector sequences (pUC8 in the case of pDHG8a, and pBLUESCRIPTII KS+ in the case of pYG12); the open boxes, argB sequences; the cross-hatched boxes, AMA1-derived sequences, and the grey boxes adD sequences. The triangles represent pUC8 polylinker sequences. Abbreviations for restriction enzymes sites: Bg, BglII; C, ClaI; E, EcoRI; P, PstI; S, SalI; Sm, SmaI.

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alongside the digests of pDHG8 are digests of an EMBL3 lambda vector with a 13.6-kb insert (LadD11) containing the adD region. Comparing the EcoRI digests it can be seen that whereas the 1.7-kb EcoRI fragment is present in both LadD11 and pDHG8, the 1.1-kb EcoRI fragment is only present in pDHG8. This corresponds to the map, where the 1.7-kb EcoRI fragment is located within the 3.5-kb library insert, and the 1.1-kb EcoRI fragment is formed by one EcoRI site within the insert, and the other in the pUC8 polylinker.

Discussion

The results described in this chapter show the successful use of cotransformation with helper plasmids to facilitate gene cloning using a gene library in an AIp vector. It has been shown that to maximise the chances of success with this method a number of measures should be taken. Firstly, the size of the two plasmid species used should be minimised, and the ratio of plasmid to chromosomal DNA maximised to facilitate plasmid rescue. The p : c ratio may perhaps be increased using a modified boiling prep procedure as described. Secondly, initial transformants should be subcultured (under selective conditions) to cure them of as much unrecombined helper plasmid as possible. During subculture, transformant instability should be checked and any stabilised integrants should be discarded. For plasmid rescue the E.coli strains used should be as deficient in recombination as possible - especially homologous recombination - to prevent resolution of the cointegrate.

A further possibility is that pHELP3 is not a suitable helper plasmid for gene cloning. Characterisation of Aspergillus transformants with pILJ23, pILJ25 and pDHG24, which contain fragments of AMA1 either lacking, or with reduced inverted repetition, showed that changes in plasmid size had occurred (data not shown). This implies that the inverted repeat structure may add to plasmid stability with respect to rearrangement. If this were so, the non-repetitive na ture of the AMA1-derived insert in pHELP3 may result in plasmid rearrangement, perhaps by non-homologous recombination.

The structure of pDHG8a shows that even where there is sequence homology between recombining plasmids non-homologous recombination may still occur. It also suggests the possibility that the properties of AMA1 plasmids in cotransformations observed may be due to stimulation of non-homologous recombination, although as can be seen in the structure of pDHG8a, the two non-AMA1 plasmids present have recombined via homologous recombination.

The disruption of two of the three pUC8 sequences conforms with its disablement as an E.coli plasmid. However, it is not easy to envisage, from this structure, how its resolution occurs to yield pHELP3, nor why the other resolution product should be disabled. It is also not clear why resolution does not give rise to free pILJ16.

CHAPTER 6

Cotransformation with linear DNA

Introduction

As described in the introduction to chapter 4, unselected linear DNA has been found to cotransform with selected cccDNAs at frequencies generally lower than those found where unselected cccDNA is used. In budding yeast 1% and 4% cotransformation of linear DNA with non-homologous replicating plasmids was reported (Siliciano and Tatchell, 1984; Rudolph et al, 1985). In both these experiments expression of coding regions on the unselected DNA was only likely to be seen where its chromosomal integration by double crossover had occurred. Whether recombination of linear and cccDNA had occurred was not investigated. In a similar study a *trpC*$^+$*pyrA*$^-$ strain of *A.niger* was transformed with linear DNA consisting of an *A.niger* *trpC* gene disrupted by insertion of a *lacZ* gene, and an integrative plasmid carrying *pyrA*$^+$ (Goosen et al, 1989). Here 0.15% of PYR$^+$ colonies were LAC$^+$TRP$^-$ and 0.016% were LAC$^+$TRP$^+$. Further studies showed that the *pyrA* plasmid had integrated at a different chromosomal site to the *trpC* locus. However, investigation of whether unexpressed *lacZ*$^+$*trpC*$^-$ sequences had integrated at the same site as *pyrA* sequences were not reported.

Cotransformation with integrating cccDNA has been used to localise genes to given restriction fragments of long stretches of cloned DNA in *A.nidulans* (Timberlake et al, 1985) and *U.maydis* (Wang et al, 1989). Maximum frequencies of cotransformation seen were 10% and 28% respectively. Whether the two DNA species had integrated at the same site was not examined in either case. These cotransformation frequencies are higher than those in the yeast and *A.niger* experiments described, and may reflect the fact that the *A.nidulans* and *U.maydis* cotransformations have resulted from non-homologous recombination of linear and plasmid DNA prior to integration. If this was the case one would predict a) integration of both DNAs at the same site in the chromosome and b) a much lower frequency of transformation with the linear DNA alone than that found in cotransformations.

Investigation of whether transforming non-homologous linear and autonomous plasmid DNA results in recombination to form cointegrates in any organism have not been reported. Nor have studies involving cotransformation of homologous linear DNA with cccDNA, integrative or autonomously replicating.

Table 6.1 Frequency of transformation of Aspergillus to TRP^+ with linear and circular DNAs

Approximately equimolar amounts of selected and unselected DNAs were added to each double transformation.

	Number of transformants/10^6 protoplasts with transforming plasmids indicated				
Strain	ARp1/ pTA11	ARp1/linear *trpC*$^+$	pTA11	linear *trpC*$^+$	pHELP1/linear *trpC*$^+$
DHG019	100	400	20	-	-
"	-	2,200	60	-	-
DHG135	300	780	0	0	-
"	200	10	2.4	0	-
"	10,000	3,000	-	0	3,000
Average	2,650	1,280	21	0	3,000

Results

6.1.1 Cotransformation of linear DNA with ARp1 and pHELP1

Restriction of plasmid pTA11 with XhoI releases a 4.4-kb DNA fragment containing the gene *trpC*$^+$ (Fig. 4.3). This linear DNA was gel purified, and used, with approximately equimolar amounts of ARp1, or pHELP1 to transform *A.nidulans* strain DHG019, or DHG135 (both *trpC*$^-$), to TRP$^+$.

ARp1 and *trpC*$^+$ linear DNA gave an average of 1,280 TRP$^+$ transformants per 10^6 protoplasts over five transformations; in one transformation with pHELP1 and *trpC*$^+$ linear DNA 3,000 transformants per 10^6 protoplasts were seen. By comparison transformations with pTA11 gave on average 21 TRP$^+$ transformants per 10^6 protoplasts (three transformations), and *trpC*$^+$ linear DNA gave no transformants at all (three transformations). Four transformations with ARp1 and pTA11 gave on average 2,650 TRP$^+$ transformants per 10^6 protoplasts.

These results show that addition of ARp1 or pHELP1 to transformations with *trpC*$^+$ linear DNA results in at least a 1,000-fold increase in transformation frequency. Addition of ARp1 to transformations with pTA11 also results in an approximately 180-fold increase in transformation efficiency. These results are summarised in Table 6.1.

6.1.2 Analysis of TRP$^+$ transformants

Three TRP$^+$ transformants of *A.nidulans* strain DHG135 (*biA1;argB2 methH2*, *trpC801*) with ARp1 and *trpC*$^+$ linear DNA were examined. Conidia were plated on MM plus biotin, methionine and arginine and the progeny used to stab inoculate one 24 colony array master plate for each of the original transformants, on the same medium. These master plates were then tested for ARG$^+$. In two cases 100% of progeny were ARG$^+$, and in the third case 96%, suggesting all three progeny were cotransformed with ARp1. It also suggested that if the TRP$^+$ phenotype were unstable selection for TRP$^+$ was resulting in coselection for ARG$^+$ - i.e. the two genes were linked.

Conidia from eight TRP$^+$ transformants of strain DHG019 (*argB2 methH2;brlA42 trpC801*) with ARp1 and *trpC*$^+$ linear DNA were plated on non-selective medium (CM plus arginine and tryptophan) and the resultant colonies used to stab-inoculate one 24 colony array master

ıble 6.2 Comparison of patterns of instability of ARG^+ and TRP^+ ıenotypes of eight ARG^+TRP^+ transformants each with either ARp1 and $\underline{trpC}^+$ linear DNA, or ARp1 and pTA11.

Asexual progeny of Transformants with ARp1 and $\underline{trpC}^+$ linear DNA			
$\%ARG^+TRP^+$	$\%ARG^+TRP^-$	$\%ARG^-TRP^+$	$\%ARG^-TRP^-$
83	0	16	0
56	0	0	44
75	0	0	25
36	0	0	64
86	0	14	0
21	0	0	79
100	0	0	0
80	0	0	20
Asexual progeny of transformation with ARp1 and pTA11			
100	0	0	0
32	0	0	68
5	10	0	84
17	0	0	82
100	0	0	0
18	6	12	65
31	0	5	63
87	4	0	9

facing page 104

wrong way

plate for each transformant. The phenotypes with respect to ARG^+ and TRP^+ were then tested. Full results are shown in Table 6.2, with the patterns of instability of ARG^+TRP^+ pTA11/ARp1 transformants shown for comparison. In 5/8 cases 18-79% of progeny were ARG^-TRP^-, 21-82% were ARG^+TRP^+ and none were ARG^+TRP^- or ARG^-TRP^+. The degree of instability of the ARG^+TRP^+ genotype is similar to that seen in ARG^+TRP^+ transformants using ARp1 and pTA11 (chapter 4, section 4.4.2). However, in these ARp1/*trpC*$^+$ linear DNA transformants no ARG^+TRP^- or ARG^-TRP^+ colonies were seen. This conforms with the view that in this case autonomously replicating cointegrates carrying both *argB*$^+$ and *trpC*$^+$ genes are being formed by non-homologous recombination of the two DNAs; such a cointegrate would be incapable of resolving and giving rise to ARG^+TRP^- and ARG^-TRP^+ progeny. Thus, the two genes behave as 100% linked.

In the case of 3/8 transformants no ARG^-TRP^- progeny were seen. In one of these 100% of progeny were ARG^+TRP^+. In the other two 16% and 14% of progeny were ARG^-TRP^+, and the rest were ARG^+TRP^+. The most plausable explanation for this result is that integration of the cointegrate occurred, and that in some cases the *argB*$^+$ gene is disrupted in the process.

These results suggest a high degree of homogeneity in the plasmid population in the transformants - i.e. only ARp1/*trpC*$^+$ linear DNA cointegrates are present, and no ARp1 alone - since no ARG^+TRP^- progeny were seen.

6.1.3 Plasmid rescue from pHELP1/*trpC*$^+$ linear DNA cotransformants

DNA was prepared from two TRP^+ transformants of *A.nidulans* strain DHG135, designated XH3 and XH5, grown under conditions selecting for the maintenance of the *argB*$^+$*trpC*$^+$ cointegrate. These DNAs were used to transform competent cells of *E.coli* strain DH5, selecting for AMP^R. Two AMP^R colonies were obtained using DNAs of strain XH3 and one from XH5. The plasmids contained in the AMP^R transformants were designated pXH3.1, pXH3.2, and pXH5.

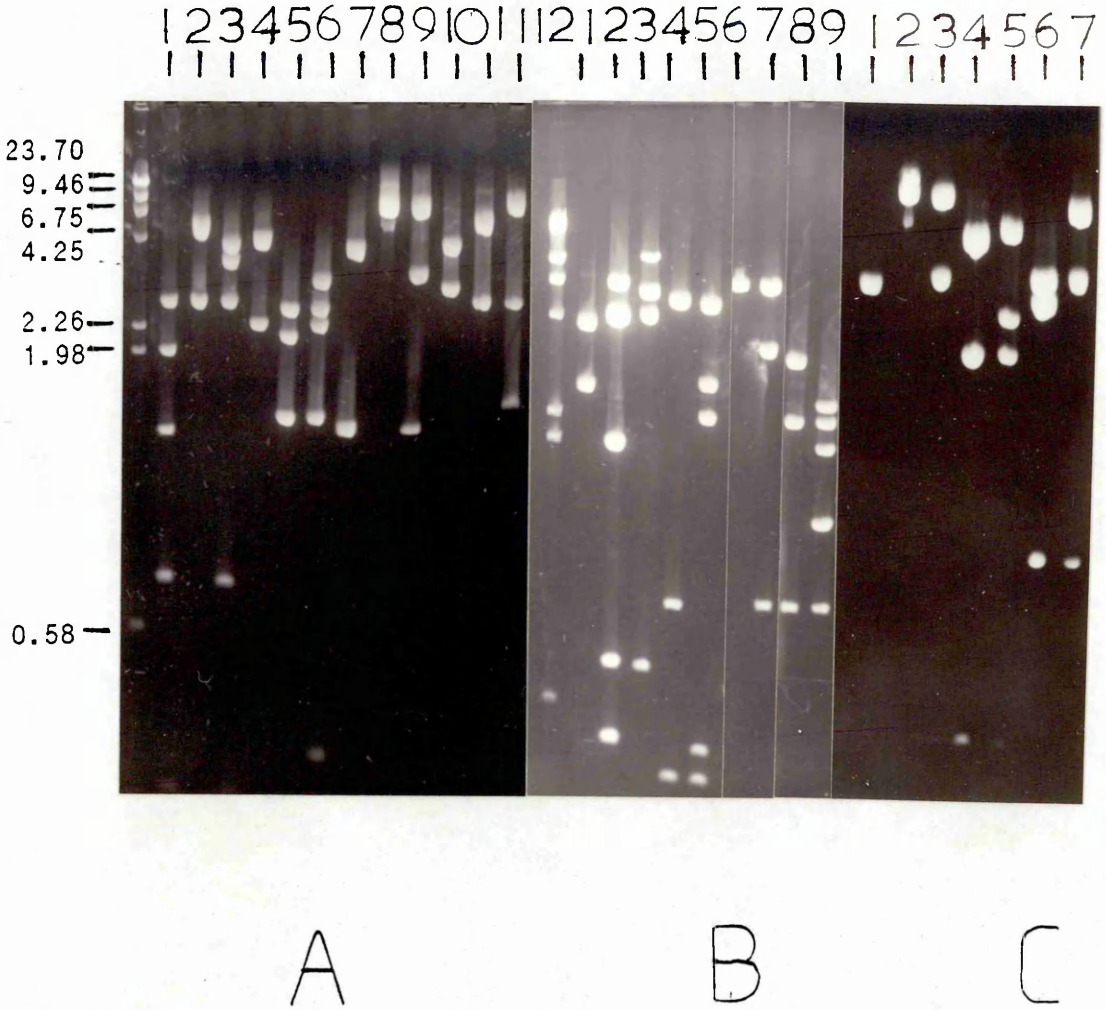

Figure 6.1. **Restriction analysis of plasmid cointegrate pXH3.1**
Only significant digests are labelled; lambda HindIII/EcoRI size standards are given in kb.
Single and double digests are as follows:

Gel A: Lane 1) pTA11, HindIII, 2) pHELP1, HindIII, 3) pXH3.1, HindIII, 4) pTA11, EcoRI, 5) pHELP1, EcoRI, 6) pXH3.1, EcoRI, 7) pTA11, BglII, 8) pHELP1, BglII, 9) pXH3.1, BglII, 10) pTA11, PstI, 11) pHELP1, PstI, 12) pXH3.1, PstI.

Gel B: Lane 1) pTA11, XhoI, 2) pHELP1, XhoI, 3) pXH3.1, XhoI, 4) pTA11, SalI, 5) pXH3.1, SalI, 6) pTA11, ClaI, 7) pTA11, ClaI, 8) pXH3.1, Cla1, 8) pTA11, EcoRI/BamHI, 9) pXH3.1, EcoRI/BamHI.

Gel C: Lane 1) pTA11, BamHI, 2) pHELP1, BamHI, 3) pXH3.1, BamHI, 4) pHELP1, SalI, 5) pXH3.1, SalI, 6) pHELP1, ClaI, 7) pXH3.1, ClaI.

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Table 6.3 Sizes of DNA fragments resulting from restriction of plasmid pXH3.1 with various enzymes

Enzyme(s)	Fragment sizes (kb)	Sum of fragment sizes (kb)
ClaI	5.9, 4.8, 1.0	11.7
BamHI	8.0, 3.5	11.5
SalI	5.4, 2.8, 2.3, 0.4, 0.37	11.27
XhoI	6.0, 4.1, 0.7	10.8
PstI	7.4, 2.6, 1.5	11.5
BglII	6.8, 3.3, 1.4	11.5
HindIII	4.1, 3.9, 2.7, 0.7, 0.2	11.6
EcoRI	3.2, 2.7, 2.6, 1.4, 1.4, 0.3	11.6
EcoRI/BamHI	2.7, 2.0, 1.8, 1.4, 1.4, 0.8, 0.3	11.6
Average:		11.3

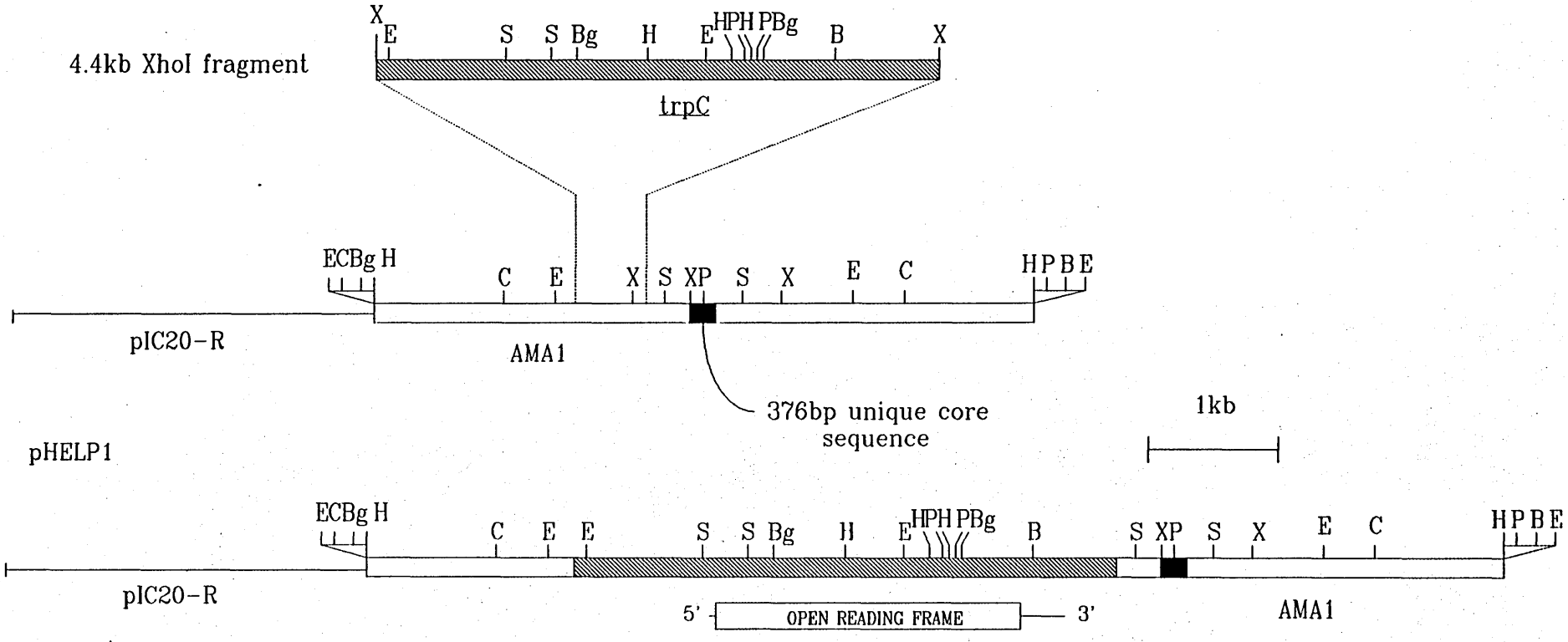

Figure 6.2. Linear restriction digest map of pXH3.1

This figure shows a schematic representation of how pXH3.1 could be formed by recombination between the 4.4-kb XhoI fragment containing trpC, and pHELP1.

Single lines represent pIC20-R sequences. Open boxes represent AMA1 sequences, and cross-hatched boxes trpC sequences. The black boxes represent the 376-bp non-repeated core sequence of AMA1. The dotted lines represent a recombination event.

Abbreviations for restriction sites: B, BamHI; Bg, BglII; C, ClaI; E, EcoRI; H, HindIII; P, PstI; X, XhoI.

6.1.4. Structure of pHELP1/*trpC*$^+$ cointegrates

Plasmid DNAs of pXH3.1, pXH3.2 and pXH5 were prepared and subjected to restriction analysis.

Firstly, pXH3.1 was examined. Fig. 6.1 shows electrophoresis gels of digests, and Table 6.3 summarises the restriction fragment sizes measured from these gels. The initial assumption was made that pXH3.1 was the product of recombination of the 4.4-kb *Xho*I fragment carrying *trpC*$^+$, and pHELP1. Digests were carried out with each enzyme of pXH3.1, pTA11 and pHELP1 in order to identify the points of recombination between the two DNAs.

With respect to pHELP1, pXH3.1 contained the 2.9-kb *Hin*dIII fragment corresponding to the pIC20-R component (Fig. 6.2), and the 2.5-kb *Pst*I fragment corresponding to the right hand repeat of AMA1 as shown (Fig. 6.1, gel A; Fig. 6.2). The 1.0-kb *Cla*I fragment of the left hand arm of AMA1 is also present (Fig. 6.1, gel C). However, gel B shows that both the 2.0-kb and 0.4-kb *Xho*I fragments of pHELP1 are absent from pXH3.1. This suggests that recombination has occurred in the region of the left hand *Xho*I site as shown, and that deletion of part of AMA1 may have occurred. The average size calculated for pXH3.1 is 11.3 kb, 0.8 kb less than the sum of the sizes of pHELP1 (7.9 kb) and the 4.4-kb *Xho*I fragment carrying *trpC*$^+$. Thus a deletion must have occurred.

With respect to the 4.4-kb *Xho*I fragment, the 2.5-kb *Eco*RI and 1.5-kb *Bgl*II fragments are present in pXH3.1 (Fig. 6.1, gel A). The 0.8-kb *Eco*RI/*Bam*HI fragment is also present (Fig. 6.1, gel B). Thus, it seems likely that recombination has occurred between the two DNAs in the left hand arm of AMA1 as shown and the distal most regions of the 4.4-kb *Xho*I fragment. The presence of the 1.6-kb *Pst*I fragment in pXH3.1 indicates the orientation of the *trpC*$^+$ gene DNA fragment and allows a model to be constructed (Fig. 6.2). The presence of a 0.3-kb *Eco*RI fragment in pXH3.1 suggests that recombination between the region of the left hand end of the *trpC* gene DNA as shown and AMA1 has resulted in the deletion of 0.8-kb in this region.

Restriction analysis of pXH3.2 was then carried out. The restriction patterns produced by pXH3.2 and pXH3.1 with a number of enzymes was compared (Fig. 6.3, gel A). It can be seen that whereas the restriction patterns of the two plasmids with *Eco*RI are indistinguishable, with other enzymes they show some differences. Fragment sizes calculated from the gels shown in Fig. 6.3 are

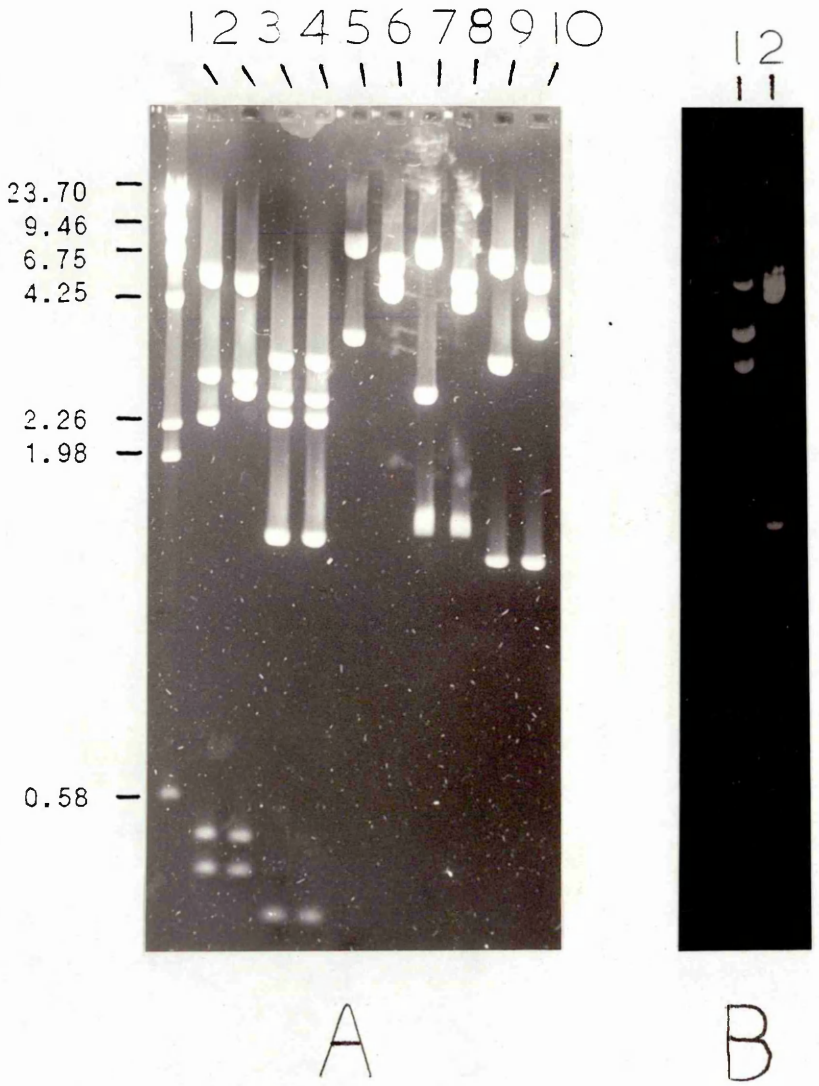

Figure 6.3. **Restriction analysis of plasmid cointegrate pXH3.2**
Only significant digests are labelled; lambda HindIII/EcoRI size standards are given in kb.
Single and double digests are as follows:

Gel A: Lane 1) pXH3.1, SalI, 2) pXH3.2, SalI, 3) pXH3.1, EcoRI, 4) pXH3.2, EcoRI, 5) pXH3.1, BamHI, 6) pXH3.2, BamHI, 7) pXH3.1, PstI, 8) pXH3.2, PstI, 9) pXH3.1, BglII, 10) pXH3.2, BglII.

Gel B: Lane 1, pXH3.1, XhoI, 2) pXH3.2, XhoI.

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Table 6.4 Sizes of DNA fragments resulting from restriction of plasmid pXH3.2 with various enzymes.

Enzyme	Fragment sizes (kb)	Sum of fragment sizes (kb)
SalI	4.8, 2.8, 2.6, 0.48, 0.42	11.1
PstI	6.3, 4.4, 1.5	12.2
BglII	6.3, 3.7, 1.4	11.4
EcoRI	3.0, 2.5, 2.3, 1.5, 1.5, 0.37	11.17
BamHI	6.0, 4.7	10.7
Average:		11.3

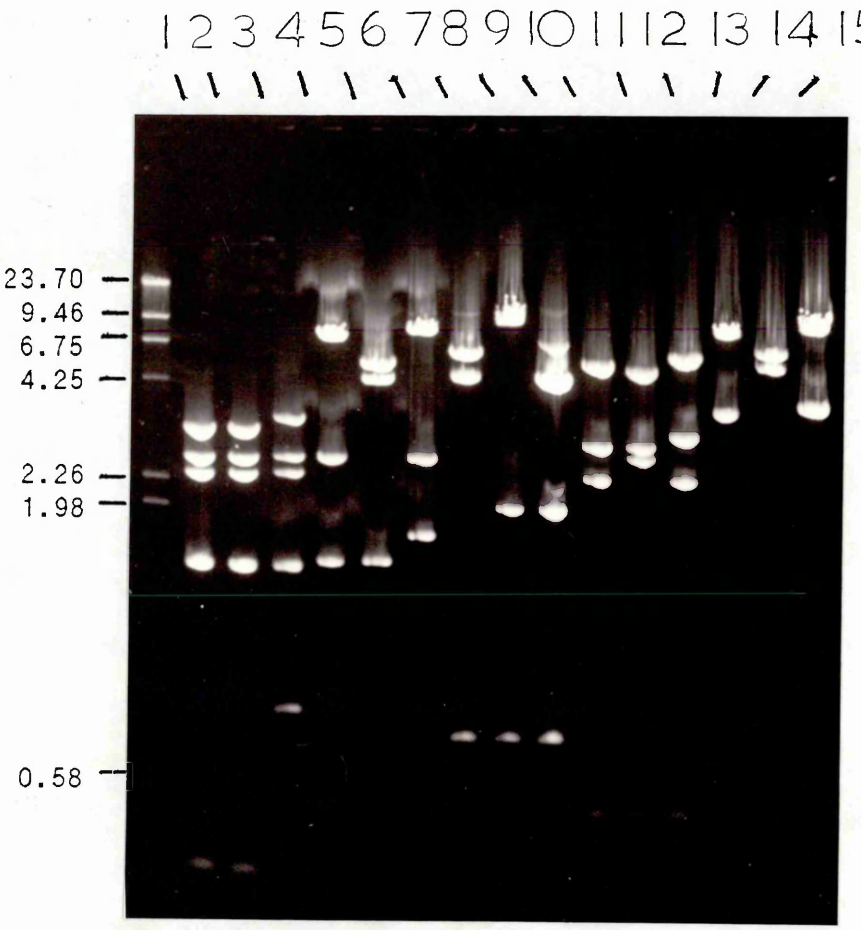

Figure 6.4. **Restriction analysis of plasmid cointegrate pXH5**
Only significant digests are labelled; lambda HindIII/EcoRI size standards are given in kb.
Single and double digests are as follows:

Lane 1) pXH3.1, EcoRI, 2) pXH3.2, EcoRI, 3) pXH5, EcoRI, 4) pXH3.1, PstI, 5) pXH3.2, PstI, 6) pXH5, PstI, 7) pXH3.1, XhoI, 8) pXH3.2, XhoI, 9) pXH5, XhoI, 10) pXH3.1, SalI, 11) pXH3.2, SalI, 12) pXH5, SalI, 13) pXH3.1, BamHI 14) pXH3.2, BamHI, 15) pXH5, BamHI.

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displayed in Table 6.4. The interpretation of this data is that the structure of pXH3.2 is identical to that of pXH3.1, except that the *trpC*$^+$ sequence is in the right arm of the AMA1 inverted repeat instead of the left. One possible explanation for the presence of the two isomeric forms of pHELP1-*trpC* linear DNA cointegrates is that in *A.nidulans* recombination between inverted repeats has occurred resulting in an reversal of orientation, or flipping, of the *trpC*$^+$ insert and AMA1 central region relative to the bacterial sequence. That this is indeed the case is confirmed by Gel B (Fig. 6.3) which shows digests of pXH3.1 and pXH3.2 with *Xho*I. If *trpC*$^+$ linear DNA had integrated at a very similar position in the right hand arm of AMA1 in pXH3.2 to that in the left arm in pXH3.1, then it would be expected that the 0.7-kb *Xho*I fragment which spans and runs right from the centre of AMA1 as shown (Fig. 6.5) would be disrupted. This is not the case, i.e. the 0.7-kb *Xho*I fragment is present, but now runs from the centre to the left of centre in AMA1 (Fig. 6.5). Thus flipping of the central region, mediated by homologous recombination between the AMA1 inverted repeats, has occurred.

Restriction analysis of pXH5 was then carried out. Results of one set of digests of pXH3.1, pXH3.2 and pXH5 are shown in Fig. 6.4. The restriction pattern with *Eco*RI of pXH5 shows a single difference from those of pXH3.1 and pXH3.2. Whereas the size of the *Eco*RI fragment covering the left hand junction between the two parent DNAs (as shown in Fig. 6.2) is 0.3 kb, in the former plasmid it is 0.84 kb. This suggests that in this case only about a 0.26-kb deletion of the left hand arm of AMA1 (as shown) has occurred; otherwise a similar recombination event has taken place, as the *Bam*HI digest shows, in the left arm of AMA1. This is confirmed by the *Sal*I and *Xho*I restriction patterns, where the central 0.4-kb *Sal*I and right of centre 0.7-kb *Xho*I fragments are both present. Restriction maps of pXH3.1, pXH3.2 and pXH5 are shown in Fig. 6.5.

In order to confirm that these cointegrates do indeed carry functional copies of the *trpC*$^+$ gene, pXH3.2 and pXH5 were transformed into *A.nidulans* strain DHG135, selecting for TRP$^+$. The two plasmids gave 16,000 and 4,600 transformants respectively per 10^6 protoplasts, whereas pTA11 alone gave four transformants per 10^6 protoplasts.

The structure of the pHELP1/*trpC* cointegrates strongly suggest that the 4.4-kb *Xho*I fragment was not ligated in vivo to form cccDNA prior to recombination with pHELP1. Rather, it appears that recombination

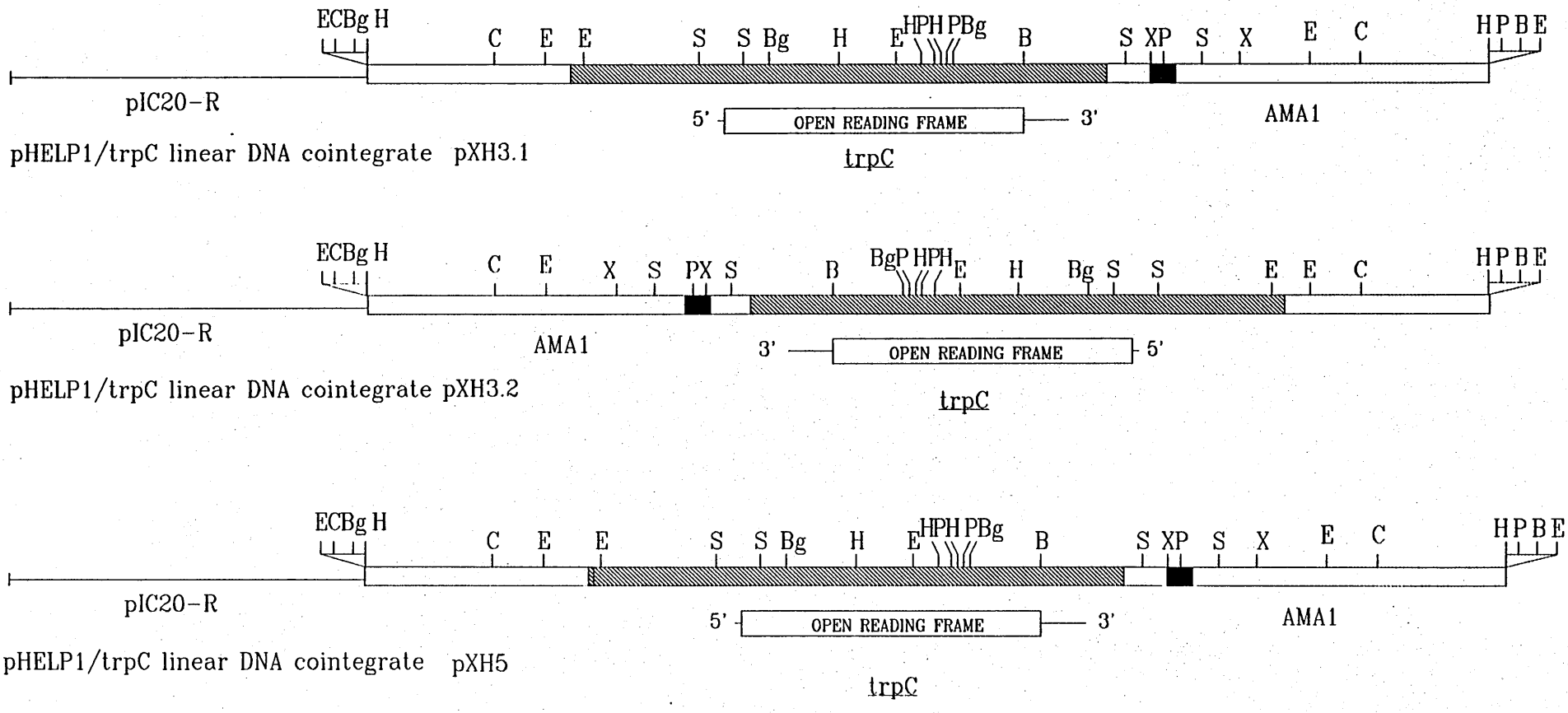

Figure 6.5. Linear restriction digest maps of plasmid cointegrates pXH3.1, pXH3.2 and pXH5
Single lines represent pIC20-R sequences. Open boxes represent AMA1 sequences, and cross-hatched boxes trpC sequences. The black boxes represent the 376-bp non-repeated core sequence of AMA1. Abbreviations for restriction sites: B, BamHI; Bg, BglII; C, ClaI; E, EcoRI; H, HindIII; P, PstI; X, XhoI.

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between the two ends of the linear DNA and two sites close together in AMA1 occurred. This resulted in a small deletion of the AMA1 sequence. The integration of the ends of the 4.4-kb fragments at similar sites in the three cases examined may be interpreted either as signifying that some limited degree of sequence homology exists between them, and that region of AMA1. Alternatively, it may be that there is a recombination hot-spot in the central region of AMA1 capable of stimulating both homologous recombination (e.g. flipping) and non-homologous recombination (integration of trpC sequences). In chapter 4 it was shown that sequences flanking the argB gene recombined with a point in the same region in cotransformations with pILJ16 and pHELP2 which conforms with the recombination hot-spot model.

An implication of the occurrence of high frequency cotransformation of autonomously replicating plasmids with non-homologous linear DNA is that gene cloning should be possible without the necessity of constructing a gene library in a plasmid vector - a procedure that can prove laborious and time-consuming. Linear DNA fragments of an optimal average size, say 5 kb, could be prepared by sonication, or by partial digestion with a restriction enzyme which cuts frequently along the DNA, such as SauIIIa. This DNA could then be used with a helper plasmid, such as pHELP1, to cotransform Aspergillus, selecting for complementation of the gene to be cloned. In the protoplasts recombination between pHELP1 and the linear DNA fragments would occur, in effect creating an instant gene bank in vivo. Any fragments carrying the entire complementing gene which did recombine with pHELP1 would then be able to replicate, and be maintained as part of a freely replicating cointegrate. This plasmid could then easily be reisolated into E.coli by transformation with uncut whole transformant DNA.

Such an "instant gene bank" technique could prove useful in cloning genes from other species of Aspergillus or related species where gene libraries have not been constructed, or for cloning dominant mutant alleles such as fungicide resistance genes.

The literature gives no indication that an approach such as this has been tried in other organisms such as S.cerevisiae, S.pombe, or cultured mammalian cells.

Table 6.5. Stability of seven ARG^+ colonies produced by transformation of Aspergillus with an instant gene bank.

Strain	Sample size	% ARG^+
IGB1	97	99
IGB2	26	38
IGB3	37	100
IGB4	23	4
IGB5	16	13
IGB6	34	35
IGB7	28	7

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6.2.1 Transformation of Aspergillus with an instant gene bank

Chromosomal DNA was prepared from A.nidulans strain G089 (fpaB37 riboA1 biA1), and 100 ug was digested to completion with EcoRI. Restriction with EcoRI generates an approximately 9-kb DNA fragment which carries the entire argB gene. The restricted DNA was extracted against phenol, and then three times against chloroform, then ethanol precipitated and redissolved in TE. This DNA was then used, together with pHELP1, to transform A.nidulans strain G34 (yA2;argB2 methH2) selecting for ARG^+. Eight transformations were carried out, in each using 2 ug of restricted DNA and 1ug pHELP1 to transform $3x10^6$ protoplasts. Seven ARG^+ colonies were obtained. In a negative control transformation with pHELP1 alone, no transformants were seen. In a positive control transformation with pHELP1 and pILJ16, 20,000 transformants per 10^6 protoplasts were obtained.

As described, a total of 16ug of EcoRI restricted DNA was used over the eight transformations. Given that the $argB^+$ gene is on a 9-kb DNA fragment, and that the A.nidulans genome size is 31,000 kb, 16ug (16,000ng) of the EcoRI digested chromosomal DNA contains 16,000 x 9/31,000 = approximately 5ng of the 9-kb $argB^+$ DNA fragment. Since the use of 500ng of $trpC^+$ linear DNA in a transformation with pHELP1 gives about 3,000 TRP^+ transformants, one might predict that using 5ng of $trpC^+$ linear DNA one might obtain about 30 TRP^+ colonies. This is close to the number of seven ARG^+ colonies obtained in this experiment.

6.2.2 Characterisation of ARG^+ transformants

The seven ARG^+ transformants, designated IGB1-7, showed slightly irregular, heterokaryonlike colony morphologies, indicating unstable transformation. All seven colonies grew when stab inoculated onto MM plus methionine, whereas none grew on MM alone, suggesting that they were not contaminants. However, on MM plus methionine it was observed that while IGB1 and IGB3 grew well, the other transformants grew weakly. All were yA^-

Conidia were harvested and plated on CM. It was found that in the cases of IGB1 and IGB3, 99% and 100% respectively of progeny were ARG^+, indicating that these colonies were stably transformed. Tne remaining transformants showed 4%-38% ARG^+ progeny, indicating that these were unstably transformed. Full results are shown in Table 6.5

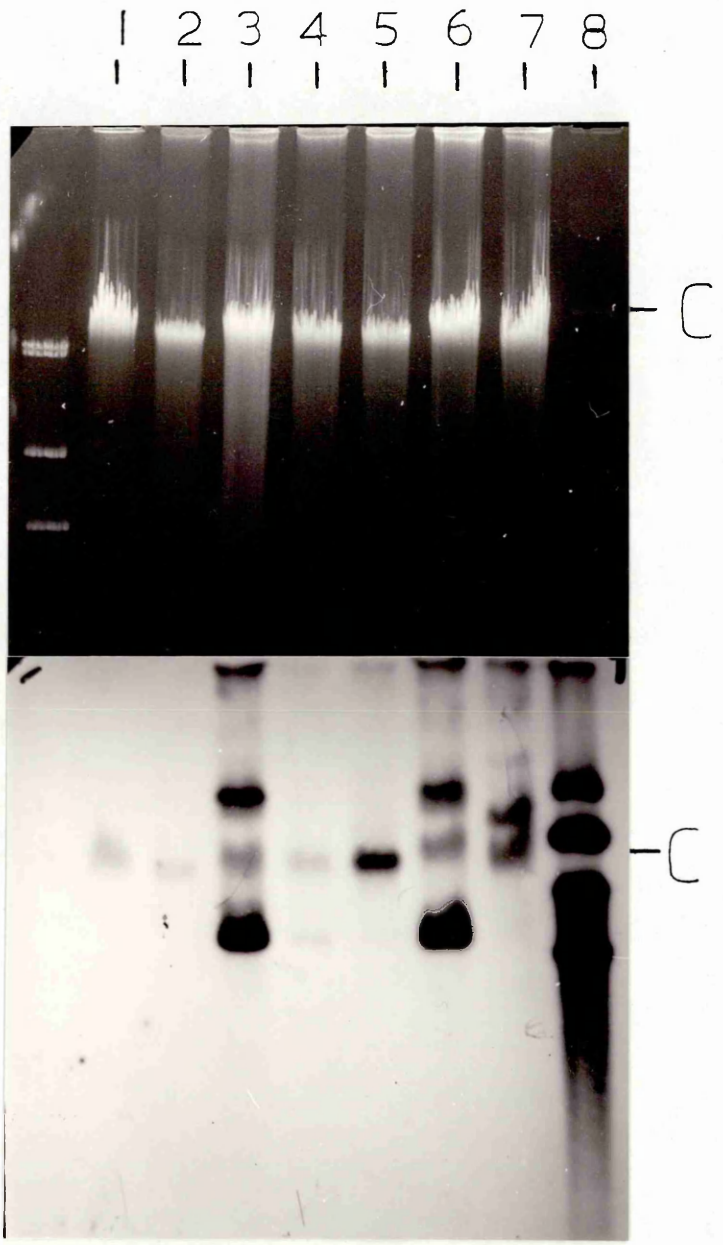

Figure 6.6 **Southern blot analysis of six ARG$^+$ Aspergillus transformants with an instant gene bank**

Uncut transformant DNA was probed with a radiolabelled 1.0-kb HindIII fragment containing argB gene sequences.

Lane 1) Untransformed A.nidulans strain 089, 2) IGB1, 3) IGB2, 4) IGB3, 5) IGB4, 6) IGB6, 7) IGB7, 8) ARp1 prepared from E.coli.
c, chromosomal DNA band.

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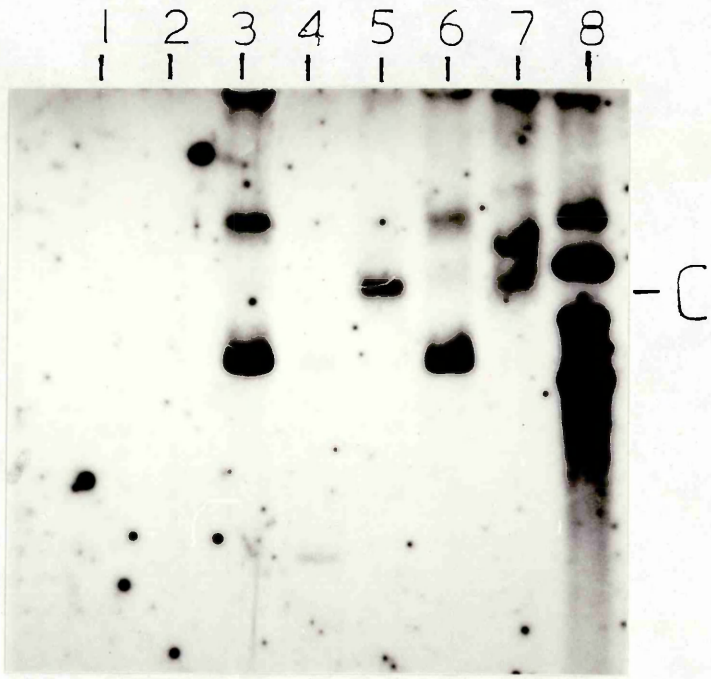

Figure 6.7 **Southern blot analysis of six ARG$^+$ Aspergillus transformants with an instant gene bank**

The Southern blot probed in Figure 6.6 was stripped and reprobed with radiolabelled pIC20-R.

Lane 1) Untransformed A.nidulans strain 089, 2) IGB1, 3) IGB1, 4) IGB3, 5) IGB4, 6) IGB6, 7) IGB7, 8) ARp1 prepared from E.coli.
c, chromosomal DNA band.

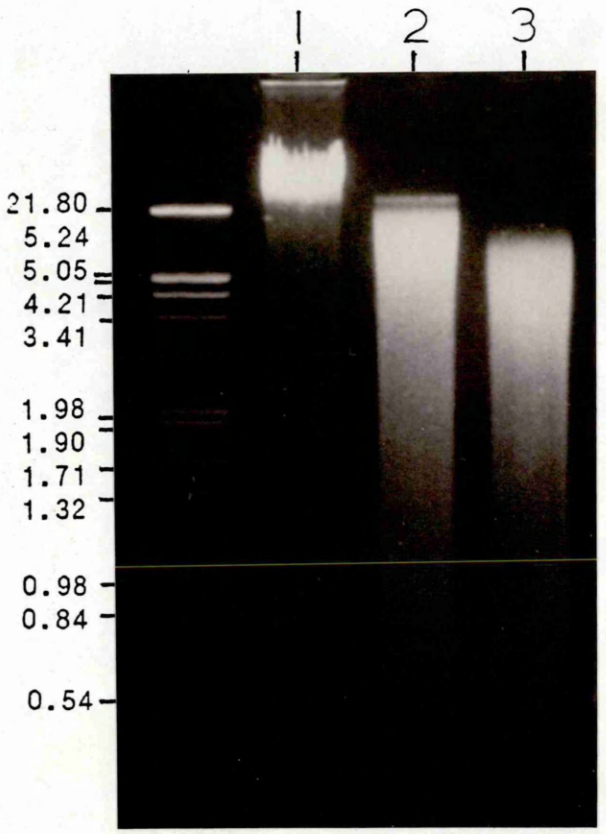

Figure 6.8. **Agarose gel electrophoresis of sonicated DNA of A.nidulans strain 089**

Lambda EcoRI/HindIII size standards are given in kb.

Lane 1) DNA prior to sonication, 2) sonicated DNA (output control 4, 1 second), 3) sonicated DNA (output control 6, 5 seconds).

The sonicated DNA shown in lane 3 was used to make an instant gene bank to clone trpB.

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6.3.3 Southern analysis of IGB strain DNAs

Chromosomal DNA was prepared from ARG$^+$ transformants IGB1, 2, 3, 4, 6 and 7, grown under conditions selecting for ARG$^+$. Approximately 5ug of each DNA was then run on a 0.8% agarose gel, and subjected to Southern blot analysis. The nylon membrane was probed first with a radiolabelled 1.0-kb HindIII DNA fragment containing argB sequences; the membrane was then stripped and reprobed with radiolabelled pIC20-R. Results are shown in Figs. 6.6 and 6.7. Where the filter was probed with argB sequences (Fig. 6.6) it can be seen that IGB2, 3, 4, 6 and 7 all contain a similar sized plasmid which is slightly larger than the ARp1 monomer run alongside - perhaps 13 - 15-kb. This plasmid varies greatly in abundance, and in IGB4 and 7 is only visible in the overexposed autoradiograph. IGB7 appears also to contain a larger plasmid which is not present in the other transformants, and which runs slightly behind the chromosomal DNA. The results of probing the filter with radiolabelled pIC20-R show hybridisation to chromosomal DNA in the case of IGB4, suggesting that plasmid integration had taken place. The lack of hybridisation to any DNA of IGB1 combined with the 100% stability of the ARG$^+$ phenotype of this transformant strain suggests either that gene conversion of the argB2 allele to argB$^+$ has occurred, or that integration of the linear DNA carrying the argB$^+$ gene taken place.

The predicted size of a cointegrate formed by non homologous recombination between a 9.4-kb EcoRI fragment and pHELP1 (7.9-kb) would be 16.9 kb. Southern analysis suggests that in the five transformants which contain them, identical plasmids of around 14 kb in size are seen. This suggests that, as in the case of recombination between trpC$^+$ linear DNA and pHELP1 a consistant deletion event may be occurring.

6.3.1 Transformation of an A.nidulans trpB403 strain with an instant gene bank

Chromosomal DNA prepared from A.nidulans strain G089 (fpaB37riboA1biA1) was sonicated to a mean DNA fragment size of approximately 5 kb. Sonication was carried out in a volume of 1ml using a DNA concentration of 200ng/ul in TE, and a Dawes Soniprobe 7532B sonicator with a microtip. This was set at output control 6, and sonication time was 5 seconds. Mean fragment size was ascertained by

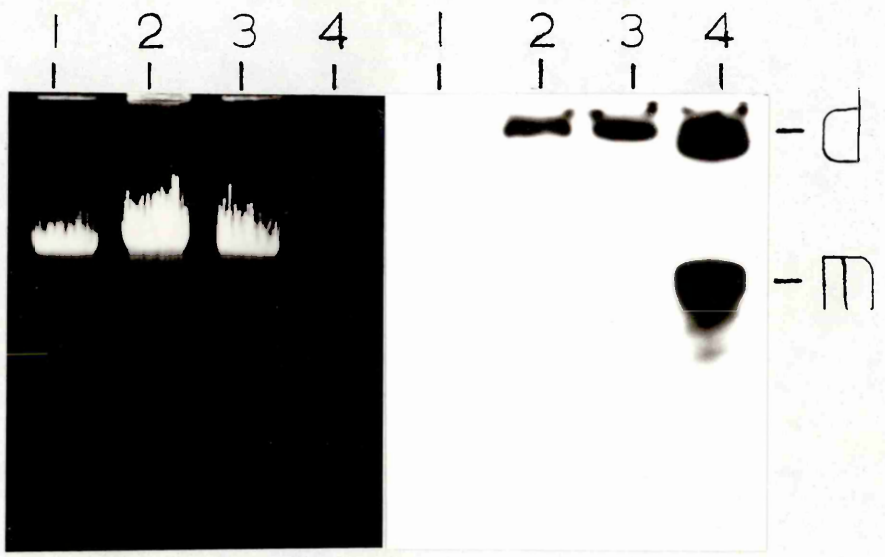

Figure 6.9. **Southern blot analysis of *trpB*$^{+}$ transformant IGBT**
Uncut transformant DNA was probed with radiolabelled pIC20-R.

Lane 1) Untransformed *A.nidulans* strain 089, 2), 3) IGBT, 4) ARp1 prepared from *E.coli*.
m, ARp1 monomer (11.5 kb); d, ARp1 dimer (23 kb).

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agarose gel electrophoresis (Fig. 6.8). This DNA was then extracted once against one volume of phenol, once against one volume of 50 : 50 phenol chloroform, and then three times against chloroform, before being ethanol precipitated and resuspended in TE at a concentration of 0.5ug/ul.

A.nidulans strain G196 (trpB403 pabaA1 yA2) was then transformed with the sonicated DNA and pHELP1 with selection for TRP^+. 16 transformations were carried out, using in each case $1x10^7$ protoplasts, 3ug of sonicated DNA and 3ug of pHELP1. One TRP^+ colony was obtained.

6.3.2 Characterisation of TRP^+ transformant

The transformant, designated IGBT, had an uneven, heterokaryonlike colony morphology, characteristic of unstable transformants with AMA1 plasmids. It grew when stab-inoculated onto MM plus PABA, but not on MM, and was $yA2^-$, suggesting that it was not a contaminant.

Conidia harvested from the TRP^+ transformant colony were plated on CM plus tryptophan, and progeny used to stab inoculate master plates, also on CM plus tryptophan. The phenotypes of the master plates were then examined, and 26% were found to be TRP^+ (sample size: 243). Thus the transforming $trpB^+$ gene is unstable, which given the results described in previous sections of this thesis, suggests that it may be present in transformants as part of an autonomously replicating plasmid, rather than integrated into the chromosome.

6.3.3 Southern analysis of IGBT DNA

DNA was prepared from strain IGBT grown under selective conditions (MM plus PABA). This was run uncut on a 0.8% agarose gel, with ARp1 and DNA from an untransformed strain of A.nidulans for comparison, and Southern blot analysis was carried out, using radiolabelled pIC20-R as a probe. Results are shown in Fig. 6.9. In the two tracks containing IGBT DNA the pIC20-R probe hybridises with a very high running plasmid, larger than the ARp1 dimer (23 kb). No hybridisation to chromosomal DNA of IGBT is seen, indicating that no pIC20-R sequences have integrated into the chromosome. No hybridisation to the DNA from the untransformed strain is seen either.

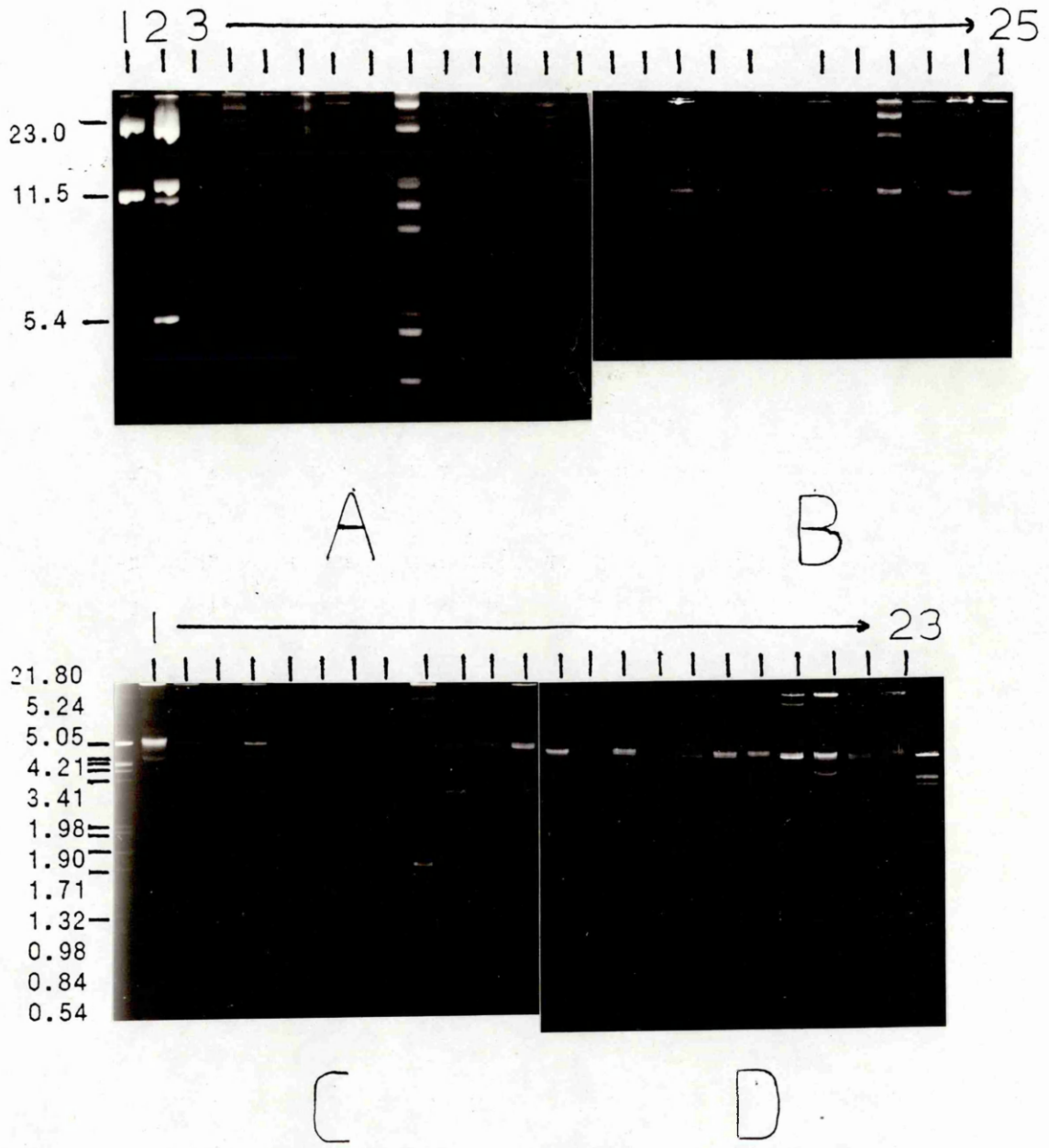

Figure 6.10. **Gel electrophoresis of plasmids rescued from IGBT**
Gels A and B: sizes of ARp1 and pILJ16 monomeric and dimeric cccDNA given in kb; gels C and D: lambda HindIII/EcoRI size standards are given in kb.

Gels A and B: Lane 1) ARp1, 2) pILJ16, 3)-25) plasmids prepared from 24 different amp^R E.coli colonies produced by transformation with uncut IGBT DNA.
Gels C and D: Lanes 1-23) The same 23 rescued mixtures of plasmids restricted with BamHI.

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6.3.4 Plasmid rescue from strain IGBT

Uncut DNA from strain IGBT was used to transform competent cells of E.coli strain DH5, selecting for AMP^R. 24 AMP^R colonies were obtained. DNA minipreps were carried out using the STET method, and DNAs were electrophoresed on agarose gels intact, and restricted with BamHI, which cuts once in pHELP1. The results, shown in Fig. 6.10, are complex, but suggest that many colonies contain as many as three different plasmids. The uncut DNA shows that the fastest running plasmid is considerably smaller than pILJ16 (5.4 kb). From the cut DNA it appears that as in the case of pDHG8 (Chapter 5) high and low abundance plasmids are present. This is clearly seen in the case of ptrpB10 (Fig. 6.10, gel C) where approximate sizes of the more or less abundant plasmids were measured as 25 kb and 8 kb respectively. The difference between the mixture of plasmids seen here, and the single high running band observed in the Southern blot suggest that, again, cointegrate resolution may have occurred, even though DH5 is a $recA^-$ strain.

Unfortunately the E.coli strain containing these plasmids became contaminated and had to be discarded, so no more plasmid DNA could be prepared.

6.3.5 Transformation of A.nidulans with rescued plasmids

STET miniprep DNAs of ptrpB1-24 were used to transform A.nidulans strain G196 selecting for TRP^+. Pools of four plasmid DNAs each were used for each transformation. Where the plasmids used were ptrpB21-24 a single TRP^+ colony was obtained. When examined it was found that 36% of progeny of this transformant were TRP^+ (sample size: 112), i.e. it appeared to be a genuine transformant.

The low frequency may reflect the relative impurity of plasmid DNAs produced using the STET miniprep method. However, the single unstable transformant obtained suggested that $trpB^+$ gene sequences were rescued into E.coli, and that the instant gene bank method is a viable technique for gene cloning.

Discussion

In chapters 4, 5, and 6 of this thesis it has been demonstrated that in transformations of *Aspergillus* with autonomously replicating plasmids and various non-replicating DNAs that cotransformation occurs at high frequency by means of recombination of the two transforming DNA species. Where no homology exists between cotransforming DNAs - e.g. in the cases of pHELP2 and pILJ16, pHELP1 and *trpC*$^+$, *argB*$^+$ or *trpB*$^+$ linear DNA - non-homologous recombination occurs. Where homology does exist, e.g. as it did between pILJ16, pILJ16 with the *adD*$^+$ gene insert, and pHELP3 in the formation of pDHG8a - homologous *or* non-homologous recombination may occur. Whether non-homologous recombination is a general property of transforming plasmids or is stimulated by AMA1 is unclear.

In transformations where AIp plasmids were cotransformed with helper plasmids maximum frequencies of transformation of 12,000 transformants per 10^6 protoplasts (pILJ16 and pHELP1) and 3,000 transformants per 10^6 protoplasts (pHELP1 and *trpC*$^+$ linear DNA) were seen. Given that of protoplasts used in transformations only 10-15% are capable of regenerating under non-selective conditions, these frequencies of transformation represent the transformations of 8.3%-12.5% and 2.1%-3.1% of all protoplasts respectively. In transformations with ARp1, 13-20% of all viable protoplasts are transformed.

As described in the introduction to chapter 4, integrative cotransformation of eukaryotic cells has been widely interpreted as signifying the existence of a small subpopulation of highly competent cells. With respect to transformation by autonomously replicating plasmids it would appear that a subpopulation of 13-20% of viable protoplasts are competent. On the other hand this figure may simply reflect the fact that in the transformation process each transformant is the product of the PEG induced fusion of, on average, 5-10 protoplasts, indicating that in fact all protoplasts capable of regeneration are competent to take up DNA.

It also appears that where near-saturating amounts of transforming DNA is used, multiple copies of transforming DNA sequences enter most cells, and that, in most cases, recombination between these sequences occurs. Given these observations, positing the existence of a tiny subpopulation of competent protoplasts to explain the occurrence of cotransformation in integrative transformation is superfluous. On the

other hand, it does not disprove the existence of a subpopulation of cells competent with respect to integration. However, it would also be sufficient to propose that there is simply a small probability that in any given cell which has taken up exogenous DNA, integration of that DNA into the chromosome will occur. In such a situation it is likely that recombination between plasmids would occur prior to such rare integration events.

In the introduction to chapter 4 of this thesis, four general observations about the mechanism of cotransformation were made. These loose rules can now be reassessed in the light of the evidence described in the results sections of chapters 4, 5 and 6. With respect to Rule I, that cotransformation usually involves recombination between cotransforming DNAs: it has been shown that in the cases examined cotransformation has occurred as the result of recombination of transforming DNAs to form plasmid cointegrates. With respect to Rule II, that homologous recombination will occur where homology exists, and that homologous recombination happens more readily than non-homologous recombination: although it has been shown that the presence of homology between cotransforming sequences does, as a rule, appear to increase cotransformation efficiency, non-homologous recombination also occurs at a high frequency. With respect to Rule IV, that unselected linear DNA will recombine with selected cccDNA resulting in cotransformation: cotransformatin of cccDNA with linear DNA does result from non-homologous recombination resulting in cointegrate formation. Since transformation of *trpC*$^+$ linear DNA alone did not give rise to transformation, it is not likely that any of the 3,000 TRP$^+$ colonies per 10^6 protoplasts seen were the result of integration of *trpC*$^+$ linear DNA into the chromosome. The results described do not have any bearing upon Rule III, that hindrances which delay homologous integration into the chromosome enhance cotransformation and multiple integration.

CHAPTER 7

Cloning the *adC*-*adD*-*ygA* gene cluster

Figure 7.1 **Mutants affecting pigmentation during conidiation.**

CONIDIOPHORE

Locus	Phenotype	Time of appearance of mRNA (hours)[1]	Gene product/ function
ivoA	*ivory*	20	AHT biosynthetic enzyme
ivoB	"	18	AHTase - cuprozinc phenol oxidase
ygA	"	?	Copper uptake/ distribution
brlA[2]	"	15	Gene regulatory Zn finger DNA binding protein
medA[2]	"	?	?

CONIDIAL

wA	*white*	30	?
wB	"	?	"
wetA	*wet-white*/autolysing conidia	25	Gene regulatory protein
yA	*yellow*	20.5	laccase I
yB	"	?	copper uptake/distribution
ygA	*yellow-green*	"	" " "
dilA	*dilute*	"	?
dilB	"	"	"
drkA	*dark*	"	"
drkB	"	"	"
chaA	*chartreuse*	"	"
bwA	*brown*	"	"
fwA	*fawn*	"	"
pA	*pale*	"	"

[1]Growth at 37°C.

[2]Also affects morphology.

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Introduction

The intensive research into the expression patterns of the brlA, abaA and wetA genes, and cDNAs produced from developmentally regulated transcripts has yielded a lot of valuable information about gene regulation. However, absolutely nothing has been discovered about the genetic basis of morphogenesis. brlA, abaA and wetA might as well be genes regulating, say, carbon catabolism. It is assumed that the genetic basis of conidiophore morphogenesis can be understood only by studying those structural genes regulated by brlA, abaA and wetA. However, mutant alleles of such genes have yet to be identified. The next best thing - models, if you like, for structural morphogenes - are the genes determining pigmentation: regulated structural genes with, in some cases, well characterised products. These include ivoA and ivoB (ivory) which are involved in conidiophore melanin biosynthesis; and yA and yB (yellow), and wA and wB (white), which give the dark-green conidial pigmentation. A seventh gene, ygA (yellow-green) affects pigmentation both of conidiophore and conidia. Genes determining pigmentation during conidiation identified to date are summarised in Fig. 7.1.

The yA gene encodes a p-diphenol oxidase (laccase I) the active form of which contains copper (Clutterbuck, 1972; Kurtz and Champe, 1982), and the production of which is developmentally regulated (Law and Timberlake, 1980). The product of wA is unknown, but wA^- mutants show a conidial wall deficiency (Claverie-Martin et al, 1986). wA mutations are epistatic to yA mutations (Pontecorvo et al, 1953), suggesting that the wA product catalyses the production of the yellow substrate of the yA gene product from a colourless substrate. However, yA is expressed before wA: only $brlA^-$ or $apsA^-$ mutants lack yA transcripts, whereas $brlA^-$, $abaA^-$, $wetA^-$ and $apsA^-$ lack wA transcripts (Yelton et al, 1985; O'Hara and Timberlake, 1989; Mayorga and Timberlake, 1990). The wB locus has only just been identified (Roper, 1990).

The ivoA/ivoB pigment, a complex polyphenolic melanin, is thought to tan the polysaccharide walls of the vesicle and sterigmata, thus consolidating it against degradation. The ivoA gene product is believed to catalyse the synthesis of N-acetyl-6-hydroxytryptophan (AHT) (McCorkindale et al, 1983). This is the substrate for the ivoB gene product, AHTase, a cuprizinc phenol oxidase. Synthesis of both mRNA and enzyme are developmentally regulated, the former appearing

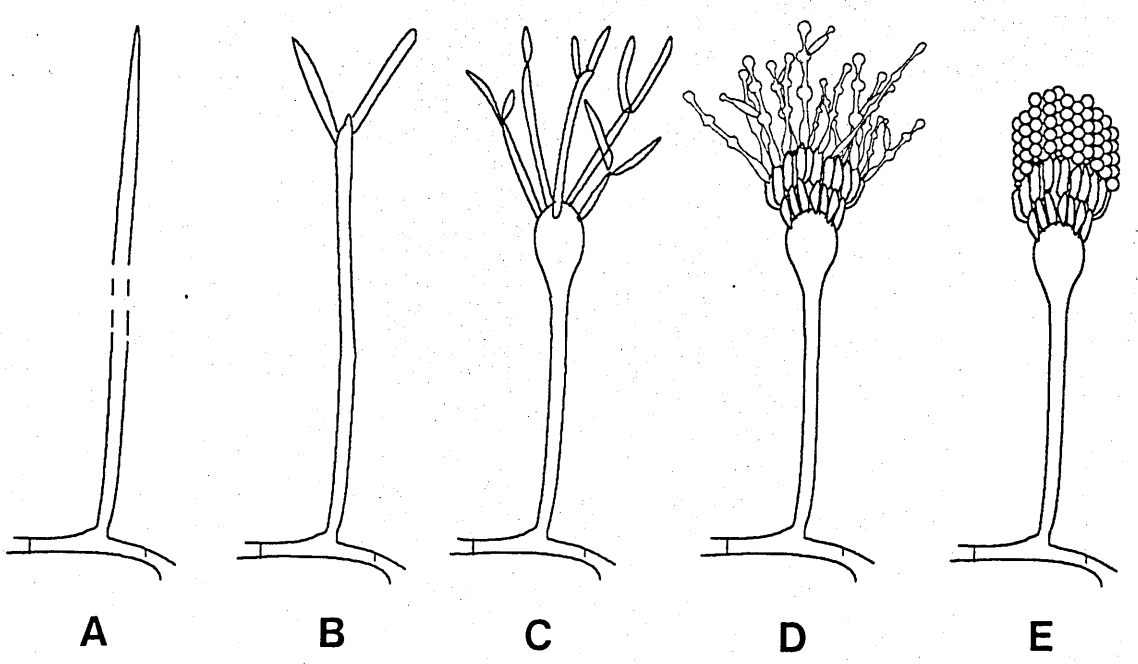
Figure 7.2 **Relationship between morphological development and AHTase and AHT accumulation in various brlA mutants.**

A: AHTase and AHT accumulation.

Relevant genotype	Morphology	Conidiophore melanin	AHTase*	AHT in ivoB63 derivative*
brlA1	E	+	16	34
brlA2	A	-	7	-
brlA3	A	-	7	0
brlA14	A	-	57	3
brlA19	A	-	85	0
brlA6	A-B	+	99	18
brlA10	B	-	72	2
brlA9	B	+	134	43
brlA7	B-C	+	192	23
brlA35	B-C	+	158	56
brlA42	C	+	100	100

* Levels calculated as percentages relative to brlA42 levels.

B: Morphology of wild-type and brlA and abaA mutants. A: brlA null mutant. B-C: Increasingly leaky brlA mutants. D: abaA mutant. E: wild-type. (Courtesy of A.J.Clutterbuck, 1990a).



after 20-24 hours of growth at 37°C - the time conidiophores first appear (Birse and Clutterbuck, 1990; 1991). *ivoA* transcript accumulation is also developmentally regulated, again, accumulating after 20 hours of growth (Griffith, pers. comm.).

One approach taken to examine the relationship between gene expression and morphogenesis has been to isolate a number of leaky *brlA* mutations resulting in a range of degrees of morphological development (Clutterbuck, 1969; 1990a). An attempt was then made to correlate precise degree of morphological development with the appearance of specific gene products and structural elements. These were AHT (produced by the *ivoA* gene product), AHTase (the *ivoB* gene product) and conidiophore melanin itself. The results are summarised in Fig. 7.2. It was found that in some mutants (*brlA*14, *brlA*19) AHTase accumulated in the absence of pigmentation or morphogenesis. In one (*brlA*6) there was pigmentation without morphogenesis. In others still (*brlA*10,*brlA*31) there was AHTase production, and a degree of morphogenesis, without pigmentation. However, none were identified which accumulated AHT, but no AHTase, i.e. where *ivoA* was identifiably expressed before *ivoB*. Unless *brlA*6 is ignored, these results preclude a simple model of *brlA* function where a single series of gradated developmental stages correspond to given degrees of *brlA* activity. Rather, they suggest that, if *brlA* activity directly regulates these other functions, it may have a separate regulatory specificity for each function.

With the cloning of the *ivoA* and *ivoB* genes, and the purification of the *ivoB* gene product, AHTase, already achieved (Birse and Clutterbuck, 1990; 1991; Griffith and Clutterbuck, pers.comm.), and identification of the *ivoA* gene product hopefully imminent, a much more precise correlation of degree of morphogenesis and developmentally regulated gene expression may be made. This can be done by examining the various leaky *brlA* mutants for transcript and gene product accumulation and, possibly, using antibody studies, developmental changes in the spatial locations of the gene products.

A range of leaky *brlA* mutants could also be used as the basis of a dissection of the conidiophore specific transcript population. cDNAs made from poly(A)$^+$RNA prepared from *brlA*1 strains (morphology A: see Fig. 7.2B) could be used in subtractive hybridisation from poly(A)$^+$RNA prepared from morphology B mutants (Timberlake, 1980). Then morphology B from morphology C, and so on. These poly(A)$^+$RNAs could then be used

a) to form cDNAs, to be cloned into plasmid vectors; or b) to prepare radiolabelled first strand cDNAs for use as probes to identify cDNA clones already made (Zimmerman et al, 1980) from conidiophore specific transcripts. Thus, cDNAs corresponding to genes activated at precise stages in conidiophore morphogenesis could be identified.

Both the medA and ygA genes also affect conidiophore pigmentation. In medA15;brlA42 ivoB63 strains accumulation of AHT is reduced by nearly 50% (Clutterbuck, 1990a), and conidiophores of medA$^-$ strains grown at 30^oC have an ivory phenotype.

Both conidial laccase and AHTase are partially deficient in ygA$^-$ mutants. Adding Cu^{++} to extracts of ygA, and ygB mutants restores full activity (Clutterbuck, 1972; 1990a; Kurtz and Champe, 1982). ivoA and ivoB mutants suppress the yellow-green spore colour phenotype of ygA, presumably because they don't excrete all the copper (AHTase is an extracellular enzyme). The ygA$^-$ spore colour phenotype is also pH sensitive: at low pH wild-type pigmentation (green) is seen; at high pH conidia are yellow. The ygA locus is located on linkage group II, 0.1 map units distal to the adD locus (Clutterbuck, 1981).

The ygA (and possibly ygB) product, then, is likely to be involved on uptake and distribution of copper ions. It was decided to clone the ygA gene primarily in order to see whether it is developmentally regulated. Although it seems likely that some system of copper distribution must exist for vegetative growth - and at least one vegetative phenol oxidase (hyphal tyrosinase) has been identified - the fact that two copper requiring enzymes are produced in large quantities during conidiation (AHTase is approximately 1% of total cell protein; Birse and Clutterbuck, 1990) suggests that a conidiation specific copper distribution system may exist. A role for transition metals in morphogenesis cannot be ruled out.

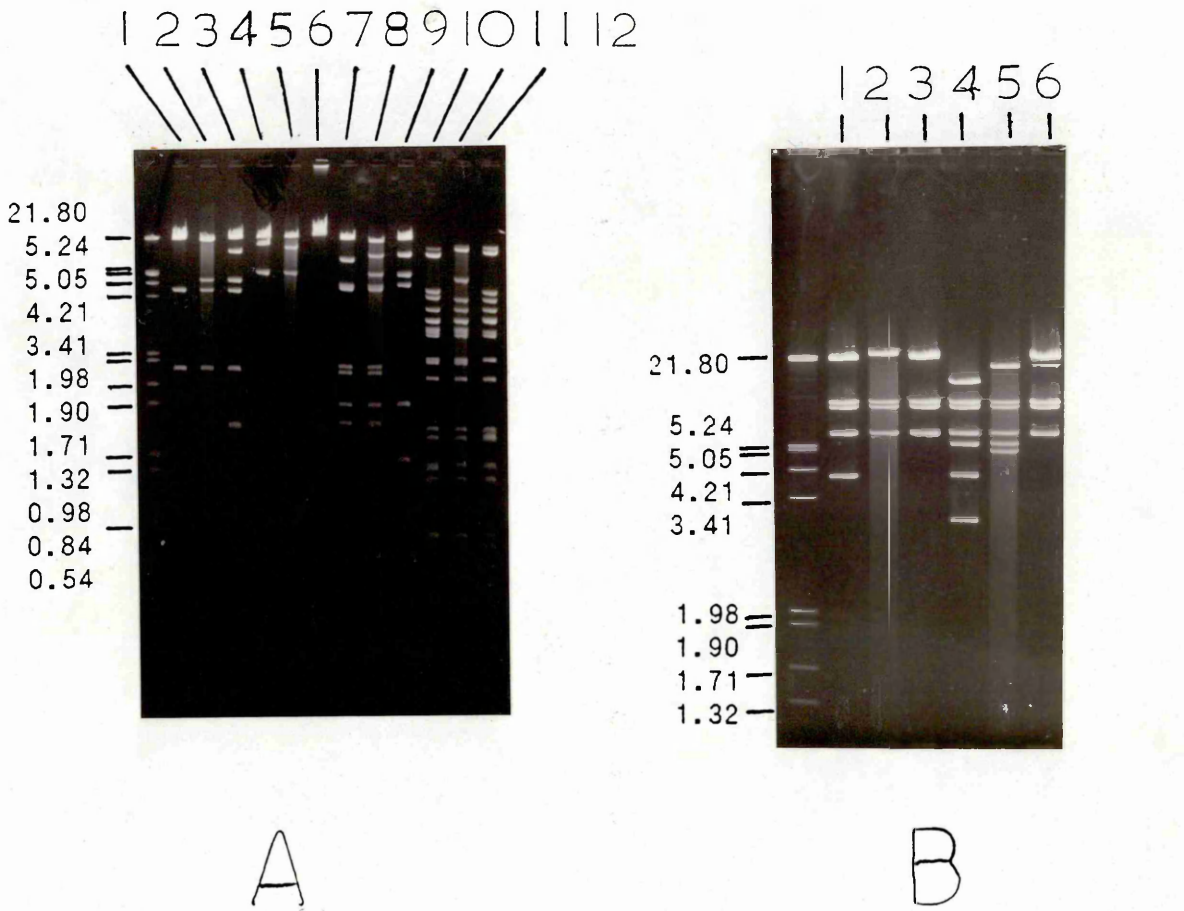


Figure 7.3. **Restriction digests of LadD6, LadD10 and LadD11**
Only significant digests are labelled. Lambda EcoRI/HindIII size standards are given in kb.

Gel A: Lanes 1 - 3) LadD6, LadD10 and LadD11, EcoRI, 4 - 6) LadD6, LadD10 and LadD11, BamHI, 7 - 9) LadD6, LadD10 and LadD11, HindIII, 10 - 12) LadD6, LadD10 and LadD11, PstI.

Gel B: Lanes 1 - 3) LadD6, LadD10 and LadD11, DraI, 4 - 6) LadD6, LadD10 and LadD11, DraI/BamHI.

facing page 118a

7.1. Screening an Aspergillus gene library in EMBL3 with pDHG8

In chapter 5 isolation of the plasmid mixture pDHG8, which contains adD gene sequences was described. A gene library of DNA from A.nidulans strain G089 was constructed in the lambda replacement vector EMBL3 (Frischauf et al, 1983) by Gareth Griffith. Phage were plated out using E.coli host strain NM621 to generate approximately 6,000 plaques on 10 x 10 petri plates. Given an average library insert size of about 20 kb this represents approximately four genomes of A.nidulans DNA. Lambda DNA was transferred to duplicate sets of nylon filters. One set of filters was probed with ARp1, and the other with pDHG8. HRP-labelled ARp1 and pDHG8 DNA was used.

Since both ARp1 and pDHG8 contain AMA1 and argB sequences, plaques hybridising to adD were identified as those which lit up with pDHG8, but not ARp1. 57 plaques lit up with both probes, i.e. hybridised with AMA1 or argB sequences. 12 plaques lit up with pDHG8 alone. The X-ray film was then aligned with the plates from which the probed filters were prepared, and the areas from which the adD-hybridising phage had come were picked (0.5 cm) and resuspended in SM buffer. These phage were then plated at a lower density (several hundred per plate) and reprobed with pDHG8. Of the original 12 areas picked, three yielded plaques which gave a signal. The other nine signals may have been caused by non-specific binding of probe. When phage from several individual plaques each of the three positive clones were plated at low density (approximately 30 per plate) all plaques hybridised with pDHG8.

DNA was extracted from these lambda clones, designated LadD6, LadD10 and LadD11.

7.2. Transformation of Aspergillus to AD^+ with LadD6, LadD10 and LadD11

A.nidulans strain G135 (yA2 luA1;adD3) was transformed separately with LadD6, LadD10 and LadD11, selecting for AD^+. In each case pHELP1 was added to the transformation. The numbers of resulting AD^+ transformants were 900, 560 and 720 per 10^6 protoplasts respectively. By comparison, a transformation with pDHG7 resulted in 2,000 AD^+ transformants per 10^6 protoplasts.

From the high frequency of transformations to AD^+ obtained it was concluded that a) all three lambda clones contain the adD^+ gene, and

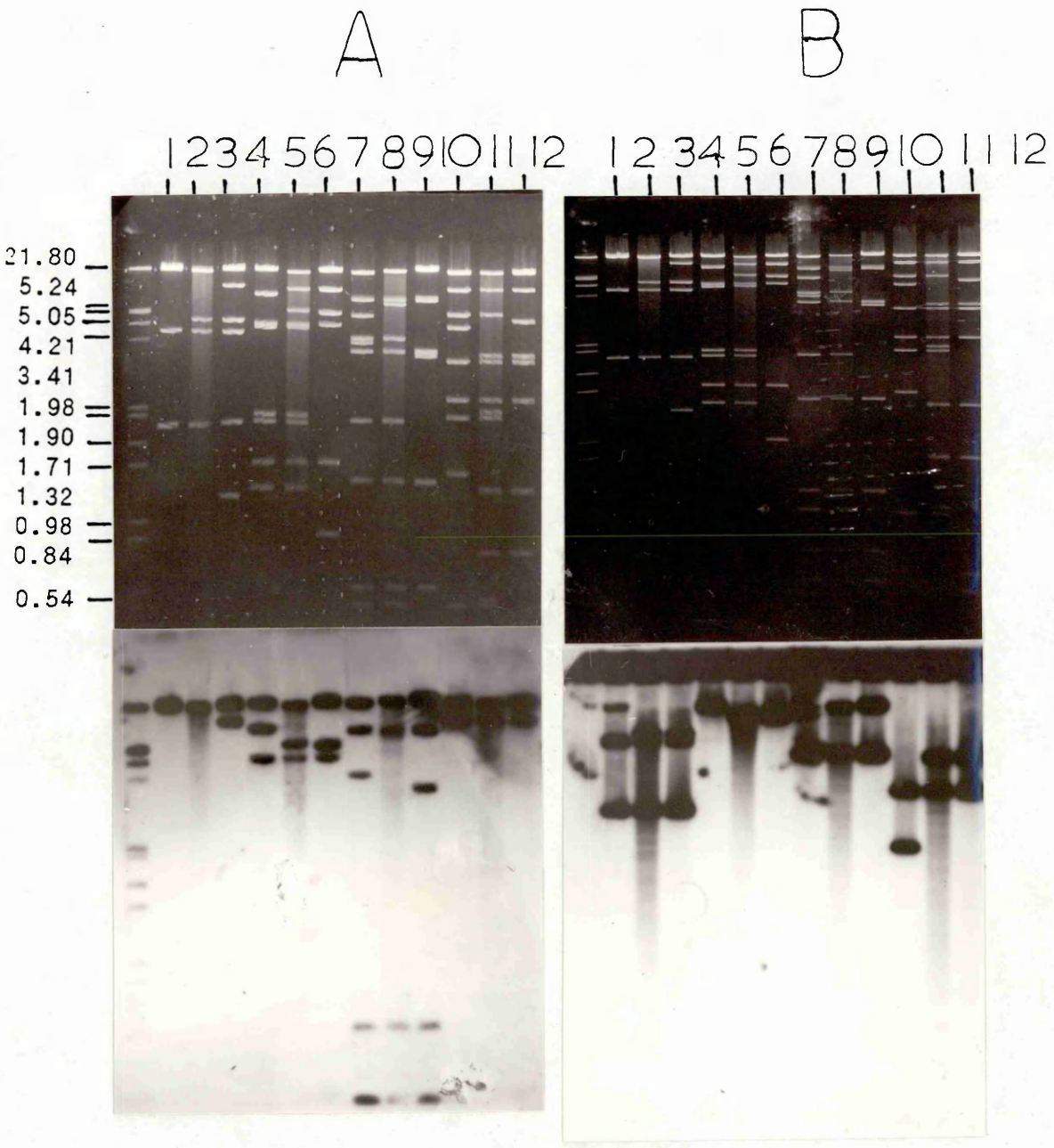


Figure 7.4. **Southern blot analysis of LadD6, LadD10 and LadD11**
Southern blots shown below correspond to gels above. Lambda EcoRI/HindIII size standards are given in kb.

A) Probe: Lambda DNA.

B) Probe: pDHG8.

In both cases restriction digests were as follows:

Lanes 1 - 3) LadD6, LadD10 and LadD11, EcoRI; 4 - 6) LadD6, LadD10 and LadD11, HindIII; 7 - 9) LadD6, LadD10 and LadD11, BglII; 10 - 12) LadD6, LadD10 and LadD11, SalI.

facing page 118b

Table 7.1. Sizes of restriction fragments of LadD6, LadD10 and LadD11 estimated from gels shown in Fig. 7.3 and Fig. 7.4.

Fragments hybridising with Lambda DNA are in bold, and those hybridising with pDHG8 and underlined. Fragments originating from the EMBL3 vector alone are bracketed.

Enzyme(s)	Restriction fragment sizes	Sum of Frag. sizes
	LadD6	
BamHI	**27, 14, 5.0, 0.5**	46.5
DraI	**19.8, (8.0), (7.6), (5.3), 3.8, (1.1), (1.0)**	46.6
SmaI	**(20), 12.5, (5.8), 4.6, 2.0, 1.8, 0.6, 0.5**	48.7
DraI/BamHI	**11.5, (8.0), (7.6), (5.3), 5.0, 4.0, 2.9, (1.1), (1.0), 0.5**	46.4
EcoRI/DraI	**10.4, (8.0), (7.6), (5.3), 3.8, 3.5, 1.7,(1.1),(1.0)**	42.5
BglII	**20, (6.4), 4.7, 3.5, 3.3, 3.0, 1.9, (1.3), (0.64), 0.58, (0.43), (0.39)**	46.14
EcoRI	**20.8, 20.6, 3.5, 1.5**	46.4
HindIII	**25, 7.4, (4.2), 4.0, 2.0, 1.9, 1.4, 1.2**	47.1
	LadD10	
BamHI	**28.8, 11.5, 5.0. 0.5**	45.8
DraI	**23, (8.0), (7.6), (5.3), (1.1), (1.0)**	46.0
SmaI	**(17), 10.0, 6.0, (5.5), 4.6, 2.0, 0.6, 0.5**	46.2
DraI/BamHI	**15.5, (8.0), (7.6), (5.3), 5.0, 4.5, (1.1), (1.0), 0.5**	48.5
BglII	**24.4, (6.4), 5.6, 3.5, 3.0, 1.9, (1.3), (0.64), 0.58, (0.43), (0.39)**	48.1
EcoRI	**18.0, 18.0, 4.3, 3.5, 1.5**	45.3
HindIII	**20, 8.0, 5.9, (4.2), 4.0, 2.0, 1.9, 1.4**	47.4
	LadD11	
BamHI	**42.7**	42.7
DraI	**18.8, (8.0), (7.6), (5.3), (1.1), (1.0)**	
SmaI	**(19.3), 9.4, (5.8), 4.6, 3.6**	43.0
DraI/BamHI	**18.8, (8.0), (7.6), (5.3), (1.1), (1.0)**	
BglII	**27.5, (6.4), 3.0, 2.9, (1.3), (0.64), (0.43), (0.39)**	42.6
EcoRI	**23, 8.8, 4.3, 3.5, 1.5, 1.1**	42.2
HindIII	**22.4, 8.0, 5.0, (4.2), 4.0**	43.6

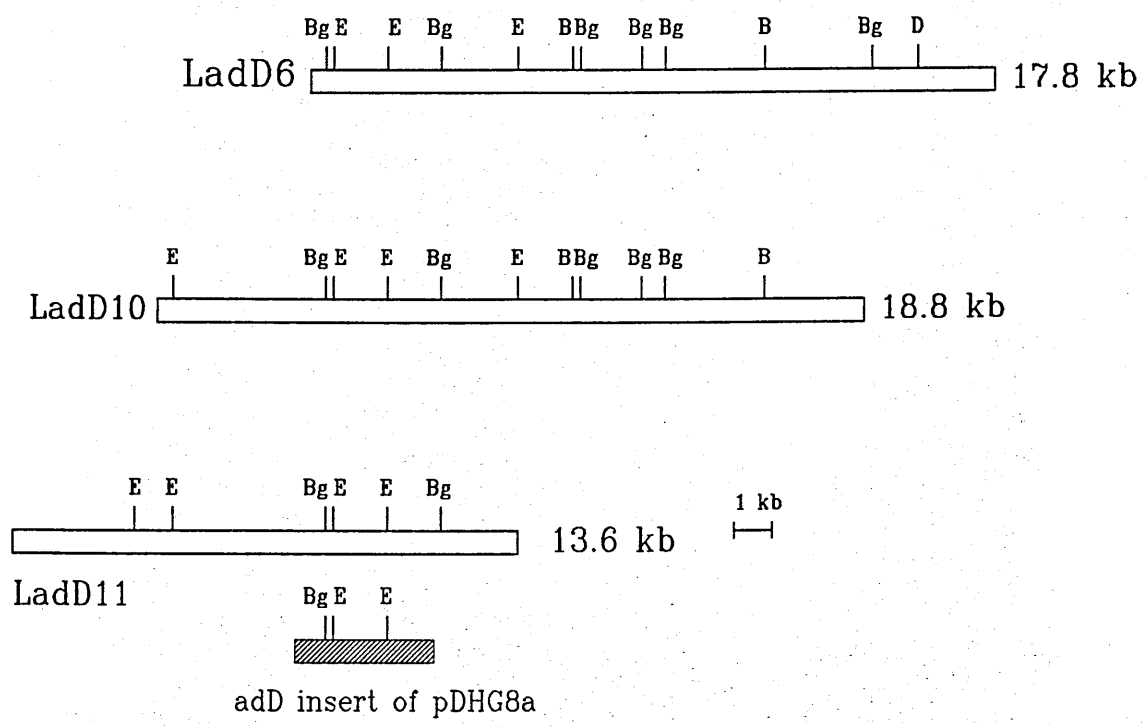


Figure 7.5. Restriction maps of Aspergillus DNA inserts of LadD6, LadD10 and LadD11

Note: LadD6 and LadD11 inserts contain a third BamHI site 0.5 kb from one of those two mapped.

Abbreviations for restriction enzyme sites: B, BamHI; Bg, BglII; C, ClaI; D, DraI; E, EcoRI; Sm, SmaI.

facing page 119a

b) ARp cointegrates were forming by recombination between linear DNA and pHELP1, implying that the adD gene on the lambda insert can function without interaction with the chromosome and therefore in each case the entire adD gene is contained within the lambda DNA insert.

In a similar manner A.nidulans strain G225 (yA2 pyroA4; adC1) was cotransformed with the three lambda clones and pHELP1, selecting for AD^+. LadD6, LadD10 and LadD11 gave 400, 120 and 500 AD^+ transformants per 10^6 protoplasts respectively. Again, it was concluded that each clone contains the adC^+ gene in its entirety.

7.3. Restriction mapping of LadD6, LadD10 and LadD11

Results of digests of LadD6, LadD10 and LadD11, and estimations of fragment sizes are shown in Fig. 7.3 and Table 7.1. Southern analysis of restricted DNA was also carried out (Fig. 7.4) using pDHG8 and lambda DNA probes.

From this data restriction maps, shown in Fig. 7.5, were constructed. LadD6, LadD10 and LadD11 contain inserts of sizes 17.8 kb, 18.8 kb and 13.6 kb respectively, all of which are in the same orientation relative to the EMBL3 vector. pDHG8 hybridises to the 3.0-kb BglII fragment which is left-most in the LadD6 insert, as shown (Fig. 7.5).

7.4. Localisation of adD and adC

pDHG7 DNA was restricted with a variety of enzymes and then used to transform A.nidulans strain G135 (adD3) to AD^+. pHELP1 was added to the transformation. That DNA cleavage had occurred to completion was checked by AGE. Results are displayed in Table 7.2. EcoRI, PstI and XhoI restriction abolished gene function, whereas HindIII and BglII did not. SalI restriction reduces gene activity by one order of magnitiude but does not entirely abolish it.

LadD6 was then restricted with SmaI, and with ClaI and, again, used to transform strain G135 to AD^+ (Table 7.2). Neither of these enzymes abolished gene function.

LadD6 was then restricted with EcoRI, SmaI and BglII and used to transform A.nidulans strain G225 (adC1) to AD^+. It was found that, like adD^+ EcoRI restriction abolished gene function, whereas SmaI and BglII did not.

That BglII does not abolish adD or adC function suggests that both

Table 7.2. Transformation of Aspergillus to AD^+ using pDHG7, LadD6 or pYG12

Two strains, G135 (adD3) and G225 (adC1) were transformed with the DNAs uncut, or cleaved with various restriction enzymes. Enzyme was removed prior to transformation by phenol/chloroform extraction which was followed by EtOH precipitation.

G135

NUMBER OF TRANSFORMANTS / 10^6 PROTOPLASTS

Transforming DNA	ENZYME									Helper
	-	HindIII	EcoRI	BglII	PstI	SalI	XhoI	ClaI	SmaI	
pDHG7	88	137	0	75	1.1	3.8	0.1	-	-	ARp1
LadD6	250	30	-	-	-	-	-	110	-	pHELP1
"	60	-	-	-	-	-	-	-	25	"
pYG12	20	-	0	-	-	6	2	-	16	"

G225

LadD6	500	-	8	120	-	-	-	-	130	pHELP1

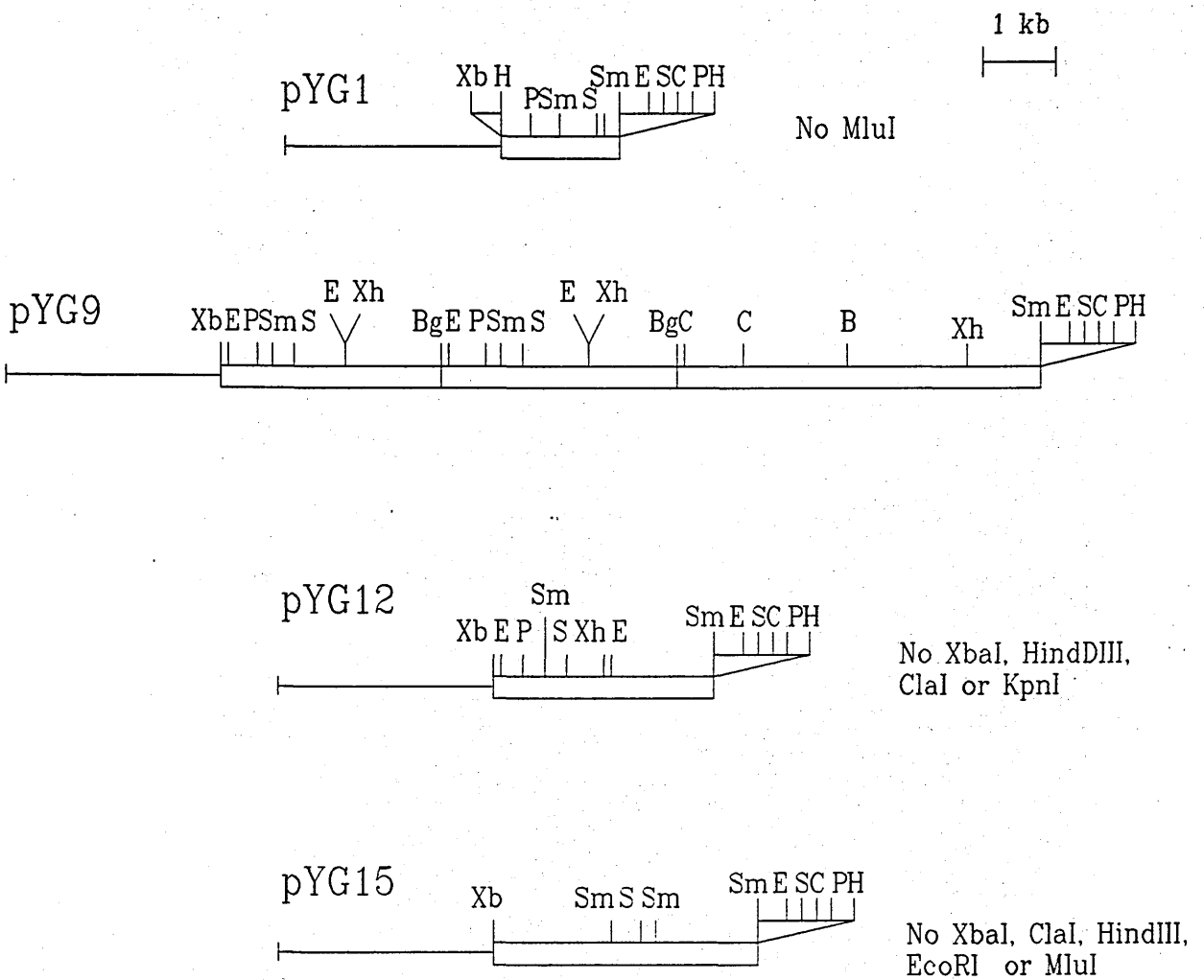


Figure 7.6. **Restriction maps of subclones of LadD6**
These subclones were produced by ligation of BglII fragments of LadD6 into the BamHI site of pBLUESCRIPTII KS+. Enzymes which did not cleave insert DNA are shown in brackets on the right. Note that pYG9 contains three BglII fragment inserts: the left hand two are the same as those in pYG12; the right hand one is that shown in Fig. 7.5.

Abbreviations for restriction enzyme sites: B, BamHI; Bg, BglII; C, ClaI; D, E, EcoRI; Sm, SmaI; Xb, XbaI; Xh, XhoI.

genes lie on a BglII fragment. Southern analysis (Fig. 7.4) suggests that both genes are contained within a 3.0-kb BglII fragment. Gareth Griffith ligated BglII fragments of LadD6 into the BamI site of pBLUESCRIPTII KS+. The ligation mix was transformed into E.coli strains DS941 selecting for white amp^R colonies containing recombinant clones on Xgal/IPTG by DHG. Four subclones of the LadD6 insert, pYG1, pYG9, pYG12 and pYG15 were isolated and restriction mapped (Fig. 7.6).

Since pYG12 contained the 3.0-kb BglII fragment where adD and adC were believed to be located, this was used to transform A.nidulans strains G135 (adD3) and G225 (adC1) to AD^+. pHELP1 was added to all transformations. pYG12 gave 20 adD^+, and 25 adC^+ transformants per 10^6 protoplasts. Transformation with pHELP1 gave no transformants.

The 3.0-kb insert of pYG12 contains both adD and adC. Since adD gene function is abolished by XhoI and EcoRI, but not by PstI or Sma, and greatly reduced by SalI, the gene appears to extend from the SalI site to some point on the right of the EcoRI site, as shown in Fig. 7.7. Similarly adC function is abolished by EcoRI, but not SmaI. This suggests that adD and adC are the same gene. However, complementation studies (Foley et al, 1965), and by DHG (data not shown) indicate that these *are* two separate genes.

7.5. Transformation of Aspergillus to ygA^+ with LadD6 and LadD10

A.nidulans strain DHG124 (ygA6; thiA4; argB2; methB3) was cotransformed with LadD6, LadD10 and LadD11, and ARp1, selecting for ARG^+. In three transformations it was found that where LadD6 and LadD10 were used, ygA^+ colonies were seen at low frequency. No ygA^+ colonies were seen using LadD11. Frequency of cotransformation to ygA^+ was difficult to estimate since ygA^+ colonies were only seen on transformation plates with confluent growth, but was generally of the order of 0.1 - 0.5%.

These results imply that a) the ygA gene is contained in its entirety in LadD6 and LadD10, and b), that it lies to the right of the 3.0-kb BglII fragment containing the adD and adC genes, as drawn in Fig. 7.5.

Further cotransformations using LadD6 DNA restricted with various enzymes, and ARp1, were carried out in an attempt to localise the ygA^+ gene. Over three cotransformations of strain DHG124 it was found

Table 7.4. Appearance of *ygA*$^{+}$ progeny of ARp1/LadD cotransformants

STRAIN	APPEARANCE
TLadD6.1	Green with yellow speckles
TLadD6.2	" "
TLadD6.3	" "
TLadD6.4	" "
TLadD10.1	Dark green
TLadD10.2	" "
TLadD10.3	Green with yellow speckles
TLadD10.4	" "

Table 7.5. Stability of asexual progeny of unstable asexual progeny of *ygA*$^+$ cotransformants

Conidia from *ygA*$^+$ cotransformants were plated at a density of approximately 50 / plate on CM, and on CM plus arginine. On CM ARG$^-$ strains do not conidiate, therefore the phenotype of ARG$^-$ progeny with respect to *ygA* is unknown.

Strain	Medium	Sample size	*ygA*$^+$	ARG$^+$	%ARG$^+$	*ygA*$^+$: % of ARG$^+$	*ygA*$^+$: % of total
TLadD10.1.1	CM	>100	>100		-	-	100
"	CM + arg	"	"		-	-	"
TLadD10.1.2	CM	"	"		-	-	"
"	CM + arg	"	"		-	-	"
TLadD10.1.3	CM	"	"		-	-	"
"	CM + arg	"	"		-	-	"
TLadD10.1.4	CM	"	"		-	-	"
"	CM + arg	"	"		-	-	"
TLadD6.1.1	CM	87	35		46	76	40
"	CM + arg	30	12		-	-	40
TLadD6.1.2	CM	77	21		21	100	27
"	CM + arg	71	15		-	-	21
TLadD6.1.3	CM	67	11		13	85	16
"	CM + arg	59	16		-	-	27
TLadD6.1.4	CM	49	12		40	30	24
"	CM + arg	60	18		-	-	30

that where digestion with XhoI and HindIII abolished ygA$^+$ activity, ygA$^+$ colonies were found among ARG$^+$ transformants using LadD6 DNA restricted with BamHI, ClaI, BglII and SmaI at a very low frequency (,0.5%). However, when cotransformations of strain DHG124 were carried out using pYG1, pYG9, pYG12 and pYG15, and ARp1, no ygA$^+$ colonies were seen among the ARG$^+$ colonies.

7.6. Characterisation of ygA$^+$ transformants

Conidia from four ygA$^+$ transformants of strain DHG124 each with ARp1/LadD6, and ARp1/LadD10, were plated on CM, and CM supplemented with arginine. The proportions of ARG$^+$ and ygA$^+$ progeny were counted; the results are displayed in Table 7.3. In all cases it can be seen that the ygA$^+$ phenotype is mitotically unstable: <0.5 - 40% of progeny are ygA$^+$. Furthermore, the proportion of ygA$^+$ colonies scored on CM, and CM plus arginine is similar; this is so even though on CM most of the colonies are ARG$^-$, and cannot be classified with respect to ygA, strongly indicating that the ARG$^-$ colonies are also ygA$^-$. This is reflected in the high proportion of ARG$^+$ ygA$^+$ colonies.

Variation in appearance of ygA$^+$ colonies was also observed (Table 7.4). In six out of eight cases ygA$^+$ progeny appeared green with yellow speckles. This was reminiscent of progeny of ARp1/pCEB218 (ivoB$^+$) cotransformants, which were brown with white speckles. This colour heterogeneity presumably reflects the heterogeneity of the colony with respect to ygA or ivoB, whichever the case may be. In two cases colonies were dark green, indicating that stabilisation of the ygA$^+$ phenotype may have occurred.

This was confirmed by testing the stability of the ygA$^+$ phenotype in four progeny each of TLadD6.1, TLadD6.4 and TLadD10.1. The results are displayed in Table 7.5. As can be seen all progeny of TLadD10.1 progeny were ygA$^+$, whereas all progeny of TLadD6.1 and TLadD6.4 were unstable (average: 28% ygA$^+$). The stability of the ygA$^+$ phenotype in the case of progeny of TLadD10.1 may reflect integration of ygA$^+$ sequences into the chromosome.

Table 7.3. Analysis of progeny of *ygA*$^+$ cotransformants with ARp1 and LadD6 and LadD11

Conidia from *ygA*$^+$ cotransformants of strain DHG124 were plated at a density of approximately 50 / plate on CM, and on CM plus arginine. On CM ARG$^-$ strains do not conidiate, therefore the phenotype of ARG$^-$ progeny with respect to *ygA* is unknown.

Strain	Medium	Sample size	ARG$^+$	*ygA*$^+$	*ygA*$^+$: % ARG$^+$	*ygA*$^+$: % total
TLadD6.1	CM	300	NR[1]	7	-	2
"	CM + arg	30	-	1	-	3
TLadD6.2	CM	160	NR	1	-	0.6
"	CM + arg	-	-	NR	-	-
TLadD6.3	CM	49	8	1	13	2
"	CM + arg	250	-	2	-	0.8
TLadD6.4	CM	115	12	11	92	10
"	CM + arg	115	-	12	-	10
TLadD10.1	CM	25	11	10	91	40
"	CM + arg	120	-	40	-	43
TLadD10.2	CM	40	10	3	30	7.5
"	CM + arg	159	-	1	-	0.6
TLadD10.3	CM	36	8	3	38	8
"	CM + arg	200	-	0	-	<0.5
TLadD10.4	CM	200	NR	4	-	2
"	CM + arg	-	-	NR	-	-

[1]NR: not recorded.

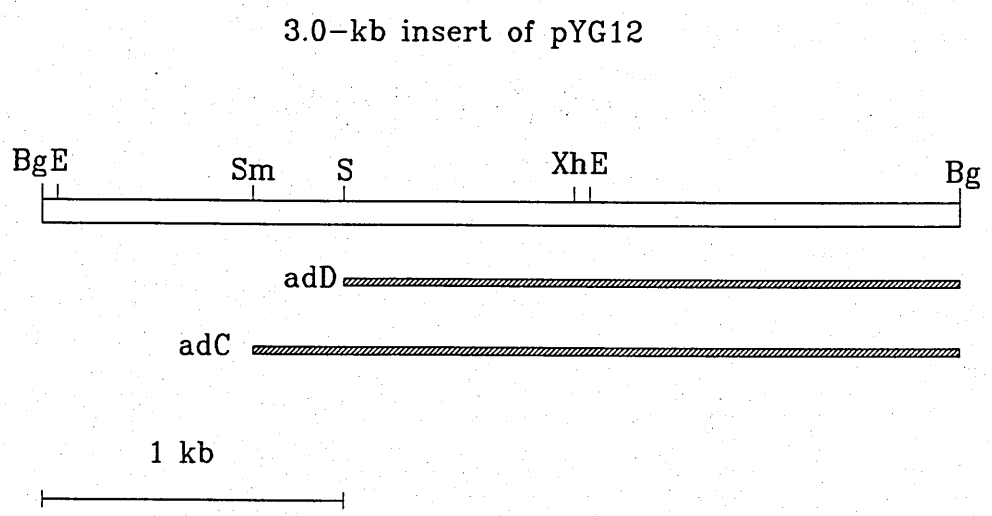


Figure 7.7. Restriction map of pYG12 A.nidulans DNA insert showing extent of region which contains adD and adC.

The open box represents the 3.0-kb insert of pYG12, and the cross-hatched boxes the minimum regions in which adD and adC lie.
Abbreviations for restriction sites: Bg, BglII; E, EcoRI; S, SalI; Sm, SmaI; Xh, XhoI.

Discussion

This chapter describes the isolation of three lambda library elements with homology to adD gene sequences. All three contain the adD and adC genes. Two, LadD6 and LadD10, also contain the conidiophore pigmentation gene ygA.

The A.nidulans DNA insert of pYG12 contains both adD and adC, apparently in the same stretch of DNA. This suggests that adD and adC are the same gene, encoding the enzyme AIR carboxylase, but that this enzyme has more than one active site. Thus mutations at adD and adC lead to loss of function at only one of the two sites, allowing complementation between adD3 adC^+ and adD^+ adC^+ mutants in heterokaryons. Alternatively, it may be that AIR carboxylase is dimeric, or oligomeric. In this case combination of mutant adD and adC monomer might result in a functional enzyme.

The frequency of cotransformation to ygA^+ using adD6 and adD10, and ARp1 was unusually low (,0.5%), possibly indicating that an incomplete copy of the ygA gene had been cloned. Two considerations speak against this: firstly, transformation with LadD10 gives ygA^+ colonies, and the LadD6 insert extends further rightward than LadD10 (as shown in Fig. 7.5). This suggests that if LadD10 did not contain the entire gene, then LadD6 would - unless the latter contains a double insert of Aspergillus DNA. Secondly, in stability studies ARG^+ ygA^+ cotransformants both LadD6 and LadD10 behave as if they contain ARp1/LadD plasmid cointegrates. If this is so, to complement the ygA6 mutation both LadD6 and LadD10 must contain the entire ygA gene. Given these two considerations, the reason for the low frequency of cotransformation to ygA^+ is unclear.

Given this low frequency of cotransformation, a practical way to subclone the ygA gene would be to use LadD6 or LadD10 to construct a DNA minilibrary. This could be done by ligating LadD DNA, partially digested with SauIIIa, into the BamHI site of pILJ16 or pDHG25. Plasmids containing the ygA gene could then be identified either by transformation of DHG124 to ARG^+ ygA^+ with large numbers of different plasmid minipreparations, or by sib selection.

CHAPTER 8

sthA and sthB: polygenes determining conidiophore morphogenesis

Introduction

A central, essentially unsolved problem of biology is how morphogenesis is determined. It is supposed that information determining morphology is contained within the genome, expression of which occurs during development, thus determining morphogenesis. This idea underlies the main approach of the molecular genetics to the investigation of morphogenesis, which is as follows. If genes determine morphology, then mutation in those genes may result in abberant morphogenesis, and large numbers of morphological mutations have indeed been identified. Using molecular biological methodology such genes can be cloned, and they and their protein products characterised. It is hoped that an understanding of such protein products, their biochemistry and effects on cell biology should reveal the processes which underlie morphogenesis. This approach has been entirely successful in understanding virion morphogenesis, e.g. that of the tobacco mosaic virus (TMV) and bacteriophage T4.

This has also been the main thrust of research into the development of the Aspergillus conidiophore. In addition Timberlake (1980) compared poly(A)$^+$RNA populations of vegetative mycelium with developing Aspergillus cultures, and found that where 6,000 possible mRNA transcripts were present in the former, an additional 1,200 appeared in the latter, 200 of which were present only in conidia. Given the above arguments, it could be postulated that among the 1,000 possible mRNAs which appear during growth of the conidiophore, some are transcribed from genes specifically involved in determining its morphology.

Martinelli and Clutterbuck (1971) estimated the number of loci involved in conidiation by comparing the frequency of mutation at such loci with those of other loci with known functions. 45-150 genes involved specifically in conidiation, and not vegetative functions were counted. When a large number of such mutants were examined, very few possessed clearly defined alterations of morphology which one might predict from alteration or destruction of the function of a morphology determining gene. 85% were found simply to conidiate rather poorly (oligosporogenous mutants) without clear alterations of morphology. However, a few such loci producing interesting alterations in mutants were identified, e.g. medA, stuA, apsA and apsB (see main Introduction). 13% did not produce conidiophores at all. 2.3% were

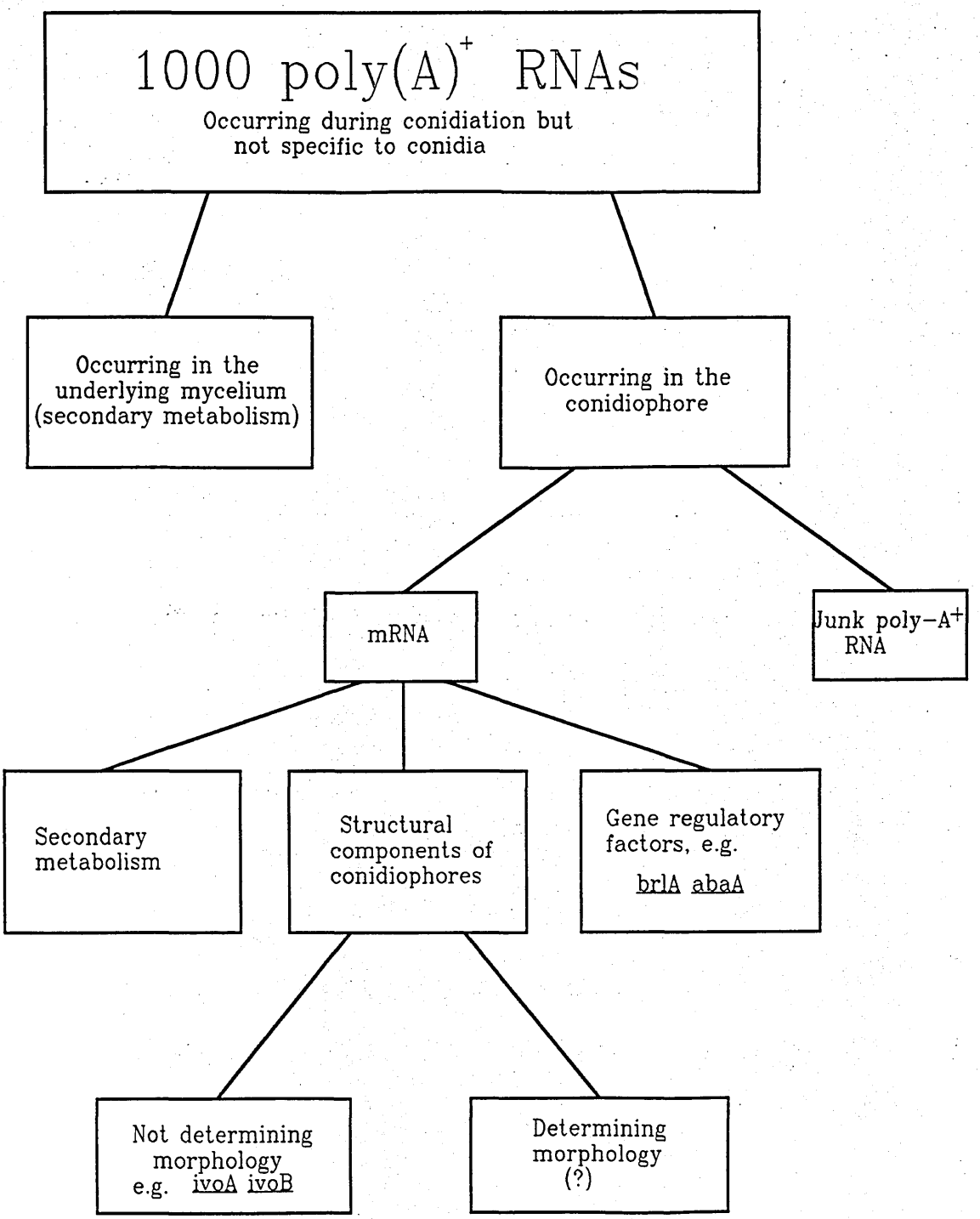


Figure 8.1. **Scheme showing possible identities of 1,000 mRNAs appearing during Aspergillus conidiophore development**

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affected in conidial germination or pigmentation. Only 0.3% showed clear morphological transformations resulting in an aconidial conidiophore. These were the loci brlA and abaA (see thesis Introduction). Both brlA and abaA were isolated separately at least 30 times, without any other mutations falling into the same category being identified, an indication that there are no more to be found.

The brlA and abaA genes have both been cloned (Johnstone et al, 1985; Boylan et al, 1987) and sequenced (Adams et al, 1988; Mirabito et al, 1989). Sequence data suggests that the brlA and abaA genes encode DNA binding proteins which regulate other genes, rather than interacting with structural elements of the cell in such a way as to exert a morphogenetic effect.

The large discrepancy between the number of poly(A)$^+$RNA transcripts appearing during conidiation, but not specific to the conidia (1,000), and the number of conidiophore morphology mutants was unexpected. What are the possible identities of the genes from which these 1,000 poly(A)$^+$RNAs are transcribed? Firstly, many may be concerned with secondary metabolism, both in the conidiophore, and in the underlying mycelium (Fig. 8.1). Secondly, some may encode structural proteins, changes of expression of which either a) cause morphogenesis, or b), like ivoA and ivoB, do not affect morphology. Thirdly, genes which regulate the expression of such structural genes - such as brlA and abaA. Finally, some transcripts may not have any function - i.e. be pseudo-mRNAs. This possibility is raised by the fact that the deletion of the SpoC1 gene cluster, from which 14 developmentally regulated mRNAs are transcribed, does not result in any detectable alteration of phenotype (Aramayo et al, 1989).

Given that morphogenesis is clearly a complex process it is perhaps surprising that no mutants of conidiophore morphology-determining structural genes have been clearly identified. There are a number of possible explanations for this. Firstly, that they may not exist. In this case the simple molecular genetic approach to morphogenesis is invalid. In their analysis of mutational frequencies, Martinelli and Clutterbuck (1971) initally estimated that 300-1,000 loci were concerned with conidiation. However, further examination showed that 85% of such mutants were also defective in vegetative growth. This suggests that genetic determination of morphogenesis involves changes in synthesis, processing and distribution of the same structural

components that determine hyphal morphology. This would mean that, of the 1,000 transcripts mentioned, only those from regulatory genes like brlA and abaA are important to morphogenesis.

A second possible explanation is that the determination of morphology is polygenic. Mutation at such loci might result in mild, oligosporogenous phenotypes, or no phenotype at all. However, the presence of mutations in several such genes in a single organism might result in major morphological alterations. One possible candidate for such a gene is medA (medusa, see main Introduction). The medusa mutant phenotype is unusual: rather than being blocked at a particular stage in conidiogenesis, a slight delay in the development of phialides from metulae occurs, as if the mutation were leaky, rather than null. However, no null mutations at medA have ever been found.

In this chapter the identification and characterisation of a group of gene loci exerting similar effects on conidiophore morphology, which appear to interact with medA, is described. Some of their properties indicate that they may be polygenes determining morphology.

ras

An oncogene is a gene the mutation of which causes cellular transformation, which may result in the formation of a tumour, benign or malignant. ras Oncogenes were first identified as the oncogenic components of Harvey and Kirsten murine (rat) sarcoma viruses, designated v-ras^H and v-ras^K respectively (Ellis et al, 1982). The proto-oncogene c-ras has been detected in a wide variety of vertebrate species, and encodes a 21,000 molecular weight protein ($p21^{ras}$). $p21^{ras}$ is a plasma membrane bound GTPase which has a role in signal transduction pathways used by several growth factors, including platelet derived growth factor (PDGF). Signal transduction occurs via the deactivation by phosphorylation of a GTPase-activating protein (GAP) by the tyrosine kinase of the membrane bound growth factor receptor. Normally GAP depresses the activity of $p21^{ras}$ by catalysing conversion of the mitogenic p21-GTP to the inactive p21-GDP (Molloy et al, 1989). Mutated forms of ras have been identified in a number of human cancers, which are unable to convert p21-GTP to p21-GDP at all, and thus remain fixed in the mitogenic form.

Two genes closely related to ras, RAS1 and RAS2, have been identified and isolated from S.cerevisiae (DeFeo-Jones et al, 1983),

and homologies to three others have been identified. RAS proteins from yeast exhibit many of the properties of their vertebrate counterparts e.g. GDP-GTP binding and GTPase activity and location in the plasma membrane. Furthermore a modified yeast RAS1 gene has been shown to transform NIH-3T3 cells, which subsequently led to metastasis in nude mice (Bradley et al, 1986). In yeast RAS1 and RAS2 proteins have been found to be positive effectors of cAMP dependant protein kinase activity.

ras1 ras2 mutants of yeast fail to germinate. Studies of the changes of levels of RAS1 and RAS2 mRNA and protein during growth suggest that these genes are involved in regulating cell growth (Breviario et al, 1988). RAS1 ras2 mutants hypersporulate in rich medium, indicating that the RAS2 protein may prevent sporulation in non-starvation conditions.

Since ras genes have been shown to be involved in growth of organisms as unrelated as yeast and man it seems quite likely that they have a role in the growth and development of Aspergillus.

v-myc was first isolated from chicken myelocytomatosis virus MC29 (Graf and Beng, 1978). c-myc encodes a nuclear protein, and it is believed that its expression confers proliferation competence to various endodermal and mesodermal cell types, and is switched off during terminal differentiation (Schmid et al, 1989). c-myc is homologous to the delilah gene of Antirrhinum majus, which regulates genes involved in flower pigment biosynthesis (Cathy Martin, pers.comm.)

v-rel was isolated from an oncogenic reticuloendotheiosis virus of turkeys (Theilin et al, 1966). v-rel is homologous to the dorsal gene of D.melanogaster which regulates the development of dorsoventral polarity in the developing embryo.

Results

8.1.1 Isolation of a *medA*^{ts} mutant

In order to characterise the *medusa* mutation it was decided to generate a large number of new *medA* mutants and look for variations in the strength of the mutant phenotype. It has been observed that on four day old colonies of *brlA*42 mutant strains grown at 37^{o}C, large numbers of spontaneous *medA* mutants appear as white tufts growing out of the surface of the colony (Clutterbuck, pers.comm., Fig. 8.2). 19 such outgrowths were picked from a four day old colony of *A.nidulans* strain AJC9.4 (*pabaA*1; *argB*2; *brlA*42 *ivoB*63) using a sterile dissection needle, and plated on CM plus arginine. These were subcultured three times at 37^{o}C in order to purifiy the *medA*$^{-}$ strain, and designated DHG031-DHG049. In order to obtain *medA*$^{-}$ strains against a *brlA*$^{+}$ background, and to check that these mutants were indeed defective with respect to the *medA* gene, DHG031-DHG049 were crossed with *A.nidulans* strain G12 (*fpaB*37 *adF*17 *pabaA*1 *yA*2). The *fpaB* locus is located 2.7 map units distal to the *medA* locus.

In all cases the *medusa* phenotype was seen amongst the progeny, and analysis of the frequency of progeny types showed close linkage between the *fpaB* locus and the new mutant gene, confirming that it was *medA* in all cases. Master plates were then inoculated with one of each of the new *medA* mutants, denoted *medA*1-*medA*19. When these had grown, replica plates were incubated at 30^{o}C and 37^{o}C on CM plus adenine and arginine. 18 out of 19 strains appeared identical to previous *medA*$^{-}$ strains examined, with an aconidial phenotype at 30^{o}C, and a leaky *bristle* type phenotype at 37^{o}C. Strain DHG051 (*medA*11 *pabaA*1 *yA*2) however, had a *medA*$^{+}$ phenotype at 30^{o}C, and a *medA*$^{-}$ phenotype at 37^{o}C. This allele was designated *medA*11^{ts}. No other variation between mutants was detected.

8.1.2 Isolation of *ultramedusa* mutants

It was observed that similar white outgrowth appeared out of the surface of *medA*$^{-}$ colonies grown at 37^{o}C for more than four days (Fig. 8.2). 17 of these were picked from the surface of three *A.nidulans* strains, DHG054 (*medA*13 *adF*17 *pabaA*1), DHG055 (*medA*14 *adF*17 *yA*2) and DHG056 (*medA*17 *adF*17).These were purified three times by subculture onto CM at 37^{o}C.

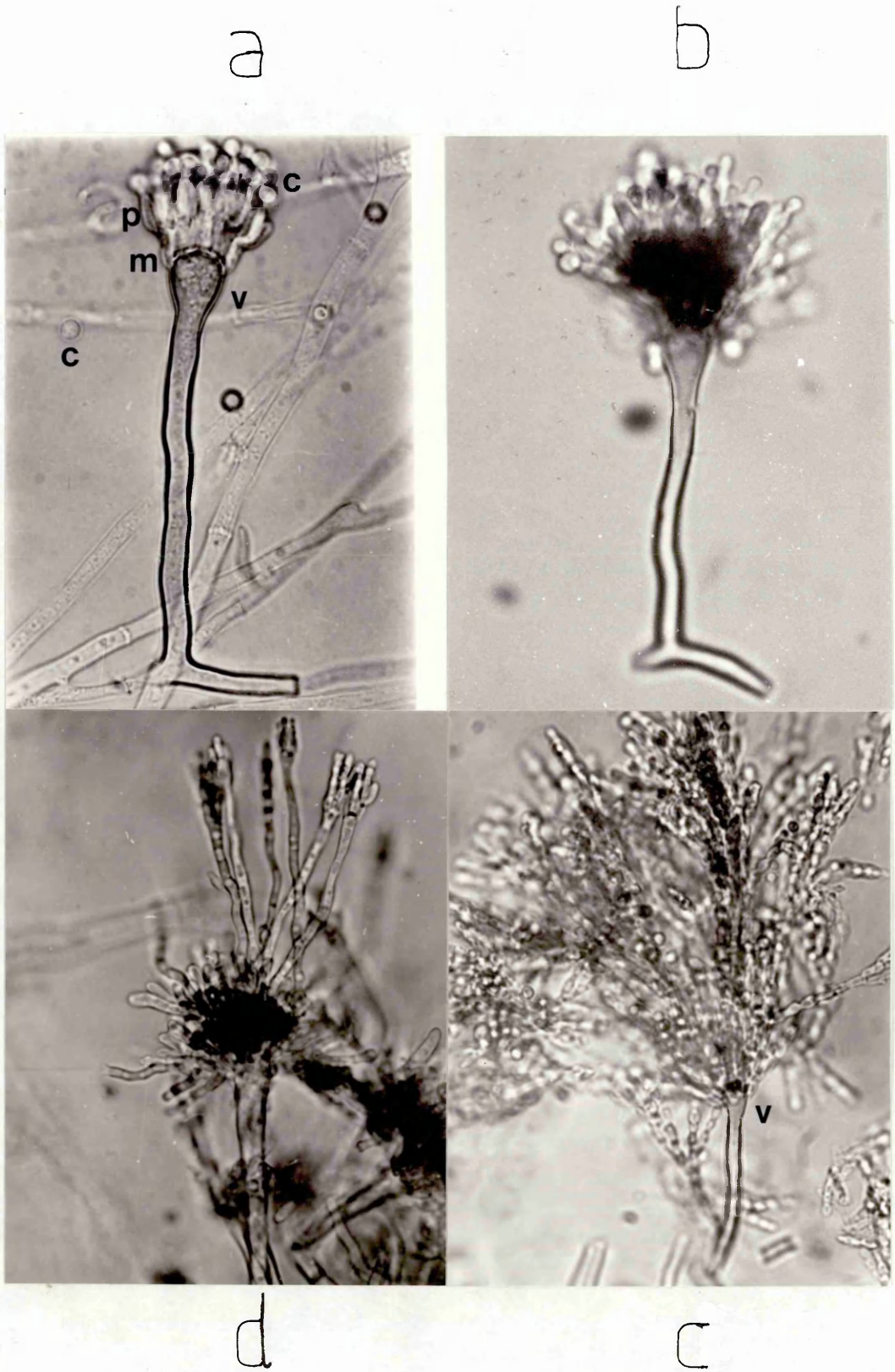

Figure 8.3. **Morphological mutants of** A.nidulans

All grown at 37°C.

a) wild type, b) medusa, c) ultramedusa, d) brlA42.

c, conidium; m, metula; p, phialide; v, vesicle.

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Light microscopy at 700x magnification showed that in all cases the conidiophores of these new mutants had an extreme morphological abnormality (Fig 8.3C). In medusa mutants, instead of the normal two tiers of metulae underlying the sporogenous phialide cells, upward of five tiers are seen, with multiple branching, topped by phialides and conidia. In these new mutants normal vesicles formed on which grew large, extremely elongated metulae with no phialides or conidia, although colonies take on a greenish hue after about three days growth at 37°C. Sometimes at the tips of these enlarged metulae, vesicles formed out of which grew more enlarged metulae (Fig. 8.3D). This mutant phenotype, designated "ultramedusa", resemblanced the leaky bristle mutant brlA42, grown at 37°C (Fig. 8.3E). The gene(s) mutation of which changes the medusa to the ultramedusa phenotype was designated "sthenyo" (sth). In ancient Greek mythology Sthenyo was the name of one of the sisters of the gorgon Medusa (Riley, 1851).

8.1.3 Genetic analysis of the sthenyo mutation

In order to see whether sth$^-$ mutants have a detectable phenotype against a medA$^+$ background, two ultramedusa strains were crossed with a medA$^+$ strain GO112 (methB3, pyroA4, yA2). These strains were DHG072 (yA2; pyroA4; medA17 sth-1), and DHG060 (medA13 adF17 pabaA1 sth-2). Ascospores were plated on CM. It was found that in the case of the cross with DHG072, 28% of progeny were medusa, 17% were ultramedusa, and 56% had a wild-type conidiophore morphology. In the case of the cross with DHG060, 13% of progeny had a medusa phenotype, 38% an ultramedusa phenotype, and 50% had a wild-type conidiophore morphology. The ratio of morphological phenotypes seen (approximately 25%, 25% and 50%) strongly suggested that the medA$^+$, sth$^-$ progeny have a wild-type morphology.

In order to test this hypothesis, two progeny of the DHG072 x GO112 cross with wild-type morphology, and four from the DHG060 x GO112 cross were back crossed with a medA$^-$ strain DHG054 (medA13 adF17 pabaA1). Ultramedusa colonies were seen among the progeny of one of the two strains derived from DHG076, and two of the four derived from DHG060. Thus medA$^+$, sth$^-$ strains do indeed have a wild-type morphology. No new nutritional mutant phenotype was detected in these strains. The strains with wild-type appearances identified by the back crosses described were designated DHG082 (sth-1 methB3) and DHG080 (sth-2 methB3 yA2).

Table 8.1 **Haploidisation mapping of the sth-1 and sth-2 loci.**

Analysis of segregants formed from master strain G695, and DHG087 (medA17,sth-1) and DHG088 (medA13,sth-2). Only medA$^-$ segregants, where the genotype with respect to sth may be known, were scored.

Linkage group	I		II		III		IV		V		VI		VII		VIII	
	yA		acrA		galA		pyroA		facA		sB		nicB		riboB	
Progeny	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
STH-1$^+$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
STH-1$^-$	55	0	24	31	30	25	30	25	19	36	35	20	35	18	28	22
STH-2$^+$	12	0	4	8	3	9	6	6	0	12	7	5	0	12	7	5
STH-2$^-$	12	0	7	5	8	4	6	6	12	0	5	7	12	0	6	6

By elimination, the sth-1 appears to be on linkage group I. The sth-2 locus is either on linkage group V or VII.

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It was also observed that all three medA$^+$ sth$^-$ strains identified, if grown at 30^{o}C, gave rise to white outgrowths, presumably spontaneous medA$^-$ mutations. These were not seen in any of the sth$^+$ strains. Thus, it is possible to identify sth$^-$ medA$^+$ mutants.

8.1.4 Gene mapping of the two sth loci

Diploids were prepared from a master strain (MSF), carrying markers on all linkage groups: G095 (yA2 suA1adE20 adE20; acrA1; galA1;pyroA4;facA303; sB3;nicB8;riboB2, McCully and Forbes, 1965), and two ultramedusa strains, DHG087 (medA17 sth-1 adF17) and DHG088 (medA13 adF17;sth-2). These latter strains were progeny from the crosses with strain G0112 described in section 8.1.3. They were used because they are genetically pure, each being derived from a single ascospore.

The results of the analysis of the products of haploidisation of the two diploids are presented in Table 8.1. In the case of the haploid segregants from the G695 + DHG087 diploid, all of the ultramedusa segregants were yA$^+$. This is hardly surprising since the medA and yA loci are both on linkage group I. However, since no wild-type allele of the markers identifying the other seven linkage groups was found to segregate exclusively with medA17 and sth-1, and since no medA$^-$ sth-1$^+$ progeny were seen at all, it was surmised that the sth-1 locus is located on linkage group I.

sth-2 appeared to segregate with the facA$^+$ and nicB$^+$ alleles, and it was inferred that the sthB locus was likely to be situated either on linkage group V or VII. A further possibility was that a linkage group V-VII translocation had occurred.

These results show that sth-1 and sth-2 are not allelic; therefore sth-1 was redesignated sthA1, and sth-2, sthB1.

Sexual crosses were then carried out. sthA1 strain DHG090 (medA17 sthA1 biA1;pyroA4) was crossed with a number of strains carrying mutations in genes on linkage group I. These were G197 (lysF88 suA1adE20 adE20 pabaA1 biA1), G141 (proA1,adF9, pabaA1,biA1) and G115 (riboA1 adG14 luA1 yA2). The results are summarised in Table 8.2.

No clear linkage was seen between sthA and riboA, adG or luA. However, weak linkage is seen to yA and proA, and strong linkage to lysF, adF and pabaA, where average recombination frequencies of 8.9%, 3.4% and 2.9% respectively were seen. This suggests that the

Table 8.2 Recombination between the *sthA* gene and linkage group I markers.

Cross parents	Cross markers	RECOMBINANTS STH^+	RECOMBINANTS STH^-	PARENTALS STH^+	PARENTALS STH^-	% Recomb.	Chi^2	P.
DHG090 x G115	*riboA*	7	12	3	21	44.2	0.58	>0.05
DHG090 x G115	*adG*	11	32	3	18	67.2	28.90	<0.001
DHG090 x G115	*luA*	5	10	1	12	53.6	0.14	>0.05
DHG090 x G115	*yA*	1	-[a]	8	-[a]	11.0	5.44	<0.05
DHG090 x G115	*proA*	3	1	1	11	25.0	5.05	<0.05
DHG090 x G141	*proA*	6	5	-[b]	49	9.3	24.70	<0.001
DHG090 x G197	*lysF*	2	15	12	161	8.9	128.08	<0.001
DHG090 x G141	*adF*	-[b]	2	-[b]	51	3.8	45.30	<0.001
DHG090 x G141	*adF*	-[b]	4	-[b]	128	3.0	116.48	<0.001
DHG090 x G141	*pabaA*	-[b]	2	-[b]	52	3.7	46.30	<0.001
DHG090 x G197	*pabaA*	-[b]	4	-[b]	186	2.1	174.34	<0.001

[a]Phenotype with respect to *yA* not clear.

[b]*medA*$^+$*sthA*$^+$ and *medA*$^-$*sthA*$^+$ not readily distinguishable in this case.

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Table 8.3 **Progeny of a four marker sexual cross involving group I markers, and sthA.**

adF$^-$ progeny were ignored since adF$^-$ acts as a partial suppressor of the medA$^-$ phenotype.

	A	B	C			
	proA	adF	pabaA	sthA	Number scored	Cross-over
	+	+	+	+	5	C
	+	+	+	−	47	P
	+	+	−	+	1	B
	+	+	−	−	0	BC
	−	+	+	+	0	AC
	−	+	+	−	3	A
	−	+	−	+	1	AB
	−	+	−	−	0	ABC

A,B and C represent the following crossover events:

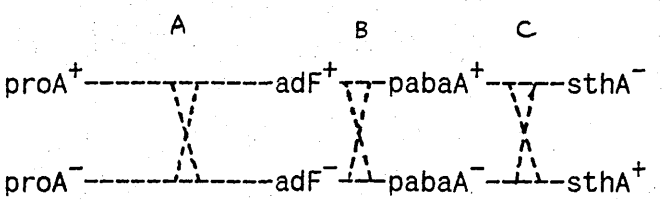

Table 8.4 Recombination between the sthA gene in crosses with linkage group V and VII markers.

LINKAGE GROUP V

Cross parents	Cross markers	Recombinants STH^+	Recombinants STH^-	Parentals STH^+	Parentals STH^-	% Recomb.	Chi^2	P.
DHG085 x G53	nicA	15	8	49	32	22.1%	32.35	<0.001
DHG085 x G76	riboD	10	14	12	11	51.0%	0.02	>0.05
DHG085 x G53	facA	6	12	9	13	45.0%	0.40	>0.05
DHG085 x G52	lysB	32	12	15	11	62.9%	4.63	<0.05
DHG085 x G523	cysA	2	5	15	25	14.9%	23.17	<0.001

LINKAGE GROUP VII

Cross parents	Cross markers	Recombinants STH^+	Recombinants STH^-	Parentals STH^+	Parentals STH^-	% Recomb.	Chi^2	P.
DHG085 x G712	nicB	24	35	19	23	58.4%	2.86	>0.05
DHG085 x G712	malA	6	10	1	15	50.0%	0.00	>0.05

Linkage is seen with group V markers nicA and cysA. No linkage is seen with the two group VII markers studied.

sthA locus is situated 2.9 map units distal to the pabaA locus (Fig. 8.4).

This was confirmed by examination of the phenotypes of progeny of a four-marker cross between strains DHG090, and G141 (Table 8.3). Here it can be seen that the phenotypes of 8/10 recombinant progeny correspond to those predicted from single cross-over events between the four genes arranged in the order proA-adF-pabaA-sthA. One recombinant corresponding to a double crossover is also seen. Most of the progeny scored were $sthA^-$, since it was not easy to distinguish $medA^-sthA^+$ and $medA^+sthA^+$. All adF^- had to be ignored as it was found that adF^- acts as a partial suppressor of the $medA^-$ phenotype.

To map sthB, sexual crosses were carried out between strain DHG085 (medA14 biA1;pyroA4;sthB1) and a number of strains carrying mutations on linkage groups V and VII. These were G53 (yA1; nicA2 pA3 facA303 riboD5; methB3), G52 (adG14 yA2;lysB5) and G523 (proA1 pabaA1 yA2;cysA1), and G712 (yA2; palD8 nicB8 wetA6 malA1). The results are summarised in Table 8.4.

Genetic linkage was detected to linkage group V markers nicA (22.1%) and cysA (14.9%), but not to lysB, which lies 4 map units proximal to nicA. Thus the sthB locus is likely to lie distal both to the cysA and nicA loci (Fig. 8.4).

8.1.5 Preliminary gene mapping of a further five sth genes.

A further five ultramedusa isolates were crossed with strains carrying markers located in the vicinity of the sthA and sthB loci. DHG065 (medA14 yA2 biA1;sth-65) and DHG066-70 (all medA13 biA;sth) were crossed with G197, G151, G141, G53, G122 and G175 (see section 8.1.4 for genotypes). The results are displayed in Table 8.5, where the unmapped sth^- alleles are denoted sth-65-sth-70.

Recombination frequencies show 27% genetic linkage between nicA (linkage group V) and sth-68; this compares with a 23% linkage between nicA and sthB1, suggesting that sth-68 is allelic with sthB1. sth-68 was therefore provisionally redesignated sthB2.

sth-69, like sthA1, showed strong linkage to adF, pabaA, and proA, indicating that sth-69 is allelic with sthA1. sth-69 was therefore redesignated sthA2. sth-67 and sth-70 both showed a similar degree of linkage to pabaA: 24% and 19% respectively. This suggests that

Table 8.5 Recombination between sth-66 -sth-70 and linkage groups I and V markers.

Cross markers	RECOMBINANTS sth-65$^+$sth-65$^-$		PARENTALS sth-65$^+$sth-65$^-$		% Recombs.	Chi^2	P.
nicA	0	16	0	21	43%	0.67	>0.05
adE	1	15	0	8	62%	2.13	>0.05
proA	1	14	0	8	65%	1.64	>0.05
	sth-66$^+$sth-66$^-$		sth-66$^+$sth-66$^-$				
nicA	19	46	40	12	56%	1.44	>0.05
adF	3	21	0	24	50%	0.00	>0.05
pabaA	11	36	15	23	55%	0.95	>0.05
	sth-67$^+$sth-67$^-$		sth-67$^+$sth-67$^-$				
nicA	3	14	4	18	44%	0.13	>0.05
pabaA	2	4	0	13	24%	2.58	>0.05
	sth-68$^+$sth-68$^-$		sth-68$^+$sth-68$^-$				
nicA	11	23	8	83	27%	0.46	>0.05
adF	8	13	0	15	57%	1.00	>0.05
pabaA	14	25	21	16	51%	0.05	>0.05
	sth-69$^+$sth-69$^-$		sth-69$^+$sth-69$^-$				
nicA	9	14	7	9	59%	1.26	>0.05
proA	2	0	0	36	5.2%	30.42	<0.001
adF	1	0	0	32	3%	30.03	<0.001
pabaA	1	5	37	32	8%	52.92	<0.001
	sth-70$^+$sth-70$^-$		sth-70$^+$sth-70$^-$				
nicA	5	4	1	9	47%	0.04	>0.05
pabaA	0	7	0	29	19%	13.44	<0.001

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these may both be alleles of a third sth locus. sth-67 and sth-70 were provisionally designated sthC1 and sthC2. sth-65 and sth-66 behaved as unlinked to all the markers examined. Thus it would appear that mutations of genes at at least four loci give rise to sthenyo-type enhancement of the medusa phenotype. The isolation of two mutants each of sthA, sthB, and sthC suggests that there are a limited number of sth loci.

8.1.6 Complementation studies of sthA and sthB

Heterokaryons were formed between strain DHG076 (medA17 sthA1 yA2;methB3;argB2) and DHG088 (medA13 adF17;sthB1). All colonies had ultramedusa morphology: no conidiation was seen. Selection for diploids was carried out , but none were seen, i.e. the complementation test failed. However, lack of conidiation in heterokaryons may reflect limitation of STHA and STHB gene products to the nucleus.

Given the polygene hypothesis described in the chapter introduction it was of interest to know the phenotype of sthA1;sthB1 double mutants. These might be expected to have some sort of medusa-like phenotype. Five attempts made to obtain hybrid cleistothecia from strains DHG081 (yA2 adF17;sthB1) and DHG082 (sthA1;methB3); all failed, although heterokaryons grew in all cases. In other crosses involving these strains a very low incidence of cleistothecia was observed. Thus, it would appear that sthA and sthB, like medA, exert an effect on sexual as well as asexual development in Aspergillus.

Outgrowths were also seen on the surface of four day old ultramedusa colonies. These were subcultured, and had an even more extreme phenotype than ultramedusa. The gene mutation of which is responsible for this extreme ultramedusa phenotype was provisionally designated "euryale" (eur). Euryale was the second sister of the gorgon Medusa (Riley, 1851).

8.1.7 Two mutations which each give no detectable phenotype combine to produce morphological changes

brlA42 is a temperature sensitive mutation affecting conidiophore development. At 30°C (permissive temperature) conidiation occurs normally; at 37°C development is blocked after vesicle formation and

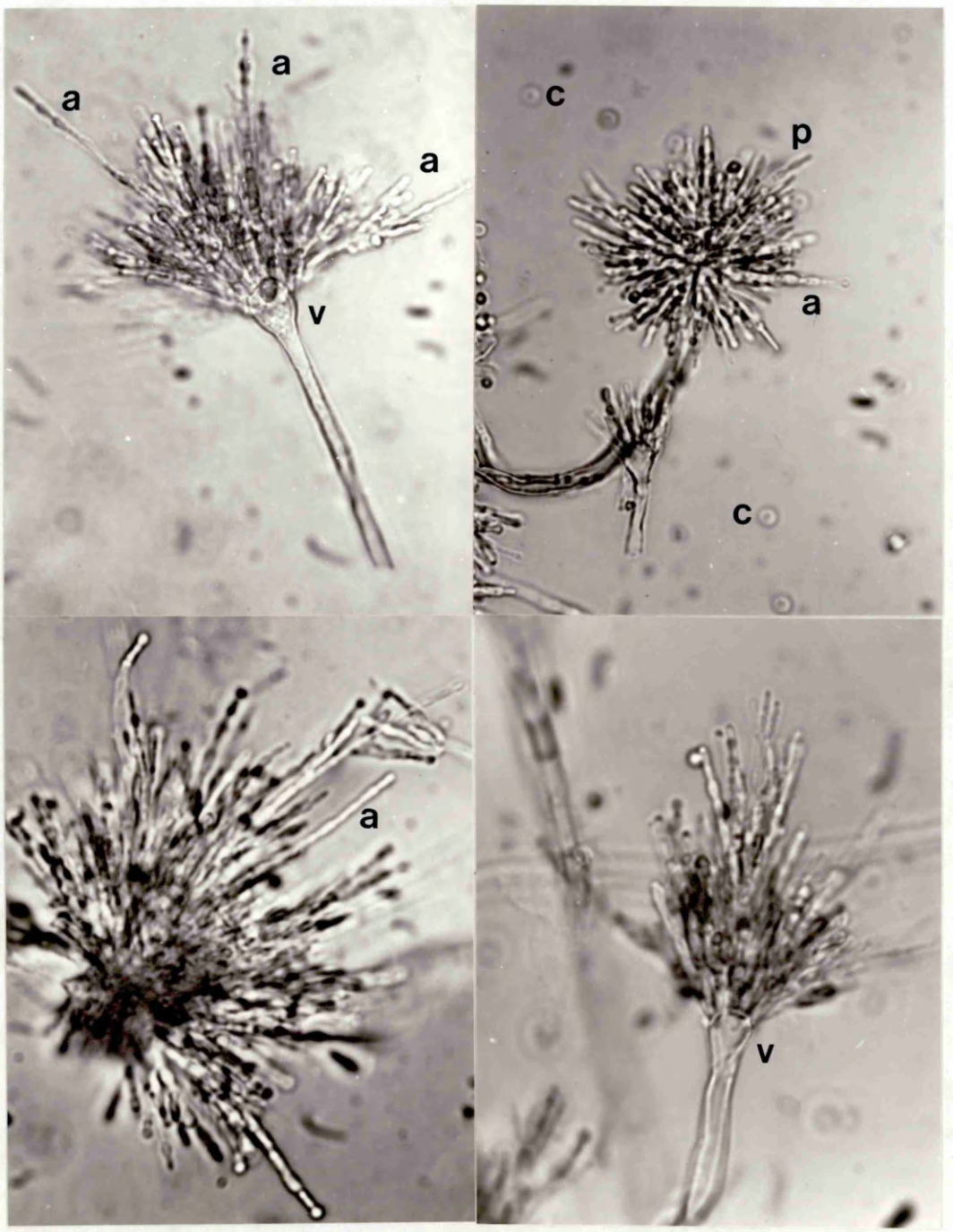

Figure 8.5. Abacoid mutant phenotype of A.nidulans.
Abacoid strains are brlA42 sth^{-}, grown at 30^{o}C. All four plates show different views of abacoid conidiophores.
a, abnormal "bead on a stick" phialide-derived structure; c, conidium; m, metula; p, phialide; v, vesicle.

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growth of the first metulae (Fig. 8.3D), resulting in an aconidial phenotype. A.nidulans strain AJC9.4 (pabaA1; argB2; brlA42 ivoB63) was crossed with DHG082 (sthA1;methB3). Aconidial (brlA42) colonies among progeny grown at 37°C were picked, and subcultured at 30°C. Although approximately half of these developed the wild-type conidiophore morphology characteristic of the temperature sensitive brlA42 strain grown at 30°C, the rest displayed a phenotype resembling that of the aconidial abacus ($abaA^-$) phenotype. In this case, however, metulae were also elongated, and although the characteristic "beads on a stick" structures of serial phialide growth was seen, some conidia were also seen (Fig. 8.5). Abnormally large numbers of selfed cleistothecia were also seen. This leaky abacus phenotype was designated "abacoid.

8.2 Aspergillus development and autoinhibition of growth

Conidiophore growth is believed to be partly supported by nutrients released by autolysis of the underlying mycelium. To what extent, and how this self-destruction is programmed genetically is unknown.

It has been observed that Aspergillus colony growth on medium overlayed with a layer of cellophane starts off normally, then dies off when the colony has reached a diameter of 4-6cm. If, however, a second layer of cellophane is placed over the initial inoculum, the colony will grow to the edge of the plate. A second effect of covering with cellophane is complete inhibition of conidiation (Clutterbuck, pers. comm.). This suggests that conidiophore development may stimulate mycelial autolysis, or that the decision to conidiate and to autolyse are subject to the same mechanism of control. Hypothetically, in growth on one layer of cellophane, as conidiation occurs autolysis inducing agents ("autolysins") are released into the underlying mycelium. Such autolysins, which would normally diffuse into the substratum, accumulate at the cellophane barrier and inhibit colony growth. On the other hand, the cellophane is sufficiently porous to allow diffusion of nutrients from the medium to the mycelium.

If release of such autolysins is under the control of the brlA or other late acting genes, then such autoinhibition of growth would not be expected of brlA mutants.

A number of morphological mutants were grown on solid medium on one, or between two layers of sterile cellophane. Conidia from

Table 8.6 Autoinhibition of colony growth of various developmental mutants on cellophane.

Strain	Developmental mutation	Number of layers of cellophane	T (^{o}C)	Autoinhibition?
G034	wt	1	30	Yes
G034	wt	2	30	No
AJC7.1	*brlA*1	1	37	No
AJC7.1	*brlA*1	2	37	No
AJC9.4	*brlA*42	1	30	Yes
AJC9.4	*brlA*42	2	30	No
AJC9.4	*brlA*42	1	37	Yes
AJC9.4	*brlA*42	2	37	No
DHG055	*medA*14	1	37	Yes
DHG033	*medA*3;*brlA*42	1	37	Yes
DHG087	*medA*17;*sthA*1	1	37	Yes
AJC6.1	*abaA*1	1	37	Unclear

conidiating strains were first embedded in minimal medium, $1mm^3$ cubes of which were used as inocula. This gave well defined single colonies. The results are summarised in Table 8.6.

The results show that whereas in a null brlA1 mutant, no autolysis is occurring, in all other mutants studied, even brlA42 strains grown at 37^oC, autoinhibition of growth does occur on a single layer of cellophane. abaA$^-$ mutants may be an exception : autoinhibition was seen in some cases and not others. It was also observed that, when cut with a scalpel, the mycelial mat from brlA1 colonies was much tougher than in all the others, with the exception of ultramedusa colonies.

This suggests that autolysis is a function of a gene, or genes, epistatic to brlA. It may be the products of such genes that accumulate and inhibit growth of brlA$^+$ strains grown on a single layer of cellophane.

8.3 Sequences homologous to ras and myc in Aspergillus.

Chromosomal DNA of A.nidulans strain G34, and A.niger strain 530.55 was digested to completion with EcoRI and BamHI, separated by gel electrophoresis, and Southern blotted. A radiolabelled 0.45-kb EcoRI DNA fragment containing v-ras^H, was used as a hybridisation probe under conditions of low stringency (adapted from DeFeo-Jones et al, 1983; see materials and methods). Stringency conditions used favoured hybridisation between DNA sequences with 75% homology and above.

Results are shown in Fig 8.6. Hybridisation is clearly seen, both to A.nidulans and A.niger DNA. In the lane corresponding to EcoRI digested A.nidulans DNA, one strongly hybridising band (B), and three less strongly hybridising bands (A, C, and D) are seen. Similarly, in both lanes corresponding to A.niger DNA, three strongly hybridising bands are seen. This suggests that it is possible that at least three genes with homology to v-ras^H are present in Aspergillus species.

Similar hybridisations were carried out using v-rel and v-myc oncogene sequences as probes. When filters carrying restricted genomic DNA of Aspergillus (as described) was probed with a radiolabelled 1.1-kb PstI DNA fragment containing v-rel sequences, no hybridisation was detected. In the case of v-myc, where a 3.4-kb SstI/SalI DNA fragment was used, hybridisation was seen after a 20-day exposure (Fig 8.7. The low stringency hybridisation conditions used were identical to

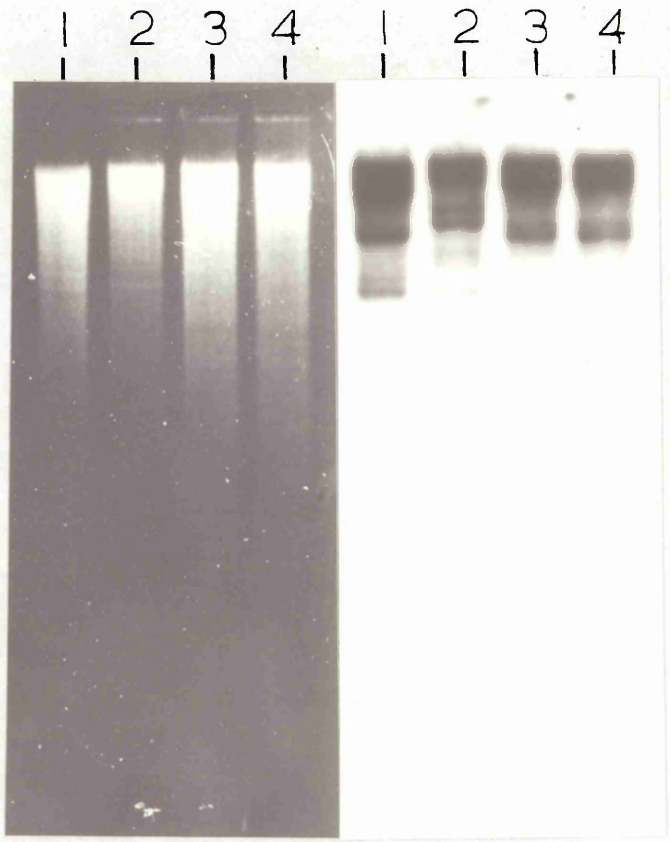

Figure 8.6. **Southern blot analysis of A.nidulans and A.niger DNA using a v-ras probe.**
Hybridisation was carried out under conditions of low stringency favouring hybridisation of sequences with 75% homology and above. Exposure time: 28 days.

Lane 1) A.nidulans DNA, EcoRI, 2) A.nidulans DNA, BamHI, 3) A.niger DNA, EcoRI, 4) A.niger DNA, BamHI.

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those used for v-*ras*^{H}. In this case, a *Hin*dIII/*Eco*RI digest of lambda DNA was run alongside the *Aspergillus* DNA as DNA fragment size markers. This lane was probed separately with radiolabelled lambda DNA under conditions of high stringency. The sizes of the *Aspergillus* DNA fragments hybridising with v-*myc* were calculated, and are displayed in Table 8.7. The strength of the hybridisation is much weaker than that with v-*ras*^{H}. This and the patterns of bands suggest that there may be 2-3 genes present in *Aspergillus* with not much more than 75% sequence homology with v-*myc*.

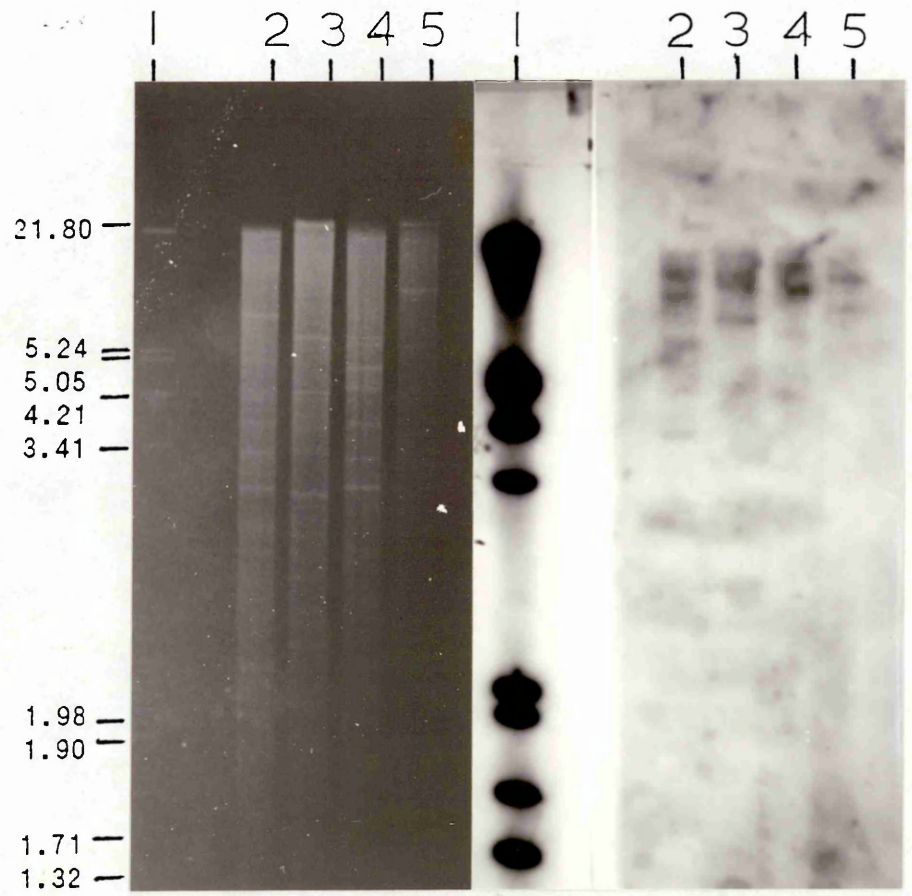

Figure 8.7. **Southern blot analysis of A.nidulans and A.niger DNA using a v-myc probe.**

Hybridisation was carried out under conditions of low stringency favouring hybridisation of sequences with 75% homology and above. Exposure time: 20 days. Lambda HindIII EcoRI size markers are given in kb.

Lane 1) lambda HindIII EcoRI, 2) A.nidulans DNA, EcoRI, 3) A.nidulans DNA, BamHI, 4) A.niger DNA, EcoRI, 5) A.niger DNA, BamHI.

facing page 135b

Table 8.7 Sizes of Aspergillus DNA fragments hybridising to v-myc under conditions of low stringency.

Fragment sizes were calcualted from the Southern blots shown in Fig. 8.7.

	A.nidulans
Enzyme	Fragment sizes (kb)
EcoRI	16, 11, 7.4, 4
BamHI	14, 12, 8.6

	A.niger
Enzyme	Fragment sizes (kb)
EcoRI	16, 12, 7.4
BamHI	14, 7.4

Discussion and Future Prospects

The sthA1 mutation has been shown to interact with two mutations, medA$^-$ and brlA42, which result in abnormal conidiophore morphology. The medA$^-$ mutation appears to retard the switch from metula to phialide growth. In brlA42;sthA1 strains grown at 37^{o}C, when brlA42 is epistatic to sthA1, the brlA42 mutation results in normal conidiophore vesicle formation, and then the growth of elongated metulae. No phialides are seen. Normally brlA42 strains have a wild-type phenotype at 30^{o}C. However, at 30^{o}C brlA42 and sthA1 strongly interact, to produce abacus-like structures. These interactions with medA$^-$ and brlA42 suggest that sthA may have a function in phialide growth.

Polygenes

The term "polygene" was coined by Mather (1943), and defined as one of a group of genes with a small effect on a particular character that can supplement each other to produce observable quantitative changes. Prior to that, however, the idea of polygenes, usually referred to then as "multiple factors", was in use. Gregor Mendel observed polygenes in genetic studies involving flower colours in beans. White and purple flowered strains were crossed, and the F_1 all had purple flowers. Mendel might have hypothesised that there was one dominant allele for purple flowers, and one recessive allele for white ones, in which case about 25% of the F_2 plants would have white flowers. In fact out of 31 F_2 plants only one had white flowers. The rest had flowers with a range of different shades of purple.

Most definable, genetically determined traits in complex eukaryotes, especially morphological ones, are determined polygenically; it is possible that most genes are polygenes. Mendel's discoveries were, in part, the result of a fortunate choice of genetic traits to study. However, since most classical and molecular genetic methods cannot be used to study polygenes - e.g. they cannot be genetically mapped - almost all present research involves those genes mutation of which, perhaps exceptionally, result in a clearly identifiable mutant phenotypes.

Most of the studies of polygenically determined traits have involved selective breeding or familial inheritance studies. These include those of the crossveinless (Waddington, 1965) and scute (Rendel and

Sheldon, 1960) phenotypes of D.melanogaster; vibrissa number in mice (Dun and Fraser, 1958); polydactyly in guinea pigs (Wright, 1934); and skin colour in people (Stern, 1960). Examples of other known polygenic traits are far too many to enumerate, but include height, eye-colour, hair colour, and predisposition to certain deseases such as cancer and heart desease.

The following characteristics of the sthA1 mutation concur with the hypothesis that sthA (and by inference the other sth genes) are members of a polygene family. 1) In the absence of other developmental mutations sth mutants have no detectable phenotype. 2) Interaction with other developmental gene mutations results in conversion of mild into severe oligosporogenous mutants; in the case of the abacoid phenotype, two mutations which normally do not result in abnormal conidiophore morphology, sthA1, and brlA42 at 30°C, interact to cause an extreme morphological abnormality. 3) In as much as the latter resemble phenotypes resulting from mutations in other alleles (brlA42, abaA1) they show partial redundancy with these gene loci. 4) There would appear to be quite a lot of them.

Should it be confirmed that sth genes are developmental polygenes, the method of identifying polygenes by looking at secondary mutations in leaky mutant strains may finally allow proper genetic and molecular genetic studies of polygenes to be carried out.

Whether or not sth genes are developmental polygenes can only be demonstrated when a) they have been cloned and their patterns of transcription characterised, and b) strains carrying multiple sth^- mutations have been constructed and their phenotypes examined.

A search for such polygenic interactions might allow assignation of phenotypes to genes identified by reverse genetics, otherwise uncharacterisable, such as those in the SpoC1 cluster (Timberlake and Barnard, 1981; Aramayo et al, 1989).

Possibly such polygenicity is a common characteristic of structural genes which determine morphology.

Modifiers

Two definitions of "modifier" or "enhancer" mutations are:
1) Secondary mutations with subtle multiple pleiotropic effects - e.g. affecting metabolism or overall gene expression. These could be e.g.

leaky auxotrophic, or ribosomal gene mutations.
2) Secondary mutations with a single clear phenotype whose alteration of a previously existing mutant phenotype do not reflect any function in wild-type development.

With respect to the first definition, sthA1 does not appear to have multiple pleiotropic effects: its effect seems to be limited to phialide development. With respect to the second definition: it is impossible to rule out that sthA has no role in normal development. However, it is hard to imagine exactly how a gene, mutation of which has such a drastic effect on development, has nothing to do with development. To put it another way: sthA *is* a developmental mutation of medA$^-$ strains.

Fungal proto-oncogenes

The presence of Aspergillus DNA sequences with homology to v-ras^H and v-myc suggests that it may be possible to combine elucidation of the molecular genetics of Aspergillus development with cancer research. Whether or not hybridisation patterns seen do represent multiple c-ras^{AN} and c-myc^{AN} genes could be investigated by using v-ras^H and v-myc as hybridisation probes for the A.nidulans DNA library constructed in the lambda replacement vector EMBL3 (Griffith, 1990). Cloned homologous sequences could then be a) sequenced and compared with known ras and myc sequences; b) used to look for developmental regulation of transcripts and c) to disrupt resident copies in Aspergillus and look for mutant phenotypes. As stated in the introduction, yeast RAS genes appear to have a role in sporulation.

If c-ras^{AN} does exist it would be interesting to carry out site directed mutagenesis to try convert it to the oncogenic activated form, and see whether transformation of Aspergillus with such a sequence would result in hyphal tumour formation. It is known that activated mammalian ras-encoded proteins of have a mutation at amino acid 12 of the ras protein and that yeast RAS-encoded protein with the same mutation at amino acid 12 is oncogenic in transformations of mammalian cells (Bradley et al, 1986). In some ways the fluffy (flu) mutants of A.nidulans, which produce aerial hyphae in copious amounts, resemble hyphal tumours, although there is evidence that fluffy strains are the result of heritable changes in patterns of DNA methylation (Tamame et al, 1983a; 1983b). Conceivably such hyphal tumours could be used as models for the action of anti-cancer agents.

CHAPTER 9

Concluding remarks

Discussion

Although the primary aim of this project was to study Aspergillus development, early on it was decided to attempt to extend the repertoire of recombinant DNA techniques available for Aspergillus. The result is four new facets of Aspergillus gene manipulation: A) the use of ARp vectors (Johnstone, 1985a; 1985b), e.g. for gene bank construction and shotgun cloning, and gene expression studies. B) The use of cotransformation for plasmid engineering in vivo simultaneous with transformation, e.g. for defining the boundaries of genes on linear DNA fragments. C) Gene cloning by cotransformation with integrating libraries and helper plasmids. And D), gene cloning with instant gene banks: linear chromosomal DNA fragments cotransformed with helper plasmids.

ARps

Although a number of groups are using ARp1-based plasmids for gene bank construction (Hynes, pers.comm; Kinghorn, pers.comm.) a better understanding of the basis of the activity of AMA1 is required before its widespread use in gene manipulation is advisable. This involves a number of questions: does the function of AMA1 require a second activity besides that of an ori? If so, what is it, and what is the role of the resident copy in the Aspergillus genome? What is the signifigance (if any) of the inverted repeat? Is the part of AMA1 which exists in multiple copies in the genome the same as that conferring free replication? If so, what is the significance of the existence of multiple copies? inverted repeats, multiple copies: is AMA1 (part of) a transposon? An understanding of AMA1 may also allow construction of biotechnologically useful higher copy number derivatives.

Besides the shotgun cloning of genes, a gene bank in an ARp plasmids could be used as the basis of a screen for centromeric sequences. If such a gene bank was transformed into Aspergillus transformants with centromeric sequences could be identified by screening for increased stability of the transforming phenotype. ARp plasmids could also be used in studies of telomeric sequences, e.g. to see whether telomeres from other organisms such as Tetrahymena would function as such in Aspergillus; or to screen for Aspergillus telomeric sequences.

Cotransformation

The knowledge that cotransformation reflects the occurrence of recombination between transforming plasmids often obviates the necessity to construct certain types of plasmid. This particularly applies to plasmids which contain more than one DNA insert in a bacterial plasmid vector. For example, Robertson (pers.comm) is planning to sequence the AMA1 region, and to study the behaviour of AMA1 subclones to precisely localise the site conferring autonomous replication. Normally it would be necessary to construct two sets of subclones - one for sequencing, and the other in a plasmid with a selectable Aspergillus marker for functional analysis. However, given that plasmid recombination occurs, it is hoped that localisation of the AMA1 active region will be possible by cotransforming pILJ16 with the sequencing constructs, making construction of a second set of constructs unnecessary. Similarly, cotransformation of gel purified DNA fragments with ARp plasmids can be used to identify gene boundaries without the need to construct subclones.

Other groups are now testing the cotransformational cloning (Sealy-Lewis, pers.comm.) and instant gene bank cloning (Batt, pers.comm) methods.

The use of all four of these new tools/methods for gene manipulation in Aspergillus require further investigation and fine tuning. Once this has been achieved it is hoped that Aspergillus may approach the degree of versatility and convenience for molecular biological studies as it already possesses for classical genetic studies.

What are polygenes?

"Polygene" is an old term used to discuss work mostly carried out by geneticists before the existence of current molecular models of the gene. As described in chapter 8, it was largely used to describe results of selective breeding experiments. Among the possible molecular definitions of polygenes are the following: A) a group of functionally identical, or fully redundant genes, e.g. rRNA genes. B) A group of genes showing partial redundancy, such that a loss of function in any one would result in a very slight phenotypic change.

How does one distinguish between polygenes and enhancers of mutant phenotype? Take sthA and medA as examples. It is possible that the MedA polypeptide is involved specifically in modulating the development of phialides. That loss of medA function delays conidiation suggests the possibility that other gene functions eventually compensate for the lack of functional MedA polypeptide. If sthA is one of these genes, sthA and medA are polygenes: they have distinct but overlapping functions.

It is possible that the sthA1 mutation acts as an enhancer of the $medA^-$ phenotype. This implies that whereas medA is involved in wild-type development, sthA has no such role; the apparent role of sthA in normal development is an artefact of secondary mutational analysis.

Yet there is a second sense in which sthA could be an enhancer: although sthA may have no role in wild-type morphogenesis, it might have such a role in $medA^-$ strains. In other words, it is possible that only in a $medA^-$ strain does sthA take over the medA function. In such a situation sthA shows induced redundancy of function to medA in $medA^-$ strains, and could be described as facultatively redundant to medA. Thus, hypothetically, whereas medA shows full facultative redundancy in $sthA^-$ strains, sthA shows only partial facultative redundancy in $medA^-$ strains. Thus $medA^-sthA^+$ strains have a distinct phenotype, whereas $medA^+sthA^-$ ones do not. this suggests a third possible molecular definition if polygenes: a group of facultatively redundant genes - i.e. where gene function is distinct in the wild type, whereas when loss of function of one gene occurs, activity of sister polygenes wholly or partially compensates for the loss.

Confirmation of the identity of sthA as a polygene involved in normal conidiophore development, or even as an induceably redundant polygene, would strongly indicate that a new approach to the molecular genetic analysis of morphogenesis may be necessary to identify the relevant genes: the isolation and characterisation of polygenes as secondary mutations. Aspergillus conidiophore development is particularly suited for this approach due to the availability of leaky brlA mutants blocked at all stages of development from before vesicle formation to phialide development (Clutterbuck, 1990a). It may be possible to identify polygenes involved in normal development at all the different stages by screening for secondary mutations in the appropriate leaky brlA mutant.

Ending on an optimistic note

An important starting point of molecular genetics was the one gene, one polypeptide hypothesis, which pointed to the fact that gene function must be understood in terms of the biochemistry of a protein product. Early molecular genetic studies involved genes where phenotype gave a strong indication of the biochemical function of the polypeptide product. For example in A.nidulans argB$^-$ mutants are auxotrophic with respect to arginine, indicating that the polypeptide product of argB is probably involved in arginine biosynthesis. Subsequently it was shown that argB$^-$ mutants lack functional ornithine transcarbamoylase. From this it can be seen that the study of mutants, e.g. argB, enables understanding of the disabled function, e.g. arginine biosynthesis.

In molecular genetic studies of morphogenesis, phenotype usually gives no indication whatsoever of the biochemical function of the gene product. This introduces uncertainties: the normal function of a gene which results in morphological abnormalities when mutated, may have nothing to do with the determination of morphogenesis, or at least, the two may not be causally related in a straight forward way, as are argB and arginine prototrophy. Such a state of affairs can lead to the unfortunate "clone and groan" situation, where the sequence of a cloned gene and its putative polypeptide give no clue as to its function. This possibility is underscored by the fact that it is not clear whether cellular morphogenetic events *can be characterised at all* in terms of a biochemistry specific to morphogenesis.

In the absence of any knowledge of the biochemistry of morphogenesis there is no guarantee that the study of genes mutation of which result in morphological abnormalities will lead to understanding of the normal genetic determination of morphogenesis, any more than those identified as developmental cDNAs. Molecular genetics may be of little use without the biochemistry to complement it.

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