Transformation and Gene Targeting in *Aspergillus nidulans*

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Abstract

Transformation of a haploid *Aspergillus nidulans pyrG* auxotrophic strain (1-85) was optimised for the vector pGM32 containing the heterologous *Neurospora crassa pyr4* gene. The resulting uracil-independent transformants could be classified into two main groups based on morphology. The minority were morphologically very similar to the parental strain, easily purified and mitotically stable. The majority (10 times more frequent) were irregular in shape and shown to be heterokaryons that could not be resolved into transformed homokaryons. Analysis of the transformant types suggested regulation of multiple copies of the gene for OMPdecase (*pyr4 and pyrG*) resulted in the titration/inactivation of essential trans-acting factors. The heterokaryon state was therefore a requirement for the survival of transformants containing multiple copies of the integrated vector.

The effect of altering the conditions of transformation on the efficiency of gene targeting in filamentous fungi was studied. The *A. nidulans niaD* and *amdS* genes, both involved in nitrogen source utilisation, were selected as target loci. Insertional inactivation vectors were constructed (based on pGM32) and parameters shown to have an effect on the targeting frequency at the *niaD* locus were subsequently tested at the *amdS* locus. A dramatic difference in targeting was observed between the *niaD* and *amdS* loci with targeting of *niaD* being much more efficient than *amdS* for the parameters tested. The level of gene targeting using circular DNA was found to correlate with the size of the homologous segment at both loci. Similarly the level of targeting was shown to increase at both loci when vectors were linearised within the region of homology. Unexpectedly the level of targeting was unaltered at the *niaD* locus when transcription was induced at different stages in the transformation procedure. Likewise targeting was unaffected by altering the amount of DNA in the reaction mix. The regeneration temperature, however, did appear to have an effect on targeting, with enhanced targeting observed at the lower temperature.

Gene replacement by transformation was used to disrupt the *cycA* gene in diploid and haploid *A. nidulans* strains. The first completely deficient *cyc* mutant in a filamentous fungus was isolated and shown to be non-lethal. Haploidisation analysis of the diploid transformant localised the chromosomal position of *cycA* to chromosome I.

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Abbreviations

AMM	*Aspergillus* minimal media
AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
bp	base pair(s)
χ	chi squared or chromosome
CTP	cytidine triphosphate
DNA	deoxyribonucleic acid
FAD	flavin adenine dinucleotide
GTP	guanosine triphosphate
hr	hour(s)
kb	kilobase pairs(s)
min	minute(s)
NADP	nicotinamide adenine dinucleotide phosphate
PCR	Polymerase Chain Reaction
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
sec	second(s)
TE	Tris/EDTA buffer
TTP	thymidine triphosphate
UTP	uridine triphosphate

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Introduction

1.1 Overview

The genetic modification of micro-organisms is a powerful tool. Along with many commercial applications it is an important tool for research into the understanding of genes, gene regulation and genomic organisation. This is made possible by the concurrent development of the techniques of molecular biology, which have allowed precise DNA constructs to be made, and transformation systems, which have allowed the DNA to be introduced into organisms of choice.

Transformation was first achieved in prokaryotic systems. Success, particularly with the prokaryotic model organism *Escherichia coli*, allowed many biological, biochemical and genetic questions to be answered. The potential applications of transformation to other systems, particularly eukaryotic systems, was obvious. A need to develop effective and efficient transformation procedures was therefore recognised and research in this area has since been intense.

Micro-organisms offer an ease of manipulation that make them suited to scientific study. Fungi are a eukaryotic micro-organism which form a very diverse group whose beneficial and detrimental biological activities have a major impact on human affairs. Filamentous fungi are recognised as having many important commercial applications as producers of food products, enzymes, primary metabolites and secondary metabolites. They also have a major negative economic impact in the form of plant pathogens. Fungi offer a system that has the complexity of eukaryotes and the relative ease of manipulation of micro-organisms. The filamentous fungi *Neurospora crassa* and *Aspergillus nidulans*, along with the non-filamentous yeast, *Saccharomyces cerevisiae*, have served as model systems for genetic and biochemical studies of eukaryotes.

1.2 Biology of Filamentous Fungi

The biology and genetics of fungi has been studied extensively and is well reviewed (Smith *et al.*, 1983). A brief description of fungal biology, with an emphasis on areas relevant to later discussions, is provided.

The biology of filamentous fungi is quite distinct from that of single-celled yeasts as they grow as branching filaments called hyphae, collectively known as mycelium. Growth is by

elongation at the tips of the hyphae, while nuclei, which are usually haploid, divide mitotically in the leading cells. The hyphae are commonly septate and septa in many species have pores through which nuclei can pass. This results in a common cytoplasm containing many nuclei.

Filamentous fungi have variably sized genomes, generally about 7 times the size of an *E. coli* genome, 2 times that of a yeast and 1% that of a human haploid germ cell. For reference, *A. nidulans* has a 26,000 kb genome on 8 chromosomes while *N. crassa* has a 27,000 kb genome on 7 chromosomes.

Fungi may have several haploid nuclei per cell, each of which is surrounded by a nuclear envelope. If all the nuclei contained in the cytoplasm of the mycelium are genetically identical the fungus is a homokaryon, if not then it is a heterokaryon. Heterokaryosis arises from hyphal fusion, spontaneous mutations or transformation.

The fungi are often able to reproduce sexually and asexually. To reproduce asexually the vegetative mycelium differentiates and produces spores that contain nuclei from mitotic divisions. The spores germinate and continue the asexual cycle. Sexual reproduction is the similar for filamentous and non-filamentous fungi. Two haploid nuclei fuse and undergo meiosis. The diploid phase is transient in most fungi. If the four products of meiosis (frequently duplicated) are contained in a sac called an ascus (sexual spores contained in the ascus are called ascospores), the fungus is an Ascomycete (eg *Neurospora* species and *Emericella nidulans*), or externally on structures called basidia, the fungus is a Basidiomycete.

In homothallic species (eg *Emericella nidulans*) genetically identical nuclei can go through karyogamy and meiosis, this is termed selfing. Heterothallic species (eg *N. crassa*) require genetically distinct haploid nuclei with different mating type loci to undergo nuclear fusion and then meiosis.

The ecological niche of fungi is as saprophytes or parasites. This is due to their ability to degrade complex substrates by the secretion of enzymes and absorption of breakdown products. This has been enhanced through classical strain improvement techniques and recombinant DNA technology to be exploited commercially.

1.3 *Emericella/Aspergillus nidulans*

The *Aspergillus* taxon was first described by Micheli, a Florentine priest mycologist, in 1729. The genus was characterised by the conidiophore which is a long hyphal cell culminating in a globe-like spore bearing structure. The genus is classified among the Deuteromycetes and consists of asexual (anamorphic) molds. The sexual (telomorphic) stage, observed in some

members, are classified among the Ascomycetes. *Emericella nidulans* is the sexual form of *Aspergillus nidulans*.

Aspergilli are recognised as one of the most widely distributed and abundant groups of living organisms and have a major role in the natural world. Their metabolic versatility has both positive and negative economic consequences. They are major agents of decomposition and decay and the enzymes that facilitate these biodegradative activities are exploited for food fermentation and industrial processes. Some species cause serious diseases (mycosis) or produce toxic metabolites (mycotoxins). Others can be grown in controlled fermentations in order to harvest small molecules of industrial importance.

Examples of some medically and industrially important species are:

A. flavus	-aflatoxin production
A. fumigatus	-etiological agent in aspergillosis
A. nidulans	-model system for genetic studies
A. niger	-citric acid production
A. oryzae	-saki and soy sauce production

Classically *Aspergillus nidulans* has proven to be a valuable model system for genetic studies. It forms a bridge between researchers working on bacteria and bacteriophages, and those working on higher organisms. The genetic system has been well reviewed: from Pontecorvo in 1953, who laid down the foundations, through to Clutterbuck in 1992, who gave a summary of the sexual and parasexual genetics of *A. nidulans*. Due to the solid classical foundations the molecular genetics of fungi is now well advanced.

The important features of the life-cycle of *A. nidulans* are illustrated in Figure 1. Conidia are the asexual spores which determine the colour of a sporulating colony by the pigmentation of the spore wall. They are generally haploid and always uninucleate with a spore diameter of approximately 3 μm. The ascospores are the sexual spores and are found inside large, black, thick walled spheres called perithecia (or cleistothecia) on the surface of the colonies. The sexual cycle is similar to higher organisms with an alternation of haploid and diploid phases. The diploid phase is derived from the haploid phase by nuclear fusion, and the haploid phase from the diploid by meiosis.

The *Aspergillus* somatic tissue can be propagated indefinitely without recourse to the sexual cycle, which requires specific environmental conditions. Clonal propagation and selective techniques similar to those used for bacteria can therefore be employed, thereby making experimental manipulation easier.

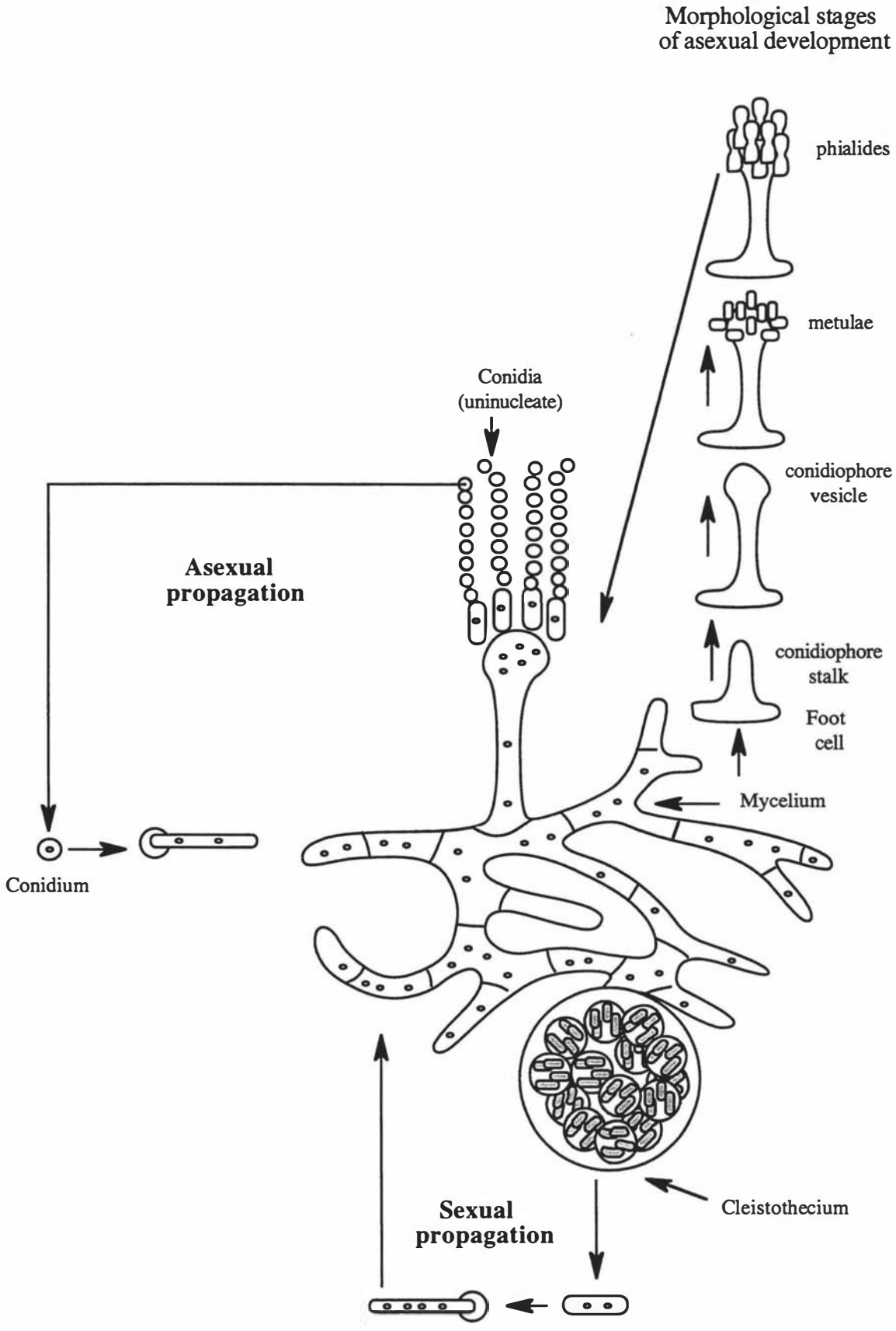


Figure 1 Asexual and Sexual Life Cycle of *Aspergillus/Emericella nidulans*

Another feature of *Aspergillus* is the ability to form heterokaryons from two genetically different strains. This may result from hyphal fusion whereby the hyphae contain nuclei from both strains (as opposed to a homokaryon containing identical nuclei). As *Aspergillus* is homothallic (has no mating types) zygotes can be formed from fusion of any two nuclei. If nuclear fusion occurs in the mycelium, albeit very rarely, the diploid nuclei do not undergo meiosis. They give rise to diploid colonies which are stable and can be isolated and maintained. These diploid cultures have many uses, such as gene mapping and the study of mitotic recombination, which have been explored and exploited.

A study of the *A. nidulans* genome (Timberlake, 1978) showed that the genome size is 2.6 x 10^7 bp or 0.028 pg DNA/haploid nucleus. It consists of approximately 97 to 98 percent unique, and 2 to 3 percent reiterated, sequences as shown by DNA-DNA reassociation experiments.

1.4 Transformation

The commercial and academic contributions of fungal transformation have been well documented since it was first achieved in the early 1970's (Fincham 1989). *Saccharomyces cerevisiae* provided the initial model system after successful transformation with integrating plasmids and then, with the identification and inclusion of autonomously replicating sequences (ARS's), autonomously replicating plasmids. Consequently it has been possible to clone many genes by complementation with autonomously replicating plasmids. Integration of DNA in *S. cerevisiae* is predominantly by homology so targeted gene disruptions or replacements have been achieved allowing the function of cloned genes to be studied in detail.

The initial success with non-filamentous fungal systems was extended to filamentous systems in the early 1980's by the transformation of *Neurospora crassa,* and soon afterwards, *Aspergillus nidulans.* As functional ARS's for filamentous fungi have only recently been identified the early work was with integrative plasmids. Integration in filamentous fungi can be at homologous or heterologous sites. The transformation frequencies were soon improved by the optimisation of the transformation techniques, development of more efficient plasmids and selection systems, and the identification of sequences which improve transformation frequencies. These improved transformation frequencies allowed the screening of libraries to clone genes and promoter elements. Genes could be cloned by the complementation of a specific mutation or by disruption using heterologous plasmids. The cloned genes could then be studied using transformation to correct mutations, or make defined mutations, as with *S. cerevisiae.* The disruption of essential genes in filamentous fungi was also possible as the nuclei containing the disruption could be maintained as a heterozygous diploid, or as a

heterokaryon.

High transformation frequencies are required to clone genes and study them in filamentous fungi. If a gene is to be cloned by disruption using a heterologous plasmid, with subsequent rescue of the plasmid and surrounding genomic sequences, it is important to know if integration of the plasmid is random. If the plasmid is shown to target specific sites its value for cloning by this method may be diminished or negated. Likewise, if a gene disruption or replacement is required it is important to know if the vector will integrate at the selected homologous site, and how frequently. It may be possible to optimise for a specific integration event (homologous or heterologous) when designing the transforming vector and determining the conditions for transformation. Knowledge such as this will ensure transformation systems are more effective and efficient.

1.5 Aims

Due to the many applications of fungal transformation and gene targeting it was decided to study the frequency of integration events upon fungal transformation. Filamentous fungi provide an intermediate level of gene targeting (homologous integration) to that observed in yeast (very high) and humans (very low). *Aspergillus nidulans* was chosen as a model filamentous fungus due to its ease of manipulation and the wealth of literature available on it. Complementation of a mutation in the orotidine-5'-phosphate decarboxylase gene (*pyrG*) provided a relatively inexpensive selection system which has been employed by many research groups. The genes for nitrate reductase (*niaD*) and acetamidase (*amdS*) were targeted as they have been well studied, and the homologous and heterologous integrants could be selected on the basis of phenotype. Due to the interest by our research team in the cytochrome *c* gene of *A. nidulans* (*cycA*), this provided an example of the applications of this technology.

Thus the aims of this investigation were:

(1) To optimise the conditions for the isolation of protoplasts from, and transformation of, *A. nidulans* (Chapter three);
(2) Analyse the transformants (Chapter four);
(3) Investigate the effect of altering the transformation conditions on the ratio of integration (homologous:heterologous) events (Chapter five); and
(4) Investigate the disruption of the cytochrome *c* (*cycA*) gene in *A. nidulans* (Chapter six).

Chapter 2.0

Materials and Methods

2.1 Fungal Strains, Bacterial Strains and Plasmids

Fungal strains, bacterial strains and plasmids used in this study are listed in Table 1.

2.2 Water Supply and Sterilisation

The water (dH_2O) used in the preparation of all solutions, unless otherwise stated, was purified by a Millipore MilliQ Reagent Water System. Sterilisation of solutions and equipment was at 121°C for 25 to 40 min, or 110°C for 25 min, in commercial autoclaves. All solutions were sterilised in commercial autoclaves unless otherwise indicated. Glassware used for the preparation of solutions for work with DNA was acid washed and sterilised. All labware, tips and microcentrifuge tubes used for PCR were from stocks handled with clean disposable gloves.

2.3 Media

Sterilised solid media was melted then cooled to 55-60°C before the addition of supplements and pouring. Media was stored at room temperature and plates were stored at 4°C.

2.3.1 Bacterial Media

2.3.1.1 Liquid Media

Luria Broth (LB): LB media (Miller, 1972) contained (g/l): tryptone (Difco), 10; NaCl, 5.0; and yeast extract (Difco), 5.0. The pH was adjusted to 7.5 with NaOH prior to autoclaving.

SOC Medium: SOC medium (Dower *et al*., 1988) contained (g/l): tryptone, 20; yeast extract, 5.0; NaCl, 0.6; KCl, 0.2; $MgCl_2$, 0.95; $MgSO_4.7H_2O$, 2.5; and glucose, 3.6.

2.3.1.2 Solid Media

Luria Agar: Agar (Davis) was added to LB media to 15 g/l prior to autoclaving.

Table 1 Fungal and Bacterial Strains and Plasmids

Strain/ Plasmid	Relevant Characteristics	Source/ Reference
Fungal strains[a]		
Aspergillus nidulans		
1-85	Haploid *pyrG89 pabaA1 wA3 qutR16*	Clive Roberts, Leicester
MH1165	Haploid *yA1 riboB2 amdA7 amdS223*	Michael Hynes, Melbourne
A691	Haploid *biA1 niaD15*	FGSC, Kansas
2-1	Wildtype *A. nidulans* strain	John Peberdy, Notingham
D10	Diploid *pyrG89/pyrG89*, p*abaA1/+*, *yA/+*; *+/wA3*; *bgaA4/+*; *+/pyroA4*; *qutE208/+*, *+/fwA*	Streatfield and Roberts 1993
TZ24	Diploid D10 transformant: pR54 replacement of *cyc* locus	Paul Hirst
TZ1	Diploid D10 transformant: pR54 replacement of *cyc* locus	This study
TZ7	Diploid D10 transformant: pR54 integrated into χVII	This study
TZ8	Diploid D10 transformant: pR54 integrated into χIV	This study
TZ12	Diploid D10 transformant: pR54 replacement of *cyc* locus	This study
TZ17	Diploid D10 transformant: pR54 replacement of *cyc* locus	This study
R546	1-85 transformants: pR54 replacement of *cyc* locus	This study
R54	1-85 transformants: pR54 integrated at an ectopic site	This study
N1-10	1-85 N-type transformants containing pGM32	This study
H1-10	1-85 H-type transformants containing pGM32	This study
GM1-10	1-85 N-type transformants containing pGM32	This study
PG1-6	1-85 N-type transformants containing pPYRG	This study
DW1-10	1-85 N-type transformants containing pDW2	This study
Bacterial strains		
Escherichia coli		
DH1	F$^-$ *supE44 recA1 endA1 gyrA96* (Nal^r) *thi-1 hsdR17* ($r_k^-m_k^+$) *relA1 spoT1 rfbD1*	Bachmann 1987
XL1-Blue	F':: Tn*10* (tet^r) *proAB*$^+$ *lacI*^{q} *Δ(lacZ)M15/ recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* ($r_k^-m_k^+$) *supE44 relA1 lac*	Bullock *et al.* 1987

Table 1 continued

Plasmids[b]

pGM32	4.5 kb, Amp^R pUC19 based containing *pyr4* from *N. crassa* and the pBluescript II S/K +/- multiple cloning site	Greg May
p3SR2	8.8 kb, pBR322 derivative containing the *A. nidulans amdS* and *amdI* genes	Tilburn *et al.* 1983
pSTA8	11.1 kb, Amp^R contains the *A. nidulans niiA* and *niaD* genes	Johnston *et al.* 1990
pamdS1	5.1 kb, pGM32 containing a 0.68 kb internal *amdS* fragment	This study
pamdS2	5.0 kb, pGM32 containing a 0.60 kb internal *amdS* fragment	This study
pamdS3	5.7 kb, pGM32 containing a 1.27 kb internal *amdS* fragment	This study
pniaD1	5.4 kb, pGM32 containing a 0.90 kb internal *niaD* fragment	This study
pniaD2	5.7 kb, pGM32 containing a 1.24 kb internal *niaD* fragment	This study
pniaD3	6.6 kb, pGM32 containing a 2.15 kb internal *niaD* fragment	This study
pAN8-1	5.9 kb, Phleo^R Amp^R	Mattern and Punt 1988
pUC12	2.7 kb, Amp^R	
pniiA-6	6.1 kb, pUC19 containing the *A. nidulans pyrG* and the *niiA* promoter connected to the *tubC* terminator	Greg May
pPYRG	4.8 kb, pUC19 containing the *A. nidulans pyrG* gene	Greg May
pDW2	4.8 kb, pGM32 containing a replacement of the *pyr-4* promoter with the *A. nidulans niiA* promoter	This study
pDW5	3.2 kb, pUC12 containing the *pyr-4* promoter	This study
pDW6	5.9 kb, pUC12 containing the phleomycin resistance cassette from pAN8-1	This study
pDW7	6.4 kb, pUC12 containing the phleomycin resistance cassette from pAN8-1 and the *pyr4* promoter	This study
pDW8	4.3 kb, pDW6 replacement of the *gpdA* promoter with the *pyr4* promoter	This study
pR54	6.0 kb, pGM32 containing fragments 5' and 3' of the *cyc* coding region	Rosie Bradshaw

[a] *A. nidulans* strains 1-85, MH1165, A691, D10 and 2-1 were obtained from silica gel and single spore purified (Section 2.4.2.1). They were tested for all genetic markers by growth on supplemented minimal media (Section 2.3.2.3) and then stored on slants (Section 2.4.2.5) and on silica gel (Section 2.4.2.4).

[b] All plasmids were digested with restriction enzymes (Section 2.11), single and double digestion, to confirm their identity before use. The plasmids pamdS1-3 and pniaD1-3 were further tested by sequencing over the cloning sites (Section 2.15, Appendix 2).

2.3.1.3 Media Supplements

Ampicillin was supplemented at a concentration of 100 μg/ml, isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) in dimethylformnamide were both supplemented at a concentration of 40 μg/ml. Stocks of ampicillin, IPTG and X-gal were filter sterilised and stored at -20°C.

2.3.2 Fungal Media

2.3.2.1 Liquid Media

Aspergillus complete media (MYG): MYG contained (g/l): malt extract (Difco), 5.0; yeast extract (Difco), 2.5; glucose, 10.0; and $CuSO_4.5H_20$, trace.

Aspergillus minimal media (AMM): AMM contained (g/l): $NaNO_3$, 6.0; $MgSO_4.7H_2O$, 0.52; KCl, 0.052; KH_2PO_4, 1.52; glucose, 10; $FeSO_4.7H_2O$, trace; and $ZnSO_4.7H_2O$, trace. The pH was adjusted to 6.5 prior to autoclaving.

Nitrogen Source Free AMM: Nitrogen source free AMM contained (g/l): NaCl, 4.1; $MgSO_4.7H_2O$, 0.52; KCl, 0.052; KH_2PO_4, 1.52; glucose, 10; $FeSO_4.7H_2O$, trace; and $ZnSO_4.7H_2O$, trace. The pH was adjusted to 6.5 prior to autoclaving.

2.3.2.2 Solid Media

Plates: Agar (Davis) was added to liquid media to 20 g/l prior to autoclaving. Media was prepared at a 2x or 1x concentration.

Top Agar: Agar (Davis) was added to liquid media to 8 g/l prior to autoclaving

2.3.2.3 Media Supplements

For growth of strains containing the *pyrG* mutation 2.2 g/l of uracil was added before autoclaving (MU: MYG + uracil). AMM was supplemented as required to the following concentrations: arginine, 200 μg/ml; biotin, 0.02 μg/ml; nicotinic acid, 2 μg/ml; para-aminobenzoic acid, 1 μg/ml; proline, 50 μg/ml; pyridoxine, 0.05 μg/ml; riboflavin, 2.5 μg/ml

(photolabile); and uridine, 10 mM. Stocks of vitamins and amino acids were stored at 4°C.

Nitrogen sources were added to nitrogen source free AMM from 1 M stock solutions to a final concentration of: urea, 5 mM; glutamate, 10 mM; potassium nitrate, 10 mM; ammonium chloride, 10 mM; sodium nitrite, 5 mM; and acetamide 10 mM. To AMM containing 10 mM acetamide as the sole nitrogen source caesium chloride was added to a final concentration of 12.5 mM.

For protoplast regeneration 273.8 g/l (0.8 M) of sucrose was added to solid media. This was prepared as a concentrated solution (2x) and autoclaved at 110°C for 20 min. To an equal volume of molten solid media (2x) was added sucrose solution (2x) for osmotically stabilised media, and sterile dH_2O for standard plates.

2.4 Growth, Maintenance and Storage of Cultures

2.4.1 Bacterial Cultures

Pure *Escherichia coli* cultures were obtained by streaking for single colonies on LB plates and incubating at 37°C. Single colonies were selected and maintained on LB plates supplemented as required, then stored at 4°C with regular subculturing.

2.4.1.1 Storage in Glycerol

For long term storage of *E. coli* strains, 3 ml of appropriately supplemented LB medium was inoculated with a loop of culture, and incubated with shaking (~250 rpm) overnight at 30°C or 37°C. An equal volume of 100% glycerol was mixed with the broth culture and aliquots stored at -70°C in cryogenic tubes. To retrieve a culture the appropriate tube was removed and placed on ice. A scraping from the surface of the frozen culture was spread onto an LB plate.

2.4.2 Fungal Cultures

Aspergillus nidulans cultures were routinely grown at 37°C on MYG or AMM plates, supplemented as required. Alternatively siliconised flasks containing liquid MYG, supplemented as required, were inoculated with spores to a final concentration of 10^6 to 10^7 spores per ml. The flasks were incubated with shaking (~ 250 rpm) at 37°C.

2.4.2.1 Purification of *Aspergillus nidulans* Strains

A. nidulans produces uninucleate conidia (Pontecorvo *et al.*, 1953). Pure strains of *A. nidulans* were obtained by streaking for single spore isolates by using a fine glass needle to gently spread a small quantity of spores on MYG or MU plates. The plates were incubated at 37°C for 2 to 3 days. This procedure was repeated, from well separated colonies, to ensure purification.

2.4.2.2 Isolating *Aspergillus nidulans* Cleistothecia

Cleistothecia were obtained by incubating *A. nidulans* on MYG plates, supplemented as required, at 37°C until well sporulating. Plates were then sealed with laboratory film and incubated at 37°C until mature cleistothecia surrounded by Hulle cells were formed. Cleistothecia were harvested using a fine, blunted, glass needle and a compound microscope. The cleistothecia were transferred to a 5% water agar plate and individual cleistothecia were separated, using the glass needle, and cleaned of the outer Hulle cells by rolling on the agar surface. The clean cleistothecia were placed in 50 µl of dH_2O and burst to release the spores which were then plated on MU media.

2.4.2.3 Preparation of Large Scale Spore Suspensions

Using a sterile loop spores from a pure colony were suspended in dH_2O. The spore suspension was spread onto MYG or MU plates and the plates incubated at 37°C until a well sporulating lawn was formed (3 to 4 days). In a biohazard cabinet the lawn was flooded with Tween 80/saline solution (Section 2.5.23) and the spores resuspended by gently scraping the surface of the lawn with a glass spreader. The spores were harvested by centrifugation (5 min, 6 000 **g**), washed twice with dH_2O and resuspended in saline solution (Section 2.5.11). Aliquots were stored at 4°C, or frozen at -20°C or -70°C.

2.4.2.4 Long Term Storage in Glycerol and on Silica

Fungal cultures can be stored in glycerol at -70°C by homogenising mycelia in dH_2O and adding an equal volume of 100% glycerol.

A. nidulans spore suspensions, prepared in a 5% solution of non-fat milk powder, were

poured into a cooled glass jar 2/3 fill with silica gel (previously dry sterilised at 180°C for 90 min) and stood on ice for 15 min. The glass jars were placed at room temperature until crystals readily separated when shaken (~1 week). The cap was screwed down firmly and the jars stored at 4°C or room temperature.

2.4.2.5 Short Term Storage on Slants

Universal bottles were prepared with 10 ml of *Aspergillus* complete media, supplemented as required, containing 2% agar. After sterilisation the molten media was allowed to set in the universal bottle on an angle to create a slant. The slants were inoculated with *A. nidulans* spores and incubated at 37°C for 2-4 days with loosened lids. The lids were then tightened and the slants stored at 4°C. Sub-culturing to fresh slants was required every 6 to 8 months.

2.5 Common Buffers and Solutions

2.5.1 10 x Gel Loading Dye contained 2 M urea, 50% glycerol, 50 mM TAE (Section 2.5.18) and 0.4% bromophenol blue.

2.5.2 Denaturation Solution contained 0.5 M NaOH and 1.5 M NaCl (not sterilised)

2.5.3 10 x Denhardt's Solution contained 0.5 g Ficoll (Sigma), 0.5 g polyvinylpyrolidone, 0.5 g bovine serum albumin (BSA, Sigma, pentax fraction V) and dH_2O to 50 ml. The 10 x solution was filter sterilised and stored in aliquots at -20°C.

2.5.4 Depurination Solution contained 0.25 M HCl (not sterilised).

2.5.5 Hybridisation Solution for Northern analysis contained 5 x SSPE (Section 2.5.13), 5 x Denhardt's (Section 2.5.3), 0.2% sodium dodecyl sulphate (SDS) and 50 µg/ml sheared salmon sperm DNA (Section 2.5.14) (not sterilised).

2.5.6 Hybridisation Solution for Southern analysis contained 3 x SSC (Section 2.5.12), 0.02% Denhardt's (Section 2.5.3), 0.5% SDS and 50 µg/ml sheared salmon sperm DNA (Section 2.5.12) (not sterilised).

2.5.7 Phenol (Tris-equilibrated) was prepared by melting solid ultra pure phenol at 50°C. Hydroxyquinoline was added to a final concentration of 0.1% (w/v). An equal volume

of 1 M Tris-HCl (pH 8.0) was added at room temperature and the solution well mixed. The Tris-HCl phase was removed once the phases had separated. This was repeated with 100 mM Tris-HCl (pH 8.0) until the aqueous Tris-HCl phase had a pH greater than 7.8. The equilibrated phenol was retained and an equal volume of 100 mM Tris-HCl (pH 8.0) added. The equilibrated phenol solution was stored in an acid washed glass bottle covered in aluminium foil at 4°C (not sterilised).

2.5.8 Neutralisation Solution contained 1.5 M NaCl and 0.5 M Tris-HCl. The pH was adjusted to 7.4 with concentrated HCl.

2.5.9 OM Buffer contained 1.2 M $MgSO_4.7H_2O$ and 10 mM Na_2HPO_4. The pH was adjusted to 5.8 with 100 mM stock of $NaH_2PO_4.2H_2O$.

2.5.10 PEG Transformation Solution contained 40% Polyethylene glycol 4000, 1 M sorbitol, 50 mM $CaCl_2$ and 50 mM Tris-HCl (pH 8.0).

2.5.11 Saline Solution contained (g/l): NaCl, 8.

2.5.12 20 x SSC contained 3 M NaCl and 0.2 M tri sodium citrate. 2 x and 3 x SSC was prepared by diluting 20 x SSC with dH_2O (not sterilised).

2.5.13 20 x SSPE contained 3 M NaCl, 175 mM $NaH_2PO_4.2H_2O$ and 20 mM Na_2EDTA (pH 7.4).

2.5.14 Sheared Salmon Sperm DNA was prepared by dissolving 10 mg/ml of DNA (Sigma Type III sodium salt) in water at 65°C overnight. The DNA was sheared by sonication on ice and then denatured by boiling for 10 min. The DNA was cooled rapidly on ice and 1 ml aliquots were stored at -20°C.

2.5.15 ST Buffer contained 0.6 M sorbitol and 100 mM Tris-HCl (pH 8.0).

2.5.16 STC Buffer contained 1 M sorbitol, 50 mM Tris-HCl (pH 8.0) and 50 mM $CaCl_2.2H_2O$.

2.5.17 STET Buffer contained 8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM Na_2EDTA (pH 8.0) and 50 mM Tris-HCl (pH 8.0).

2.5.18 10 x TAE Buffer contained (g/l): Tris, 48.4; Na_2EDTA, 7.5; and glacial acetic acid 11.4 ml. This was diluted 10 fold to provide a working solution of 1 x TAE.

2.5.19 10 x TBE Buffer contained (g/l): Tris, 108; boric acid, 55; and Na_2EDTA 9.3. This was diluted 10 fold with dH_2O to provide a working solution of 1 x TBE.

2.5.20 TE Buffer 10/1 contained 10 mM Tris-HCl (ph 8.0) and 1 mM Na_2EDTA (pH 8.0). TE 10/0.1 contained 10 mM Tris-HCl (pH 8.0) and 0.1 mM Na_2EDTA (pH 8.0).

2.5.21 TES Buffer (10/1/100) contained 10 mM Tris-HCl (pH 8.0), 1 mM Na_2EDTA (pH 8.0) and 100 mM NaCl.

2.5.22 10 x TNE Buffer contained (g/l): Tris, 12.1; Na_2EDTA, 3.7; and NaCl, 58.4 The pH was adjusted to 7.4 with HCl. This was diluted 10 fold with dH_2O to provide a working solution of 1 x TNE.

2.5.23 Tween/Saline Solution contained 25 ml Tween 80 stock solution (10 ml Tween 80/l dissolved at 60°C) and 8 g of NaCl in a total volume of 1 l.

2.6 Plasmid DNA Isolation

2.6.1 Small Scale Plasmid Isolation by Rapid Boil Method

A 3 ml LB broth culture of *E. coli* cells was prepared by inoculating 3 ml of LB in a test tube and shaking overnight at 30°C or 37°C. A total of 1.5 ml of the broth culture was pelleted by centrifugation for 2 min in a 1.5 ml microcentrifuge tube. The supernatant was drained and the pellet resuspended in 350 µl of STET buffer (Section 2.5.17). Lysozyme [25 µl of a 10 mg/ml solution (in 10 mM Tris-HCl, pH 8.0)] was added and the tube mixed and placed in a boiling water bath for 40 sec. The tube was immediately centrifuged for 10 min and the gelatinous pellet removed using a sterile toothpick. The DNA was precipitated by the addition of an equal volume of isopropanol, mixed by inversion and then placed on ice for 10-20 min. The precipitated DNA was pelleted by centrifugation for 15 min in a microcentrifuge. The plasmid was washed once with 70% ethanol and dried for 5 min under vacuum, prior to resuspension in 30 µl dH_2O or TE 10/0.1. This method was based on that of Homes and Quigley (1981).

2.6.2 Large Scale Plasmid Isolation by Alkaline Lysis Method

A 5 ml LB broth, supplemented with the appropriate antibiotic, was inoculated with *E. coli*, containing the desired plasmid, and incubated overnight with shaking at 37°C. This culture broth was used to inoculate 1/100 (2.5 ml) of 250 ml appropriately supplemented LB media (x 2). These were incubated at 37°C with shaking until the culture was in the exponential phase (OD_{600} = ~0.4). The cells were harvested by centrifugation for 10 min at 4°C, 6 000 **g**. The pellet from 500 ml of culture was resuspended in 4 ml of GTE solution [50 mM glucose; 25 mM Tris-HCl (pH 8.0); 10 mM Na_2EDTA (pH 8.0)] and transferred to a 30 ml corex tube. 1 ml of lysozyme solution (25 mg/ml in GTE solution) was added and thoroughly mixed. After 10 min incubation at room temperature 10 ml of freshly prepared NaOH/SDS (0.2 M NaOH, 0.1% SDS) was added and then mixed until the solution became homogeneous. After standing on ice for 10 min, 7.5 ml of potassium acetate solution (29.4 g potassium acetate (3 M) and 5 ml of 90% formic acid (1.18 M) per 100 ml) was added and mixed gently until the viscosity was reduced. The precipitate was pelleted by centrifugation at 4°C, 20,000 **g**, for 10 min then the supernatant, containing the DNA, transferred to a fresh tube. The DNA was precipitated by the addition of 0.6 volumes of isopropanol and allowed to stand, after mixing, for 10 min at room temperature. The DNA was pelleted by centrifugation at 16,000 **g** for 10 min. After washing in 70% ethanol the pellet was dried under vacuum and resuspended in TE, or further purified by PEG precipitation. This method was based on Current Protocols Unit 1.7 (1989).

2.6.2.1 Purification of Plasmid DNA by PEG Precipitation

The pellet obtained by large scale alkaline lysis plasmid isolation (Section 2.6.2) was resuspended in 1 ml GTE buffer. RNAse was added, 2 µl of a 10 mg/ml stock, and the solution incubated for 20 min at 37°C. 2 ml of NaOH/SDS was added and incubated at room temperature for 5-10 min before the addition of 1.5 ml potassium acetate solution. After incubation at room temperature for 5-10 min the solution was centrifuged for 10 min at 14,500 **g**, at room temperature. The supernatant was transferred to a clean tube, extracted with phenol/chloroform (Section 2.8) and then the DNA precipitated with 95% ethanol and 1/4 volume of 10 M ammonium acetate. The DNA was pelleted by centrifugation, washed with 70% ethanol then dried under vacuum. The pellet was resuspended in 2 ml TE and 0.8 ml PEG solution (30% PEG 8000, 1.6 M NaCl) and placed at 0°C overnight. The DNA was pelleted by centrifugation at 8,000 **g**, at 4°C for 20 min. The pellet was resuspended in TE then precipitated with ethanol (Section 2.9) before final resuspension in TE. This method was based on Current Protocols Unit 1.7 (1989).

2.6.3 Isolation of DNA Fragments

DNA fragments could be recovered by electrophoresis in SeaPlaque (low melting point agarose) agarose gels. A 1% SeaPlaque gel was prepared in 1 x TAE electrophoresis buffer, melted and poured into the appropriate gel apparatus. DNA containing the desired fragment was loaded onto the gel and electrophoresed at 4°C to separate fragments (Section 2.12). The gel was stained in ethidium bromide and the bands visualised using a long wave UV lamp and then excised from the gel with a scalpel.

2.6.3.1 Extraction of DNA from Sea-plaque Agarose by Phenol Freeze

The excised DNA fragment (Section 2.6.3) was transferred to a microcentrifuge tube with the minimum amount of excess agarose. The agarose was melted at 65°C then covered with Tris-equilibrated phenol (Section 2.5.7), mixed and placed at -20°C for at least 2 hrs. The tube was then centrifuged for 10 min and the aqueous phase, containing the recovered DNA, was extracted with phenol/chloroform (Section 2.8). This is referred to as phenol freeze extraction (Thuring *et al.*, 1975).

2.6.3.2 Extraction of DNA from Sea-plaque Agarose using GENECLEAN

The GENECLEAN KIT manufactured by BIO 101 inc was used to extract DNA from SeaPlaque agarose (Section 2.6.3) according to the manufacturers instructions.

2.7 Fungal Genomic DNA Isolation

A. nidulans genomic DNA was extracted from mycelia. Approximately 2 x 10^7 spores were spread onto a cellophane disc overlaid onto an agar plate, appropriately supplemented, and incubated for 18-20 hrs at 37°C. The mycelial mat formed was harvested by scraping it away from the cellophane disc, and freeze dried.

2.7.1 Small Scale Preparation

Freeze dried mycelia (approximately 30 mg) was ground in a pre-cooled 1.5 ml microcentrifuge tube under liquid nitrogen. The powder was resuspended in 500 µl of DNA extraction buffer [100 mM LiCl, 10 mM Na_2EDTA (pH 8.0), 10 mM Tris-HCl (pH 7.4), and 0.5% SDS] by vortexing. The resuspended powder was phenol/chloroform extracted (Section

2.8), ethanol precipitated and resuspended in dH_2O or TE then quantitated (Section 2.10). This method was based on that of Yoder (1988).

2.7.2 Large Scale Preparation

Freeze dried mycelia was ground in a pre-cooled mortar and pestle to fine powder under liquid nitrogen. The powder was resuspended in 3 ml of extraction buffer (Section 2.7.1) to which an equal volume of phenol was added. The suspension was gently mixed and the aqueous phase recovered by centrifugation then phenol/chloroform extracted (Section 2.8) and ethanol precipitated (Section 2.9). This method was based on that of Yoder (1988).

2.8 Purification by Phenol/Chloroform Extraction

DNA, genomic or plasmid, could be further purified by extracting with Tris-equilibrated phenol (Section 2.5.7) and chloroform. An equal volume of Tris-equilibrated phenol and chloroform was mixed with DNA and centrifuged. The aqueous phase was re-extracted until a clear interface between the aqueous and organic phases was obtained. The aqueous phase was then extracted with one volume of chloroform to remove residual phenol. The DNA in the aqueous phase was precipitated with ethanol or isopropanol (Section 2.9). This method is based on that of Sambrook *et al.* (1989).

2.9 Precipitation of DNA with Ethanol or Isopropanol

To the DNA to be precipitated was added one tenth volume of 3 M sodium acetate and either 2.5 volume of 95% ethanol, or 0.6 volumes of isopropanol. The solution was mixed by inversion and placed at -20°C, for 10-20 min, after which time the DNA was pelleted by centrifugation for 30 min. The pellet was washed once with 70% ethanol and dried under vacuum before resuspension in dH_2O or TE. This method is based on that of Sambrook *et al.* (1989).

2.10 Quantitation of DNA

DNA could be quantified by three methods: spectrophotometrically, for concentrated pure solutions of plasmid DNA; fluorometrically, for small quantities or impure DNA; intensity of ethidium bromide fluorescence after gel electrophoresis (Section 2.12), for very low concentrations of DNA. Generally the spectrophotometric, or the fluorometric, method was used in combination with the intensity of ethidium bromide fluorescence.

2.10.1 Spectrophotometric Quantitation of DNA

Concentrated DNA was appropriately diluted and the absorbance of the solutions (in quartz cuvettes with a 1 cm light path) was determined at both 260 nm and 280 nm. The 260 nm reading allowed the concentration of nucleic acid to be determined as a reading of 1 corresponds to 50 µg/ml of double stranded DNA. The ratio of the readings for 260 nm and 280 nm (OD_{260}/OD_{280}) was used to estimate the purity of the DNA. An OD_{260}/OD_{280} of 1.8 - 2.0 was considered pure.

2.10.2 Fluorometric Quantitation of DNA

A Hoefer Scientific TKO 100 Fluorometer was used for the quantitation of impure DNA, generally fungal genomic DNA as prepared in Sections 2.7.1 and 2.7.2. DNA was quantitated in a dye solution containing 1 x TNE buffer (Section 2.5.22) and 0.1 µg/ml Hoechst 33258 dye. The fluorometer was adjusted to zero against 2 ml of dye solution containing no DNA, and the scale set to 100 after the addition of 2 µl of 100 µg/ml calf thymus DNA (100 ng/ml). This standardisation was repeated until the scale was reliably set, then 2 µl of sample DNA, diluted as required, was added to 2 ml of dye solution. The reading was recorded as the concentration of the DNA solution in ng/µl.

2.10.3 Agarose Gel Quantitation of DNA

The DNA solution was diluted, if necessary, and a series of samples separated by electrophoresis through an agarose gel (Section 2.12) alongside a series of standard DNA solutions of known concentration. Once run the gel was stained with ethidium bromide solution and photographed. The intensity of fluorescence of the DNA of unknown concentration was compared to that of the DNA standards to determine the approximate concentration. This was frequently done to check the concentrations determined by either the spectrophotometric (Section 2.10.1) or fluorometric (Section 2.10.2) methods.

2.11 Restriction Endonuclease Digestion of DNA

DNA was digested by restriction enzymes according to the manufacturers recommendations using the buffer supplied with the enzyme. Digestions of plasmid DNA were performed at the recommended temperature, generally 37°C, for a minimum of 1 hour. The efficiency of digestion was checked by running a small aliquot of the digestion mixture on an agarose gel

(Section 2.12). If digestion was incomplete then fresh enzyme was added, keeping quantities within the range recommended by the manufacturers to avoid star activity, and the reaction incubated for longer. If digestion was still incomplete the DNA was cleaned by phenol/chloroform extraction (Section 2.8) and ethanol precipitation (Section 2.9), then the digestion was repeated. Once complete the reaction was stopped by the addition of 1/5 or 1/10 volume of 10 x gel loading dye (Section 2.5.1). Digestion of genomic DNA was performed in a similar fashion except larger reaction mixtures were set up with higher than recommended amounts of restriction enzymes, and digestion times increased to a minimum of 4 hrs.

2.12 Agarose Gel Electrophoresis of DNA

Horizontal agarose gel electrophoresis was carried out in a Horizon mini gel apparatus at room temperature for approximately 1 hr, or in a Bio-Rad DNA Sub-Cell (150 x 200 mm gel bed) at 4°C overnight. The concentration of ultra pure electrophoresis grade agarose ranged from 0.4% to 1.5% depending on the size of the fragments requiring separation. Higher agarose concentrations were used for the separation of smaller fragments while the lower agarose concentrations were for the separation of larger fragments. Generally mini gels were of 0.7% or 1.0% agarose while larger gels were 1.0% agarose. The appropriate quantity of agarose was added to 1 x TBE electrophoresis buffer (Section 2.5.19) and the agarose melted in a microwave. After cooling to approximately 50°C the gel was poured and allowed to set. DNA samples (mixed with gel loading dye (Section 2.5.1) were loaded and fragments separated by electrophoresis at 80 V to 100 V (Minigels) and 35 V to 45 V (Bio-Rad Sub Cell). After electrophoresis gels were stained with ethidium bromide (2 μg/ml), by submerging for 5-20 min, depending on the size of the gel and the age of the ethidium bromide stain, and then destained in dH_2O. The gel was positioned on a UV transilluminator, visualised and photographed under UV on Polaroid type 667 film. If a negative was required then Polaroid type 665 film was used. Exposure and developing times were according to the manufacturers instructions.

2.13 Determination of Molecular Weights of DNA Fragments

The sizes of the DNA fragments (in kilobases, kb) were determined by comparison with a set of standard fragments of known size run on the same agarose gel. The distances the standard fragments had migrated from the well were plotted against the known size of the fragments on semi log graph paper, or the GELFRAGMENTSIZER program (Gilbert 1990) was employed. The distance migrated by the fragments of unknown size were measured and their sizes calculated by interpolation of the plot of the standard molecular weight markers. Standard

molecular weight markers used were generally the BRL 1 kb ladder, or λ digested with *Hin*dIII.

2.14 Cloning Procedures

Cloning procedures typically involved the ligation of the DNA fragment of interest into a suitable vector, then introduction into an *E. coli* host by transformation. Depending on the host vector combination, bacterial transformants were screened visually on selective plates containing IPTG, X-gal and ampicillin (Section 2.3.1.3), by blue/white selection (α complementation). Plasmids were isolated from transformants suspected of containing the required ligation product by the rapid boil method (Section 2.6.1). The isolated plasmids were tested by restriction endonuclease digestion (Section 2.11) and gel electrophoresis (Section 2.12).

2.14.1 CAP Treatment of DNA

Vector DNA was digested to completion by the appropriate restriction endonucleases (Section 2.11). To the restriction enzyme reaction mix 0.5 units of calf intestinal alkaline phosphatase (CAP, Boehringer) was added per 5 μg of vector DNA, and the mixture incubated a further 30 min at 37°C. The reaction was stopped by the addition of Na_2EDTA (pH 8.0) to a final concentration of 5 mM, and SDS to a final concentration of 0.5%. Proteinase K was added to a final concentration of 50 μg/ml and the mixture incubated at 56°C for 30 min. Alternatively the reaction was heated to 65°C for 5 min after incubation with CAP to stop the reaction. After either method of stopping the reaction the mixture was phenol/chloroform extracted (Section 2.8) and the ethanol precipitated DNA (Section 2.9) resuspended in dH_2O.

2.14.2 Ligation

A 10 μl ligation mixture contained 1 μl of 10 x ligation buffer (New England Biolabs), at least 20 ng of insert and 20 ng of vector (generally a 2-3 molar excess of insert:vector), 1.0 μl of a 1/10 dilution T4-DNA ligase (40 units/μl, New England Biolabs), and dH_2O. For non-directional cloning the vector was treated with calf alkaline phosphatase (Section 2.14.1). For blunt end ligations the T4 ligase was added undiluted. The ligation mixtures were left overnight at 4°C, or at room temperature for > 4 hrs.

The efficiency of ligation was checked by removing a 2 µl aliquot of the ligation mixture, prior to the addition of the T4-ligase, and adding it to 2 µl of gel loading dye (Section 2.5.1). This was examined by gel electrophoresis alongside the comparative volume of the ligation mix after incubation.

2.14.3 Bacterial Transformation by Electroporation

Electrocompetent *E. coli* (Section 2.14.3.1) cells were thawed gently on ice and divided into 40 µl aliquots in microcentrifuge tubes placed on ice. To the aliquots was added 1-2 µl of DNA (ligation reactions, or control plasmids used to check the efficiency of ligation) and the cell/DNA mixture left on ice for greater than 1 min. The Gene Pulser (Bio-Rad) was set to 25 µF and 2.5 kV, and the pulse controller to 200 Ω resistance in parallel with the sample chamber. The cell/DNA mixture was transferred to an ice-cold 0.2 cm cuvette, shaken to the bottom then placed in the sample chamber and pulsed at the above settings. A time constant of approximately 4-5 msec was recorded for successful pulses. The cells were immediately resuspended in 1 ml of SOC or LB medium (Section 2.3.1.1) and incubated at 37°C for 1 hr to aid recovery of transformed *E. coli*. The cells were diluted and plated on LB media to determine viability of the cells after transformation, and LB media, appropriately supplemented, to select for transformants.

2.14.3.1 Preparation of Electro-Competent *E. coli* Cells

Supplemented LB media, 1 l, was inoculated (1/100) with an overnight culture of the desired *E. coli* strain and grown with vigorous shaking (300 rpm) at 37°C until mid log phase (OD_{600} 0.5-1.0, approximately 3 hrs). The cells were chilled on ice and harvested by centrifugation at 4 000 g for 10 min (all subsequent steps were done on ice, and centrifugations were at 4°C). The cells were washed in ice cold dH_2O, 1 l then 500 ml, and then ice cold 10% glycerol, 20 ml. Cells were finally resuspended in 4 ml 10% glycerol and 200 µl aliquots were stored at -70°C.

2.15 Sequencing

2.15.1 Labelling Reaction

Vectors prepared by large scale alkaline lysis (Section 2.6.2) and purified by PEG precipitation

(Section 2.6.2.1) were labelled using the PRISM^{TM} Ready Reaction DyeDeoxy^{TM} Terminator Cycle Sequencing Kit. The double stranded vector DNA, 1 µg in 5 µl, was mixed with 9.5 µl Terminator Premix and 3.2 pmol primer (Table 2) in a final volume of 20 µl. The reaction mixtures were placed in the Corbett FTS-960 thermal cycler preheated to 96^{o}C, then subjected to 25 cycles of 96^{o}C for 10 sec, 50^{o}C for 5 sec and 60^{o}C for 4 min. The reaction was held at 4^{o}C until the extraction step. The volume of the reaction mix was increased by adding 80 µl of dH_2O. The DNA in the reaction mix was cleaned by phenol/chloroform extraction (Section 2.8) and the DNA concentrated by ethanol precipitation (Section 2.9).

2.15.2 Sequencing

The labelled DNA (Section 2.15.1) samples were analysed on an Applied Biosystems Model 373A DNA Sequencing System.

2.16 Preparation of *Aspergillus nidulans* Protoplasts

Protoplasts were prepared from uninucleate conidia, or multinucleate mycelia, by digestion of the cell wall with a hydrolytic enzyme, in the presence of an osmotic stabiliser, to release the fungal cell components surrounded by a cell membrane.

2.16.1 Protoplasts from Conidia

Flasks containing MU media were inoculated with spores to a final concentration of 2 x 10^7 spores/ml. The flasks were incubated with shaking at 37^{o}C until approximately 30% had formed germ tubes (approximately 7 hrs). Spores were harvested by centrifugation at 4^{o}C and resuspended in Novozyme solution (Section 2.16.2). Incubation in Novozyme was at 37^{o}C with gentle shaking for 18 hrs. Protoplasts were harvested as described in Section 2.16.2.

2.16.2 Protoplasts from Mycelia

Protoplasts were prepared using a modification of the method described by Yelton *et al.* (1984). Fungal cultures were grown by inoculating approximately 2 x 10^7 spores onto MU plates overlaid with a cellophane disc, and incubating for 18 hours at 37^{o}C. The cellophane discs, covered in fungal mycelium, were incubated in filter sterilised OM buffer (Section 2.5.9)

Table 2 Primers used in PCR and Sequencing Reactions

Primer	Size	Tm°C[a]	Sequence[b]	Source
Forward M13	22 mer	70	gCCAgggTTTTCCCAgTCACgA	Promega
Reverse M13	24 mer	70	gAgCggATAACAATTTCACACAgg	Promega
act1	20 mer	59	TgATCggTATgggTCAgAAg	This study[c]
act2	20 mer	63	TTTCACgCTCAgCggTAgTg	This study[c]
nia1	20 mer	60	gTCTTAggCTTCTTgACgCC	This study[c]

[a] Calculated as Tm = 2(A+T) + 4(G+C).
[b] 5' to 3' sequence.
[c] Custom made.

containing 10 mg/ml Novozyme 234 (Novo Industries A/S) at 37°C for 1.5 hrs with gentle shaking. The protoplast suspension was distributed into corex tubes (5 ml per tube) and gently overlaid with 1 ml of ST buffer (Section 2.5.15). Protoplasts were collected at the interphase by centrifugation at 1075 g for 5 min. The protoplasts were washed twice in STC buffer (Section 5.5.16) and resuspended in STC buffer at a final concentration of 1.25 x 10^8 protoplasts/ml.

2.17 Storage of Protoplasts

Protoplast preparations (Section 2.16) not used immediately for transformation were stored at 4°C overnight, or, after the addition of 0.25 volumes of PEG transformation solution (Section 2.18), at -70°C for up to three months.

2.18 Transformation of *Aspergillus nidulans*

Standard transformations were carried out using a procedure modified from Itoh *et al* (1994). To 40 µl of a protoplast suspension in STC buffer (Section 2.5.16) (5.0 x 10^6 protoplasts) was added 10 µl of PEG transformation solution (40% Polyethylene glycol 4000, 1 M sorbitol, 50 mM $CaCl_2$, 50 mM Tris-HCl, pH 8.0)), 2.5 µg of plasmid DNA (linear or circular) and 1.0 µl of spermidine (50 mM in H_2O). The transformation reaction mixtures were mixed gently and incubated on ice for 30 minutes, then a further 450 µl of PEG transformation solution (9 volumes) was added and incubation continued at room temperature for 15 min. The transformation mix was diluted 10-fold in STC buffer and plated on osmotically stabilised non-selective medium to determine protoplast viability after transformation, and on osmotically stabilised selective medium for selection of transformants.

2.18.1 Plating Protoplasts

Aliquots of the transformation mixture, diluted in STC buffer (Section 2.5.16) were added to 3 ml of molten top agar (osmotically stabilised selective or non-selective media, 0.8% agar) at 50°C and overlaid onto osmotically stabilised plates. The number of viable mycelial fragments in the protoplast mix was determined by diluting the protoplasts in dH_2O and plating on non-selective media that was not osmotically stabilised. Plates were incubated at 37°C. After two days incubation regenerated protoplasts on non-selective media were counted, and after 3-4

days transformants on selective media were recorded.

2.18.2 Selection and Purification of Transformants

Transformants of *A. nidulans* 1-85 transformed with the vector pGM32 were selected on osmotically stabilised MYG plates. Transformants were purified by streaking for single spore isolates (Section 2.4.2.1) on selective MYG plates and then non-selective MU plates. If the purity of the transformants was in question they were further streaked on non-selective media.

2.18.3 Identification of Integration Events

The purified strains were tested for growth on selective and non-selective media. Transformants, able to grow on selective media, were plated on minimal media containing the appropriate nitrogen source to identify gene disruptions at the *niaD* or *amdS* loci. The ploidy of the transformants was also determined (Section 2.19).

2.18.3.1 Growth on Selective Media

Purified transformants were grown on *Aspergillus* minimal media (Section 2.3.2.1) supplemented with para-aminobenzoic acid and a nitrogen source from stocks of potassium nitrate, sodium nitrite, glutamate, acetamide and ammonium chloride (Section 2.3.2.3). Growth on 10 mM nitrite was retarded so 5 mM was used. The distinction between $amdS^+$ and $amdS^-$ transformants was enhanced if 12.5 mM CsCl was included in the media (Tilburn *et al.*, 1983).

2.18.3.2 PCR

PCR analysis of transformants was performed on intact conidiospores and was optimised for the internal control primers, act1 and act2 (Table 2), which amplify a section of the actin gene. The nial and the reverse primer (Table 2) detects disruption of the *niaD* locus. For n PCR reactions a cocktail for n + 1 reactions was prepared on ice. A single reaction mix contained: 2.5 µl of 10 x *Tfl* buffer (Boehringer); 1.5 mM $MgCl_2$; 1 µl of 1.25 mM dNTPs; 10 pmol primer 1 and 10 pmol primer 2; and 1 unit of *Tfl* (Boeringer); and dH_2O to 22.5 µl. Aliquots of 22.5 µl were pipetted into 0.2 ml strip tubes for use in the Corbett FTS-960 thermal cycler. Spores were added (1 x 10^4 - 9 x 10^4 spores), in a total volume of 2.5 µl, to the appropriate aliquot and mixed gently. A negative control containing water only was included in each PCR

run and was prepared as the last reaction for each set. The reaction vessels were placed in the thermal cycler pre-heated to 94°C and incubated for 6 min at 94°C. The reaction mix was then subjected to 30 cycles of 94°C for 45 sec, 55°C for 45 sec and 72°C for 2 min, and then held at 72°C for 5 min before storage at 4°C. The reaction was checked on an agarose gel (Section 2.12). Amplification of the actin gene resulted in a product of 425 bp. Disruption of the *niaD* locus with pniaD2 or pniaD3 resulted in a product of 2.4 kb, while disruption with pniaD1 resulted in a product of 1 kb. This method was described by Aufauvre-Brown *et al.* (1993).

2.19 Determination of Ploidy

2.19.1 Tolerance to Benomyl

The ploidy of transformants was initially determined by testing tolerance to benomyl as haploid cultures are more tolerant to benomyl than diploid cultures (Upshall *et al.*, 1977). Transformants were grown on sets of plates containing between 0.8 - 2.0 µg benomyl/ml. Diploid cultures were identified as those showing comparatively reduced growth. The ploidy was confirmed by measuring the spore diameter (Section 2.19.2).

2.19.2 Measurement of Spore Diameter

Putative diploids were selected (Section 2.19.1) and their ploidy confirmed by measuring the average spore diameter. Diploid strains have larger asexual spores with a diploid to haploid spore diameter ratio of 1.3:1 (Pontecorvo *et al.*, 1953).

2.20 Southern Blotting and Hybridisation

2.20.1 Southern (capillary) Blotting

Transfer of DNA to nylon membrane was by capillary blotting. The DNA was separated on an agarose/TBE gel by electrophoresis (Section 2.12) overnight at 4°C. The gel was stained and photographed next to a ruler so that 0 mm corresponded to the position of the wells in the gel. The gel was placed in a container, covered with depurination solution (Section 2.5.4) and gently shaken for 15 min. The gel was rinsed with dH_2O, covered with denaturation solution (Section 2.5.2) then gently shaken for 30 min. This was repeated with neutralisation solution (Section 2.5.8) for 30 min and finally 2 x SSC (Section 2.5.12) for 5 min. The gel was trimmed to remove the section above the wells and any excess regions not required for DNA transfer. The final gel dimensions were recorded.

The capillary blotting apparatus was prepared by laying two sheet of 3MM chromatography paper, soaked in 20 x SSC, on a plastic or glass platform so that the ends of the sheets over reached and folded into a trough containing 20 x SSC. Gladwrap was used to cover the 3MM paper and the trough, and a section the size (less 2 mm from each side) of the gel removed. The treated gel was placed on the exposed 3MM paper so that it overlapped the Gladwrap. A piece of nylon membrane (Hybond-N, Amersham) the size (plus 2 mm on each side) of the gel was wetted in 2 x SSC and laid on the gel. At each stage care was taken to remove any air bubbles that may have been trapped between the layers. Two pieces of 3MM paper the same size as the section removed from the Gladwrap, and pre-soaked in 2 x SSC, were placed on the nylon membrane. This was repeated using dry 3MM sheets and then a stack of dry paper towels was placed on top of the 3MM paper. This was followed by a flat perspex tray and a weight of approximately 350g. The DNA was left to transfer overnight (> 4 hrs) and the apparatus disassembled. The position of the wells, and orientation, was recorded on the membrane which was washed in 2 x SSC for 5 min, then baked at 80°C for 2 hrs under vacuum. This was based on the method of Southern (1975).

2.20.2 Preparation of [α-^{32}P]dCTP-Labelled Probe

DNA was labelled according to the manufacturers instructions using 3-5 µl of [α-^{32}P]dCTP, at 3000 Ci/mmol, and the following kits: Ready-to-go kit from Pharmacia, High prime kit from Bio-Rad and Rad-Prime kit from Life Technologies.

2.20.3 Removal of Unincorporated Nucleotides

Unincorporated nucleotides were separated from labelled DNA using freshly prepared minispin columns or commercial columns.

2.20.3.1 Minispin Column Chromatography

Minispin columns were prepared in a 1 ml plastic, disposable, Termo, Tuberculin syringe. The syringe was plugged with siliconised glass wool and then filled, to the 1 ml graduation, with Sephadex G-50 resin, equilibrated in TES (10/1/100, Section 2.5.21). The end of the syringe was placed in a hole in the top of a microcentrifuge tube and spun at approximately 1,500 **g** for 3 min in a swinging bucket rotor. Additional resin was added, and the column spun, until the total volume of resin was 0.9 ml, and did not alter after spinning. The column was equilibrated by adding 100 µl of TES (10/1/100) and centrifuging as above. This step was repeated twice.

TES (10/1/100) was added to the radio-labelled DNA (Section 2.20.2) to bring the total volume up to 100 µl. This was added to the column and collected in a fresh microcentrifuge tube by centrifuging as above.

2.20.3.2 Commercial Columns

The unincorporated nucleotides were separated from the labelled DNA using commercial columns according to the manufacturers instructions. The commercial columns used were: ProbeQuant^{TM} G-50 Micro Columns, from Pharmacia Biotech; and Quick Spin^{TM} Columns, from Boehringer Mannheim.

2.20.4 Hybridisation of Radio-Labelled DNA to Southern Blots

Southern blots were prehybridised for at least 2 hrs at 65^{o}C in approximately 50 ml of hybridisation buffer (Section 2.5.6) in a sealed glass tube. After prehybridisation the hybridisation buffer was poured off leaving approximately 7 ml in the tube. The probe DNA was boiled for 3 min, placed on ice for 2 min and then added to the hybridsation solution remaining in the tube. The blot and probe were hybidised overnight at 65^{o}C and then unbound probe removed by washing three times in 0.1 to 3 x SSC, at 65^{o}C, for 20-30 min. The blot was removed and wrapped in Gladwrap, while still damp, then exposed to X-ray (Kodak or Fuji Medical) film. Exposure was in the presence of a Cronex intensifying screen in a X-ray cassette at -70^{o}C. After exposure to the film, the film was developed.

2.20.5 Stripping Hybridised DNA from the Southern Blot

2.20.5.1 Boiling SDS

The bound probe DNA was removed from the membrane by pouring a boiling solution of 0.5% SDS directly onto the membrane. After cooling to room temperature this was repeated twice. The stripped membrane was checked by autoradiography (Section 2.20.4). If stripping was not completely successful then the membrane was stripped using the alkali method (Section 2.20.5.2).

2.20.5.2 Alkali

The bound probe DNA was removed from the membrane by incubating the blot in 0.4 M

NaOH for 30 min at 45°C. The blot was transferred to a solution of 0.1 x SSC, 0.1% SDS and 0.2 M Tris-HCl (pH7.5) and incubated a further 15 min. The stripped membrane was checked by autoradiography (Section 2.20.4).

2.21 Techniques for Working with RNA

Equipment and reagents used for work with, or preparation of, RNA were specially treated and stored separately. Glassware was acid washed and baked in a dry oven at 180°C. Plastic labware (tips and microcentrifuge tubes) were handled with clean disposable gloves and autoclaved in glassware (prepared as described). All pipetting of solutions was with a set of dedicated pipettors which were used exclusively for RNA work. Solutions were prepared from dedicated stocks and autoclaved.

2.21.1 RNA Isolation from Fungal Cultures

Freeze dried mycelium (Section 2.7) was ground to a fine powder under liquid nitrogen in a pre-cooled mortar and pestle. The powder was transferred to 1 ml of 80°C phenol extraction buffer [containing: 100 mM Tris-HCL, pH 8.0; 10 mM Na_2EDTA, pH 8.0; and 1% SDS, mixed with 1 volume of Tris-equilibrated phenol (prepared as in Section 2.5.7 except hydroxyquinoline was excluded)] and mixed by vortexing. The homogenate was incubated at 80°C for 5 min and an equal volume of chloroform added. The solution was phenol/chloroform extracted (Section 2.8). To the aqueous phase 1/2 a volume of 8 M LiCl was added, the mixture was vortexed and then incubated at -20°C overnight. The precipitated RNA was pelleted by centrifugation at 17 000 **g** for 30 min at 4°C. The RNA was washed with 1 volume of 2 M LiCl (in 50 mM Na_2EDTA, pH 8.0) and recentrifuged. The pellet was washed twice with 3 M sodium acetate (pH 5.5) then resuspended in TE (10/1). This method was based on that of Shirzadegan *et al.* (1991).

2.21.2 Northern Blotting of RNA and Hybridisation to [α-^{32}P]dCTP-Labelled Probes

Based on the method of Bradshaw and Pillar (1992).

2.21.2.1 Glyoxalation of RNA

To RNA in a 4 µl volume was added 16 µl of a mixture of 40% deionised glyoxal (Sigma, G-3140, deionised under light mineral oil on a mixed bed resin [Bio-Rad AG 501-X8] and stored at -80°C in aliquots), 20 x MOPS buffer [400 mM 3-(N-morpholino) propane-sulfonic acid, 60 mM sodium acetate and 20 mM Na_2EDTA, pH 7.0], and dimethylsulfoxide (DMSO, Fluka, analytical grade, 41640) in a ratio of 50:8:100. The mixture was incubated at 50°C for 1 hr and under a layer of light mineral oil (Sigma M-3516).

2.21.2.2 Glyoxal Gel

A 1% agarose gel in 1 x MOPS buffer (Section 2.21.2.1) was poured on a 12.5 x 10 cm glass plate and allowed to form by surface tension. All 20 µl of the glyoxalation reaction (Section 2.21.2.2) was loaded, and 10 x gel loading dye (Section 2.5.1) loaded in an adjacent lane. The gel was run at 70 V at 4°C until the marker dye had run 2/3 the length of the gel. The gel was then stained and photographed (2.12).

2.21.2.3 Northern Blotting and Hybridisation

RNA from the glyoxal gel (Section 2.21.2.2) was transferred to a nylon membrane (Amersham, Hybond-N) by capillary blotting, as in Section 2.20.1, except the gel was not pre-treated, and the transfer was at 4°C overnight. The membrane was baked, without washing, at 80°C for 2 hrs in a vacuum oven to reverse the glyoxalation, and to fix the RNA to the membrane. Preparation of the [α-^{32}P]dCTP-labelled probe DNA, hybridisation and autoradiography was performed as in Sections 2.20.2 to 2.20.5.

2.22 Enzyme Extraction

2.22.1 Determination of Mid-log Phase of Fungal Cultures

Siliconised flasks (500 ml) containing 100 ml of selective (for transformants) or non-selective (for parental strain 1-85) media were inoculated with spores to a final concentration of 10^6 spores/ml and incubated with shaking (250 rpm) at 37°C. At 12.25, 13.5, 15, 18, 21, 24 and 48 hours 10 ml samples were transferred to Falcon tubes and the mycelium harvested by centrifugation, then snap frozen in liquid air. After all the samples were collected they were freeze dried and the weight of the mycelia determined. Plots of mycelial dry weight vs time were drawn and from them the incubation time to mid log phase calculated.

2.22.2 Crude Protein Extraction from Fungal Mycelia

Mycelium from 100 ml mid-log phase cultures (Section 2.22.1) was harvested by centrifugation, washed once with dH_2O and then freeze dried. The freeze dried mycelium, 140 to 250 mg, was ground in liquid nitrogen and extracted in 3 ml of potassium phosphate buffer (pH 7.5, 1 mM dithiothreitol). The extract was cleared of debris by centrifugation for 15 min at 12 000 g. All steps were carried out at 4°C. Aliquots were stored at 4°C, or snap frozen and stored at -20°C. Method modified from Myers *et al.* (1995).

2.22.3 Determination of Protein Concentration

Protein concentration was determined using the Bio-Rad Protein Assay according to the manufacturers microassay procedure. A standard curve was prepared using BSA stock solution of 0.5 mg/ml. Standards were prepared for 2.5 µg/ml, 5 µg/ml, 7.5 µg/ml and 10 µg/ml, in duplicate, and the absorbance at 595 nm recorded after incubation at room teperature for 5 min. The concentration of the unknowns was determined by comparing the absorbance at 595 nm to the standard curve obtained.

2.23 Enzyme Assays

Orotidine 5'monophosphate decarboxylase activity was measured by following a decrease in absorbance at 285 nm at 25°C. In a quartz cuvette with a 1-cm light path was placed 0.5 ml 100 mM potassium phosphate buffer (pH 6.0); 0.01 ml of 100 mM 2-mercaptoethanol; 10 - 150 µg of crude protein extract in a total volume of 440 µl (100 mM potassium phosphate buffer (pH 7.5), 1 mM dithiothreitol) and 0.05 ml of 1 mM orotidylate. The reaction was initiated with the addition of the orotidylate and the activity calculated based on a molar extinction coefficient of 1.65×10^{-3} $cm^{-1}M^{-1}$ for the conversion of orotidylate to UMP. One enzyme unit is described as the amount of enzyme which catalyses the decarboxylation of 1 µmole of orotidylate to UMP per minute. Method was based on that of Yoshimoto *et al.* (1978).

2.24 Statistical Analysis

Statistical differences in frequency data (numbers of ectopic and targeted transformants and the numbers of N-type and H-type transformants) were assessed using the Chi-squared test (χ^2) and were based on the null hypothesis that there was no difference in the proportions between

experiments and or treatments. The probability (P) of the null hypothesis being accepted is given in each case, along with the degrees of freedom (df).

Chapter Three

Optimisation of Protoplast Isolation from, and Transformation of, *Aspergillus nidulans*

3.1 Summary

Transformation of the *Aspergillus nidulans pyrG* auxotrophic haploid strain 1-85 was optimised for the vector pGM32 containing the heterologous *Neurospora crassa pyr-4* gene. The conditions for optimal protoplast preparation that were investigated included: molarity of $MgSO_4$ in the cell wall digestion buffer and choice of osmotic stabiliser for protoplast suspension. The conditions for the transformation reaction that were investigated included: addition of heparin; addition of spermidine; vector concentration; vector linearisation; incubation in PEG solution and the use of top agar for plating.

3.2 Introduction

Transformation of a fungal species was first reported in 1973 from the Rockefeller University laboratory of E. L. Tatum (Mishra and Tatum 1973). The transformed strain was an inositol-requiring mutant (*inl*) of *Neurospora crassa.* It was possible to select inositol-independent strains when an *inl*$^-$ mutant was transformed with DNA isolated from a wild type (*inl*$^+$)strain. The *inl* selectable marker was used in subsequent studies by Mishra *et al.* (1973; 1977; 1979). These early results were received with some scepticism at the time due to the belief that eukaryotes were impossible to transform. The results were thrown into doubt on the basis of the procedure not being readily reproducible, and the spontaneous reversion of *inl*$^-$ to *inl*$^+$ at an appreciable frequency. The transformation frequency was up to 30 times that of the spontaneous reversion rate so it was postulated by some that the revertants were somehow selected for. Mishra then transformed *inl*$^-$ mutants with a temperature sensitive *inl* allele to obtain temperature sensitive *inl* transformants which provided stronger evidence that transformation had occurred.

A major breakthrough came when Hutchinson and Hartwell (1967) devised a way of preparing protoplasts of *S. cerevisiae* by digesting the cell walls with glucanase, and stabilising with 1 M sorbitol. Hinnen *et al.* (1978) used protoplasts from a *leu2* mutant strain and transformed them to leucine independence by treatment with wild-type DNA in the presence of calcium chloride. The first shuttle vector for *S. cerevisiae* - *E. coli* was constructed by Beggs (1978) soon after this. It contained replication origins from *S. cerevisiae* and *E. coli*, along with selection

markers for both systems. This vector was also found to replicate well in *Schizosaccharomyces pombe*. Consequently initial progress with transformation procedures was with the non-filamentous fungus *S. cerevisiae*. The technique of protoplast formation was soon extended to the filamentous systems of *N. crassa*, by Case *et al.* in 1979, and *A. nidulans*, by Tilburn *et al.* in 1983. Transformation systems have since been developed for many other fungi.

3.2.1 Transformation Procedures

The optimal transformation procedure is efficient, rapid, simple to perform and does not require the use of toxic chemicals. The original protocols have been modified and improved over the years but have not fundamentally changed in most cases. Transformation of fungal systems has been well reviewed by Fincham (1989), Goosen (1992) and Rambosek and Leach (1987). This prologue focuses on work with *Neurospora* and *Aspergillus* to provide a comprehensive review of the available literature.

3.2.1.1 Protoplast Generation

Protoplast yield is affected by the nature and the molarity of the osmotic stabiliser used, the pH of the lytic medium, period of lytic digestion and age and amount of mycelium used in the digestion mix (Peberdy 1979). The choice of enzyme for digesting the cell wall is also very important. The various preparations used generally contain a complex mixture of hydrolytic enzymes, most notably 1,3-glucanases and chitinase. Some examples are Helicase or Glusulase (commercial snail stomach preparation), Zymolyase (microbiological origin) and Novozyme 234 (fungal origin, *Trichoderma viride*). Variation between enzyme preparations means the batch of enzyme used for the preparation of the protoplasts is an important consideration. Since its successful use in forming *A. nidulans* protoplasts, batches of Novozyme 234 (Hamlyn *et al.*, 1981; Akins and Lambowitz, 1985) have been routinely used for protoplast isolation from filamentous fungi. Some workers, however, still choose to work with more defined enzyme mixtures.

With filamentous fungi the protoplasts can be prepared from many different cell types depending on the species. In *Neurospora* species, germinating macroconidia are used which are predominantly multinucleate. The alternatives are young mycelium, which is also multinucleate, or uninucleate micro-conidia which can be difficult to obtain. *Aspergillus* protoplasts are prepared from germinating conidia, which are uninucleate, or young mycelium, which is multinucleate. The choice of cell type is a matter of convenience, there is little evidence to suggest one type gives better results than another. Wernars *et al.* (1985) found no difference in results when using *A. nidulans* protoplasts prepared from conidia as opposed to

mycelia. They did however find that there was an increase in the protoplast viability after transformation when protoplasts prepared from germinating conidia were used.

There are many procedures for the isolation of protoplasts from *A. nidulans*. The protoplasts isolated from mycelium have been used for fusion and recombination experiments with maximal yields of protoplasts being obtained from cultures at the end of the growth phase (Peberdy 1979). The protoplasts isolated from mycelium vary in the size and number of nuclei. The total content of a hypha between the septa can be isolated as a single protoplast. The heterogeneous nature of the protoplasts may make them less suitable for recombination and some transformation experiments as transformants are often heterokaryons (Upshall 1986). A marked heterogeneity in organelle constitution and biochemical functions is also observed. Skatrud *et al.* (1987) noticed a range of protoplast sizes for *Cephalosporium acremonium*. The larger protoplasts were more likely to be viable and when enriched for, and used for transformation, resulted in an increase in the number of transformants.

Some of these difficulties may be overcome by using the conidiospores of *A. nidulans* for the preparation of protoplasts (Bos and Slakhorst 1981). Protoplasts prepared from conidia are generally small, homogeneous and contain only one or 2 nuclei, depending on the length of the preincubation step. Protoplast isolation from conidia requires preincubation of the conidia in supplemented liquid media until germ tube emergence. The germinating conidia are then incubated in osmotically stabilised buffer containing a lytic enzyme until protoplasts form. These are then purified, washed and resuspended in osmotically stabilised buffer. They can be used for transformation at this stage or stored at 4^{o}C for several days.

Germ tube emergence in *A. nidulans* was studied in detail by Bainbridge (1971) and is shown to exhibit only partial synchrony. The spores were incubated in liquid media at a concentration of 2 x 10^{7} spores/ml. Higher spore concentrations resulted in auto-inhibition of germination. Germ-tube emergence was recorded if the germ tube was equal to half the diameter of the spore. When there was 23.5% germ-tube emergence the percentage of spores with only one nucleus was still 100 (240min). When germ tube emergence was 57% (270min), 2% were binucleate. When conidia are incubated until germination, for use in protoplast preparation, it is therefore important to stop incubation early to ensure maximal yields of uninucleate protoplasts. Unfortunately this means sacrificing the yield of protoplasts as non-germinating conidia do not form protoplasts.

Due to the lack of a cell wall, protoplasts need to be protected and this is achieved by the presence of an osmotic stabiliser in the suspending medium. One of the most popular osmotic stabilisers is sorbitol, at a concentration of 0.8-1.2 M, which is satisfactory for most fungi. A

common alternative osmotic stabiliser for use in the digestion buffer is magnesium sulphate The optimal concentration is specific for different fungal strains with 1.2 M $MgSO_4$ common for protoplast isolation from *A. nidulans*.

Peberdy (1979) compared several osmotic stabilisers for use in preparing protoplasts for fusion experiments. When *A. nidulans* was digested with lytic enzymes in the presence of $MgSO_4$ (0.6 M in 0.01 M phosphate, pH 5.8) two classes of protoplasts were obtained. There were large vacuolate types and small dense protoplasts lacking a vacuole. These showed different sedimentation properties during centrifugation. The larger vacuolate types could be harvested by flotation after centrifugation resulting in preparations relatively free of mycelial debris. This property of buoyancy is specific to $MgSO_4$ as it does not occur with other magnesium salts or sulphates. The $MgSO_4$ method of protoplast preparation for the purpose of transformation was first used successfully by Tilburn *et al.* (1983) with *A. nidulans*.

The effect of protoplast storage on viability has been reported by various groups. Bos and Slakhorst (1981) found storage of *A. nidulans* protoplasts overnight at 4°C, before transformation, to be acceptable. Cullen *et al* (1987) reported that it is acceptable to freeze a fresh preparation of protoplasts and store them at -70°C; these frozen protoplasts yielded the most reproducible results (for the set of experiments described). Vollmer and Yanofsky (1986) reported that freshly prepared *N. crassa* spheroplasts that were stored immediately at -80°C, remain viable and competent for DNA uptake for at least 6 months. However, Penttila *et al.* (1987) found that storage of *Trichoderma reesei* protoplasts at -70°C reduced transformation frequencies by approximately 50%.

The value of protoplasts in physiological and biochemical research is based on the assumption that they are physiologically normal, retaining all the properties of the intact cells from which they were derived. This was studied in some detail by Peberdy (1979) who found that *A. nidulans* protoplasts have a different physiology from the whole cells. The importance of this to transformation is not usually considered but may be important when studying or considering the mechanism of transformation.

Also studied by Peberdy (1979) was the frequency of regeneration of protoplasts. In *A. nidulans* the proportion of protoplasts able to be regenerated was in the range of 10-30%, with no difference observed in defined and complex media. The viability is variable between fungi and the exact reasons are unknown. One factor, however, is the absence of nuclei as it has been shown by nuclear staining of protoplasts that the majority lack a nucleus and are therefore

inviable (Peberdy 1979). Another factor may be the origin of the protoplasts in relation to hyphal organisation (Barrett *et al.*, 1989).

Although relatively straightforward, protoplast preparation requires refinement initially, then careful application. To avoid this several groups have looked into alternatives to protoplasts. One approach is to use a mutant strain with a more permeable membrane, or cell wall, as a recipient for the DNA. Although success has been reported, the transformation frequencies are low (Wootton *et al.*, 1980). High concentrations of alkali metal ions have been used to induce permeability to DNA in intact cells. This involves exposure of intact cells to the transforming DNA in the presence of 0.1 M lithium acetate and was developed for, and routinely used, with *S. cerevisiae* (Ito *et al.*, 1983). *N. crassa* has been successfully transformed using this procedure (Dhawale *et al.*, 1984; Dhawale and Marzluf 1985; Kim and Marzluf 1988) although how it works is not entirely understood.

Another alternative is to physically introduce the DNA into the cells. For example suspensions of *S. cerevisiae*, in growth medium supplemented with 1 M sorbitol and DNA, have been successfully transformed by agitation at high speed, with glass beads, for 30 seconds (Costanzo and Fox 1988). Although viability was low, and so were transformation frequencies, its simplicity is attractive. At a more technically advanced level *Botryotinia fuckeliana* has been transformed to hygromycin resistance by precipitating plasmid DNA onto tungsten particles and delivering them into conidia by a helium driven gene gun (Hilber *et al.*, 1994). *A. nidulans* conidia have also been successfully transformed using a biolistic method (Fungaro *et al.*, 1995).

3.2.1.2 Conditions for DNA Uptake

In the majority of transformation systems to date (fungal, mammalian and bacterial) the universal components have been DNA and calcium ions. The typical components of a fungal transformation mixture are; protoplasts at a density of 10^8 to 10^9/ml, cloned DNA at 5 μg/ml, 10 or 50 mM calcium chloride, and a buffer of 10 mM Tris (pH 7.5 or 8.0) or 10 mM MOPS (pH 6.0). It is not known if the variation in pH 6.0 to 8.0 has an effect (Fincham 1989).

Double stranded DNA, either circular or linear, is generally used, although single stranded DNA has been used effectively in transformation of *Saccharomyces* species (Singh *et al.*, 1982) and *Ascobolus immersus* (Faugeron *et al.*, 1989). The use of linear, as opposed to circular, double stranded DNA resulted in a substantial increase in the transformation frequency of the yeasts *S. cerevisiae* (Orr-Weaver 1981) and *Candida boidinii* (Sakai *et al.*, 1991).

The effect of DNA conformation on transformation efficiency with filamentous fungi is not clear. An increase in transformation frequency has been observed in a number of different systems. Studies with *N. crassa* have shown an increase in the transformation frequency with linear DNA depending on where the vector was cut. Dhawale and Marzluf (1985) observed an increase of 3-4 fold when the vector was linearised within the homologous region, but outside the gene, *qa-2*. Linearisation within the *qa-2* gene resulted in much lower transformation frequencies and analysis of transformants indicated the DNA may recircularise in the cell before integration. When cut in the heterologous vector sequence, no change in transformation frequency was observed. Kim and Marzluf (1988) also found cutting in the homologous region, outside the selectable gene, *trp-1*, resulted in an increase in transformation frequency, whereas cutting within the coding region gave transformation frequencies no different to those with circular DNA. Similar results were found when *A. niger* (Unkles *et al.*, 1989(a)) and *A. oryzae* (Unkles *et al.*, 1989(b)) were transformed with the respective homologous *niaD* genes. Linearisation of the vector outside the *niaD* gene, but within the region of homology, resulted in an approximately 5-fold increase in transformation frequency.

Similar studies have been done with *A. nidulans*. Wernars *et al.* (1985) linearised the DNA outside and within the *amdS* coding region while Yelton *et al.* (1984) linearised within the *trpC* coding region: both observed no change in the transformation frequency when linear or circular DNA was used. When Horng *et al.* (1990) transformed *A. parasiticus* and *A. nidulans* with the *niaD* gene of *A. parasiticus* they found linearisation increased the transformation frequency in the homologous transformation system, while decreasing it in the heterologous system. More work is needed to resolve some of these conflicting results although in general it appears that linearisation within the homologous region, but outside the coding region of the selectable gene, may improve the transformation frequency. The conflicting results for linearisation in the coding region may be due to the nature of the mutation that is being complemented. The studies outlined above do not indicate the effect of linearisation on the types of integration events, this will be considered in Chapter 5.

The optimal amount of DNA to include in a transformation mix has only been studied by a few groups. One of the more extensive studies was done by Yelton *et al.* (1984) working with *A. nidulans* and transforming with the *trpC* gene. They found that increasing the amount of the selectable marker, but not the overall amount of DNA (the balance was made up with vector DNA without the selectable marker), increased the number of stable transformants. At low DNA concentrations the increase was greater than at higher concentrations. Greater than 300 transformants/μg of DNA (1.6×10^{-6} transformants per protoplast) were obtained when less than 0.05 μg of DNA was used, compared to 20 transformants/μg (1.7×10^{-4} transformants

per protoplast) when 10 μg of DNA was used. This suggests a small proportion of cells take up DNA more readily than the majority of those competent to do so. This was supported by Wernars *et al.* (1985) who found an exponential increase in transformation frequency between 2-10 μg of DNA, and a linear increase between 10-50 μg, when *A. nidulans* was transformed with the homologous *amdS* gene. They also found the major factor influencing transformation frequency was the purity of the DNA. Plessis and Dujon (1993) transformed yeast with linear DNA and also observed saturating levels of DNA. They also studied the type of transformants and found increasing amounts of DNA increased the number of tandemly repeated copies. The results suggested this was due to plasmid-plasmid recombination prior to chromosomal integration. In contrast to this Binninger *et al.* (1987) found *Corprinus cinereus* transformation was not sensitive to DNA concentrations over a very wide range.

Virtually all fungal transformation protocols call for the addition of high concentrations of polyethylene glycol following the initial period of exposure to DNA. The concentrations of buffer and calcium which were used in the protoplast preparation are maintained in the PEG solution as up to 10 volumes of 40% PEG 4000 is commonly used. The effect of the PEG is believed to cause the cells to clump, which may facilitate the trapping of the DNA (Fincham, 1989).

Other additions to transformation mixtures have been reported. Some groups working with *Neurospora*, following Case *et al.* (1979), added 1% dimethyl sulphoxide (DMSO), 0.05-0.1 mg/ml heparin (glycosaminoglycan) and 1 mM spermidine (3-aminopropyl-1,4-butane-diamine). These additives are not generally used in transformation mixtures of other fungi.

Chemically or biochemically these additives have a number of known uses and functions, although why they are used in transformation mixtures is unknown. Dimethyl sulphoxide is a trisomigen (a substance which induces segregational errors at both the first and second meiotic division) which was evaluated using the fungus *Sordaria breviollis* (Fulton and Bond 1984). When tested by Cantoral *et al.* (1987) in the transformation of *Penicillium chrysogenum* with the *N. crassa pyr-4* gene it was found to have no effect on transformation frequency. Spermidine is known to stimulate T4 polynucleotide kinase activity (Sambrook *et al.*, 1989). It is included in some plasmid cloning procedures where it is believed to stimulate DNA precipitation at lower temperatures (Promega 1991) which may relate to its role in transformation. Various functions of heparin are known, such as assisting serine protease inhibitors, although how these may influence transformation efficiency is not clear.

3.2.1.3 Electroporation

An alternative method of introducing DNA into fungi is electroporation. Richey *et al.* (1989) investigated the use of a high voltage electric pulse to transform *Fusarium solani* and *Aspergillus nidulans* protoplasts prepared from mycelium. They evaluated the effects of pulse amplitude and duration on transformation frequency and cell viability and found the high amplitude of the electric pulse was of greater importance than its duration. Under optimal conditions for *A. nidulans,* transformation frequencies for two different strains were 8.4 and 20 per μg of DNA (6.0 x 10^7 protoplasts).

Chakraborty *et al.* (1991) transformed germinating conidia of *N. crassa, Penicillium urticae, Leptosphaeria maculans* and *A. oryzae* by electroporation. Under optimal conditions it was found that only a small fraction of the cells were electrocompetent. This was thought to be due to the low permeability of the cell walls. A cell wall weakening agent, β-glucuronidase, was therefore used as it did not seriously compromise the integrity of the wall, or the viability of the competent cells. A degree of strength in the wall was found to be required for the cells to withstand electroporation. They found the transformation efficiency to be determined by both the host cell and the selectable marker employed. To ensure consistently high yields it was therefore crucial to establish precise conditions for each species and strain. Some important factors were found to be the age of conidia, stage of germination, nature of mycolytic enzymes used, duration of treatment, choice of selectable markers, and the selection medium. Under optimal conditions for *A. oryzae* 1-6 x 10^2 transformants/μg DNA (2.5 x 10^6 conidia) were obtained which is considerably higher than the earlier report of Richey *et al.* (1989). This may reflect the use of conidiospores instead of protoplasts.

The filamentous fungus *Pleurotus ostreatus* has been transformed by electroporation (Peng *et al.*, 1992; 1993). The fungus was transformed to Hyg^R at transformation frequencies of 5 to 46 resistant colonies per μg of DNA per 10^7 viable protoplasts. Electroporation was performed on protoplasts prepared from macerated mycelium and suspended in osmotically stabilised medium. The conditions follow a procedure outlined in Goldman *et al.* (1990). This was compared to the standard PEG treatment and they found no significant difference in transformation yields between the treatments. The resulting transformants were unstable to varying degrees, and from them autonomously replicating plasmids were rescued.

3.2.1.4 Regeneration of Protoplasts

To obtain growing colonies from protoplasts it is essential to maintain the osmotic stabilising conditions in the growth medium, at least until the cell wall has regenerated. Apart from studying the type of osmotic stabiliser to use, few studies have been made in this area. Generally protoplasts are added to osmotically stabilised top agar before pouring onto a regeneration plate.

Ballance and Turner (1985) found that increasing the concentration of the agar in the regeneration overlay from 0.9% to 2% resulted in a greater transformation frequency. The number of transformants increased 3-4 fold but there was no alteration in the number of protoplasts regenerated. Yelton *et al*. (1984) found that plating the protoplasts in agar overlays compared to spreading had no effect on regeneration frequency when transforming *A. nidulans* TrpC$^-$ mutants with *trpC*.

3.2.1.5 Selection of Transformants

When selecting for transformants the DNA treated cells are plated on selective media. A list of selectable markers and genes used in the transformation of *A. nidulans* is given in Table 3.

Direct selection of transformants is the most convenient method of selection. This can involve the complementation of an auxotrophic marker to prototrophy, which requires the availability of a mutant recipient strain and the cloned complementary gene. Examples in *A. nidulans* are orotidine-5'-phosphate decarboxylase (*pyrG*), nitrate reductase (*niaD*) and acetamidase (*amdS*). Nitrate reductase and acetamidase selection will be considered in more detail in Chapter 5 and orotidine-5'-phosphate decarboxylase (OMPdecase) selection will be considered in Chapter 4.

Dominant selection systems employ a dominant resistant marker or a dominant mutant gene. If the resistance gene is of prokaryote origin it must be attached to a fungal promoter and termination sequences to give adequate levels of expression. Examples of such genes are *hygB* which confers hygromycin resistance and *ble* which confers phleomycin resistance (Punt and van den Hondel, 1992). Dominant mutant genes that have been used are the β-tubulin genes which encode resistance to the fungicide benomyl, and *oliC* which encodes resistance to oligomycin. These genes require that the strain be sensitive to the applied selective pressure and that the cloned gene be expressed in the recipient. The *amdS* gene can be used as a dominant marker in systems lacking this gene.

Table 3 Genes Used in the Transformation of *Aspergillus nidulans*

Gene	Name or selective feature	Source	Reference
acuD	Isocitrate lyase	*A. nidulans*	Ballance and Turner1986
amdS	Acetamidase	*A. nidulans*	Tilburn *et al.*, 1983
argB	Ornithine transcarbamoylase	*A. nidulans*	John and Peberdy, 1984
benA	β-tubulin	*A. nidulans*	May *et al.*, 1985
ble	Phleomycin resistance	*Streptoalloteichus hindustanus*	Punt and van den Hondel 1992
brlA	Dev. gene-required for conidiation	*A. nidulans*	Johnstone *et al.*, 1985
gusA	β-glucuronidase	*E. coli*	Roberts *et al.*, 1989
hygB	Hygromycin B phosphotransferase	*E. coli*	Punt *et al.*, 1987
lacZ	β-galactosidase	*E. coli*	Van Gorcom *et al.*, 1985
niaD	Nitrate reductase	*A. parasiticus*	Horng *et al.*, 1990
niaD	Nitrate reductase	*A. nidulans*	Johnstone *et al.*, 1990
niaD	Nitrate reductase	*A. oryzae*	Unkles *et al.*, 1989
nimA	Probable protein kinase	*A. nidulans*	Osmani *et al.*, 1988
oliC	Oligomycin resistance	*A. nidulans*	Ward *et al.*, 1986
prn	Proline catabolism gene cluster	*A. nidulans*	Durrens *et al.*, 1986
pyr-4	Orotidine 5'-phosphate decarboxylase	*Acremonium lolii*	Collett *et al.*, 1995
pyr-4	Orotidine 5'-phosphate decarboxylase	*N. crassa*	Ballance *et al.*, 1983
pyrG	Orotidine 5'-phosphate decarboxylase	*A. nidulans*	Oakley *et al.*, 1987
pyrG	Orotidine 5'-phosphate decarboxylase	*A. niger*	van Hartingsvelt *et al.*, 1987
pki	Pyruvate kinase	*A. nidulans*	De Graff *et al.*, 1988
riboB	Riboflavin auxotrophy	*A. nidulans*	Oakley *et al.*, 1987
SpoC1C	Sporulation specific gene cluster	*A. nidulans*	Miller *et al.*, 1987
trpC	Tryptophan synthetase	*A. nidulans*	Yelton *et al.*, 1984
tubC	β-tubulin	*A. nidulans*	May *et al.*, 1985
uapA	Uric acid-Xanthine permease	*A. nidulans*	Diallinas and Scazzochio 1989
uaZ	Urate oxidase	*A. nidulans*	Oestreicher *et al.*, 1993
wA	White conidia	*A. nidulans*	Tilburn *et al.*, 1990
yA	Laccase	*A. nidulans*	Yelton *et al.*, 1985

Other very useful markers allow visual selection for the presence of the transforming gene. For example, selection can be on the basis of conidial colour by using such genes as *yA* which codes for laccase and results in yellow conidia (Yelton *et al.*, 1985) and *wA* which results in white conidia (Tilburn *et al.* 1990). However, it is important to be careful when selecting the recipient strain as these genes are on the same pigmentation pathway leading to the wildtype green conidia; white is epistatic to yellow (Tilburn 1990). Visual selection is also possible using indicator plates. Genes such as *E. coli lacZ* (β-galactosidase) (Van Gorcom *et al.* 1985; Wernars *et al.*, 1987) or *gusA* (β-glucuronidase) (Roberts *et al.*, 1989; Monke and Shafer 1993) can be attached to constitutive, or inducible, fungal promoters.

3.2.1.6 Cotransformation

In bacterial and mammalian cells the introduction of non-selected DNA sequences can be accomplished by cotransformation with a selected gene. This negates the need to link the DNA of interest to the vector containing the selectable gene. This has also been demonstrated in fungi. It appears that when a recipient cell is exposed to two types of DNA simultaneously, there is a high probability that a cell which takes up one type will also take up the other. This can be rationalised by supposing not all protoplasts are competent to take up DNA, but those that are, do so readily (Wernars *et al.*, 1985).

Wernars *et al.* (1987) studied cotransformation of *A. nidulans* with the *amdS* gene, *trpC* gene and *trpC* linked to *E. coli lacZ*. The experiments with *amdS* and *trpC* genes indicate that the frequency of cotransformation is determined by both the absolute amount of the cotransforming vector and the ratio of the transforming and cotransforming vectors. For best results high concentrations of both vectors should be used. The maximum cotransformation frequency that can be reached depends on the nature of the cotransforming DNA: over 95% for *trpC* but only 62% for *amdS* (95% of the Amd^+ transformants were also Trp^+ and 62% of the Trp^+ transformants were also Amd^+). This observation was independent of the strain used, although strain MH1277 was more likely to have multi-copy integration, while strain WG290 had predominantly single-copy integration. Similar results were obtained using a control vector which contained both genes. Co-transformation is a feasible alternative to the sometimes difficult chore of constructing plasmids containing multiple markers.

Consideration must be given to work on transformation competency by Götelueschen and Metzenberg (1995) and Miao *et al.* (1995) working with *N. crassa* which lead to the proposal that DNA integrates preferentially into some nuclei and not others. Furthermore spheroplasts differ in their competency for specific transformation events. The results indicate that

spheroplasts competent to undergo homologous integration are not competent to undergo ectopic integration.

3.2.1.7 Purification of Transformants

The protoplasts used for transformation are likely to be multinucleate because they are formed from multinucleate tissue, such as mycelium or *N. crassa* conidia, or because of PEG fusion of protoplasts during transformation. This means the initial transformants may be heterokaryons containing transformed and un-transformed nuclei. As *A. nidulans* forms uninucleate conidia a heterokaryon may usually be resolved into its components very simply by plating (or streaking) conidia, and isolating single colonies. Generally a few rounds of isolation are performed to ensure purity. When conidia are multinucleate, as in *N. crassa*, it is more difficult, however pure transformants can be isolated by successive rounds of conidial purification. If the fungus does not form conidia, such as *Podospora* species, one way to isolate pure colonies is by crossing with an untransformed strain and re-isolating the transformed phenotype from the meiotic products. This method of transformant purification is complicated by a process called RIP (Repeat Induced Point mutations). This is covered in more detail in Chapter 4. Alternatively protoplasts can be prepared and the uninucleate protoplasts concentrated by centrifugation or filtering. Repeated protoplast isolation and selection of regenerated protoplasts has been used to prepare a homokaryon from heterokaryotic isolates of *Rhizoctonia solani* (Phillips 1993).

3.2.2 Aims

Since transformation efficiencies are influenced by the transforming strain and the selection system (Wernars *et al.*, 1985) the aim of this study was to identify the simplest and most efficient procedure for the transformation of the *A. nidulans* auxotrophic haploid 1-85 (*pyrG89*, *pabaA1*, *wA*, *qutR16*) with the vector pGM32 [contains the heterologous *N. crassa* OMPdecase (*pyr-4*) gene]. The *N. crassa pyr-4* gene has very little homology to the *pyrG* locus and is expected to integrate into the *A. nidulans* genome randomly (Ballance and Turner, 1985).

3.3 Results

3.3.1 Protoplast Preparation from Mycelia

Protoplasts are formed by the digestion of mycelial cell walls to leave only the cell membrane. As the cell wall integrity has been destroyed this has to be performed in an osmotically stabilised solution.

3.3.1.1 Osmotic Stabiliser

Protoplasts were prepared as described in Section 2.16.2. High transformation frequencies for *A. nidulans* transformed with the *N. crassa pyr-4* gene were reported using KCl buffer (Ballance and Turner 1985). The effect of washing the protoplasts in KCl buffer was thus compared to washing in sorbitol buffer (Section 2.5.16). Protoplasts prepared in KCl buffer were regenerated on plates containing KCl, while protoplasts prepared in sorbitol buffer were selected on plates containing sucrose.

The protoplast yields obtained using the osmotic stabilisers KCl and sorbitol were 2.0×10^9/ml and 7.0×10^8/ml, respectively. The percentages of viable protoplasts were 1.2% for the KCl buffer, and 14% for the sorbitol buffer. Transformation of the protoplasts prepared in sorbitol buffer with vector pGM32 (Section 2.18) resulted in a transformation frequency of 99 transformants/8.5×10^3 protoplasts/μg DNA. In contrast, no transformants were obtained from transformation of the protoplasts prepared in KCl buffer with vector pGM32 (Ballance and Turner 1985). Protoplasts were therefore prepared using buffer containing sorbitol as an osmotic stabiliser, and selected on plates containing sucrose as an osmotic stabiliser.

3.3.1.2 Molarity of $MgSO_4$ in Cell Wall Digestion Buffer

The effect of altering the molarity of $MgSO_4$ in the cell wall digestion buffer was investigated. The cell wall digestion enzyme, Novozyme 234, dissolved more readily as the $MgSO_4$ concentration decreased from 1.2 M to 0.6 M and, consequently, filter sterilisation was easier. Protoplast formation from mycelia (Section 2.16.2) was observed microscopically. The protoplast yield appeared to increase with an increase in the $MgSO_4$ concentration however the protoplasts were smaller, when compared to protoplasts formed at the lower concentrations of $MgSO_4$.

The protoplast yield, after harvesting by flotation (Section 2.16.2), increased with increased concentration of $MgSO_4$ however, the percentage protoplast viability decreased (Table 4). The proportion of viable mycelial fragments in each protoplast preparation was approximately the same at each concentration (Table 4).

The yield of viable protoplasts was greatest (2.1×10^8/ml) when the concentration of $MgSO_4$ was 1.2 M. This concentration was therefore used in subsequent protoplast preparations.

3.3.2 Protoplast Preparation from Conidia

Protoplasts formed from mycelia form a heterogeneous population due to the heterogeneous nature of mycelia. The proportion of protoplasts which are viable varies considerably and was found to be on average 32% $\pm$ 12% (n = 32). Inviability may be due to a number of reasons such as lack of nuclei or lack of cytoplasmic components. Protoplasts prepared from conidia are reported to be homogeneous and to contain one or two nuclei (Bos and Slakhorst 1981). Protoplasts were therefore prepared from the conidia of *A. nidulans* 1-85.

3.3.2.1 Germination

For efficient protoplast formation it was necessary to identify an incubation time whereby a sufficient percentage of the spores were germinating, to allow protoplast formation, while still being uninucleate.

Liquid media was inoculated with spores and incubated with shaking at 37°C and germination monitored microscopically. Germination was identified as the time point when obvious germ tubes were first observed (Table 5). The germination time for 5×10^6 and 2.5×10^7spores/ml of MU media was shown to be equal. Germination occurred earlier using uridine as a supplement rather than uracil, and later using citrate minimal media instead of complete media. Clumping of conidia was observed to occur before germination in all media at both concentrations.

The time at which 20-30% of the spores had germinated was determined, as the majority should be uninucleate (Bainbridge 1971). Inoculation of MU liquid media at a concentration of 2×10^7 spores/ml and incubation with shaking, uninterrupted, for 7 hrs had the required level of germination. This was, however, difficult to determine accurately due to clumping of the spores.

Table 4 Effect of $MgSO_4$ Molarity on Protoplast Preparation

	$MgSO_4$ concentration[a]		
	1.2 M	**0.8 M**	**0.6 M**
Protoplasts/ml[b]	5.6×10^8	3.9×10^7	1.0×10^7
Viable protoplasts/ml	2.1×10^8	3.2×10^7	7.1×10^6
Viable mycelial fragments/ml[c]	4.7×10^3	9.0×10^2	2.5×10^2
Percentage of protoplasts viable	38%	82%	71%
Percentage due to mycelial fragments	0.0022%	0.0028%	0.0035%

[a] OM buffer (Section 2.5.9) containing Novozyme 234 at a concentration of 5 mg/ml.

[b] Concentration was determined using a haemocytometer and therefore includes viable and non-viable protoplasts.

[c] The protoplast suspension was diluted in H_2O and plated on non-osmotically stabilised media to determine the number of viable cells other than protoplasts.

Table 5 Germination of 1-85 Spores in Liquid Media

Media	**Spore Concentration**	**Germination time**[a,b]
MYG + Uridine(10mM)	$5x10^6$ spores/ml	4hrs 45min
MYG + Uracil	$5x10^6$ spores/ml	8hrs
MYG + Uracil	$2.5x10^7$ spores/ml	8hrs
Citrate Minimal Media + Uracil	$5x10^6$ spores/ml	9hrs

[a] Time at which germination of 1-85 spores in liquid media was first observed.

[b] Incubation was interrupted by storage overnight at 4°C for the medias: MYG + uracil and citrate minimal media + uracil. This resulted in longer incubation times to germination as uninterrupted incubation of 2×10^7 spores/ml, in MYG + Uracil, resulted in 20-30% germination after 7 hours.

3.3.2.2 Protoplast Preparation and Transformation

Germinating spores were harvested and washed in preparation for protoplast isolation (Section 2.16.1). Initially a modification of a method by Oakley *et al.* (1985) was used with KCl as an osmotic stabiliser in buffers and plates. The protoplasts were harvested by centrifugation, resulting in a high level of contamination with spores. Transformation of these protoplasts was not successful.

Protoplast preparation by incubating the germinating spores overnight in Novozyme solution (Section 2.16.2) was successful. Using $MgSO_4$ as the osmotic stabiliser allowed the protoplasts to be harvested by flotation. Although the yield and viability were good, as found by Wernars *et al.* (1985), the transformation efficiency was not (25 transformants/2.8 x 10^4 viable protoplasts/µg DNA and 65 transformants/1.4 x 10^4 viable protoplasts/µg DNA).

3.3.3 Protoplast Storage

Due to the heterogeneous nature of protoplasts, and the variability between prepared batches, it was desirable to use the same batch of protoplasts for a set of experiments. As these were to be performed over extended periods of time the storage of protoplasts was investigated.

Protoplasts were stored, as described in Section 2.17, and protoplast viability and competency to be transformed was determined over a period of approximately 200 days. Viability decreased, along with competency to be transformed with the pGM32 vector (Table 6). Protoplasts could be successfully stored at -70°C for use in transformations for a limited period (few months) only.

3.3.4 Transformation

The transformation procedure investigated was initially developed for *A. nidulans* (Yelton *et al.*, 1984) and modified for use with *Acremonium lolii* (Itoh *et al.*, 1995). The transformation mixture included: protoplasts, PEG transformation solution, spermidine, heparin and vector DNA and was incubated as described in Section 2.18.

3.3.4.1 Incubation in PEG Transformation Solution

The effect of incubation time after the addition of 9 volumes of PEG solution was investigated. Transformation reactions were set up with and without pGM32 and incubated on ice (30 min).

Table 6 Protoplast Viability and Transformation Efficiency After Storage at -70°C

Expt.	Days frozen -70°C (days)	Protoplast viability[a]	Viable mycelial fragments[b]	Transformation efficiency[c]
1[d]	0	3.5×10^7	1.4×10^4	
2[e]	0	2.1×10^7	1.5×10^4	404
3	65	2.8×10^7	1.3×10^4	
4	70	3.7×10^7	1.3×10^4	250
5	81	1.3×10^7	1.9×10^4	125
6	103	2.3×10^7	1.2×10^4	50
7	147	2.6×10^7	1.0×10^4	58
8	176	9.5×10^6	1.2×10^4	40
9	213	6.7×10^6	1.1×10^4	

[a] Viable protoplasts per ml. Duplicate dilutions were plated and the average calculated. The initial, haemocytometer counted, concentration was 1×10^8 protoplasts/ml.

[b] Viable mycelial fragments per ml.

[c] Transformants per μg of DNA.

[d] Fresh protoplasts.

[e] Protoplasts stored overnight at 4°C.

These were split into 7 tubes and 9 volumes of PEG solution was added to each tube. The reaction mixes were further incubated for 0 to 60 minutes at room temperature (approximately 10 minute intervals). No effect on protoplast viability or transformation frequency was observed. This was repeated with the same result.

The PEG solution did not appear to be toxic, but rather improved the storage of transformed protoplasts. Although viability was reduced, transformation mixtures were also stored successfully for 3 to 4 days at 4°C after the addition of 9 volumes of PEG solution. If the PEG solution was diluted 10 fold in STC buffer prior to storage at 4°C, storage was not successful. It was therefore possible to plate a sample of the transformation mix and store the remainder until the success, or otherwise, of the transformation procedure was determined.

3.3.4.2 Addition of Heparin and Spermidine

As many transformations were to be performed, and comparisons between them made (Chapter 5), it was desirable to have a simple transformation procedure, to reduce possible sources of variation. The requirement for the inclusion of heparin and spermidine to the reaction mix was therefore investigated.

A set of transformation reaction mixtures were set up, from different batches of protoplasts, differing in the addition of heparin or spermidine. The results indicate the addition of heparin and spermidine has no effect on the transformation efficiency or protoplast viability (Table 7). However, not obvious from these results is the effect of spermidine or heparin on the proportion of transformants which were stable. The addition of spermidine appeared to increase the proportion of 'stable' transformants so was retained in the transformation mix. This is considered in more detail in Chapter 4.

3.3.4.3 Vector Conformation

The effect of vector linearisation was investigated. The vector, pGM32, was linearised within the multiple cloning site with *Xba*I, therefore outside the *pyr-4* gene. To ensure any differences in transformation efficiencies were due to whether the vector was linearised (and not due to the post-linearisation purification step), two reaction mixtures were prepared with only one containing the restriction enzyme. The reaction mixtures were treated in the same way.

The preparations of circular and linear DNA were checked on an agarose gel (data not shown).

Table 7 Effect of Vector Linearisation, and the Addition of Spermidine and Heparin, on the Efficiency of Transformation

Expt.	Mixture[a]	Transformants/ µg DNA	Transformants/ viable protoplast
1[b]	+Circular DNA +S&H	260	2.4×10^{-3}
	+Circular DNA +S	380	2.9×10^{-3}
	+Circular DNA +H	200	2.7×10^{-3}
	+Circular DNA	340	4.0×10^{-3}
2[c]	+Circular DNA	25	8.9×10^{-4}
	+Circular DNA +S&H	65	4.6×10^{-3}
3[d]	+Circular DNA	24	2.0×10^{-3}
	+Circular DNA + S&H	16	1.3×10^{-3}
4[b]	+Circular DNA	303	1.5×10^{-3}
	+Circular DNA +S	404	2.2×10^{-3}
	+Linear DNA	168	8.4×10^{-4}
	+Linear DNA +S	174	9.1×10^{-4}
5[b]	+Circular DNA (stock)[e]	438	2.2×10^{-3}
	+Circular DNA	519	2.2×10^{-3}
	+Linear DNA	176	1.1×10^{-3}

[a] Abbreviations: S=spermidine; H=heparin.

[b] Protoplasts prepared from mycelia and used fresh (Section 2.16.2).

[c] Protoplasts prepared from conidia (Section 2.16.1)

[d] Protoplasts prepared from conidia (c) and stored frozen for 6 days. Freezing was by placing the aliquots of protoplasts directly at -70°C.

[e] Stock DNA refers to the original aliquot of pGM32 before treatment to obtain linear and circular DNA.

The efficiency of linearisation was tested by transformation of *E. coli* (Section 2.14.3). The number of *E. coli* transformants obtained with linear DNA (5.7 x 10^3/µg DNA) was compared to that with circular DNA (3.7 x 10^6/µg DNA). Assuming transformants obtained with the linear vector were from uncut, circular, DNA, the results indicate that 99.85% of the vector molecules had been linearised with *Xba*I.

A decrease in the overall transformation efficiency was observed when the linearised vector was used (Table 7). However, as with the addition of spermidine to the transformation mix an increase in the proportion of 'stable' transformants was observed with use of the linear vector. This is considered in more detail in Chapter 4.

3.3.4.4 Vector Concentration

The effect of the concentration of vector in the transformation mix was investigated. The number of transformants per viable protoplast is approximately constant from 0.5 to 2.5 µg of DNA per 25 µl reaction mix (the standard amount of DNA for this volume is 1.25 µg) while the number of transformants per µg of DNA decreased (Table 8). If the amount of DNA was reduced further, to 0.25 µg, a decrease in the number of transformants per viable protoplast was observed (Table 8).

3.4 Discussion and Conclusions

The protoplast preparation and transformation procedures were studied to determine the optimal conditions for the *A. nidulans* strain, 1-85, and vector, pGM32, combination.

Due to confusion in the literature as to the concentration of the osmotic stabiliser, $MgSO_4$, to be used in the cell wall digestion buffer [Peberdy (1979) used 0.6 M while Tilburn *et al.* (1983) used 1.2 M] the optimal molarity of $MgSO_4$ was investigated. The optimal concentration of $MgSO_4$ in the cell wall digestion buffer was determined to be 1.2 M as this resulted in the highest yield of viable protoplasts. However, the percentage of viable protoplasts was greater at lower $MgSO_4$ concentrations and the protoplasts were observed to be larger. This is consistent with reports that indicate larger protoplasts are more likely to be viable (Rodriguez and Yoder 1987; Skatrud *et al.*, 1987).

Table 8 Effect of Vector Concentration on the Efficiency of Transformation

Expt.	Transforming DNA added (μg)	Transformants/ μg DNA	Transformants/ viable protoplasts
1[a]	0.5μg	665	1.4×10^{-3}
	1.0μg	250	1.0×10^{-3}
	1.5μg	167	$\sim 9.8 \times 10^{-4}$
	2.0μg	~66	$\sim 9.3 \times 10^{-4}$
	2.5μg	~57	$\sim 5.2 \times 10^{-4}$
2[b]	0.25μg	215	2.6×10^{-4}
	0.5μg	233	5.1×10^{-4}
	0.75μg	183	5.4×10^{-4}
	1.0μg	125	5.2×10^{-4}

[a] Protoplasts were stored at -70°C for 70 days. Due to the plates drying out at 37°C the results indicated (~) are underestimates.

[b] Protoplasts were from the same batch as in [a] stored at -70°C for 81 days.

As $MgSO_4$ was used as the osmotic stabiliser in the cell wall digestion buffer the protoplasts were able to be harvested by flotation. Ballance and Turner (1985) described a method of protoplast preparation and transformation using the osmotic stabiliser KCl, which was reported to result in high frequencies of Pyr^+ *A. nidulans* transformants. This was compared to the use of the osmotic stabiliser sorbitol for the preparation of protoplasts for transformation. Transformants were obtained from protoplasts prepared with sorbitol but not KCl. Sorbitol was therefore selected as the best osmotic stabiliser for the preparation of protoplasts for transformation.

Protoplasts could be stored successfully for short periods at 4°C (Bos and Slakhorst 1981) and for longer periods at -70°C (Cullen *et al.*, 1987). Protoplast viability was determined to be greatest if PEG transformation solution was added to the protoplasts and freezing was regulated. The transformation efficiency decreased with time at -70°C, along with protoplast viability, although at a much greater rate. This suggests protoplast viability may not be a good determinant of protoplast competency. The frozen protoplasts remained competent for transformation for only a few months.

Storage of the transformation mixture at 4°C was successful provided the mixture was not first diluted with STC buffer. This was surprising as there were concerns that the PEG solution would be toxic (Anne and Peberdy 1976) and that this may reduce the viability of the protoplasts. The addition of 9 volumes of PEG solution to the transformation mix, and incubation at room temperature, is believed to facilitate clumping of the protoplasts and thereby trapping DNA close to the surface of the protoplasts (Fincham 1989). The length of the incubation time had no effect on the transformation frequency or the protoplast viability.

The conformation and concentration of the vector DNA was investigated for an effect on the transformation frequency. As the vector, pGM32, was entirely heterologous it was linearised within the multiple cloning site. A decrease in transformation efficiency was observed with vector linearisation. This is in contrast to the literature which reports no change in the transformation efficiency with vector linearisation, or an increase in transformation efficiency provided the vector was linearised within a region of homology. The observed result, of a decrease in transformation efficiency, may be a feature of the vector being entirely heterologous.

It is suggested that competent protoplasts become saturated when a certain amount of DNA is used in the transformation mix (Yelton *et al.*, 1984, Wernars *et al.*, 1985). This means that the addition of more DNA does not result in an increase in the number of transformants. The effect

of the concentration of vector in the transformation mix was investigated to determine if this would be observed for *A. nidulans*, and if so, what the saturating level of DNA would be. The results indicated that if more than 0.5 µg of DNA was included in a 25 µl transformation mix the available competent protoplasts were saturated. As protoplast batches are variable, and the proportion which are competent to be transformed could not be determined, prior to transformation, the amount of DNA used per 25 µl reaction mix was standardised at 1.25 µg (5 µg per 100 µl protoplast solution). This excess of DNA was used to ensure the most efficient utilisation of all protoplast batches, some of which may have a high proportion of competent protoplasts.

Relatively simple protoplast preparation and transformation procedures were thus optimised for *A. nidulans* strain 1-85 and the vector pGM32. The transformants on the selective plates were obvious after 2 days incubation and were selected after 3 days incubation. The colonies on the selective plates could be classified on the basis of morphology. Analysis of the types of transformants obtained is covered in detail in Chapter 4.

Chapter Four

Analysis of Transformants

4.1 Summary

Transformation of a haploid *Aspergillus nidulans pyrG* auxotrophic strain (1-85) with a vector containing the heterologous *Neurospora crassa pyr-4* gene (pGM32) resulted in uracil-independent transformants that could be classified into two main groups based on morphology. The minority were morphologically very similar to the parental strain, easily purified and mitotically stable (N-types). The majority (10 times more frequent) were irregular in shape and shown to be heterokaryons (H-types) that could not be resolved into transformant homokaryons.

Southern analysis of a random sample of both types of transformants indicated the difference was due to the number of copies of the integrated vector in a head to tail tandem repeat. Combined with spore germination patterns it appeared the heterokaryons (H-types) contained a mixed population of nuclei of which a small proportion contained the vector integrated in long tandem repeats, while the N-types had a relatively low number of integrated copies in all nuclei. It appeared that multiple copies of *pyr-4* inhibited the growth of germinating spores, leading to a requirement for these transformants to exist as heterokaryons. This was investigated by analysing transformants obtained from vectors, containing different promoter and selection system combinations, for morphology, growth rate and orotidine 5' monophosphate decarboxylase activity.

4.2 Introduction

The use of transformation as a tool for making specific changes to fungal genomes, or for cloning fungal genes, requires a reliable level of stability. Stable integration of the transforming DNA is essential if the aim is to construct a strain with specific genomic features for study, or use in the field. If, however, the aim is to clone a gene by complementation then instability in the form of autonomous replicating plasmids has its advantages. The stability of transformants has therefore been the focus of considerable research.

4.2.1 Autonomously Replicating Plasmids

The first shuttle vector for *S. cerevisiae* employed the origin of replication from the 2 μm

plasmid (Beggs 1978) which was found to be non-functional in filamentous fungi. During the same period the sequence conferring autonomous replication to a plasmid in *S. cerevisiae* was determined by Stinchcomb *et al.* (1979). This is referred to as the replication origin or autonomously replicating sequence (ARS). The *S. cerevisiae* ARS is not entirely sufficient for stable maintenance of a plasmid, but it is necessary and has improved the transformation frequency of *S. cerevisiae* 1000 fold. Unfortunately this ARS does not function well in other species, including *Schizosaccharomyces pombe*, which can host the 2 μm plasmid. This ARS is, however, functional in the filamentous fungus *Ashbya gossypii* which was transformed to geneticin resistance (Wright and Philippsen 1991). The transformants were mitotically unstable on non-selective media and contained the transforming DNA as autonomously replicating plasmids.

The obvious benefit of such sequences to transformation in filamentous fungi has lead to an extensive search for ARS-like sequences in many species. The search in *A. nidulans* was initially carried out by Ballance and Turner (1985). They screened *A. nidulans* DNA for sequences that improved the transformation frequency of *S. cerevisiae*. One sequence was discovered which gave a 50-100 fold improvement in the transformation frequency and was termed *ans1*. When this sequence was used to transform *A. nidulans* the transformation frequencies improved, as did the stability of the transformants, but the DNA was shown to integrate into the genome rather than be maintained autonomously. It was possible to recover plasmids from the transformants, but these were shown to have undergone structural rearrangements and to have acquired sequences from the recipient strain. This is more consistent with integration and excision rather than with autonomous replication.

The *ans1* sequence has been localised to linkage group I near the centromere. It consists of a 1.8 kb fragment which has an 81% A + T composition containing a match to the *S. cerevisiae* ARS (TTTTATATTTA). Subcloning showed that the ARS-like region and others, that do not contain an exact match, are able to increase the transformation frequency. However, the feature of AT richness can not account for this activity as many areas of the mitochondria are AT rich and do not enhance transformation (Cullen *et al.*, 1987). Further analysis of *A. nidulans* revealed *ans1*-like homologous sequences present in multiple copies throughout the genome.

After initially being shown to enhance transformation frequencies of *pyr-4* based vectors in *A. nidulans, ans1* was shown to do the same to *argB* and *trpC* based vectors (Cullen *et al.*, 1987) although not as dramatically. This effect was apparent when the *ans1* sequence was included in the vector construct with the selectable marker, or added on a different vector and introduced by cotransformation (Cullen *et al.*, 1987). By increasing the amount of the cotransformation vector containing *ans1* in the reaction, the transformation frequency was improved.

The mechanism by which transformation frequency is improved with *ans1* is unknown. Evidence suggests it does not involve homologous recombination as only a low proportion of preferential homologous recombination at *ans1*-like sites is observed. Also, addition of ribosomal regions to transformation vectors does not affect transformation frequency, and these are also present in many copies in the genome (Tilburn *et al.*, 1983). It has been speculated that *ans1* may help stabilise the plasmid early on so it is maintained for a longer period and is therefore more likely to integrate (Cullen *et al.*, 1987). It is also possible it promotes integration at unrelated sites that are difficult to detect.

The effect of the *ans1* sequence on transformation of various other filamentous fungi has been studied since its discovery. The frequency of transformation in *Penicillium chrysogenum* was found to only increase 3-fold, whereas the stability of the transformants increased from 15% to 70% (Cantoral *et al.*, 1987). The lower number of unstable abortive transformants suggests the sequence promoted early integration. In contrast, Unkles *et al.* (1989), found that the *ans1* sequence did not improve the transformation frequency when *A. niger* was transformed with the homologous *niaD* gene. When Whitehead *et al.* (1989) transformed *P. chrysogenum* using the *A. niger niaD* vectors used by Unkles *et al.* (1989), they also found no enhancement in transformation frequency with the presence of *ans1*. It is interesting to note that with this selection system (*niaD*) there tend to be no abortive transformants so the percentage of stable transformants is already high.

The *ans1* sequence has an interesting, if unknown, effect on transformation frequency and stability of transformants. It does not, however, confer autonomous replication to the vectors. There are considered to be four potential sources of replication origins: nuclear plasmids, chromosomal DNA, mitochondrial DNA and mitochondrial plasmids. Examples of ARS's functional in filamentous fungi show that the sequence for the replication origin can come from any one of these sources.

Attempts to isolate an ARS from *A. nidulans* have met with mixed results since the early work by Ballance and Turner who isolated the *ans1* sequence. More recently Gems *et al.* (1991) have isolated a plasmid from an unstable *A. nidulans* colony which resulted from transformation with an *A. nidulans* gene bank. The transformants were selected on the basis of their slower growth. The isolated plasmid (ARp1) transforms at a frequency of 20 000 transformants per 10^6 protoplasts at near saturation levels (term used instead of DNA quantity) of transforming DNA. This represents a 250-fold enhancement of transformation efficiency over that found for typical integrative vectors. They analysed the 6.1 kb insert (*AMA1*) and confirmed it existed in free form, not integrated into the chromosome, at a mean copy number of 10-30 per haploid

genome. The plasmid is confined to the nucleus and is mitotically unstable, being lost from 65% of the asexual progeny of transformants. This instability was sustained through subculturing on selective media indicating the plasmid remained unintegrated. Further confirmation, by linkage analysis, showed the *argB* selective marker used in the transformation construct remained unlinked to all chromosomal markers tested. The structure of *AMA1* shows a short unique sequence flanked by long inverted repeats, with the region conferring autonomous maintenance lying in each arm of the inverted repeat. The sequence originates from *A. nidulans* genomic DNA, where it is present in multiple copies, and was shown to function in a similar manner in *A. niger* and *A. oryzae*, with an increase in transformation frequency of 25-fold and 30-fold respectively (Gems *et al.*, 1991).

It was argued that transformation with an autonomously replicating plasmid could result in reduced stability of the transforming sequence by comparison with integrative transformation. This might cause a reduction in transformation colony size and/or alteration in colony morphology. In addition, such free plasmids ought to be easy to re-isolate by transforming *E. coli* (Gems *et al.*, 1991). The transformation systems discovered so far with autonomous replicating plasmids have indeed shown high levels of mitotic instability. These systems can be employed for the study of transient gene expression of reporter gene constructs, and the cloning of genes by complementation.

The *AMA1* sequence can increase transformation frequency in *A. nidulans* when included in the transforming vector, or on a separate vector, pHELP1, by cotransformation (Gems and Clutterbuck 1993). The ability to increase transformation frequency *in trans* is believed to result from recombination between the transforming DNA and the replicating vector to create a hybrid vector. It has since been found that plasmid rearrangement is frequent during transformation but not vegetative growth. During sexual reproduction, autonomous plasmids exhibit increased recombination, which results in both plasmid concatamerisation and integration into the genome (Aleksenko and Clutterbuck 1995). This feature of plasmid rearrangement during transformation has been used to construct 'instant gene banks' by cotransforming *A. nidulans* mutants with pHELP1 and linear fragments from other fungi. Genes have been successfully cloned by selecting for complementation of an *A. nidulans* mutation, followed by rescue of the plasmids in *E. coli* (Gems *et al.*, 1994; Bowyer *et al.*, 1994; Verdoes *et al.*, 1994). Problems with rescuing the plasmids have been reported and this is believed to be due to rearrangement of the hybrid plasmids (Gems *et al.*, 1994). Despite these problems this technique offers a rapid and simple method of gene cloning which overcomes the problems associated with the construction and amplification of conventional gene libraries.

4.2.2 Mitotic Stability

As a general rule purified transformants of filamentous fungi are mitotically stable in the sense that there is not a rapid loss of the transformed phenotype. This is tested by the growth of transformants on non-selective media through rounds of subculturing, then transfer to selective media to check for the retention of the transformed phenotype. This stability was quantified by Dunne and Oakley (1988) who developed a method for determining the rates of mitotic recombination of an interrupted duplication, which is the form often taken by integrated DNA. Although the recombination frequencies were for one locus, and it is possible recombination frequencies vary among loci, they were very low which accounts for the high mitotic stability observed for *A. nidulans* transformants.

Mitotic instability of transformants has been observed and studied in a number of fungal systems. The instability seems to be correlated with a number of different factors. A detailed study of the mitotic stability of *Cochliobolus heterostrophus* transformed to *hygB* resistance was carried out (Keller *et al.*, 1991). The stability of the integrated DNA was tested after successive cycles of subculturing and pathogenesis on maize. It was demonstrated that the mitotic stability was dependent on the configuration of the DNA and that instability, when observed, caused complete deletion of individual copies. However, in no case were all copies deleted. Integration occurred at either ectopic or homologous sites, as a single copy or multiple copies in tandem repeat form. All transformant types were stable after 5 rounds of subculturing, or 7 rounds of pathogenesis. The least stable configuration was a single copy integrated at an homologous site and the most stable a single copy integration at an ectopic site. Transformants with multiple copies integrated at an ectopic or homologous sites showed intermediate levels of stability.

Mitotic stability was shown to be affected by the selection system used for the plant fungal pathogen *Pseudocercosporella herpotrichoides* (Blakemore *et al.*, 1989). When transformed to hygromycin B resistance mitotic stability was high, while when transformed to benomyl resistance mitotic stability was very low. Both the vectors were shown to integrate at one or more sites in multiple copies.

High mitotic instability is also observed when the transformant was initially a heterokaryon. Transformants can assume a heterokaryotic nature for a number of different reasons, for example the multinucleate nature of the cells being transformed, as in the conidia of *N. crassa* and the mycelia of *A. nidulans*. The initial transformants may be heterokaryons, with some nuclei transformed and some not, or the nuclei transformed in different ways. Heterokaryotic transformants have been reported for *N. crassa* (Paietta and Marzluf 1985; Davis 1984) and *A.*

nidulans (Upshall 1986) and can be resolved in a number of different ways as described in Chapter 3.

It is suggested that it is possible to avoid heterokaryons by preparing protoplasts from uninucleate conidia (Miller *et al.*, 1985) or selecting for uninucleate protoplasts prepared from mycelia by filtering (Faugeron *et al.*, 1989). However, even if the cells being transformed are uninucleate heterokaryons can still be formed. This may be due to the maintenance of the plasmid through several rounds of replication before being stably integrated or may stem from the use of the cell-clumping agent, PEG, in the transformation protocol.

The heterokaryotic nature of transformants has been observed in different systems. Transformation of *A. giganteus* (Wnendt *et al.*, 1990) and *Septoria nodorum* (Cooley *et al.*, 1988) to hygromycin B resistance resulted in heterokaryotic transformants. However, these transformants were mitotically stable after purification and this stability was maintained.

In *A. nidulans* heterokaryotic transformants were characterised by Upshall (1986). The selection system was *argB* as Arg^- and Arg^+ conidia are easily distinguishable by colour, providing the parental strain is green. Initially all the transformants (vigorously growing colonies on selective media) were mosaic (light and dark green conidia) which indicated heterokaryosis. These were put through 2 rounds of single colony purification and of 33 Arg^- to Arg^+ transformants, only 2 continued to showed high levels of mitotic instability. These two were taken through 5 rounds of purification and at each stage a high proportion of Arg^- colonies segregated out, although the frequency varied. Only mosaics were recovered on selective media. It was suggested that this was a consequence of heterokaryosis rather than instability of the transformation event. One of the two transformants produced a stable mitotic segregant that was shown to be a heterozygous diploid (as were two of the stable transformants). The heterokaryon condition may thus be required for survival of the haploid transformed phenotype. This indicates the lethal disruption of a gene in these transformants. This feature has been used to disrupt essential genes for study by creating heterokaryotic transformants (Osmani et al., 1988).

There have been a few reports of mitotic instability of fungal transformants which may be due to the formation of heterokaryons, although this is not always suggested. Wernars *et al.* (1985) transformed an *amdS A. nidulans* mutant strain with the homologous gene and selected for Amd^+ transformants. Transformants were observed that showed continuous growth and normal sporulation. There was a variation in the number of conidiospores with the Amd^+ phenotype of 0.1-100%. However, after the first round of subculturing all were homogeneous

for Amd^+.

Barnes and MacDonald (1986) transformed an *A. nidulans pyrG* mutant with a vector containing the *N. crassa pyr-4* gene. Two of the transformants isolated and analysed were found to be mitotically unstable with a high proportion of Pyr^- conidia segregating during vegetative growth. The mitotic instability of Pyr^+ *A. nidulans* transformants when transformed with the *pyr-4* gene (and *ans1*) was also observed by Cullen *et al.* (1987). The Pyr^+ phenotype was detected in <20% of the conidia isolated from transformants on selective plates. Some of these stabilised during subculturing. Using the same selection system Cantoral *et al.* (1987) transformed *Penicillium chrysogenum* to Pyr^+ with and without the *ans1* sequence. The *ans1* sequence increased the number of mitotically stable transformants from 15% to 70%. The *pyr-4* gene was used again by Woloshuk *et al.* (1989) to transform *A. flavus*. Of the two transformants analysed, one with single copy integration and one with multicopy integration, mitotic instability was observed. During five consecutive transfers on non-selective media approximately 20% uracil dependent colonies were observed at each stage. When Berges and Barreau (1991) transformed *Trichoderma reesei ura3* and *ura5* mutants with the cloned *ura3* and *ura5* genes transformation was very efficient (>10^4 transformants/μg DNA). The transformation efficiencies were quite difficult to estimate as regenerating colonies rapidly invaded the transformation plates. They found DNA integrated at the homologous or non-homologous locus and that some of the transformants grown on non-selective medium exhibited a high mitotic instability when the conidia were transferred to selective medium. The reason for this was not clearly determined. Although Southern analysis of unstable transformants showed the transforming plasmid seemed to be integrated, while some 'free copies' of the plasmid were detected by transformation of ura^- protoplasts with transformant genomic DNA. These 'free copies' could not be rescued in *E. coli* (Gruber *et al.*, 1990).

4.2.3 Meiotic Stability

Purified transformants which show a high level of mitotic stability do not necessarily show meiotic stability. Analysis of progeny from sexual crosses of *N. crassa* transformants showed a frequent loss of the transformed phenotype. Despite the low 5-methylcytosine content of *N. crassa*, Bull and Wootton (1984) found the amplified DNA of transformants to be heavily methylated and rearranged. The significance of this observation, and many similar observations, regarding the meiotic instability of transformants, was not understood until the work of Selker *et al.* (1987, 1988).

It was shown that crossing *N. crassa* transformant containing a duplication of a normally single copy gene, with any other strain, resulted in a proportion of meiotic products having both copies extensively changed. The copy in the untransformed strain used for the cross remained unaltered. These changes appear to occur within the duplicated sequences, without rearrangement of the genomic position. They were shown to occur before DNA replication, but after the initial association of a single pair of nuclei at fruiting body initiation. This phenomenon was termed the RIP (Repeat Induced Point mutations) effect and has been well reviewed (Selker 1990[a], 1991). The following is a summary of what is known about RIP in *N. crassa* and other filamentous fungi.

The RIP effect is triggered by duplications of foreign as well as native DNA and the mutations are generally confined to the duplicated sequences. The changes in the primary structure of the DNA that result from RIPing are polarised transition mutations in which G:C pairs are replaced by A:T pairs, with 10% or more of the G:C pairs being replaced in a single cross (Cambareri *et al.*, 1989). Sequences altered by RIP are sensitive to additional mutations in subsequent crosses, as long as there are similar sequences present (Cambareri *et al.*, 1991; Foss *et al.*, 1991). Although these sequences appear to mutate independently the mutations are site specific. The process is confined to G:C pairs and some G:C pairs are affected more than others.

Typically sequences altered by RIP display DNA methylation although the role of this methylation is unknown. Apparently it does not seem sufficient to promote high frequency mutation (Selker 1991) and does not protect the DNA from further RIP (Cambareri *et al.*, 1991). Evidence suggests G:C to A:T mutations resulting from RIP somehow make the sequences susceptible to DNA methylation. The extent of DNA methylation of foreign sequences in *Neurospora* was observed to depend on the sequence context it was in (Selker 1990). It is speculated that methylation is the default state of chromosomal DNA. Sequence specific DNA-binding proteins are indirectly responsible for preventing DNA methylation and these may be displaced by RIP mechanisms. DNA methylation and the contribution of transformation of filamentous fungi to understanding this phenomenon has been well reviewed by Selker (1990[b]).

The proportion of meiotic tetrads that undergo the RIP effect seems to be a function of the degree of proximity of the duplicated sequences (Selker *et al.*, 1987). If the sequences are unlinked the frequency of RIP may be of the order of 50% or less, whereas if they are adjacent there is little chance of going through a cross without being affected by RIP. The degree of similarity has also been shown to influence the efficiency of the RIP process (Cambareri *et al.*, 1991). The RIP phenomenon may therefore serve both to preserve the gross structure of the

genome and to generate sequence diversity.

Since the RIP effect is not selective it may be used for gene disruptions. DNA homologous to the sequence to be mutagenised can be introduced into the organism by transformation to form a duplication. Transformants are then crossed and the progeny are screened for inactivation of the sequence (Selker *et al.*, 1987; Selker and Garrett, 1988; Marathe *et al.*, 1990; Harkness *et al.*, 1994).

Faugeron *et al.* (1989) worked with *Ascobolus immersus* to study the premeiotic inactivation process by transforming a mutant *met-2* strain with the homologous *met-2* gene. After meiosis the transformed phenotype was frequently lost. This inactivation was caused by *de novo* methylation at a stage of the sexual phase which precedes meiosis, and was spontaneously reversible after mitotic divisions. The mutations associated with the RIP effect observed in *N. crassa* were not present so this was distinct from 'RIPing' and was called 'MIP' (Methylation Induced Premeiotically).

Premeiotic inactivation in *N. crassa* (Yamashiro *et al.*, 1992), and *A. immersus* (Faugeron *et al.*, 1990) can also be triggered by introducing multiple copies of the exogenous *amdS* gene from *A. nidulans*. If two ectopic copies were present in *A. immersus* then either both or neither were inactivated (Faugeron *et al.*, 1990). Inactivation occurred in approximately 50% of nuclei if the copies were at ectopic locations, and 90% of the nuclei if they were tandemly repeated. If the strain contained 3 copies then none, two or all three copies were inactivated. It is proposed that a prerequisite for inactivation of each of the ectopic copies is a premeiotic pairing of repeated sequences, and that each copy can undergo successive rounds of pairing. Pairing can occur between methylated and unmethylated copies. This inactivation is also observed for the endogenous *met-2* gene in *A. immersus* (Faugeron *et al.*, 1989), and the *am* gene of *N. crassa* (Fincham *et al.*, 1989). Analysis of triplicate *am* mutants of *N. crassa* also showed inactivation of none, two or all three copies.

Mooibroek *et al.* (1990) working with *Schizophyllum commune* (Basidiomycete) showed that donor DNA is not indiscriminately methylated, but the origin of the donor DNA determines the occurrence and level of methylation. Integrated homologous DNA, and its non-homologous flanking DNA, is not methylated whereas integrated non-homologous DNA is. They propose that heterologous sequences are integrated into methylated regions of the genome, while homologous sequences are integrated into non-methylated regions.

To obtain transformants that will be stable through meiosis it is necessary to use a marker that will not result in duplication of an exogenous sequence, and to select a transformant with a

single integrated copy. Yamashiro *et al.* (1992) used the *amdS* gene of *A. nidulans* to transform *N. crassa* and selected for the ability to use acetamide as a sole nitrogen source. Mitotically stable transformants with a single copy of the gene were also meiotically stable. Another marker that is not vulnerable to RIP in *N. crassa* is the *trpC-hph* construct.

A phenomenon to be aware of when studying stability of markers in transformants is that of 'quelling'. This is the transient inactivation of gene expression in *N. crassa* by transformation with a homologous sequence (Romano and Macino 1992). It was found that the presence of exogenous sequences (which were randomly integrated in ectopic locations) provoked a severe impairment in the expression of the endogenous *al-1* or *al-3* genes. This effect was found to be spontaneous and progressively reversible, leading to wildtype or intermediate phenotypes (after a number of vegetative subcultures). This reversion correlated with loss of the ectopic copies and was monodirectional.

Quelling is similar to a phenomenon called co-suppression which occurs in plants (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). It appears plants and fungi have the capacity to search for and modify integrated duplicated sequences in the genome. The mechanism of quelling has not been determined although methylation has not been entirely ruled out.

4.2.4 Abortive Transformants

In the transformation systems developed for filamentous fungi, a common observation is the presence of 'abortive' transformants. These are colonies that initially grow on selective media but fail to grow upon subculturing. One theory is that the DNA has not been stabilized by integration, or by autonomous replication, but is transiently expressed. Therefore there is initial growth until the gene product is diluted out, then growth ceases. Tilburn *et al.* (1983) observed that transformed DNA is present in abortive transformants after growth has ceased. After prolonged incubation sectors of vigorous growth could develop from an abortive colony. These sectors were shown to have been stabilised by integration of the transforming DNA. These observations have also been made in *A. nidulans* (Wernars *et al.*, 1985) and *Trichoderma reesei* (Penttila *et al.*, 1987).

The frequency of abortive transformants differs depending on such factors as the selection system used for detection of the transformants. The *niaD* selection system produces no abortive transformants therefore selection of transformants is less confusing (Unkles *et al.*, 1989). The *amdS* marker tends to produce many abortive transformants and true transformants that range considerably in size. This has been observed for *A. nidulans* (Tilburn *et al.*, 1983; Wernars *et al.*, 1985), *A. niger* (Kelly and Hynes 1984) and more diverse species such as *Trichoderma*

reesei (Penttila *et al.*, 1987) and *Cochliobolus heterostrophus* (Turgeon *et al.*, 1985).

When van Hartingsveldt *et al.* (1987) transformed *A. niger* with the homologous *pyrG* gene, abortive transformants were observed when the full *pyrG* gene was used, but not if only part of the gene (with the complementary sequence to the mutation) was present. This supports the theory that abortives are due to the transient gene expression before integration, as only the full gene copy in the construct would allow this. The presence of the *ansl* sequence, which was shown to improve transformation frequency, also had the effect of decreasing the number of abortive transformants present (Ballance and Turner 1985). Similarly Cantoral *et al.* (1987) observed a lowering of the number of abortive transformants, and an increase in the number of stable transformants, when *Penicillium chrysogenum* was transformed with a vector containing the *ansl* sequence. This supports the theory that *ansl* promotes early stable integration of the DNA into the genome.

4.2.5 Pyrimidine Biosynthetic Pathway

The pyrimidine biosynthetic pathway involves the conversion of glutamine, carbon dioxide and the phosphate group of adenosine triphosphate (ATP) to uridine monophosphate (UMP) through six enzyme catalysed steps (Fig. 2, Table 9). This pathway has been studied in the filamentous fungus *N. crassa* (Radford *et al.*, 1985). The first step of the pathway, the formation of carbamoyl phosphate, is common to the arginine biosynthetic pathway. As with animals, fungi possess two carbamoyl-phosphate synthase (CPSase) genes, one regulated by arginine and the other by pyrimidine. The pyrimidine enzyme is bifunctional, having both CPSase and aspartate carbamoyl transferase (ACTase) activities.

Unlike yeast, which have only one pool of carbamoyl phosphate (CP), filamentous fungi and animals have two pools of this precursor, one for each pathway, with effective feedback control on the CPSase enzyme (Davis 1975). This channelling involves compartmentalisation with the arginine-specific pool in the mitochondria and the pyrimidine-specific pool in the nucleus. The fusion of CPSase and ACTase activities in a bifunctional protein may also aid in channelling.

The product of the pyrimidine biosynthetic pathway, UMP, is the precursor of all pyrimidine nucleotides and therefore essential. Blockages in the pathway lead to the requirement for growth of an exogenous supply of pyrimidine nucleotide, nucleosides or bases (Radford *et al.*, 1985).

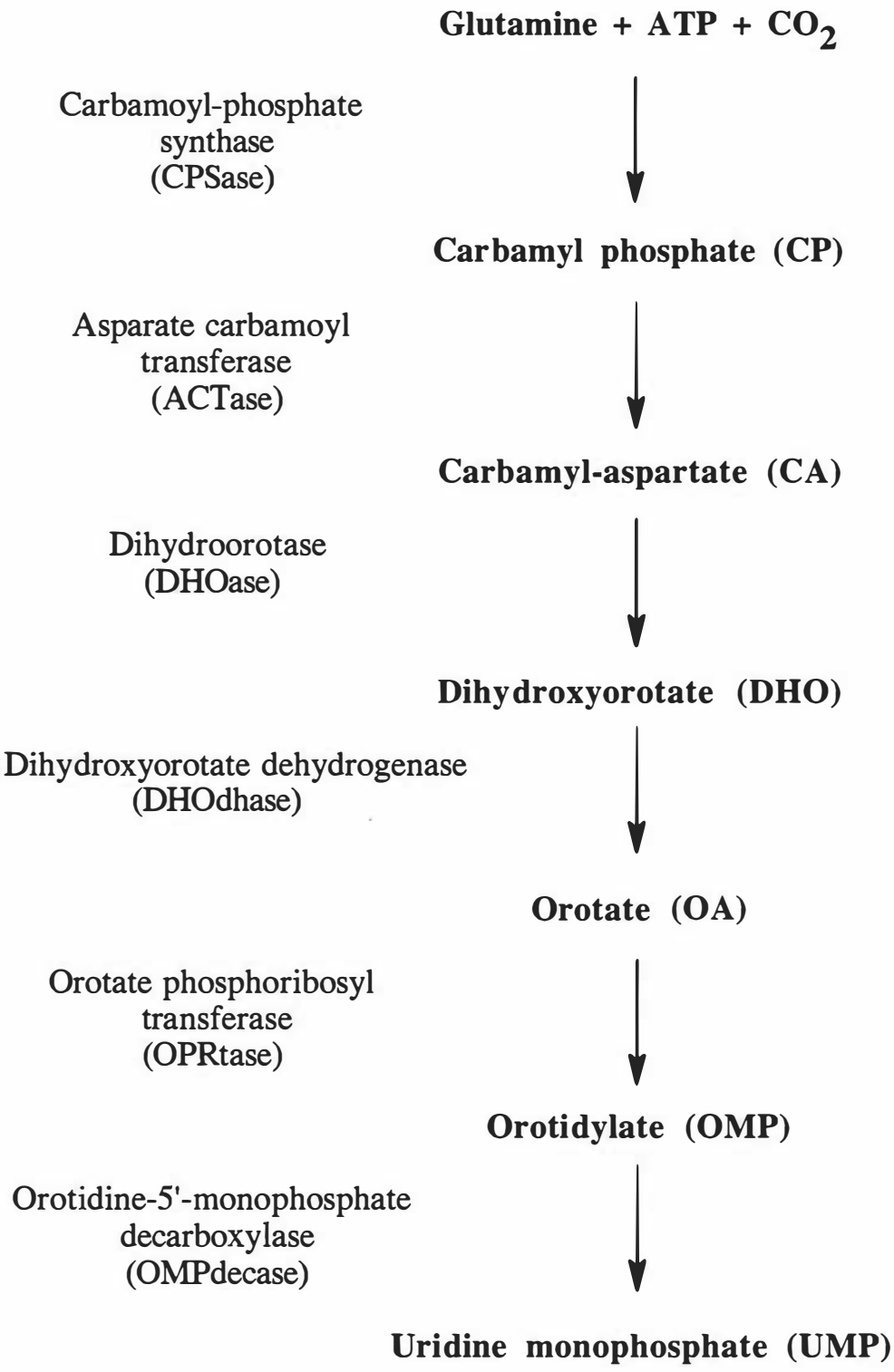


Fig 2 The Pyrimidine Biosynthetic Pathway

Table 9 Enzymes and Corresponding Genes of the Pyrimidine Biosynthetic Pathway

Enzyme	***E. coli***[a]	***S. cerevisiae***[b]	***N. crassa***[c]	***A. nidulans***[d]
CPSase[e]	*carA/carB*	*(URA2B)*	*pyr3*	*pyrA (pyrN)*
ACTase[e]	*pyrB,I*	*(URA2A)*	*pyr3*	*pyrB/pyrC(pyrN)*
DHOase	*pyrC*	*URA4*	*pyr6*	*pyrD*
DHOdhase	*pyrD*	*URA1*	*pyr1*	*pyrE*
OPRtase	*pyrE*	*URA5*	*pyr2*	*pyrF*
OMPdecase	*pyrF*	*URA3*	*pyr-4*	*pyrG*

[a] Radford *et al.*, 1985

[b] Mortimer and Hawthorn 1966

[c] Radford *et al.*, 1985

[d] Palmer and Cove 1975

[e] CPSase and ACTase may be fused to form an enzyme complex. Fusion is indicated by brackets

4.2.5.1 Regulation of the Pyrimidine Biosynthetic Pathway in Fungi

Regulation of the pyrimidine biosynthetic pathway is complicated and has been studied most extensively in the yeast *S. cerevisiae* (Losson and Lacroute 1983; Losson *et al.*, 1985; Roy *et al.*, 1990) and the filamentous fungus *N. crassa* (Radford *et al.*, 1985). In both fungi the pathway is reportedly affected by the presence of uracil, feedback inhibition and substrate induction.

In *N. crassa* the CPSase and ACTase genes are de-repressed 4-5 fold via the regulatory gene *fdu-2* during uracil starvation. CPSase is also subject to end product inhibition by UTP. The build up of the substrate dihydroxyorotate (DHO) induces dihydroxyorotate dehydrogenase (DHOdhase). In *N. crassa* this pathway is also affected by nitrogen metabolite regulation as nitrogen starvation leads to a 50-fold increase in the rate of uracil uptake, and to a lesser extent uridine uptake (Radford *et al.*, 1985).

In *S. cerevisiae* the orotidine-5'-monophosphate decarboxylase (OMPdecase) gene is induced 3-5 fold in response to uracil starvation. Substrate induction is also observed as the build up of the pathway intermediate DHO leads to the activation of a regulatory gene, *PPR1*, which acts via the 5' flanking region to increase the activity of DHOdcase (6-8 fold) and OMPdecase (3-5 fold) (Losson *et al.*, 1985). The *PPR1* gene product is not required for a basal level of expression of *URA3*. The structural genes *URA1* and *URA3* are known to specify mRNA classes with different 5' terminal sequences. Induction, by *PPR1* activation, of *URA3* leads to an increase in the synthesis of only a subset of the transcripts.

4.2.5.2 Orotidine-5'-Monophosphate Decarboxylase as a Selectable Marker

Selection of auxotrophic mutants for use in transformations can be difficult but is, in some cases, facilitated by the availability of a positive selection system. One such system, that allows for selection of mutations in the pyrimidine biosynthetic pathway, is resistance to the toxic compound 5'-fluoro-orotic acid (5-FOA) (Boeke *et al.*, 1984). Due to the relative ease of selection of *ura*$^-$ mutants (uracil- or uridine-requiring) in this way, many transformation systems have been developed based on this selection system, and genes involved in this pathway have been cloned from many fungi (Akileswaran *et al.*, 1993; Banks and Taylor 1988; Berges and Barreau 1991; Boy-Marcotte *et al.*, 1984; Buxton and Radford 1983; Collett *et al.*, 1995; Goosen *et al.*, 1987; Oakley *et al.*, 1987; Skory *et al.*, 1990; Smit and Tudzynski 1992; van Hartingsveldt *et al.*, 1987).

The final step in the pyrimidine biosynthetic pathway, in which orotidylate (OMP) is converted

to uridine monophosphate (UMP), is catalysed by OMPdecase which has been used as a selectable marker in transformations of a wide range of fungal species (Ballance *et al.,* 1983; Oakley *et al.,* 1987; van Hartingsveldt *et al.,* 1987). In *A. nidulans* OMPdecase is coded for by the *pyrG* gene (Table 9).

4.2.5.3 Orotidine-5'-Monophosphate Decarboxylase as a Fusion Protein

The OMPdecase gene has been identified as a good candidate for a reporter gene in filamentous fungi. The common *E. coli* reporter genes β-galactosidase, β-glucuronidase and hygromycin B phosphotransferase do not function in all fungi, even when expressed from a native promoter. The use of *URA3* as a reporter gene has been demonstrated in *S. cerevisiae* where fusion to the *GAL1* promoter resulted in expression levels regulated by carbon source (Alani and Kleckner 1987). In addition to tolerating extensive amino terminal modification, OMPdecase activity is easily assayed either biochemically (Yoshimoto *et al.*, 1978) or by the growth-phenotype conferred by its expression. The latter characteristic is particularly useful due to the availability of positive and negative selection schemes for *URA3* expression (Boeke *et al.,* 1984).

Fusion of the promoter and 90 codons of the *C. albicans CEF3* gene to the native *URA3* has been reported. Integration of a single copy into the *ADE2* locus, to avoid problems of position effect, resulted in a 30-fold increase in OMPdecase activity over wild-type levels (measured biochemically) and appropriate regulation of expression (Myers *et al.,* 1995).

4.2.6 Aims

In determining the optimal conditions for transformation of *A. nidulans* strain 1-85 with the vector pGM32, containing the *N. crassa pyr-4* gene, we observed morphologically distinct types of transformants. A high number of abortive transformants were observed, along with true transformants that could be classified into two main groups based on morphology. The majority of the transformants were heterokaryons that could not be resolved into stable transformed homokaryons. Initially the aim of this study was to identify ways of purifying the heterokaryons so they could be included in the gene targeting analysis (Chapter 5). The heterokaryon transformants represent an interesting population which were analysed in detail, alongside stable transformants, in an attempt to understand why they were formed.

4.3 Results

4.3.1 Transformation of *Aspergillus nidulans pyrG* Mutant with the *Neurospora crassa pyr-4* Gene

A. nidulans strain 1-85 was transformed with *Xba*I-linearised and circular DNA (pGM32). The restriction enzyme *Xba*I cuts the vector once in the multiple cloning site, outside the *pyr-4* gene. The transformation frequencies per µg of linear and circular DNA were 180 and 520 transformants/ 2 x 10^5 viable protoplasts respectively.

Transformants were identified after 3 days at 37°C as strong growing colonies on osmotically stabilised selective media and observed to form two distinct groups based initially on morphology (Fig. 3). The majority were irregular in shape and, although initially small, once established grew into large sporulating colonies. These were labelled H-type transformants due to their resemblance to heterokaryons. The remainder were fast growing, large and resembled the parental type with a regular shape. These were labelled N-type ('Normal') transformants.

Ten transformants of each of the two morphologically distinct types were selected at random for further analysis. Of each set of ten transformants, 5 were from transformation with linear DNA (H1-H5 and N1-N5) and 5 from transformation with circular DNA (H6-H10 and N6-N10). The transformation mix containing no DNA, and plated on osmotically stabilised non-selective media, provided control strains (C1-C10).

The transformants and the control strains were transferred to osmotically stabilised selective media (MYG + sucrose), selective media (MYG) and finally non-selective media (MU) (Fig. 4). As expected the H-type and N-type transformants grew on all media, being uracil independent by virtue of the *N. crassa pyr-4* gene used in the transformation. The control strains (untransformed) were unable to grow on selective media. The morphological differences between the H-type and N-type transformants were most apparent on osmotically stabilised selective media, although differences were still apparent on selective media without osmotic stabiliser (Fig. 4).

4.3.1.1 Purification and Mitotic Stability of Transformants

The mitotic stability of the transformants was tested by the ability to maintain the transformed phenotype (growth on selective media) after growth on non-selective media. The N-type transformants were mitotically stable whereas the H-type transformants were not. The only

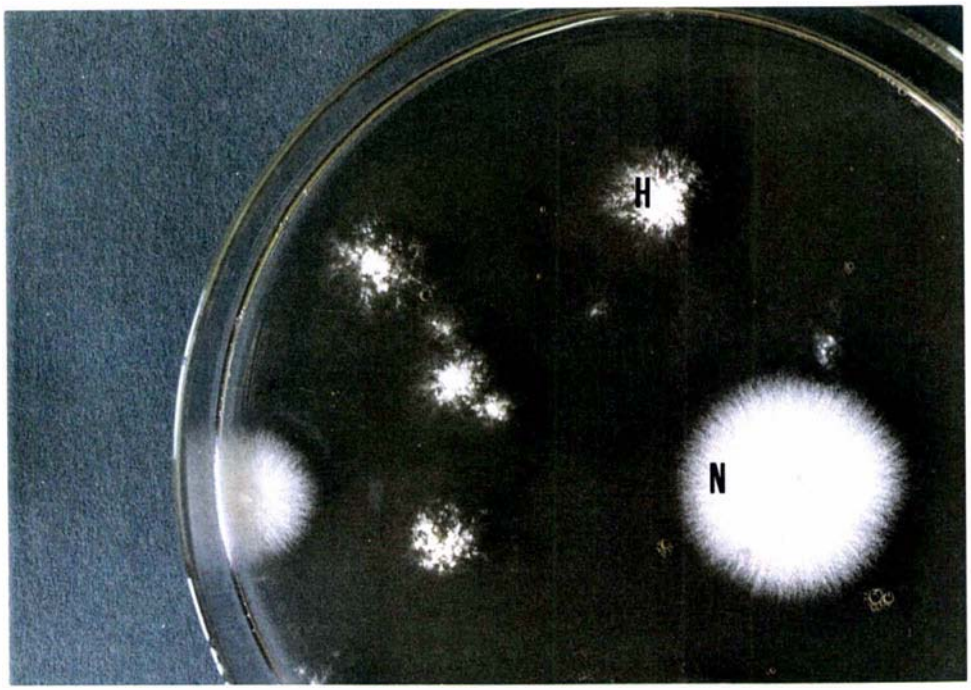
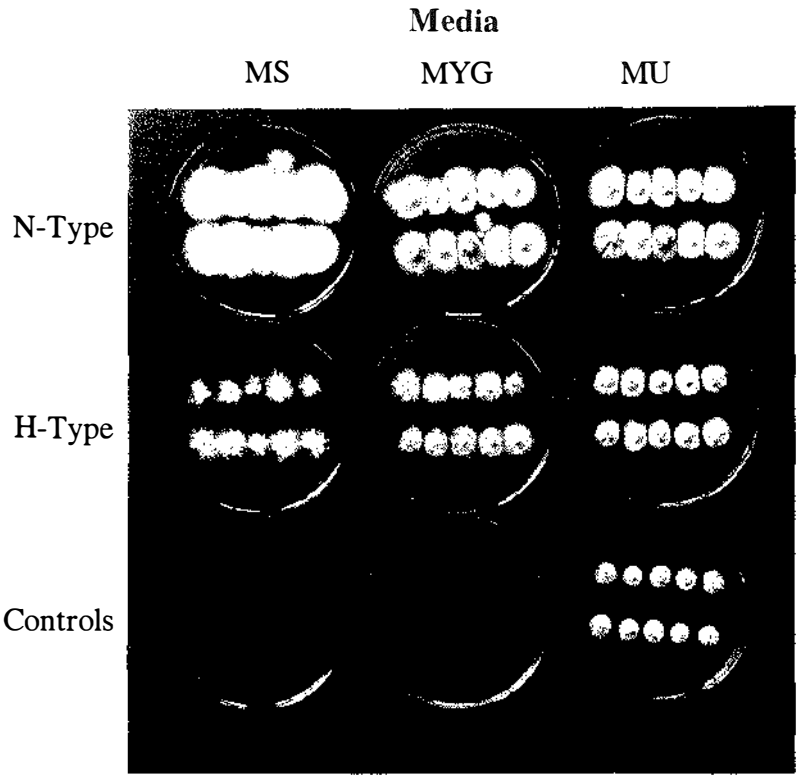


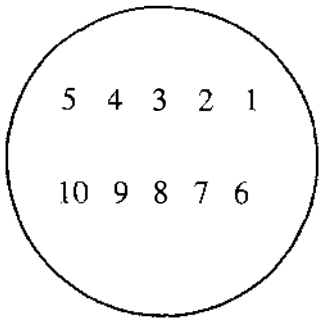
Fig 3 Morphology of H-type and N-type Pyr^+ Transformants

Transformant selection plate with *A. nidulans* strain 1-85 transformed with pGM32 and selected on osmotically stabilised complete media lacking uracil. The two phenotypically distinct types of transformants, H-type (H) and N-type (N), are indicated.

A



B



N1-5 and H1-5 transformed with linear DNA

N6-10 and H6-10 transformed with circular DNA

Fig 4A-B Growth of Transformed and Untransformed Strains on Selective and Non-selective Media.

A Transformants N1-N10 (N-type), H1-H10 (H-type) and untransformed 1-85 controls (C1-C10) grown for two days at 37^{o}C on, from left to right, selective media osmotically stabilised with sucrose (MS), selective media (MYG) and non-selective media (MU). **B** Numbering of cultures on plates.

way to maintain the H-type transformants was with selective pressure.

The N-type transformants were easily purified by streaking for single spores (Section 2.4.2.1). This was repeated to ensure purification. The purified transformants were taken through 12 rounds of subculturing on non-selective media then transferred to selective media. All 10 N-type transformants retained the transformant phenotype so maintained mitotic stability.

The H-type transformants could not be purified by streaking for single spores on selective media (the single spore isolates obtained were Pyr^-) so were subcultured twice on selective media.

The ploidy of each transformant was determined by measuring the average diameter of spores (Table 10). Of the twenty selected only one, N8, was classified as a diploid. This culture grew faster than the others, although it sporulated poorly.

The control diploid strain was TZ24 (Pyr^+) and had an average spore diameter of 4.16 μm while the haploid control, 1-85, had a spore diameter of 3.28 μm. This is in agreement with the published diploid:haploid spore diameter ratio of 1.3:1 (Pontecorvo *et al.*, 1953). Five strains obtained from regenerated protoplasts from the negative control plate of the transformation of 1-85 (C1-C5) were also measured and were all haploid having a spore diameter of 3.26 $\pm$ 0.054 μm. The spore diameters were more variable with the N-type transformants with an average spore diameter for the 9 haploids of 3.3 $\pm$ 0.1 μm. Similarly the H-type transformants spore diameters were variable with an average spore diameter for the 10 haploids of 3.44 $\pm$ 0.12 μm, which is slightly larger than that observed for the N-type transformants.

4.3.1.2 Analysis of Spore Germination Patterns of Transformants

Spore suspensions were prepared from the transformants (N1-10 and H1-10) and plated onto non-selective media. The ratio of transformant (Pyr^+) and parental (Pyr^-) spores could be assessed accurately by scoring the number of large (Pyr^+) and small (Pyr^-) colonies (Fig. 5). Colonies that could not be easily classified as Pyr^+ or Pyr^- on the basis of size were tested by growth on selective and non-selective media.

Table 10 Transformant Ploidy

Controls[a]	Spore Diameter[b,c] (μm)	N-Type Trans.	Spore Diameter[c] (μm)	H-Type Trans.	Spore Diameter[c] (μm)
TZ24	4.16 (D)	N1	3.38 (H)	H1	3.38 (H)
1-85	3.28 (H)	N2	3.22 (H)	H2	3.44 (H)
		N3	3.38 (H)	H3	3.34 (H)
C1	3.22 (H)	N4	3.50 (H)	H4	3.18 (H)
C2	3.22 (H)	N5	3.18 (H)	H5	3.38 (H)
C3	3.28 (H)	N6	3.28 (H)	H6	3.58 (H)
C4	3.22 (H)	N7	3.34 (H)	H7	3.50 (H)
C5	3.34 (H)	N8	3.92 (D)	H8	3.50 (H)
		N9	3.22 (H)	H9	3.58 (H)
		N10	3.18 (H)	H10	3.50 (H)

[a] Control strains include TZ24 (diploid) (Chapter 6), 1-85 (haploid) and C1-C5 (from regenerated protoplasts on negative control plate).

[b] Average spore diameter in μm ($n > 20$).

[c] The ploidy classification for each transformant is given: (H) haploid and (D) diploid.

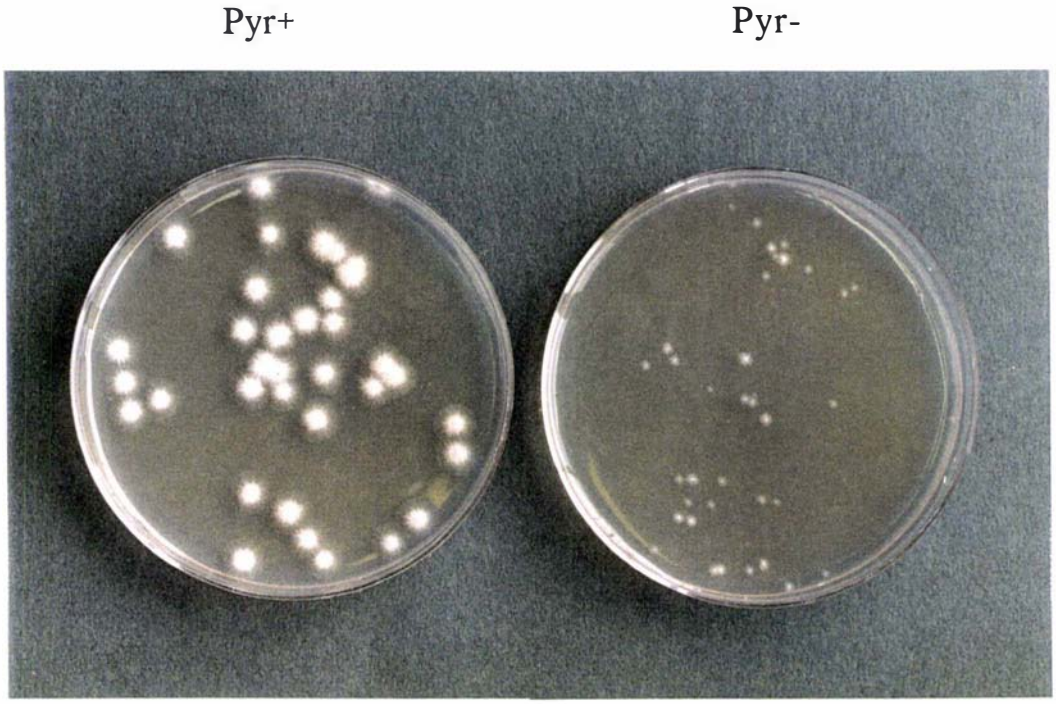


Fig 5 Growth of Pyr^+ and Pyr^- Colonies

Growth of representative Pyr^+ (obtained from transformant N1) and Pyr^- (obtained from transformant H1) colonies from spore suspensions after incubation for 27 hours at 37°C, and 15 hours at 30°C, on non-selective media.

Before single spore purification the majority of the N-type transformants were homokaryons. Only three (N4, N8 and N9) appeared to be heterokaryons, having a small proportion (8.0%, 0.9% and 0.7% respectively) of Pyr^- spores in the spore suspensions. After purification spores from all the N-types were 100% Pyr^+, with the exception of the diploid transformant N8. The purified N8 spore suspensions yielded some Pyr^- colonies which, on the basis of their unusual morphology, were classified as aneuploids. The majority of the N-type transformants were thus deemed to be true homokaryons after the purification procedure.

In contrast, plating H-type transformant spores on non-selective media yielded only Pyr^- colonies (Fig. 5). On selective media it was only possible to get a Pyr^+, or transformant, colony by plating a large number of spores ($>10^4$). Due to the large number of spores plated it was probable that the Pyr+ colonies originated from viable (heterokaryotic) mycelial fragments rather than single spores. It was therefore necessary to analyse germination directly on the plate using an inverted microscope.

Spore suspensions from a selection of N-type transformants (N1 and N5), H-type transformants (H1, H3, H5, H7 and H9), and the parental strain (1-85) were plated on selective and non-selective media and examined for germination after approximately 10 hours incubation at 37°C. Spore viability was determined by the proportion of spores forming germ tubes on the non-selective plates; this ranged from 76-98% for all spore suspensions tested (Table 11). On selective media viable spores from the Pyr^- parental strain were observed to swell but not germinate (Fig. 6A), while it appeared all viable spores from the Pyr^+ N-type transformants germinated (Fig. 6B), as expected. Interestingly, only 7-29% of the viable H-type spores formed germ tubes on selective media while, as with the parental spores, swelling was observed for the remainder of the viable spores (Fig. 6C, Table 11). This clearly indicated the presence of both Pyr^+ spores (germinating) and Pyr^- spores (swelling) in the H-type spore suspension, lending support to the theory that the H-types were heterokaryons. Upon further incubation the Pyr^+ H-type germinating spores did not appear to grow (Fig. 6D).

4.3.1.3 Southern Analysis of Transformants

The number of copies of pGM32, and pattern of integration, was determined by Southern analysis. DNA was isolated from transformants N1-10 (N-type), H1-10 (H-type), the parental strain (1-85), and two Pyr^- auxotrophs isolated from H-types H1 (A1) and H10 (A10). The latter were obtained as single spore isolates, from H-type transformants, by streaking for single

Table 11 Spore Germination Patterns on Selective and Non-selective Media

Strain	Non-selective media			Selective media			Viable Pyr+
	Germ.	Non-germ.	Viable (%)[a]	Germ.	Non-germ.	Pyr+ (%)[b]	spores (%)[c]
1-85	177	4	**98**	0	-	-	-
N1[d]	364	43	**90**	23	3	**88**	**100**
N5[d]	92	14	**86**	21	2	**91**	**100**
H1	134	38	**78**	19	192	**9**	**12**
H3	182	57	**76**	42	337	**11**	**14**
H5	146	12	**92**	38	269	**18**	**20**
H7	117	7	**94**	56	154	**27**	**29**
H9	125	13	**91**	14	220	**6**	**7**

[a] The percentage of viable spores was calculated from the number of germinating (Germ.) and non-germinating (Non-germ.) spores on non-selective media.

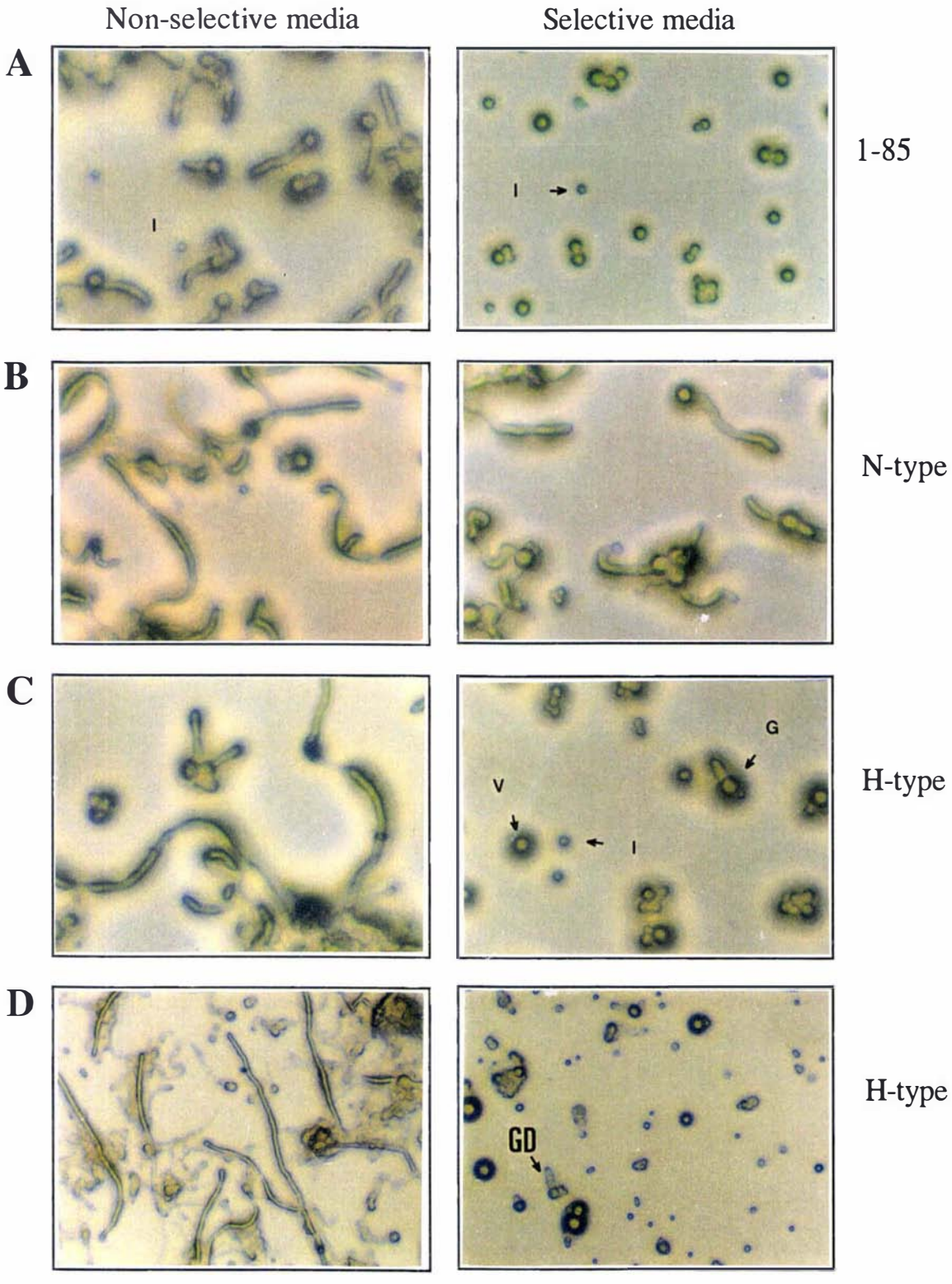
[b] The percentage of Pyr^+ spores was calculated from the number of germinating (Germ.) and non-germinating (Non-germ.) spores on selective media.

[c] The percentage of viable spores that were Pyr^+ was calculated from the percentage of viable spores and the percentage of Pyr^+ spores.

[d] The number of germinating and non-germinating spores for N1 and N5 are estimates due to overcrowding of the germinating spores.

Fig 6A-D Spore Germination Patterns

Germination of representative spore suspensions on selective and non-selective media. **A** Parental strain 1-85 incubated for 10 hrs at 37°C (400x magnification). **B** N-type transformant incubated for 10 hrs at 37°C (400x magnification). **C** H-type transformant incubated for 10 hrs at 37°C (400x magnification). **D** H-type transformant incubated for 30 hrs at 37°C (100x magnification).
Arrows indicate a swollen viable spore (V), a germinating spore (G), an inviable spore (I) and a germinating spore that has stopped growing (GD).


Non-selective media
Selective media
A
I
I
1-85
B
N-type
C
V
G
I
H-type
D
GD
H-type

spore isolates on non-selective media (Pyr^-).

Southern blots of undigested DNA probed with pGM32 confirmed the plasmid had integrated into the genome of the transformants as no bands consistent with the presence of autonomous plasmid DNA were observed (Fig. 7A). It is however a possibility that free plasmids were present in a form, such as concatamers, that runs with undigested DNA. Probing the stripped blot with actin allowed loading comparisons to be made to assist in copy number determination (Fig. 7B). The DNA from parental strain 1-85 and the Pyr^- auxotrophs A1 and A10 did not hybridise with the pGM32 probe indicating the vector DNA shares very little homology with the parental strain, and that A1 and A10 contained no vector DNA.

The restriction enzyme *Cla*I cuts once in the vector therefore integration of the vector in head to tail tandem arrays would result in a hybridising band the size of the vector (4.5 kb). A Southern blot of transformant and control DNA, digested with *Cla*I, was probed with pGM32 (Fig. 8A) and then, after stripping, probed with the *A. nidulans* actin gene (Fig. 8B).

Of the N-type transformants six out of ten gave a band the size of the vector (4.5 kb) indicating the presence of multiple copies of the vector in tandem arrays. The bands of other sizes represent the regions bordering the integrated vector and rearrangement of the vector DNA. The diploid transformant, N8, gave a very intense signal with a hybridisation pattern that was very complicated and therefore difficult to interpret, but appeared to have multiple copies integrated at multiple sites. The approximate number of copies integrated into each of the transformants was determined (Table 12). The signal intensity after hybridisation with pGM32 was compared to the signal intensity after hybridisation to actin to correct for the amount of DNA loaded in each lane. The signal intensities were then compared to each other to estimate the copy number. The results indicated the N-type transformants contained between 1 and 15 copies integrated per haploid nucleus (Table 12).

In contrast to the N-type transformants all ten of the H-type transformants had a hybridising band the size of the vector (4.5 kb) so all contained multiple copies integrated in tandem arrays. For six transformants border sequences were obvious after longer exposure of the autoradiograph. The remaining four had only a single band the size of the vector. The low intensity of the signal for the border sequences, compared to the 4.5 kb signal, suggested that tandem arrays in the H-type transformants contained many copies of the vector. Moreover, the low intensity of the border regions for the H-types, when compared to the N-types, suggested that only a fraction of the H-type nuclei had the plasmid integrated. The H-type transformants therefore contained less than one to nine copies of the vector integrated, per haploid nucleus, when averaged over the transformed and untransformed nuclei (Table 12). However, as only a

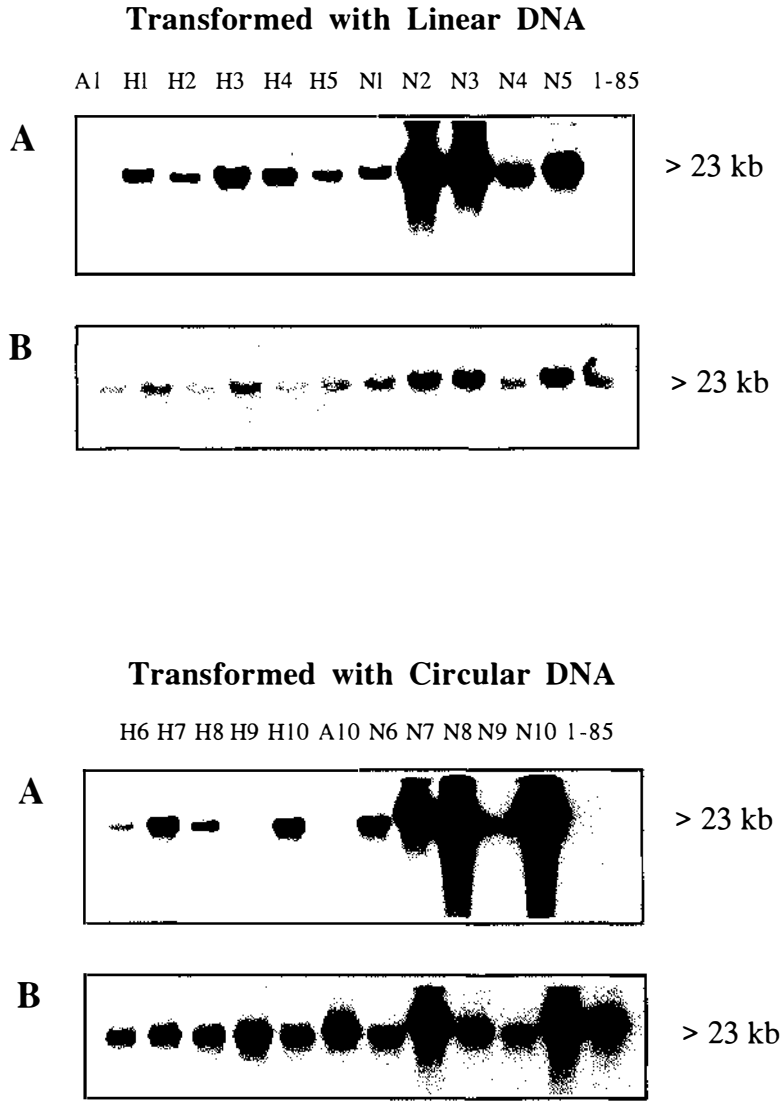


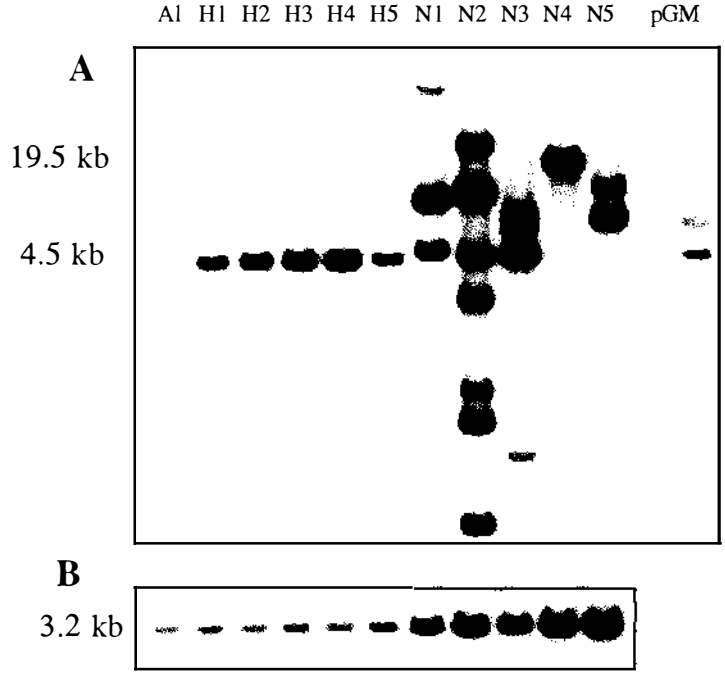
Fig 7A-B Southern Blot of Uncut 1-85 and Transformant DNA

Hybridisation of [α-^{32}P] dCTP-labelled pGM32 and *A. nidulans* actin fragment to genomic DNA of *A. nidulans* transformants. **A** Autoradiograph of a Southern blot of uncut genomic DNA run on a 1% agarose gel and probed with pGM32. **B** Autoradiograph of blots shown in **A** probed with actin. Transformants were obtained using linear or circular vectors as indicated.

Fig 8A-B Southern Blot of *Cla*I Cut 1-85 and Transformant DNA

Hybridisation of [α-^{32}P] dCTP-labelled pGM32 and *A. nidulans* actin fragment to genomic DNA of *A. nidulans* transformants. Numbers on the left of the figures indicate the size of the DNA fragments. **A** Autoradiograph of a Southern blot of *Cla*I digested DNA run on a 1% agarose gel and probed with pGM32. **B** Autoradiograph of blots shown in **A** probed with actin. Transformants were obtained using linear or circular vectors as indicated.

Transformed with Linear DNA



Transformed with Circular DNA

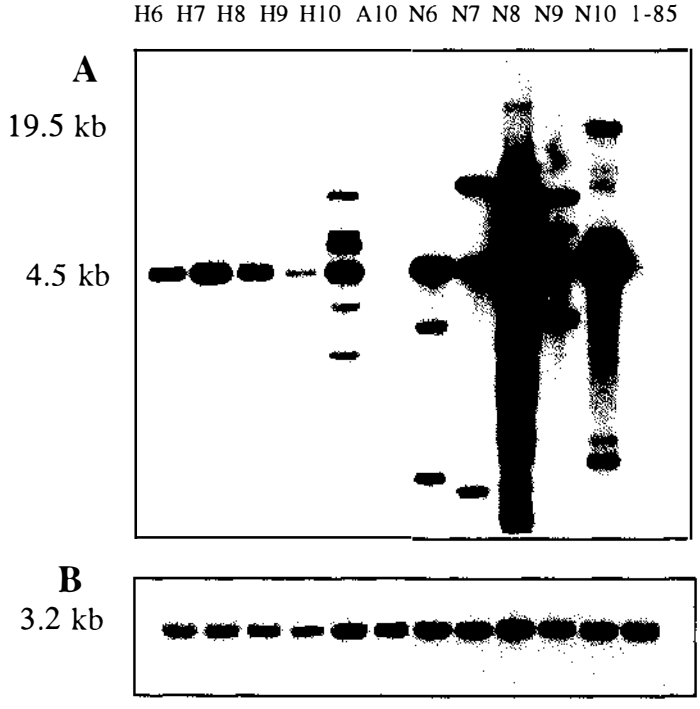


Table 12 Estimation of Vector Copy Number in Transformants

Trans.	Copy Number Range[a]	Number of Bands ClaI Southern	Tandem Repeats *Cla*I [b] Southern	Number of Bands *Sph*I[c] Southern	Max Number of Copies of pGM32 *Sph*I [c]	Average Copy Number[d] Ranged (per nucleus)	Copy Number Range[e] (per Transformed nucleus)
H1	1-2	4	+	1	>10[f]	1-2	**8-17**
H2	2-4	2	+	1	>10[f]	2-4	NC[g]
H3	1-4	1	+	1	>10[f]	1-4	**7-29**
H4	3-9	1	+	1	>10[f]	3-9	NC[g]
H5	<2	1	+	1	>10[f]	<2	**<10**
H6	1-2	1	+	1	>10[f]	1-2	NC[g]
H7	2-4	1	+	1	>10[f]	2-4	**7-14**
H8	1-2	5	+	1	>10[f]	1-2	NC[g]
H9	<1	1	+	1	>10[f]	<1	**<14**
H10	3-6	7	+	1	>10[f]	3-6	NC[g]
N1	1-3	2	-	1	≤2	1-2	**1-2**[j]
N2	3-7	7	+	1	≤10	3-7	**3-7**
N3	2-4	4	+	1	≤7	2-4	**2-4**
N4	1-3	1	-	1	≤3	1-3	**1-3**
N5	1	2	-	1	≤2	1	**1**
N6	4	3	+	1	≤7	4	**4**
N7	3-5	4	+	2	≤4 & ≤6	3-5	**3-5**
N8	ND[h]	ND[h]	+	ND[h]	ND[h]	ND[h]	ND[h]
N9	3-5	3	-	1	≤4	3-4	**3-4**[j]
N10	7-15	5	+	ND[i]	ND[i]	7-15	**7-15**

[a] Estimate of the number of copies of pGM32 integrated. This was determined by comparing the signal intensities of the autoradiographs of uncut and *Cla*I digested DNA probed with actin and pGM32. The signal intensity was determined using the ISO-1000 Digital Imaging System from Alpha Innotech Corporation. If the signal was too intense to be determined accurately an autoradiograph with a lower exposure was used. Once determined, the signal obtained with the pGM32 probe was compared to that with the actin probe to compensate for loading discrepancies between lanes. The copy number for transformants N1 to N5 and H1 to H5 were standardised against N5. N5 was assumed to have a copy number of one as only two bands were observed on the Southern containing *Cla*I digested DNA and probed with pGM32, indicating integration of a signal copy. The copy number for transformants H6 to H10 and N6 to N10 were standardised against N6. N6 was assumed to have a copy number of four based on the hybridisation pattern for the *Cla*I digest. The intensities of the border sequences were compared to the intensity of the tandem repeat at two different exposures to determine an approximate copy number.

[b] Integration as a tandem repeat was determined by the presence (+) or absence (-) of a band the size of the vector (4.5 kb), when cut with a restriction enzyme that cuts once in the vector (*Cla*I).

[c] As *Sph*I does not cut within the transforming vector the bands represent the entire genomic region containing the integrated vector (vector plus flanking region). The size of these bands was used to determine the maximum number of copies of the 4.5 kb pGM32 vector that could be integrated.

[d] Estimate of the number of copies integrated into each transformant by combining all the available information. As the number of nuclei transformed for H1 to H10 are only a proportion of the total number of nuclei this represents the average over all nuclei for these transformants (Table 11).

[e] Estimate of the copy number per transformed nuclei. The estimate for H1, H3, H5, H7 and H9 was calculated assuming that the proportion of Pyr^+ spores (Table 11) represents the proportion of transformed nuclei. The H-type spores were prepared by growing the mycelia on plates of selective media until a well sporulating lawn was formed. The spores were harvested according to the method in Section 2.4.2.3. The DNA was extracted from young mycelia grown on cellophane discs on plates of selective media (transformants) according to the method in Section 2.7. Therefore spores and DNA were from cultures plated on selective media.

[f] The absolute maximum number of copies of the vector integrated could not be determined as the size of the hybridising band could not be calculated. The size of the hybridising band was however greater than 50 kb therefore the maximum number of copies was greater than 10.

[g] The copy number per transformed haploid nucleus could not be calculated as the percentage of Pyr^+ spores was not determined for these transformants.

[h] Due to the high signal intensity and complicated banding pattern observed for transformant N8 the copy number was not determined (ND).

[i] Due to the degradation of the *Sph*I digested DNA for transformant N10 this value could not be determined (ND).

[j] The maximum number of copies integrated was determined by the *Sph*I digests. If this was lower than the maximum copy number determined by the autoradiograph signal intensity the new value was included. For example the range for N1 of 1-3 copies changed to 1-2 copies as the maximum number able to be integrated was 2 (determined by the *Sph*I digest).

fraction of the H-type nuclei were transformed (Table 11) the copy number could be recalculated **per transformed haploid nucleus**. The range was determined to be from 7 to 29 copies per transformed haploid nucleus (Table 12).

The restriction enzyme *Sph*I does not cut the vector pGM32 so digestion of transformant genomic DNA should yield a fragment containing the entire integrated vector. The size of the hybridising fragment represents the vector DNA plus any bordering genomic DNA. As the size of the vector is 4.5 kb the maximum number of tandem copies that could be contained in the fragment could be determined (Table 12). Each transformant was digested with *Sph*1 and the digests run on a 0.4% agarose gel for Southern blotting. For the N-type transformants the size of the pGM32 hybridising fragments ranged from 9.7 kb to 49 kb [Excluding N8 (large intense signal) and N10 (degraded)] (Fig. 9A). The number of integrated copies was therefore estimated to range from one to nine (Table 12). The H-type transformants, however, all had hybridising fragments of greater than 50 kb, which was the limit of resolution of the 0.4% gel. The maximum number of tandem copies was therefore estimated to be greater than 10. The lower intensity of these bands, when compared to the *Cla*I digests, may be due to shearing of the high molecular weight DNA, or less efficient transfer to the membrane.

4.3.1.4 Factors Which Affect the Ratio of Transformant Types

The number of copies of the integrated vector per transformed nucleus thus appeared to be different for the N-type (1-15 copies) and H-type (7-29 copies) transformants. Since vector conformation and the amount of DNA in the reaction mix are believed to affect the number of copies integrated, their effect on the ratio of the two types of transformants was investigated.

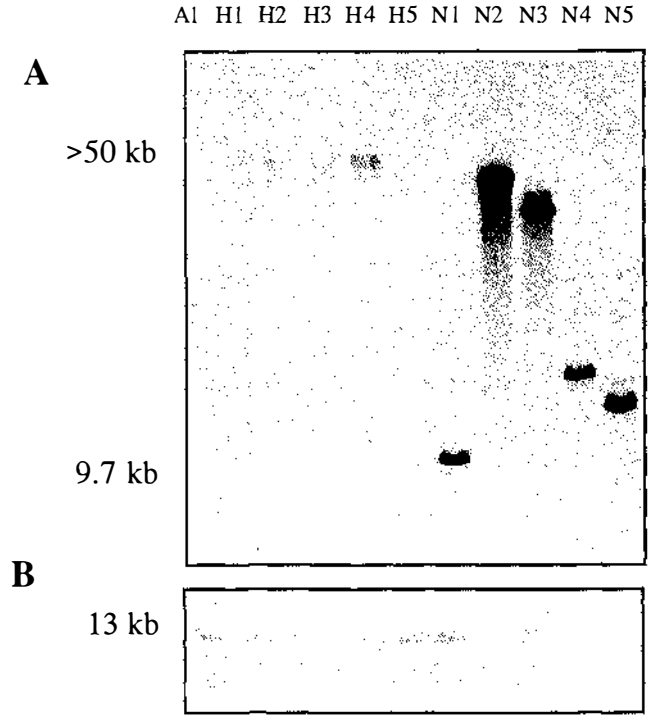
Using the pGM32 vector and a standard protocol containing spermidine and circular DNA (Section 2.18) the transformation frequency ranged from 200 to 520 transformants/2 x 10^6 protoplasts/µg DNA. The percentage of transformants that could be classified as N-type transformants ranged from 9% to 17%, with an average of 13%. However, if the vector was linearised, the percentage of N-type transformants increased up to 4-fold (Table 13). This was tested in three separate experiments and the increase was statistically significant (Appendix 3).

Increasing the amount of DNA in the transformation mix decreased the proportion of N-type transformants (Table 14), even though the proportion of N-type transformants was comparatively low for this set of transformations compared to earlier transformations. As the number of N-type and H-type transformants were not statistically different between the two experiments the results were combined. The number of H-type and N-type transformants

Fig 9A-B Southern Blot of *Sph*I cut 1-85 and Transformant DNA

Hybridisation of [α-^{32}P] dCTP-labelled pGM32 and *A. nidulans* actin fragment to genomic DNA of *A. nidulans* transformants. Numbers on the left of the figures indicate the position and size of DNA fragments. **A** Autoradiograph of a Southern blot of *Sph*1 digested DNA run on a 0.4% agarose gel and probed with pGM32. **B** Autoradiograph of blot shown in **A** probed with actin. Transformants were obtained using linear or circular vectors as indicated.

Transformed with Linear DNA



Transformed with Circular DNA

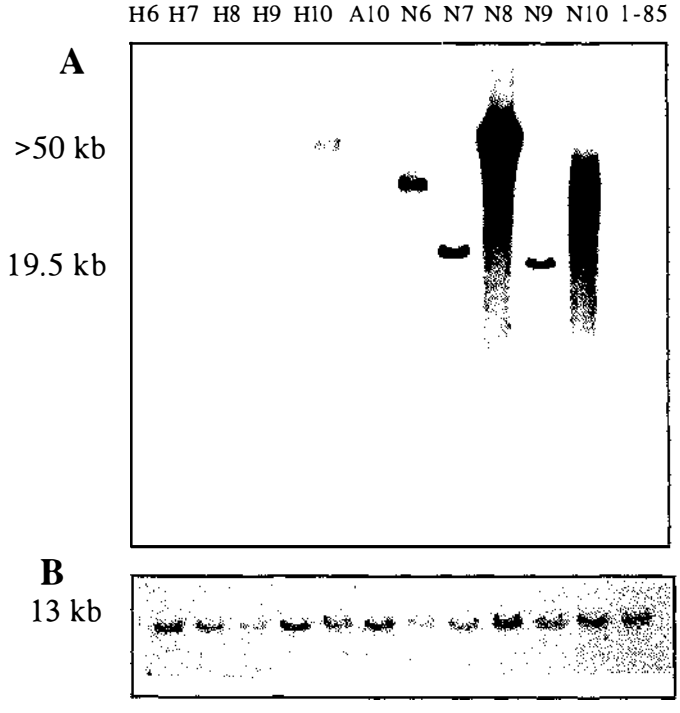


Table 13 Effect of Vector Conformation and the Addition of Spermidine on the Percentage of N-type transformants

Expt.[a]	Vector	Spermidine	N-Types	H-Types	Percentage of N-Types[b]
1	Circular	+	18	180	9.1%
	Linear	+	34	44	44%
2	Circular	-	18	135	12%
	Linear	-	18	53	25%
3	Circular	-	10	202	4.7%
	Linear	-	15	60	20%

[a] Protoplasts were prepared from mycelia (Section 2.16.2) for each experiment and transformation conditions kept standard (Section 2.18) apart from the inclusion or exclusion of spermidine (1 mM).

b The increase in the percentage of N-type transformants with vector linearisation in the three separate experiments was statistically significant (Expt. 1: $\chi^2 = 43.6$, df = 1, $P < 0.001$; Expt. 2: $\chi^2 = 14.3$, df = 1, $P < 0.001$; Expt. 3: $\chi^2 = 17.4$, df = 1, $P < 0.001$). See Appendix 3 for details of these calculations.

Table 14 Effect of the Amount of Vector DNA on the Percentage of N-type Transformants

Experiment	Amount of DNA	N-types	H-types	Percentage of N-types[c]
1[a]	0.5 μg	14	104	11.9%
	1.0 - 2.5 μg	19	379	4.8%
2[b]	0.25 - 0.5 μg	13	116	10.1%
	0.75 - 1.0 μg	10	162	5.8%

[a] Protoplasts were stored frozen for 70 days.

[b] Protoplasts were from the same batch as [a] but stored for 81 days.

[c] The number of N-type and H-type transformants were not statistically different between the two experiments (χ^2 = 0.46, df = 1, P = 0.5). The number of N-type and H-type transformants for 0.25 to 0.5 μg of DNA, and 0.75 to 2.5 μg of DNA, were not statistically different (0.25 to 0.5 μg: χ^2 = 0.511, df = 5, P > 0.9; 0.75 to 2.5 μg: χ^2 = 0.131, df = 2, P > 0.9). The data was therefore pooled and the difference in the proportion of N-type transformants in each group was determined to be significant (χ^2 = 9.216, df = 1, 0.01 > P > 0.001). See Appendix 3 for details of these calculations.

observed for 0.25 to 0.5 μg of DNA, and 0.75 to 2.5 μg of DNA, were tested and found not to be statistically different so were also pooled. The difference in the proportion of N-type transformants in each group was, however, significantly different (Appendix 3).

4.3.2 Expression and Regulation of *pyr-4*

The question of why a high number of copies of *pyr-4* in a tandem repeat form would only be able to be sustained in a heterokaryon was raised. Why would Pyr^+ conidia from such heterokaryons be unable to continue growing after germination?. Possible explanations focus on the expression and regulation of *pyr-4*.

4.3.2.1 Construction of pDW2

To determine if the level of expression of *pyr-4* was responsible for the H-type transformant phenomenon, the *pyr-4* promoter in pGM32 was replaced with the *niiA* promoter. The vector pDW2 was constructed by cloning the *niiA* promoter into pGM32, from which the *pyr-4* promoter was deleted (Fig. 10). The *niiA* gene of *A. nidulans* encodes nitrite reductase which has a low basal level of expression which is induced approximately 10 fold in the presence of nitrate.

4.3.2.2 Construction of pDW6, pDW7 and pDW8

An alternative selection system was required to determine if the transformant morphologies were specific for OMPdecase selection. It was also necessary to determine if the observed morphologies were due to the *pyr-4* promoter. The *pyr-4* promoter was therefore combined with phleomycin selection to produce pDW6, pDW7 and pDW8 (Table 15, Fig. 11).

4.3.3 Transformation of 1-85 with the OMPdecase Selection Vectors

1-85 was transformed with the circular vectors pGM32, pPYRG (contains *A. nidulans pyrG* as the selectable marker) and pDW2. Transformants were selected on media with and without nitrate. Transformants were obtained from pGM32 and pPYRG on both media. A mixture of N-types and H-types were observed for both transformations, although for the pPYRG vector the proportion of H-types was comparatively low (Fig. 12). The transformation frequency was very low for pDW2 and the transformants were only of the N-type morphology. The majority of the transformants were obtained on osmotically stabilised media containing nitrate, one was obtained on complete media without nitrate, and no transformants were obtained on osmotically stabilised minimal media without nitrate for pDW2.

Fig 10A-C Construction of pDW2

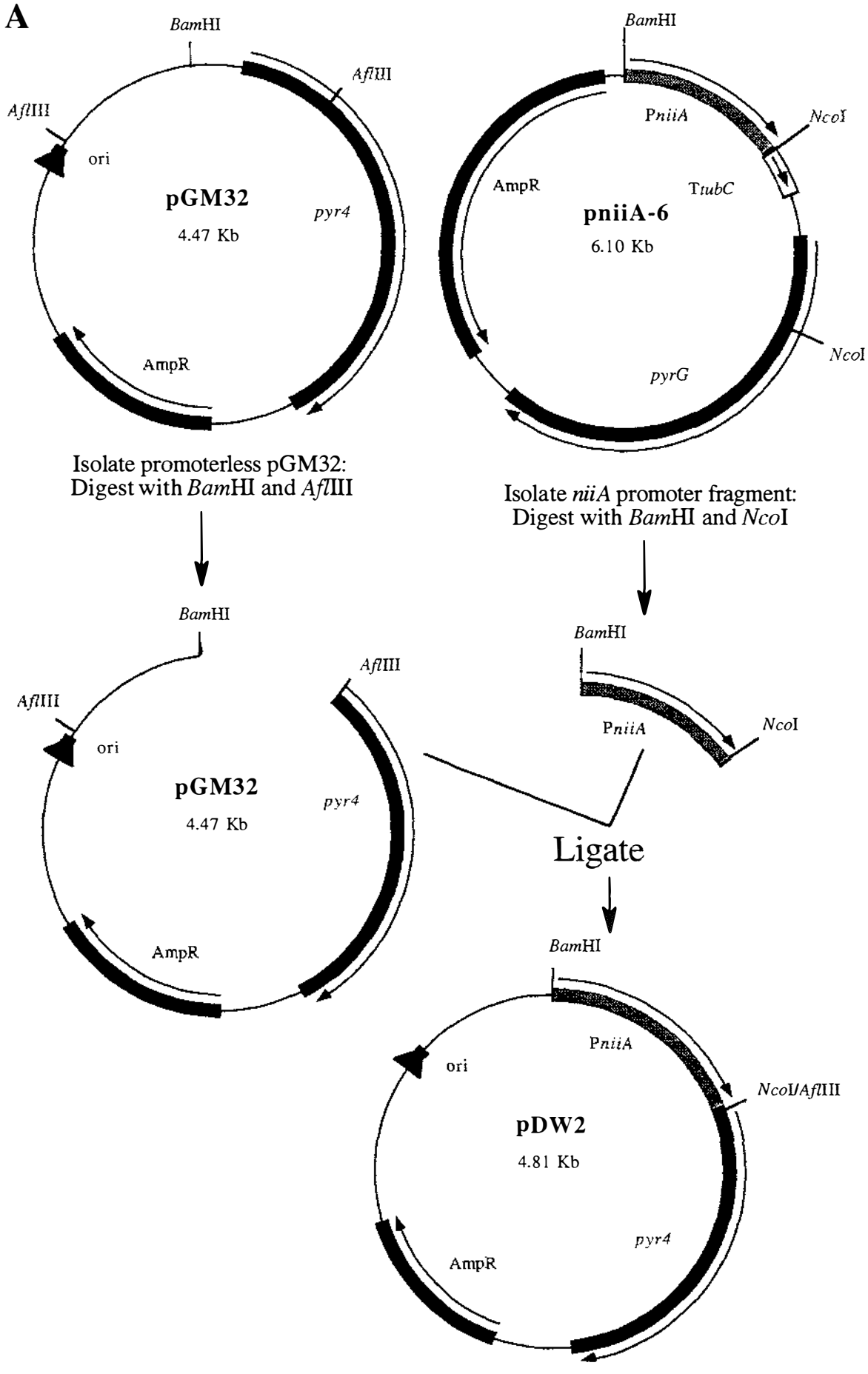
A Flow diagram of the construction of pDW2:

Vector pniiA-6 contains an altered *niiA* promoter. The promoter was produced by PCR to incorporate the native *Bam*HI site and a translation initiation site which incorporated an *Nco*I site. The *niiA* promoter was isolated from pniiA-6 as a 930 bp *Bam*HI/*Nco*I fragment. Due to a second *Nco*I site being present in pniiA-6 the vector was first digested with *Nco*I and the 1 kb *Nco*I/*Nco*I fragment isolated before digestion with *Bam*HI. The *Nco*I/*Bam*HI *niiA* promoter was then purified.

The *pyr-4* promoter could be removed from pGM32 by digestion with *Bam*HI and *Afl*III. Due to the presence of a second *Afl*III site in pGM32 it was necessary to digest pGM32 to completion with *Bam*HI prior to a partial digestion with *Afl*III. The *Bam*HI/*Afl*III promoter-less vectors were isolated and ligated with the *Bam*HI/*Nco*I *niiA* promoter to form pDW2.

B Sequence surrounding the ligation site (and the translation initiation codon) of the *niiA* *Bam*HI/*Nco*I promoter fragment cloned into promoter-less *Bam*HI/*Afl*III pGM32.

C Comparison of the sequences surrounding the translation initiation codon of: native *niiA*; *niiA* in pniiA-6, native *pyr-4* and *pyr-4* in pDW2, to the Kozak sequences described for *N. crassa* (Ballance 1991), *S. cerevisiae* (Cigan and Donahue 1987) and mammals (Kozak 1987). The translation initiation codons and -3 positions are highlighted in bold. Due to the insertion of two cytosines (CC) the highly conserved (**A**) at -3 (87% in *N. crassa* (Bruchez *et al.*, 1993)) is no longer conserved in pDW2. However, there are examples of *N. crassa* genes with a (**C**) at the -3 position (Bruchez *et al.*, 1993) which suggests the (**A**) is not absolutely required for translation.


A
BamHI
AflIII
AflIII
ori
pGM32
4.47 Kb
pyr4
AmpR
BamHI
PniiA
NcoI
AmpR
TtubC
pniiA-6
6.10 Kb
NcoI
pyrG
Isolate promoterless pGM32:
Digest with BamHI and AflIII
Isolate niiA promoter fragment:
Digest with BamHI and NcoI
BamHI
AflIII
AflIII
ori
pGM32
4.47 Kb
pyr4
AmpR
BamHI
PniiA
NcoI
Ligate
BamHI
PniiA
ori
NcoI/AflIII
pDW2
4.81 Kb
pyr4
AmpR

B

Vector: promoter-less *Bam*HI/*Afl*III pGM32

pyr-4 gene

C**ATG**TCGACAA----------*Bam*HI

AGCTGTT----------*Bam*HI

Insert: promoter fragment; *niiA Bam*HI/*Nco*I

niiA promoter

*Bam*HI----------CATCC

*Bam*HI----------GTAGGG**TAC**

Ligation of cohesive *Nco*I and *Afl*III sites:

niiA promoter *pyr-4* gene

*Bam*HI----------CATCC**ATG**TCGACAA----------*Bam*HI

*Bam*HI----------GTAGGG**TAC**AGCTGTT----------*Bam*HI

C

native: *pyr-4*

CAACAGCCA**ACATGT**CGACA *Afl*III [A/C(A,G)(T,C)GT]

native: *niiA*

CGCCCGCCATCATGCCGTTGC (No restriction site)

pniiA-6: *niiA*

CCCGCCATC**CCATGG**GAATT *Nco*I (C/CATGG)

pDW2

CCCGCCATCCCATGTCGACAA (No restriction site)

Kozak sequence of *N. crassa* where N= A, T,G or C and u=A,C or G:

CNNNCA$_{A/C}$u**AUGGC**

Kozak sequence of *S. cerevisiae*:

$_{A/Y}$**A**$_{A/U}$**AAUGUC**

Kozak sequence mammals where M=A or C:

CAMMAUGGC

pDW2:

CAUCCCAUGTC

Table 15 Summary of Vector Constructs

Vector	Vector base	Promoter[a]	Selectable Marker[b]	Extra Promoters[c]
pGM32	pUC19	P*pyr-4*	*pyr-4*	-
pAN8-1	pUC	P*gpdA*	*ble*	-
pPYRG	pUC19	P*pyrG*	*pyrG*	-
pDW2	pGM32	P*niiA*	*pyr-4*	-
pDW6	pUC12	P*gpdA*	*ble*	-
pDW7	pUC12	P*gpdA*	*ble*	P*pyr-4*
pDW8	pUC12	P*pyr-4*	*ble*	-

[a] The promoter controlling the selection gene.

[b] For *pyr-4* and *pyrG*, selection is for uracil prototrophy. For *ble*, selection is for phleomycin resistance.

[c] Fungal promoters included in the vector but not controlling the expression of any gene.

Fig 11A-C Construction of a Vector Series Based on Phleomycin Resistance

A Phleomycin cassette in pUC: pDW6

The *Eco*RI/*Xba*I 3.2 kb phleomycin cassette from pAN8-1 was cloned directly into the *Eco*RI and *Xba*I sites of pUC12, to form pDW6.

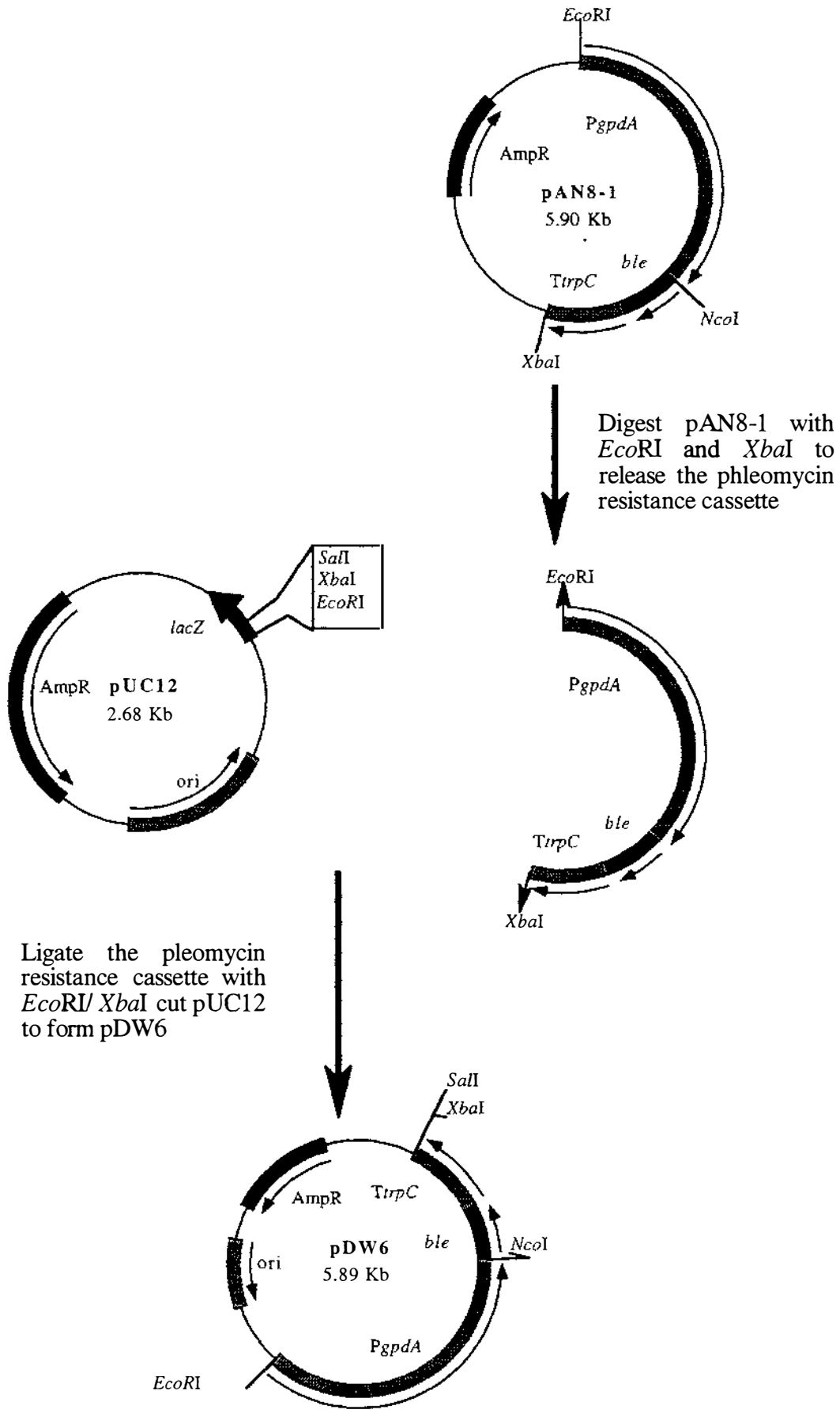
B Phleomycin cassette and *pyr-4* promoter in pUC: pDW7

The *pyr-4* promoter region was isolated as a 600 bp *Sal*I fragment. This was cloned into the *Sal*I site of pUC12 to form pDW5. The *Eco*RI/*Xba*I phleomycin cassette (**A**) was then cloned into the *Eco*RI and *Xba*I sites of pDW5 to form pDW7.

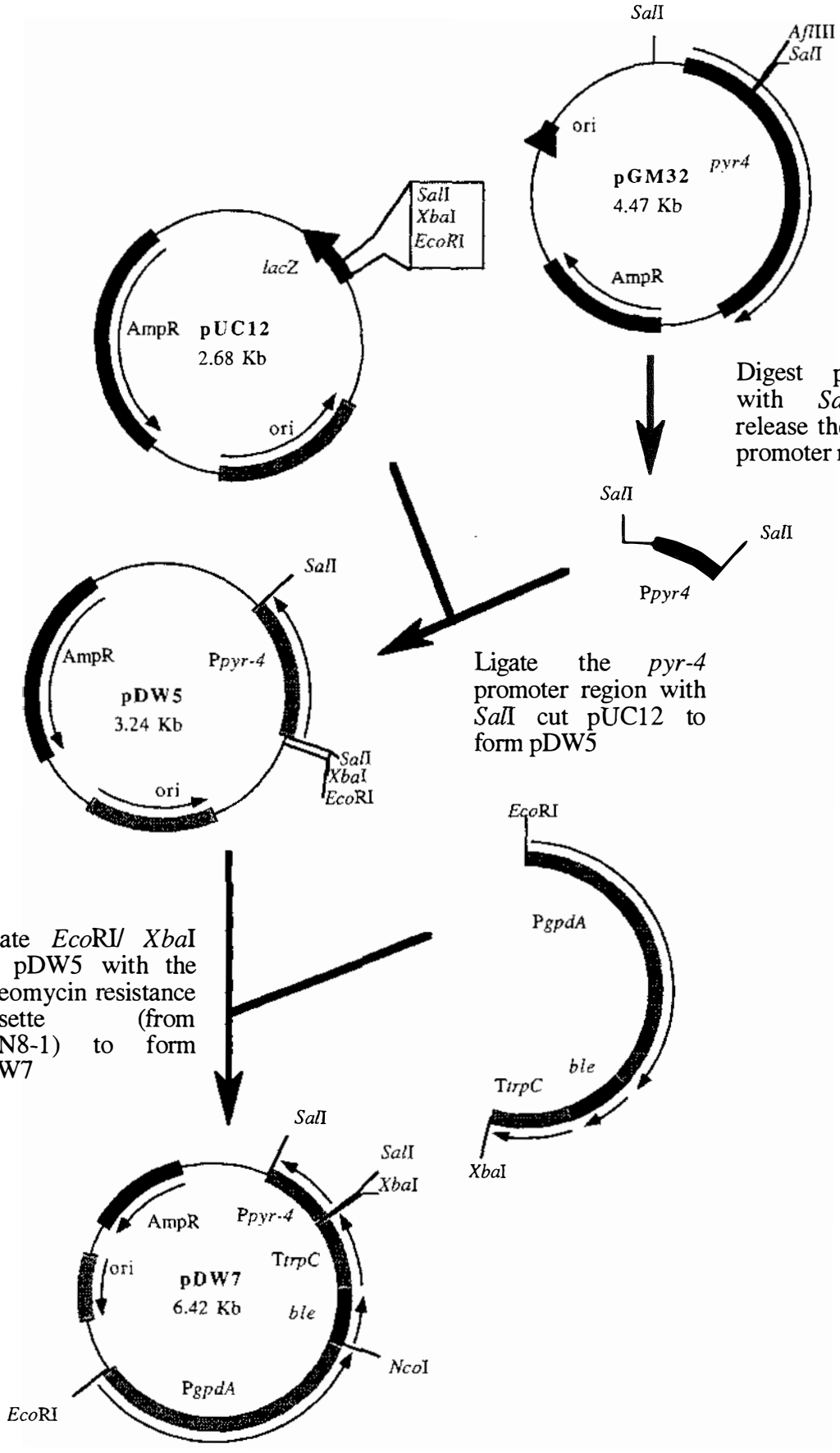
C Replacement of the *gpdA* promoter with the *pyr-4* promoter in the phleomycin cassette, in pUC: pDW8

The *pyr-4* promoter was isolated as a 600 bp *Eco*RI/*Afl*III fragment from pGM32. The *gpdA* promoter was removed from pDW6 by digesting with *Eco*RI and *Nco*I. The *pyr-4* promoter was cloned directly into these sites to form pDW8.

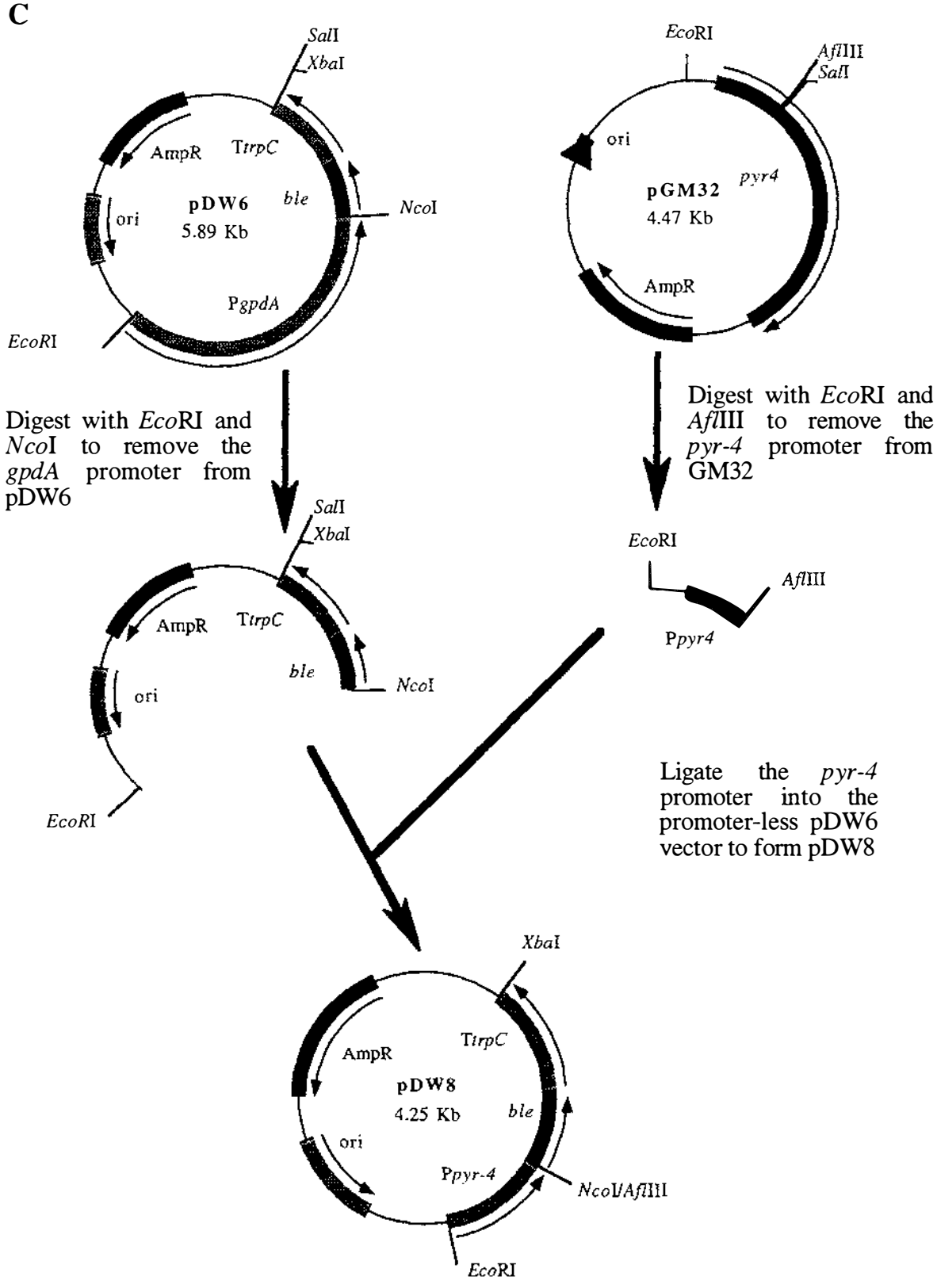
A


EcoRI
PgpdA
AmpR
pAN8-1
5.90 Kb
ble
TtrpC
NcoI
XbaI
Digest pAN8-1 with EcoRI and XbaI to release the phleomycin resistance cassette
SalI
XbaI
EcoRI
lacZ
AmpR pUC12
2.68 Kb
ori
EcoRI
PgpdA
ble
TtrpC
XbaI
Ligate the pleomycin resistance cassette with EcoRI/ XbaI cut pUC12 to form pDW6
SalI
XbaI
AmpR
TtrpC
pDW6
5.89 Kb
ble
NcoI
ori
PgpdA
EcoRI

B


SalI
AflIII
SalI
ori
pGM32
4.47 Kb
pyr4
AmpR
SalI
XbaI
EcoRI
lacZ
AmpR pUC12
2.68 Kb
ori
Digest pGM32 with SalI to release the pyr-4 promoter region
SalI
SalI
Ppyr4
SalI
AmpR
Ppyr-4
pDW5
3.24 Kb
ori
SalI
XbaI
EcoRI
Ligate the pyr-4 promoter region with SalI cut pUC12 to form pDW5
EcoRI
PgpdA
ble
TtrpC
XbaI
Ligate EcoRI/ XbaI cut pDW5 with the phleomycin resistance cassette (from pAN8-1) to form pDW7
SalI
SalI
XbaI
AmpR
Ppyr-4
TtrpC
ori
pDW7
6.42 Kb
ble
NcoI
PgpdA
EcoRI

C


SalI
XbaI
AmpR
TtrpC
pDW6
5.89 Kb
ble
ori
NcoI
PgpdA
EcoRI
EcoRI
AflIII
SalI
ori
pGM32
4.47 Kb
pyr4
AmpR
Digest with EcoRI and AflIII to remove the pyr-4 promoter from GM32
Digest with EcoRI and NcoI to remove the gpdA promoter from pDW6
EcoRI
AflIII
Ppyr4
SalI
XbaI
AmpR
TtrpC
ble
NcoI
ori
EcoRI
Ligate the pyr-4 promoter into the promoter-less pDW6 vector to form pDW8
XbaI
AmpR
TtrpC
pDW8
4.25 Kb
ble
ori
Ppyr-4
NcoI/AflIII
EcoRI

pGM32

pPYRG

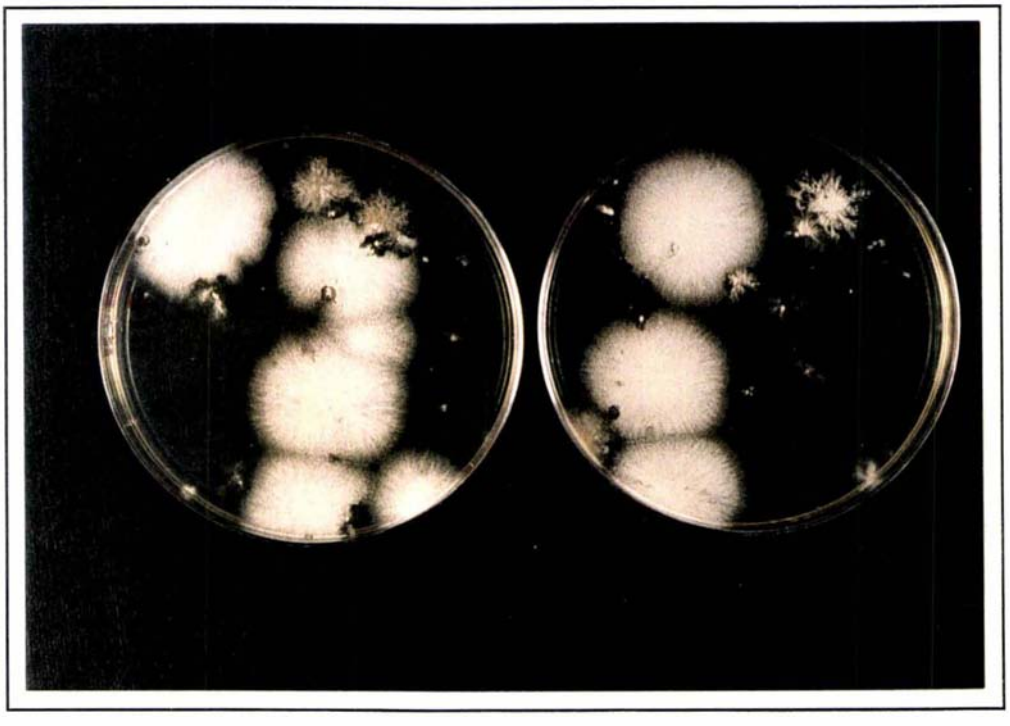


Fig 12 Morphology of pGM32 and pPYRG Transformants of 1-85

Transformation selection plates (osmotically stabilised MYG) for pGM32 (*pyr-4*) and pPYRG (*pyrG*) transformants of *A. nidulans* 1-85.

N-type transformants were randomly selected for further analysis: 10 from pGM32 (GM1-GM10); 6 from pPYRG (PG1-PG6) and 10 from pDW2 (DW21-DW210). The selected N-type transformants were purified by streaking for single spore isolates on selective media and then non-selective media (Section 2.4.2.1). Those transformants able to be purified were analysed for growth characteristics on different media, and integration of the transforming DNA was confirmed by Southern analysis. A selection of transformants were assayed for levels of OMPdecase activity.

4.3.3.1 Purification of Transformants

Single spore isolates were obtained on both selective and non-selective media for pPYRG transformants. In contrast the single spore isolates of a proportion of the pGM32 transformants on selective media required an extra days growth compared to non-selective media, this was due to a much smaller colony size on selective media. Single spore isolates were only obtained on non-selective media for pDW2 transformants. On selective media growth was observed but not from single spores. Nitrate was not required for growth of pDW2 transformants on selective or non-selective media.

Amongst the pPYRG and pGM32 transformants a sub-class of N-type transformants were observed which could not be purified. Streaking these transformants on non-selective media always resulted in a mixture of large (Pyr^+) and small (Pyr^-) colonies. This sub-class represents unstable transformants and have been described in the literature (Upshall *et al.*, 1986; Barnes and MacDonald 1986). They are distinct from the H-type heterokaryons described as when H-type transformant spore suspensions were plated on non-selective media only Pyr^- colonies were observed. This sub-class of unstable transformants were not analysed further.

4.3.3.2 Growth Rates

Spore suspensions were prepared for the parental strain, 1-85; pGM32 purified transformants, N1, N3, N5, N6, N7, N9, N10 (from Section 4.3.1) and GM1-GM10; pPYRG purified transformants PG1, PG2, PG4, PG5 and PG6; pDW2 purified transformants, DW22, DW24, DW25, DW26, DW27, DW28, DW29 and DW210.

Spores were plated onto selective and non-selective plates with and without nitrate. The plates were incubated and the average colony diameter determined at 48, 72 and 96 hrs (Tables 16 and 17). Table 16 is a summary of the minimum and maximum colony diameters observed for each set of transformants.

Table 16 Growth of 1-85 and Transformants of pGM32, pPYRG and pDW2

Strain	Incubation Time at 37°C			
Media[a]	48 hours Diameter (mm)	72 hours Diameter (mm)	96 hours Diameter (mm)	Rate mm/hr[d]
1-85[b]				
MYG	0	0	0	0
MU	3.0±0.4	9.7±0.6	19±0.8	0.28
MYG+N	0	0	0	0
MU+N	3.5±0.4	10±0.8	17±1.2	0.27
Transformants:[c]				
N1, N3, N5, N6, N7, N9 and N10				
	Min-Max	Min-Max	Min-Max	
MYG	1.3-11	12-24	25-37	0.50±0.04
MU	11-14	23-27	34-37	0.49±0.04
MYG+N	3-12	15-27	28-43	0.59±0.05
MU+N	11-15	23-28	35-41	0.53±0.04
GM1, GM2, GM3, GM4, GM5, GM6, GM7, GM8,GM9 and GM10				
	Min-Max	Min-Max	Min-Max	
MYG	1-11	6-24	16-34	0.48±0.05
MU	10-14	19-25	32-36	0.41±0.09
MYG+N	1-11	7-23	20-37	0.51±0.04
MU+N	11-14	24-26	33-41	0.52±0.04
PG1, PG2, PG4, PG5 and PG6				
	Min-Max	Min-Max	Min-Max	
MYG	15-17	27-28	37-42	0.48±0.04
MU	15-16	24-29	38-40	0.44±0.06
MYG+N	16-17	29-30	40-44	0.56±0.02
MU+N	15-16	28-30	39-41	0.55±0.03
DW2, DW4, DW5, DW6, DW7, DW8, DW9 and DW10				
	Min-Max	Min-Max	Min-Max	
MYG	0-0.7	0-6.3	0-17.4	-
MU	5-7	16-18	28-31	0.43±0.04
MYG+N	0-2.4	0-14	0-29	-
MU+N	8.4-10	21-23	34-36	0.54±0.02

[a] Growth on selective media (MYG), non-selective media (MU), selective media plus nitrate (MYG+N) and non-selective media plus nitrate (MU+N).

[b] Average diameter ± one standard deviation

[c] The minimum and maximum average colony diameter for each set of transformants is shown

[d] The rate of growth was calculated over a 24 hr period: 48 hrs to 72 hrs was chosen to avoid the lag phase and because the larger colonies (96 hrs) were difficult to measure accurately due to over crowding of the plates.

Table 17 Growth of Selected Transformants

Strain	Media[a]	Incubation Time at 37°C 48hours Diameter[b]	72hours Diameter[b]	96hours Diameter[b]	Rate mm/hr[c]
N5					
	MYG	5.8±1.4	18±1.2	31±2.0	0.51
	MU	12±0.5	23.6±1.7	34±4.4	0.48
	MYG+N	6.3±0.2	20±0.4	37±0.5	0.57
	MU+N	12±0.4	26±0	41±0.9	0.58
N7					
	MYG	11±1.0	21±1.6	32±1.5	0.44
	MU	14±0.6	25±0.5	34±2	0.42
	MYG+N	9.9±1.2	25±1.5	39±0.9	0.62
	MU+N	13±0.8	25±0.2	41±0.5	0.50
N10					
	MYG	1.3±0.4	13±1.3	25±2.4	0.49
	MU	11±0.4	23±0.5	35±0.5	0.50
	MYG+N	3±0.5	15±1.3	28±0.5	0.50
	MU+N	12±0	23±0.5	>27	0.46
PG2					
	MYG	17±0.5	28±1.5	42±1.6	0.46
	MU	16±0	26±0.5	39±0.9	0.42
	MYG+N	16±0.7	30±0.9	44±1.1	0.58
	MU+N	16±0.5	29±0.5	41±0.8	0.54
PG4					
	MYG	15±1.1	27±1.6	41±1	0.50
	MU	15±0.8	25±1.2	38±0.9	0.42
	MYG+N	17±0.5	30±0.2	40±0.5	0.54
	MU+N	15±0.7	29±0.8	39±0.5	0.58
DW27[d]					
	MYG	*0.7±0.2*	*6.3±2.6*	*17.4±5.2*	*0.23*
	MU	6.6±0.8	17±1.0	30±1.1	0.43
	MYG+N	*2.4±0.8*	*14±1.4*	*29±1.6*	*0.48*
	MU+N	8.4±0.2	21±1.5	35±1	0.53
DW28[e]					
	MYG	*0*	*0*	*0*	*0*
	MU	6.2±0.2	17±0.4	30±0	0.45
	MYG+N	*0*	*0*	*0*	*0*
	MU+N	8.9±0.4	22±0.7	36±0.8	0.54

[a] Growth on selective media (MYG), non-selective media (MU), selective media plus nitrate (MYG+N) and non-selective media plus nitrate (MU+N).

[b] Average diameter ± one standard deviation.

[c] The rate of growth was calculated over a 24 hr period: 48 hrs to 72 hrs was chosen to avoid the lag phase and because the larger colonies (96 hrs) were difficult to measure accurately due to over crowding of the plates.

[d] There were less colonies on MYG (±N) media than on MU (±N) media. The colonies on selective media do not represent the entire population of spores. These figures are italicised.

[e] There was no obvious growth from single spores on selective media.

As expected no growth was observed on media without uracil for the uracil requiring parental strain 1-85. The size of the colonies after 48 and 72 hours incubation were different for the sets of transformants. Transformants obtained using the vector pPYRG (PG1-6), containing the homologous *pyrG* gene, had colonies of similar sizes on all media. In contrast the transformants obtained from pGM32 (N1-10 and GM1-10) varied considerably in size, with colonies being much smaller on selective than non-selective media. Growth on non-selective media was less variable than on selective media.

The pDW2 transformants (DW2-10) had unusual growth characteristics. Although purified by single spore isolation on non-selective media, most were unable to grow from single spores on selective media. The colonies on non-selective media were slightly larger than those observed for the parental 1-85 spores, but much smaller than for pPYRG and pGM32 transformants on the same media after the same incubation period.

On media containing nitrate the colonies of pGM32 and pPYRG transformants and 1-85 were slightly larger. The pDW2 transformants were, however, considerably larger on nitrate than on nitrate free media.

These trends were also observed for the individual transformants which were selected for further analysis (Table 17).

4.3.3.3 Southern Analysis

Southern analysis of a representative range of transformants confirmed integration of the vector DNA (data not shown). The transformant PG2 contained a gene replacement of the mutant *pyrG* gene with the functional *pyrG* gene and was therefore selected as a positive control. Transformant PG4 had a complicated integration pattern consistent with integration of pPYRG vector at an ectopic site, although not as a tandem array. The signal intensity indicated PG4 had approximately 5 copies integrated. The pDW2 transformants all had very complicated integration patterns. The transformant DW28 appeared to have multiple copies at multiple sites while DW27 appeared to have multiple copies in a tandem array at an ectopic site (pDW27 contains more copies than pDW28).

4.3.3.4 OMPdecase Activity

The transformants N5, N7, N10, PG2, PG4, DW27 and DW28 were assayed for OMPdecase activity. Crude protein extracts were prepared from liquid cultures at mid log phase (Table 18; Sections 2.22.1 and 2.22.2).

Table 18 Incubation Time to Reach the Middle of the Log Growth Phase

Strain	Liquid media[a]			
	MYG	**MU**	**MYG+N**	**MU+N**
1-85		17 hrs		17 hrs
N5	17 hrs	13 hrs	17 hrs	
N7	13 hrs			
N10	13 hrs			
PG2	13 hrs	13 hrs	13 hrs	
PG4	13 hrs			
DW27	18 hrs		18 hrs	
DW28	18 hrs	17 hrs	18 hrs	

[a] Flasks containing liquid media [selective media (MYG), non-selective media (MU), selective media plus nitrate (MYG+N) and non-selective media plus nitrate (MU+N)] were inoculated and incubated for the time indicated (Section 2.22.1).

The crude protein extracts were assayed for OMPdecase activity (Table 19, Section 2.22.4). The enzyme activities of transformants PG2 (homologous *pyrG* gene replacement) and N5 (single integrated copy of the pGM32 vector) were higher when growth was in media without uracil, compared to media with uracil. The OMPdecase activity of transformant N5 was 3-fold higher than PG2 while the size of the PG2 colonies on selective media (without uracil) was 3-fold greater than N5 colonies at the same time. On non-selective media (with uracil) N5, with the lower enzyme activity, had larger colonies, although they were smaller than PG2 colonies on selective or non-selective media. This indicates the *pyr-4* gene is functional, although there is still a uracil requirement, despite the higher activity.

The PG4 multicopy transformant had a 2-3 fold higher activity when compared to the PG2 transformant grown in the same media. The activity of N7 in MYG was slightly higher than that of N5 in MYG media while the activity of N10 was approximately 2-fold lower. The activities correlate with the colony size but not the number of integrated copies as N10 has more copies than N7 which has more copies than N5.

The enzyme activity for the pDW2 transformants (DW27 and DW28) was very high, compared to pPYRG and pGM32 transformants, with an increase observed in media containing uracil or nitrate. Higher enzyme activity was observed in transformant DW27 compared to DW28 (contains fewer vector copies integrated).

4.3.4 Transformation of 1-85 with Phleomycin Resistant Vectors pAN8-1, pDW6, pDW7 and pDW8

1-85 was transformed with vectors containing the *ble* gene conferring resistance to phleomycin (Table 15). Transformants were obtained for all vectors: pAN8-1, 2680/2 x 10^6 protoplasts/μg DNA; pDW6, 457/2 x 10^6 protoplasts/μg DNA; pDW7, 220/2 x 10^6 protoplasts/μg DNA; pDW8, 243/2 x 10^6 protoplasts/μg DNA. The highest transformation frequency was obtained for the caesium chloride purified vector, pAN8-1, compared to the PEG purified pDW vector series. The transformants for each vector were similar in their morphologies (Fig. 13). No vector produced transformants with a morphology similar to the H-type transformants from pGM32 and pPYRG. Successful transformation with pDW8 indicates the *ble* gene is being expressed from the *pyr-4* promoter.

Table 19 Orotidine-5'-monophosphate Decarboxylase Activity of 1-85 and Selected Transformants

Strain	Growth media[a]	Activity[b] units/mg	Relative to N5[c] (MYG)	Activity[d] units/mg	Relative to N5[c] (MYG)	Average Relative to N5	Diameter 48 hrs[e] (mm)
1-85	MU	ND		ND			3.0
1-85	MU+N	ND		ND			3.4
N5	MYG	0.0097±0.0010	(1.00)	0.0158±0.0006	(1.00)	1.00	5.8
N5	MU	0.0058±0.0007	(0.60)	0.0071±0.0020	(0.45)	0.53	12
N5	MYG+N	0.0115±0.0016	(1.19)	0.0153±0.0018	(0.97)	1.08	6.3
N7	MYG	0.0117±0.0011	(1.21)	0.0158±0.0011	(1.00)	1.11	11
N10	MYG	0.0052±0.0008	(0.54)	0.0091±0.0005	(0.58)	0.56	1.3
PG2	MYG	0.0034±0.0004	(0.35)	0.0085±0.0006	(0.54)	0.45	17
PG2	MU	0.0023±0.0012	(0.25)	0.0051±0.0000	(0.32)	0.29	16
PG2	MYG+N	0.0024±0.0005	(0.25)	0.0069±0.0004	(0.44)	0.35	16
PG4	MYG	0.0118±0.0013	(1.22)	0.0151±0.0000	(0.96)	1.09	15
DW27	MYG	0.2789±0.0328	(28.8)	0.3515±0.0191	(22.2)	25.4	0.7
DW27	M+N	1.1295±0.1635	(116)	1.3735±0.1294	(86.9)	102	2.4
DW28	MYG	0.1703±0.0171	(17.6)	0.2530±0.0184	(16.0)	16.8	0
DW28	MU	0.4132±0.0318	(42.6)	0.563[f]	(35.6)	39.1	6.2
DW28	MYG+N	0.8037±0.0847	(82.9)	0.877[f]	(55.5)	69.2	0

[a] Liquid Media: selective media (MYG), non-selective media (MU), selective media plus nitrate (MYG+N) and non-selective media plus nitrate (MU+N).

[b] Activity measured three times from a fresh crude protein preparation: average ± one standard deviation.

[c] Activity comparisons were relative to N5 (MYG) rather than PG2 (MYG). This was because the activity of PG2 was very low, almost too low to detect. The PG2 activity measurements were therefore less accurate than for the more active N5 (MYG).

[d] Repeat protein concentration and activity measurements of frozen aliquots of the protein preparation in [b].

[e] Average colony diameter from a single spore after 48 hrs growth on the media indicated.

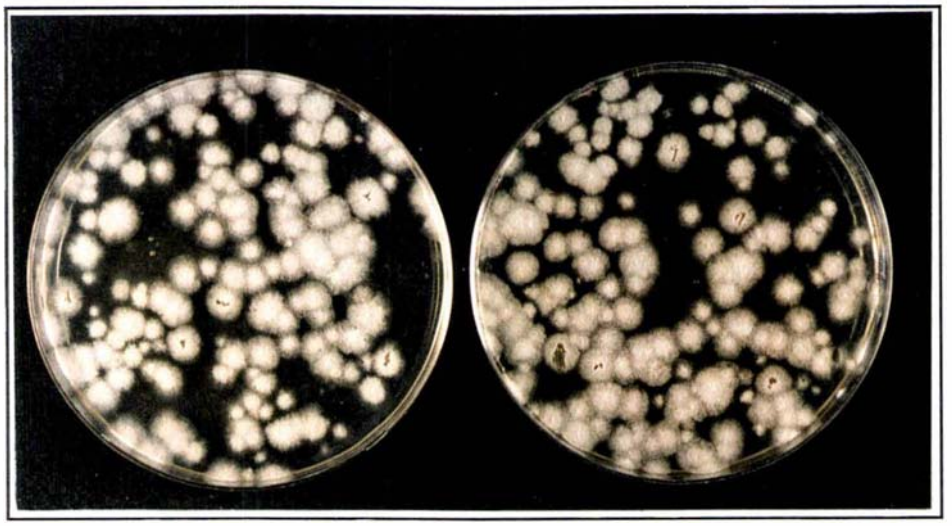
[f] Single enzyme assay

Fig 13A-E Phleomycin Resistant Transformants

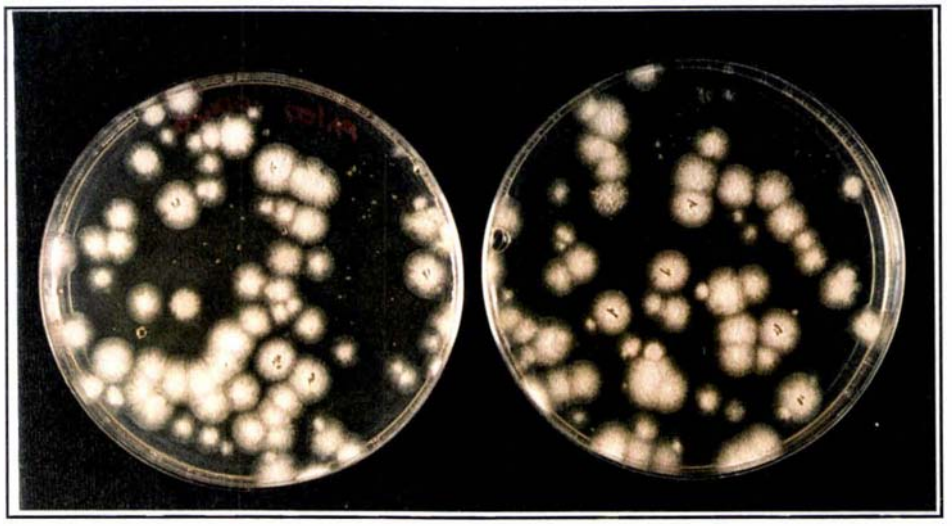
Transformant selection plates of 10 μg/ml and 25 μg/ml phleomycin incubated for 2 or 3 days respectively: **A** Transformed with pAN8-1; **B** Transformed with pDW6; **C** Transformed with pDW7; **D** Transformed with pDW8; **E** No DNA control.

10ug/ml 25ug/ml

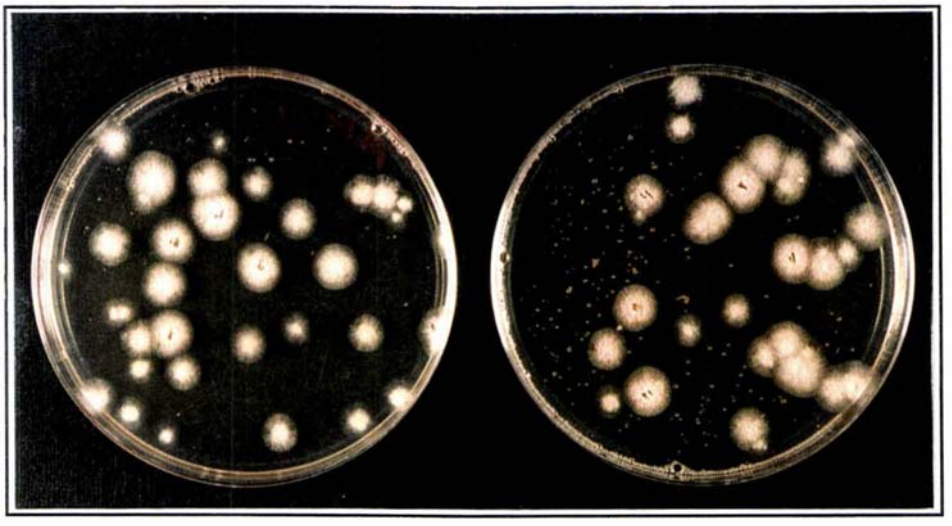
A



B

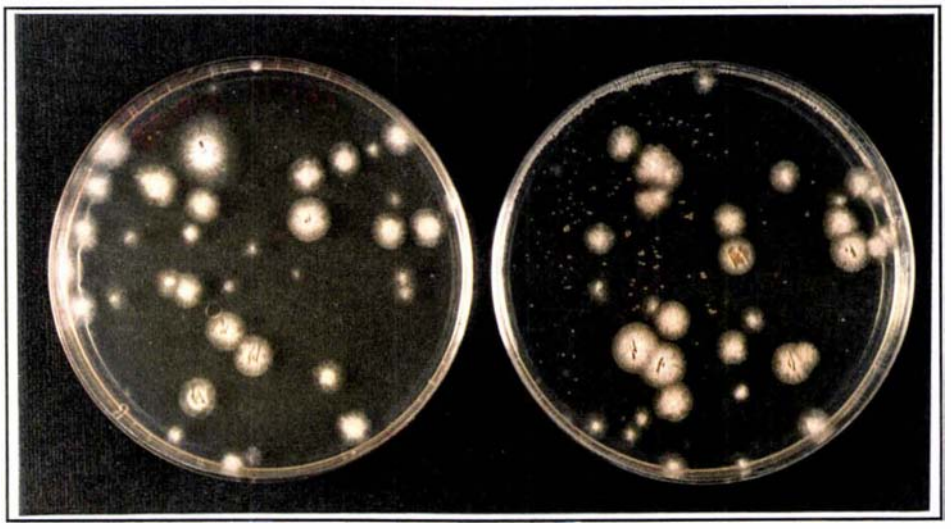


C

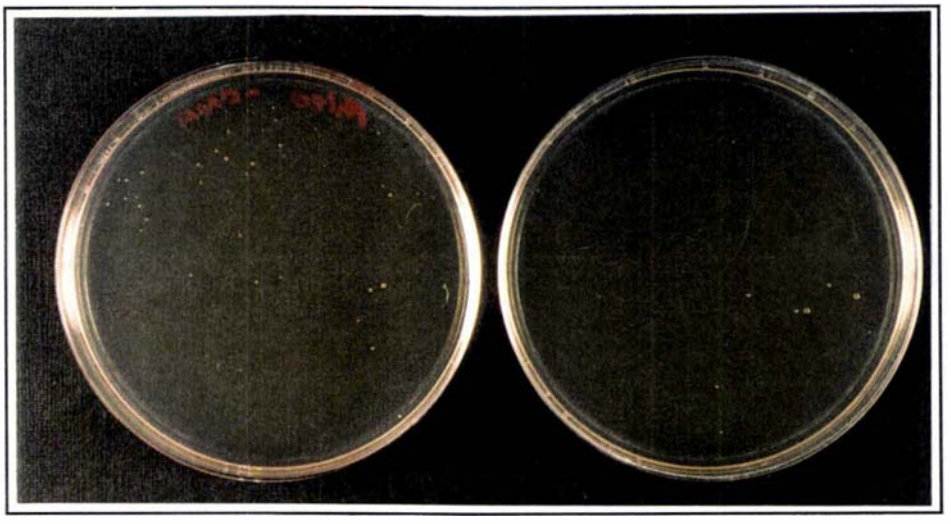


10ug/ml 25ug/ml

D



E



4.4 Discussion

Transformation frequencies for *A. nidulans* 1-85 mycelial protoplasts with the pGM32 vector ranged from 200 to 520/2 x 10^6 protoplasts/μg DNA. Of these transformants approximately 13% were phenotypically similar to, although showing more vigorous growth than, the parental strain (1-85). The vigorous growth was due to the correction of the *pyrG* mutation in the parental strain as this mutation results in relatively weak growth, even in the presence of uracil or uridine. These transformants, designated N-type transformants, were able to be single spore purified and were mitotically stable. The remainder of the transformants, designated H-type transformants, since they resembled heterokaryons in their morphology, were irregular in shape and could not be single spore purified. These were distinct from abortive transformants as, unlike abortive transformants, they were able to be maintained on selective media.

The presence of heterokaryons among transformants has been reported (Upshall 1986). Provided the heterokaryon state is not required for survival, such as when an essential gene is disrupted, it is generally possible to isolate pure homokaryons from heterokaryotic transformants. The presence of heterokaryons among transformants is expected due to the nature of the transformation procedure used. Polyethylene glycol (PEG), which is a central component of the transformation procedure, causes protoplasts to fuse into multicellular aggregates. The use of protoplasts derived from mycelium exacerbates this since many of these are already multinucleate. The possibility that the observed H-types are due to vector integration into an essential gene can not be entirely ruled out, however this seems unlikely as up to 95% of the transformants can be classified as H-types. This would suggest the heterologous pGM32 vector disrupts essential genes with a 95% hit rate. The *N. crassa pyr-4* gene has a low level of homology with *pyrG* and is reported to integrate randomly into the *A. nidulans* genome (Ballance and Turner, 1985).

The presence of mitotically stable and unstable transformants has been reported by other groups working with the OMPdecase transformation system (Ballance and Turner 1985; Cullen *et al.*, 1987; Oakley *et al.*, 1987). The unstable transformants are generally only commented upon while those able to be purified, and shown to be mitotically stable, are analysed further. In this study 10 transformants of each of the two morphologies observed were randomly selected and analysed. The results are summarised and discussed in point form:

1. N-Type Transformants

(i) pGM32

N-type transformants of pGM32 (heterologous *pyr-4* selectable marker) generally contained between 1 and 10 copies of the vector integrated. Although generally able to be single spore purified to form stable homokaryons, a sub-class of N-type transformants were observed which were unable to be purified and had instability consistent with disruption of essential genes.

Growth of the purified stable transformants was better on non-selective than selective media suggesting *pyr-4* does not completely complement the *pyrG* mutation. The size of the colonies of individual transformants on selective media was variable, as was the activity of OMPdecase. The colony size showed a positive correlation with OMPdecase activity but not the number of copies of the vector integrated. However, if the copy number was relatively low a positive correlation of vector copies, with activity and size, was observed. At a higher vector copy number the OMPdecase activity was very low and the growth very poor.

An increase in the OMPdecase activity was observed for growth in selective media compared to non-selective media. This is in agreement with the literature which suggests that OMPdecase activity is induced by uracil starvation (Radford *et al.*, 1985; Losson *et al.*, 1985).

(ii) pPYRG

N-type transformants of pPYRG (homologous *pyrG* selectable marker) were also generally able to be single spore purified to form stable homokaryons, although the sub-class of N-type transformants unable to be purified were also observed.

The stable pPYRG transformants showed much less variation in growth on selective and non-selective media, between transformants and between media, compared to pGM32 N-type transformants. Therefore *pyrG* effectively complements the *pyrG* mutation, as expected. The activity of OMPdecase was less than for the N-type pGM32 transformants so the more effective complementation was not due to increased enzyme activity. A positive correlation between the copy number and level of OMPdecase activity was observed for the two transformants tested. Again higher OMPdecase activity was observed for growth in selective media compared to growth in non-selective media, indicating regulation by uracil.

(iii) pDW2

N-type transformants of pDW2 (heterologous *pyr-4* selectable marker controlled by the homologous and inducible *niiA* promoter) were able to be single spore purified on non-selective media only. Once purified they could be grown on selective media but not from single spores. The OMPdecase activity of the two transformants tested correlated positively with copy number. The OMPdecase activity was very high which suggests the cells can tolerate very high levels of activity. Growth on the inducing agent nitrate resulted in an increase in activity and slightly better growth. The very slow growth suggests the enzyme, although functional, may not be readily available for use by the cells. An increase in OMPdecase activity was observed in the presence of uracil, indicating induction of the *niiA* promoter by uracil as well as nitrate.

2. H-Type Transformants

(i) pGM32

H-type transformants of pGM32 (*pyr-4* selectable marker) consisted of untransformed and transformed nuclei. Transformants contained between 1 and 10 copies of the vector integrated per nucleus (transformed plus untransformed). As the transformed nuclei formed less than one third of the total nuclei the copy number per transformed nucleus was higher (7-29). The transformed phenotype was rapidly lost if transformants were grown on non-selective media but was maintained by growth on selective media. The transformed and untransformed nuclei were distinguished by the germination of uninucleate spores on selective media. Spores containing untransformed nuclei would swell while spores containing transformed nuclei would germinate. The germinating spores would not continue to grow. The heterokaryon environment appeared to be essential for the maintenance of the transformed nuclei.

(ii) pPYRG

H-type transformants of pPYRG (*pyrG* selectable marker) were observed. The proportion of transformants classified as H-types was lower than for pGM32. This may be due to the presence of homologous DNA in the transforming vector as homologous integration appears to result in a lower number of copies integrated. The average copy number integrated may therefore be lower for pPYRG transformants compared to pGM32 transformants.

(iii) pDW2

H-type transformants of pDW2 (*pyr-4* selectable marker controlled by the inducible *niiA*

promoter) were not observed. This suggests the formation of H-type transformants may require the presence of the promoter for the OMPdecase gene.

3. Phleomycin Resistant Transformants

Transformants obtained from vectors pDW6 (phleomycin resistance cassette), pDW7 (phleomycin resistance cassette plus the *pyr-4* promoter), pDW8 (phleomycin resistance controlled by the *pyr-4* promoter) appeared to be of one morphology. This suggests the presence of *pyr-4* promoter alone is not responsible for the formation of the H-type transformants observed for vectors pGM32 and pPYRG.

* * * * *

The H- and N-type transformants appear to differ in the average number of vector copies integrated per transformed nucleus. This is supported by the observation that vector conformation, and the amount of DNA in the transformation mix, affect the ratio of the two types of transformants. Circularisation of the vector results in an increase in the number of H-type transformants, or transformants with a higher copy number. It is speculated that integration of multiple tandem repeats results from homologous recombination of the transforming vector before integration (Plessis and Dujon 1993). The use of a circular vector would facilitate the formation of concatamers, by homologous recombination. These concatamers would integrate, resulting in a higher vector copy number in transformants. Hence the increase in the proportion of H-type transformants with a circular vector is consistent with this proposal. It is also worth noting that even within my small sample of N-type transformants, those derived using a circular vector (N6-N10) appear to have slightly higher copy numbers than those derived using a linear vector (N1-N5).

It is possible that increasing the amount of DNA in the transformation mix would result in competent protoplasts taking up more DNA molecules (Wernars et al., 1985), possibly leading to an increase in the number of integrated copies. This was observed by Pleissis and Dujon (1993) and Walz and Kuck (1993). It was therefore predicted that an increase in the amount of DNA would result in an increase in the proportion of heterokaryons. This prediction was borne out as transformation with increasing amounts of DNA resulted in an increase in the proportion of H-type transformants, while the number of transformants per viable protoplast remained constant.

The integration of many copies of the vector DNA has been reported. For example, up to 100 integrated copies of the *amdS* gene have been recorded and the *amdS* gene was shown to have

a titrating effect on trans-acting regulatory proteins (Kelly and Hynes 1987). It appears, from my studies, that the integration of a relatively high number of copies of the OMPdecase gene in a tandem repeat prevents the transformant existing as a Pyr^+ homokaryon. At no stage were the H-type transformants observed to stabilise in any way. Transformants containing large changes to the genome are relatively stable (Xuei and Skatrud 1994) and loss of tandemly repeated sequences by mitotic recombination between repeated sequences occurs at a low frequency. This suggests the observed instability of H-type transformants is not due to the physical insertion of large fragments of vector DNA in tandem repeat form into the genome.

The reason why multiple copies of the *pyr-4* gene cannot be maintained in a homokaryon is unclear. It appears that both the OMPdecase gene and promoter are required for the formation of H-type transformants, as shown by transformation with the vectors pDW2 (*pyr-4* selectable marker controlled by the inducible *niiA* promoter) and pDW8 (phleomycin resistance controlled by the *pyr-4* promoter). The gene or promoter alone are not enough to cause the formation of H-types.

There have been reports that expression is more or less proportional to the number of integrated copies (Goosen *et al.*, 1992) and also that the position of integration is very important for developmentally specific expression (Miller *et al.*, 1987). Due to the often complicated integration patterns observed on Southern blots it can be difficult to determine the number of functional copies of the gene integrated. Integration of the vector in head to tail tandem repeats assures integration of complete copies of the gene, although the expression of these copies may still be position dependent.

In this study a positive correlation between the number of copies of the vector, and the activity of the enzyme, was observed. Only for transformant N10, which had a relatively high number of copies of pGM32 integrated, was the positive correlation not observed. The OMPdecase gene is involved in the regulation of the pyrimidine biosynthetic pathway (Radford *et al.*, 1985; Losson *et al.*, 1985) and, although induced during uracil starvation, has a basal level of expression.

Given the results discussed it is proposed that H-type transformants are formed due to promoter specific titration and inactivation of trans-acting factors. For pGM32 it is proposed that, at lower copy numbers (1-5), an increase in the copy number results in an increase in OMPdecase activity due to the binding of positive trans-acting factors. Consequently, at higher copy number (5-10), there is a build up of an effector molecule, due to the high OMPdecase activity, which binds to the positive trans-acting factor causing its inactivation. The OMPdecase activity therefore no longer increases and, when the copy number is very high, decreases. At

an even higher copy number (>10) the positive trans-acting factors may become limiting and, if required for the expression of essential genes, the transformant will be unable to survive as a homokaryon. The high copy number transformant may survive as a heterokaryon due to the expression of the essential genes in the untransformed nuclei. This model is one hypothesis to explain the available data and is summarised in Fig. 14.

As the H-type Pyr^+ spores were unable to grow on selective or non-selective media, the proposed trans-acting factors may be independent of the regulation of OMPdecase by uracil. Promoter analysis of *S. cerevisiae URA3* suggests the promoter regions responsible for the basal level of expression, and that induced by uracil starvation, are separate (Roy *et al.*, 1990).

The titration of trans-acting factors by the integration of multiple copies of vector DNA has been reported. Kelly and Hynes (1987) report that the expression of more than 100 copies of the *amdS* gene (in tandem head to tail repeats) limits the availability of the *amdR* regulatory gene product. The transformants with multiple copies of *amdS* therefore had reduced growth on certain substrates. Similarly Davies (1991) found an increase in the copy number of interferonα2, under control of the *alcA* promoter, resulted in an approximately linear increase in yield up to 7 to 10 copies, but not thereafter. The level of intrinsic *alcA* and *ADH1* was also observed to be reduced. It was proposed that multiple copies of the *alcA* promoter titrated out the *alcR* product. This was also observed by Verdoes *et al.* (1994) for the *glaA* promoter in *A. nidulans*. Similarly in *S. cerevisiae* the introduction of a high copy number plasmid containing the 5' end of the *SPS2* gene (sporulation specific gene), led to a reduction in the efficiency of spore formation (Percival-Smith and Segall 1987). It was speculated that the accumulation of a protein containing the amino-terminal portion of the SPS2 gene product prevented ascus formation.

As with the titration of trans-acting factors there are examples of high level gene expression being toxic in fungal systems (Le Dall *et al.*, 1994; Osmani *et al.*, 1988). This seems unlikely for OMPdecase as high levels were observed in pDW2 transformants and, although the mutation was not effectively complemented, this did not appear to be toxic. The use of OMPdecase as a fusion protein has also been reported and the resulting 30-fold increase in OMPdecase activity was not observed to be detrimental (Myers *et al.*, 1995).

The lack of relatively high levels of multi copy integration of heterologous OMPdecase genes has been reported in the literature (Smit and Tudzynski 1992, Le Dall *et al.*, 1994). In support of a heterokaryon state being required for maintenance of multiple copies of the gene for OMPdecase was work done by Horiuchi *et al.* (1995). When the heterologous *pyr-4* of the

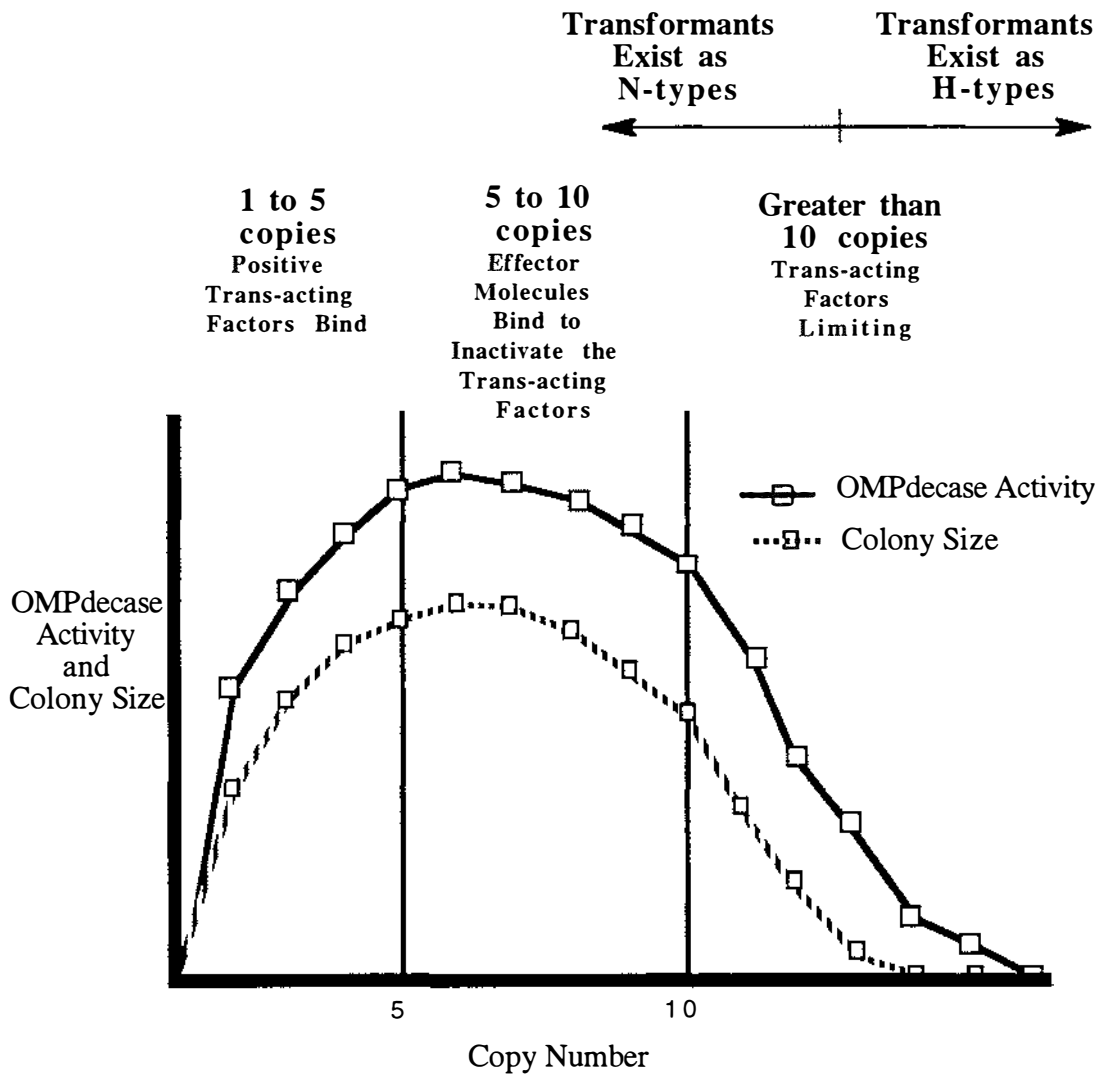


Fig 14 Model: Effect of Copy Number on OMPdecase Activity and Colony Size

Model: An increase in the number of integrated copies is proportional to an increase in OMPdecase activity and colony size, when the copy number is relatively low. This is due to the binding of positive trans-acting factors. As the OMPdecase activity increases an effector molecule is formed which binds to inactivate the trans-acting factor, eventually resulting in a decrease in OMPdecase activity. If the trans-acting factors are required for the expression of essential genes then the transformants are unable to exist as homokaryons (N-types) when the copy number is very high. These transformants may only exist as heterokaryons (H-types).

filamentous fungus *Rhizopus niveus* was used to transform a *pyr*⁻ *R. delemar* strain the vector was observed to integrate in multiple tandem arrays. All transformants were heterokaryons, due to the nature of transformation of this fungus, so required purification. They were unable to obtain homokaryons and suggested that growth of homokaryons containing multiple copies of *pyr-4* may be impaired.

4.5 Conclusion

As large numbers of transformants were required for the gene targeting experiments the aim of this work was to analyse the transformant types and identify ways of purifying them so that all transformants could be included in the analysis. The N-type transformants were generally able to be purified, although a sub-class of unstable transformants was identified. The H-type transformants were unable to be purified which appeared to be a feature of the selection system. It was decided that the H-type transformants, and the unstable class of N-type transformants, would not be considered in the populations analysed for gene targeting events (Chapter 5).

In the light of these results, where integration of multiple copies of a non-selectable gene is required, it may be advisable to use an alternative selection system to OMPdecase. Similarly, screening for the disruption of an essential gene by selection of heterokaryotic transformants, that are unable to be resolved into homokaryons, could be complicated using the OMPdecase selection system. There would be a background level of non-resolvable heterokaryons, due to a high number of copies of the OMPdecase gene, which may not be distinguishable from the non-resolvable heterokaryons due to disruption events. Consequently this work has implications for those using the OMPdecase gene as a selectable marker.

Chapter 5

Optimisation of Gene Targeting in Filamentous Fungi using *Aspergillus nidulans* as a Model Organism

5.1 Summary

The effect of altering the conditions of transformation on the efficiency of gene targeting in filamentous fungi was studied using *Aspergillus nidulans* as a model organism. The *A. nidulans niaD* and *amdS* genes, both involved in nitrogen source utilisation, were selected as target loci. Homologous recombination of transforming DNA at these loci resulted in *niaD* and *amdS* mutants with an impaired ability to utilise nitrate or acetamide as the sole nitrogen source respectively. Vectors were constructed which contained the *Neurospora crassa pyr-4* gene as a selectable marker and an internal segment of the *amdS* (0.6 kb to 1.27 kb) or *niaD* (0.9 kb to 2.15 kb) genes. The parameters investigated for their effect on gene targeting included: (a) length of homologous DNA in the disruption cassette, (b) conformation of the transforming vector (circular or linear), (c) concentration of DNA in the transformation mix, (d) transcriptional status (on/off) of the targeted gene and (e) temperature of incubation of the transformation reaction and protoplast regeneration on selective media. Parameters shown to have an effect on the targeting frequency at the *niaD* locus were subsequently also tested at the *amdS* locus. The level of gene targeting using circular DNA was found to correlate with the size of the homologous segment at both loci. Similarly the level of targeting was shown to increase at both loci when vectors were linearised within the region of homology. Unexpectedly the level of targeting was unaltered at the *niaD* locus when transcription was induced at different stages in the transformation procedure. Likewise targeting was unaffected by altering the amount of DNA in the reaction mix over the concentration range tested. The regeneration temperature, however, did appear to have an effect on targeting, with enhanced targeting observed at 25°C compared to 37°C. A dramatic difference in targeting was observed between the *niaD* and *amdS* loci with targeting of *niaD* being more efficient than *amdS* under all the conditions tested.

5.2 Introduction

The transforming DNA supplied to a recipient fungus may or may not have an origin of replication. If it does then the DNA can be stably maintained, independent of integration, as autonomously replicating DNA (refer to Chapter 4). If not then the maintenance of the DNA depends on its integration into the genome of the recipient.

5.2.1 DNA Integration

Transformation with DNA lacking an autonomous replicating sequence results in integration of the transforming DNA. Pioneering work of Hinnen *et al.* (1978) demonstrated the transformation of a *leu2* mutant strain of *S. cerevisiae* with a plasmid containing the *LEU2*$^+$ gene but lacking a *S. cerevisiae* ARS. By restriction analysis of transformant DNA, Hinnen *et al.* distinguished three types of integration events. Type 1 integration events, or homologous additive integration, contain the introduced sequence at the resident site along with the plasmid sequence. This type of integration has been attributed to homologous crossing over (Fig. 15A). Type 2 integration events, or ectopic integration, contain the introduced DNA integrated at a random site in the genome. This type of integration has been attributed to a single cross-over event at a non-homologous site (Fig. 15B). Type 3 integration events, or homologous replacement integration, contain the introduced sequence which has completely replaced the homologous resident sequence, with no additional plasmid sequence. This type of integration has been attributed to a double cross-over event, or gene conversion that may or may not involve crossing over (Fig. 15C).

Later it was shown that virtually all integration of DNA into *S. cerevisiae* occurs at homologous sites. The type 2, ectopic transformants, obtained with the plasmid containing *LEU2* were due to the presence in the transforming vector of two sites that are present in multiple copies in the *S. cerevisiae* genome: the 0.25 kb terminal repeat of the *S. cerevisiae* Ty1 transposon (δ), which is present in about 100 dispersed copies, and the leucine tRNA gene, which is present in several copies (Fincham 1989). It was reported early on that even small amounts of sequence divergence can have a statistically significant effect on the frequency of integration (Smolik-Utlaut and Petes 1983). More recently it has been demonstrated that *S. cerevisiae* possesses a recombination system which is sensitive to very small amounts of homology (Schiestl and Petes 1991). Transformation of *S. cerevisiae* with *Bam*H1 fragments in the presence of the *Bam*H1 restriction enzyme resulted in the majority of the fragments integrating into *Bam*H1 sites (GGATCC). These events have been called restriction-enzyme-mediated integration (REMI) events and have been used to target a gene in a *Dictyostelium* sp. (Kuspa and Loomis 1992). If the *Bam*H1 enzyme was not included, the *Bam*HI fragments were inserted into GATC sites in all three of the transformants analysed. Schiestl *et al.* (1993) studied this in more detail by transforming *S. cerevisiae* with DNA that shares no homology with the genome, and analysing 44 independent transformants. Three classes of transformants were observed. The most common class appeared to have required pairing of the target sequence with the terminal few base pairs of the transforming DNA fragment. In the second class no such homology was detected, and the transforming DNA was integrated next to a CTT or GTT in the target. It was suggested that these integration events are mediated by

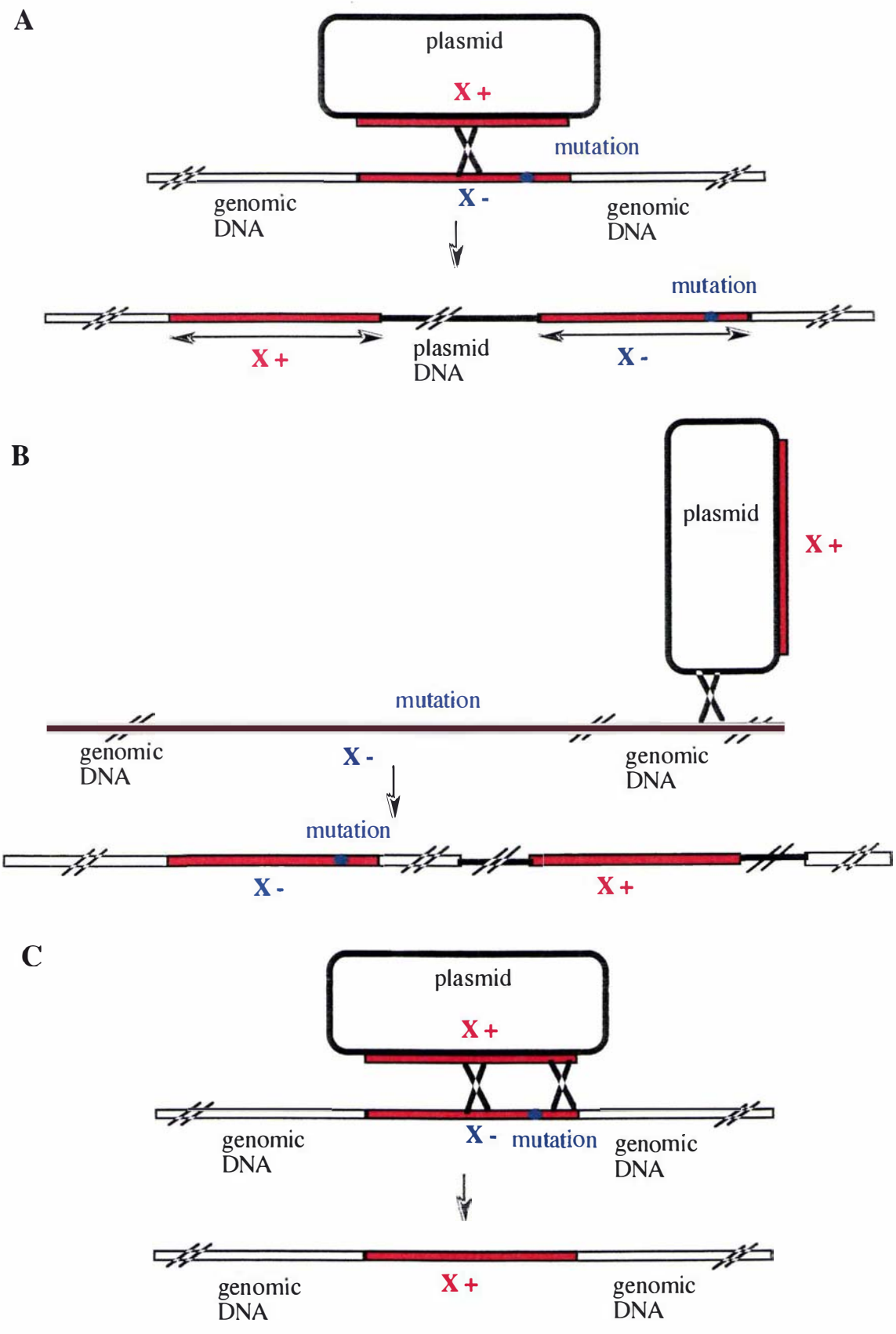


Fig 15 Integration Events

Diagram of integration events as described by Hinnen *et al.* (1978) **A** Type I. **B** Type II. **C** Type III.

topoisomerase I as these are preferred sites of cleavage for mammalian topoisomerase I. The final class showed ligation to mitochondrial DNA which may contain rearrangements. It is suggested this involved the *in vivo* ligation of transforming DNA with nucleus-localized linear fragments of mitochondrial DNA.

When the goal is to target a specific gene in the yeast genome two kinds of integrative vectors can be constructed. These depend on the type of event required and are termed insertion or replacement vectors. Insertion vectors are designed to carry the exogenous DNA adjacent to a yeast sequence that provides the homology for insertion at a specific chromosomal locus. Transformation with the vector linearised at a unique site within the region of homology leads to targeted integration of the entire plasmid (Orr-Weaver *et al.*, 1981; 1983). Replacement vectors contain exogenous DNA cloned within the yeast sequence to give a region of homology to the chromosomal locus at both ends. Homologous pairing with both sides of the chromosomal target sequence results in the replacement of the chromosomal gene with the exogenous sequence. The latter approach leads to a genetically more stable integration product since no direct repeats are left flanking the insertion site.

5.2.1.1 Mechanisms of Recombination and Integration

Details of the mechanisms by which integration and homologous recombination occur are still being deciphered. Many models have been proposed to explain the data available and they remain controversial. The following is a summary of research on recombination in fungi, and the development of possible models to explain the data. This has been well reviewed by Orr-Weaver and Szostak (1985) from which the diagrams were adapted.

Research into meiotic recombination was initially performed by tetrad analysis of the yeast *S. cerevisiae*. This research was later simplified by using filamentous fungi, such as *Ascobolus* and *Sordaria* species, as large numbers of meiotic events could be scored by the visual analysis of 8 spored asci. A diploid cell heterozygous for marker A would be expected to produce 4 'A' spores and 4 'a' spores (Mendelian segregation). When analysed there were found to be various classes of aberrant segregation patterns. A simplified version of the 3 classes is outlined in Figure 16. A segregation pattern of 6+:2- or 2+:6- demonstrates a gene conversion event where information on one chromosome is lost and replaced by information from another. A 5+:3- or 3+:5- pattern forms when information on two strands differs resulting in heteroduplex DNA. This is detected after the next round of DNA replication so is called postmeiotic segregation. The third class is recognised by an aberrant 4:4 segregation pattern which occurs when there is a double postmeiotic segregation event. Information on the frequencies of these classes of patterns, and the biases towards specific conversion events, has

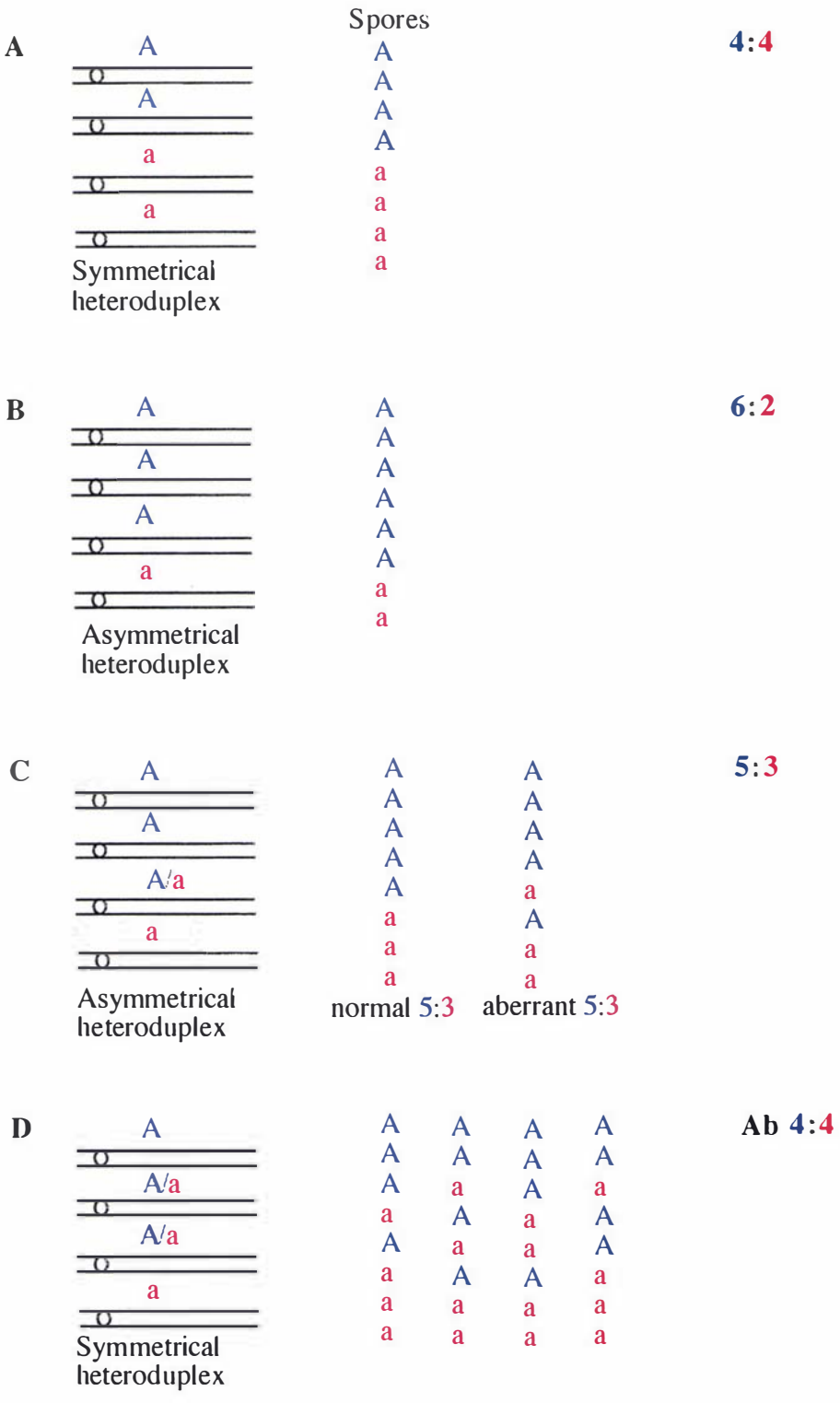


Fig 16 Segregation Patterns Observed for Lower Fungi

The spore formation in Ascomycetes allows determination of the genetic constitution of each of the DNA strands involved in meiosis. **A** Normally heterozyzous marker A will segregate 4A:4a. **B** Segregation of 6:2 indicates a gene conversion event. **C** Segregation of 5:3 indicates postmeiotic segregation event. **D** Aberrant 4:4 indicates heteroduplex DNA on two chromatids, provided no mis-match corrections occur, this is a double postmeiotic segregation event. Diagram reproduced in part from Orr-Weaver and Szosak (1985).

been collected in attempts to develop models to explain the information. The models need to include explanations for such observations as the polarity of recombination. This is an observed gradient in gene conversion frequency from one end of a gene to the other and has been studied extensively for the *ARGB* locus of *S. cerevisiae*. Polarity can be explained by initiation of recombination at specific sites (Fincham and Oliver 1989) and such an initiation site for gene conversion has been identified in the promoter region of the *S. cerevisiae ARGB* gene (Nicolas *et al.*, 1989).

Studies of recombination would not be complete if only meiotic recombination was considered. It is also important to consider mitotic recombination which was initially described as resulting from crossing over. It has since been demonstrated that heteroallelic mitotic recombination occurs predominantly by gene conversion (Roman and Fabre 1983). Most of the work done in this area has been with the yeast *S. cerevisiae*. In contrast to meiotic recombination which occurs after DNA replication most mitotic gene conversion occurs before DNA replication (Fabre 1978). Also the observed polarity of meiotic recombination is abolished in mitosis (Orr-Weaver and Szostak 1985). Gene conversion is associated with crossing over, but the degree depends on the type of heteroduplex DNA. The frequency of crossing over is much higher for symmetric heteroduplex DNA than asymmetric (Fig. 16). An association between meiotic and mitotic recombination is supported by the observation that inducing agents increase both types of recombination events. However, the mechanisms for meiotic and mitotic recombination may differ as these inducing agents alter the frequencies of each to different degrees. Evidence for different mechanisms comes from the analysis of recombination mutants. These tend to have a differential effect on mitotic and meiotic recombination (Orr-Weaver and Szostak, 1985). One mutation, *RAD52*, shows there may be two pathways of mitotic recombination. A *RAD52* dependent pathway that results in gene conversion and reciprocal recombination, and a *RAD52* independent pathway that just results in reciprocal recombination (Orr-Weaver *et al.*, 1981).

Recombination is fundamental to the process of meiosis. Mitotic recombination on the other hand may be a secondary result of DNA repair, or DNA replication past single stranded nicks. Models on the mechanism of recombination need to recognise these differences.

The first model developed which attempts to explain the earlier observations is the Holliday model (Holliday 1964). This model (Fig. 17) introduced the idea that heteroduplex DNA may be generated by the breaking and rejoining of chromatids, and that the segregation ratios result from the repair, or lack of repair, of heteroduplex DNA. Gene conversion events are observed when the DNA is repaired. This model proposes the formation of Holliday junctions to account for the association of crossing-over with aberrant segregation. This is illustrated in Figure 17 where crossing-over depends on which strands are nicked.

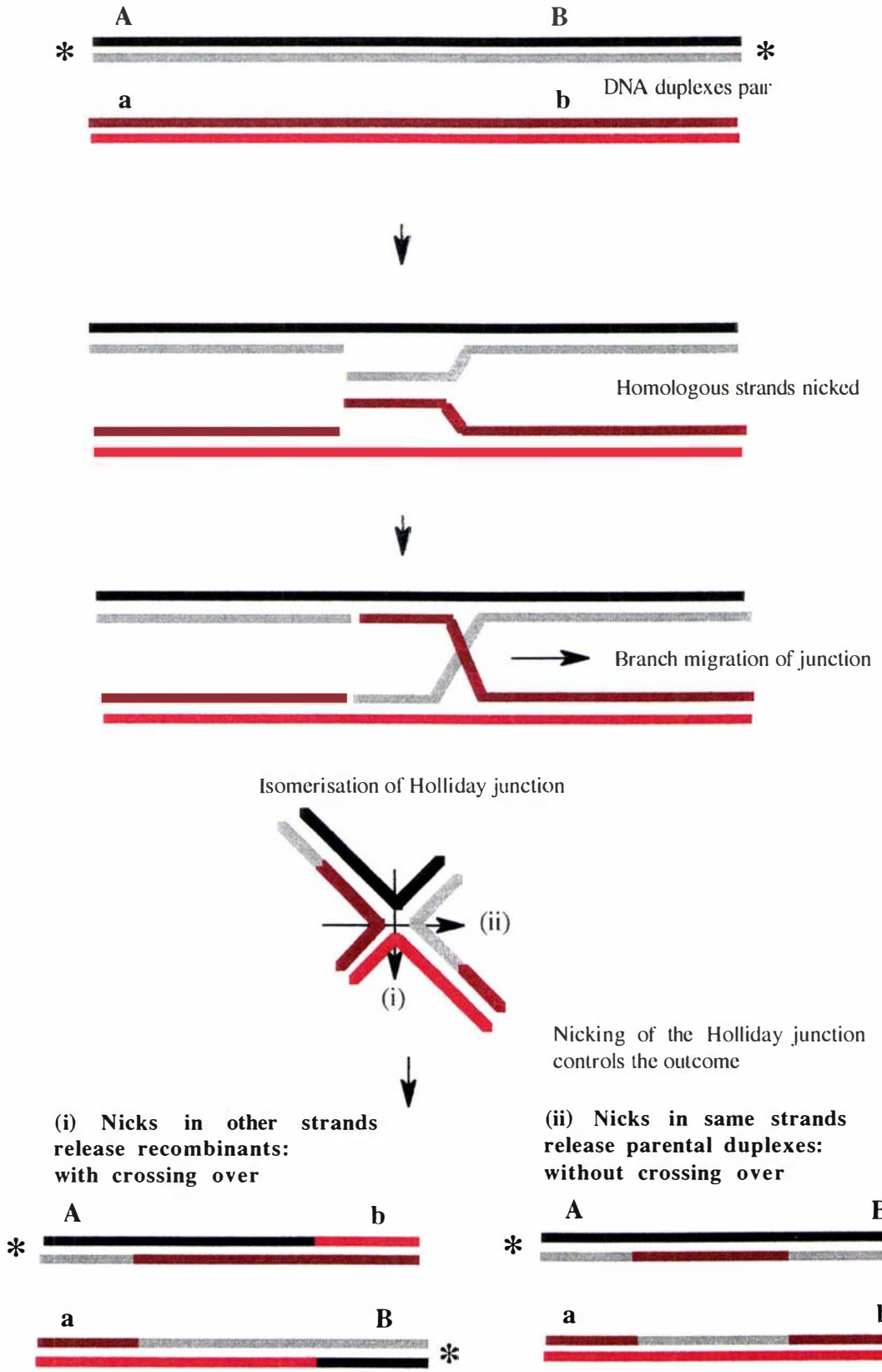


Fig 17 Holliday Model for Recombination

Strands are nicked at homologous sites then exchanged to produce symmetric heteroduplex DNA. The Holliday junction can be resolved with (i), or without (ii), crossing over. Figure adapted from Orr-Weaver and Szostak (1985).

The Holliday model does not explain the genetic demonstration of asymmetric heteroduplex DNA so it was extended to form the Meselson-Radding model (Meselson and Radding 1975). This model is outlined in Figure 18 and shows recombination to be initiated by a single stranded nick. This serves as a primer for DNA synthesis which displaces a single strand which can then pair with the homologous chromatid. Heteroduplex DNA is created by branch migration, and the ligation of nicks forms a Holliday junction. Again crossing-over depends on the resolution of the Holliday junction, and gene conversion is by DNA repair of heteroduplex DNA. A later version of the model by Radding initiates recombination at a single stranded gap in the recipient DNA instead of a single stranded nick in the donor DNA.

The use of yeast transformation as a model system for the study of recombination (Orr-Weaver *et al.*, 1981) allowed an investigation of the way circular and linear DNA recombine with homologous chromosomal sequences. The discovery that the frequency of targeted transformation in *S. cerevisiae* can be increased by using linear instead of circular DNA (Orr-Weaver *et al.*, 1981) not only facilitated genetic investigations, but stimulated investigations into the role of such breaks in chromosomal recombination. This led to the development of the double-strand break repair model (Szostak *et al.*, 1983) which is outlined in Figure 19. This differs from the Meselson-Radding model in a number of ways. In this model recombination is initiated by a double stranded break in the recipient chromatid, rather than a single stranded break in the donor DNA. Exonucleases attack this break and extend it into a gap flanked by extensive 3'-overhanging single stranded DNA. Such double stranded breaks and 3'-overhangs have been identified at the *ARG4* recombination initiation site in *S. cerevisiae* (Sun *et al.*, 1989; 1991). This 3'-single stranded DNA can invade a homologous duplex region to repair the gap. The gap repair process and the formation and migration of Holliday junctions form asymmetric and symmetric heteroduplex DNA. Gene conversion therefore results from repair of heteroduplex DNA, as in the Meselson-Radding model, but also by transfer of two strands of information. The region of gene conversion is flanked by two Holliday junctions which again determine if crossing-over occurs by their resolution. As shown in Figure 19 resolution of the two junctions in the opposite sense results in crossing over, while resolution in the same sense results in no crossing over.

The Meselson-Radding and double strand break repair models have been well reviewed and compared over the years (Orr-Weaver and Szostak 1985; Fincham 1989; Fincham and Oliver 1989; Hastings 1988). Both models can be used to explain the segregation patterns observed in fungi, including the differences between filamentous and non-filamentous fungi. In some cases the double-stranded break model provides a more simple explanation without the need to impose constraints. In other instances the Meselson-Radding model is favoured. As a general rule the double stranded break repair model is supported, and continues to be supported with

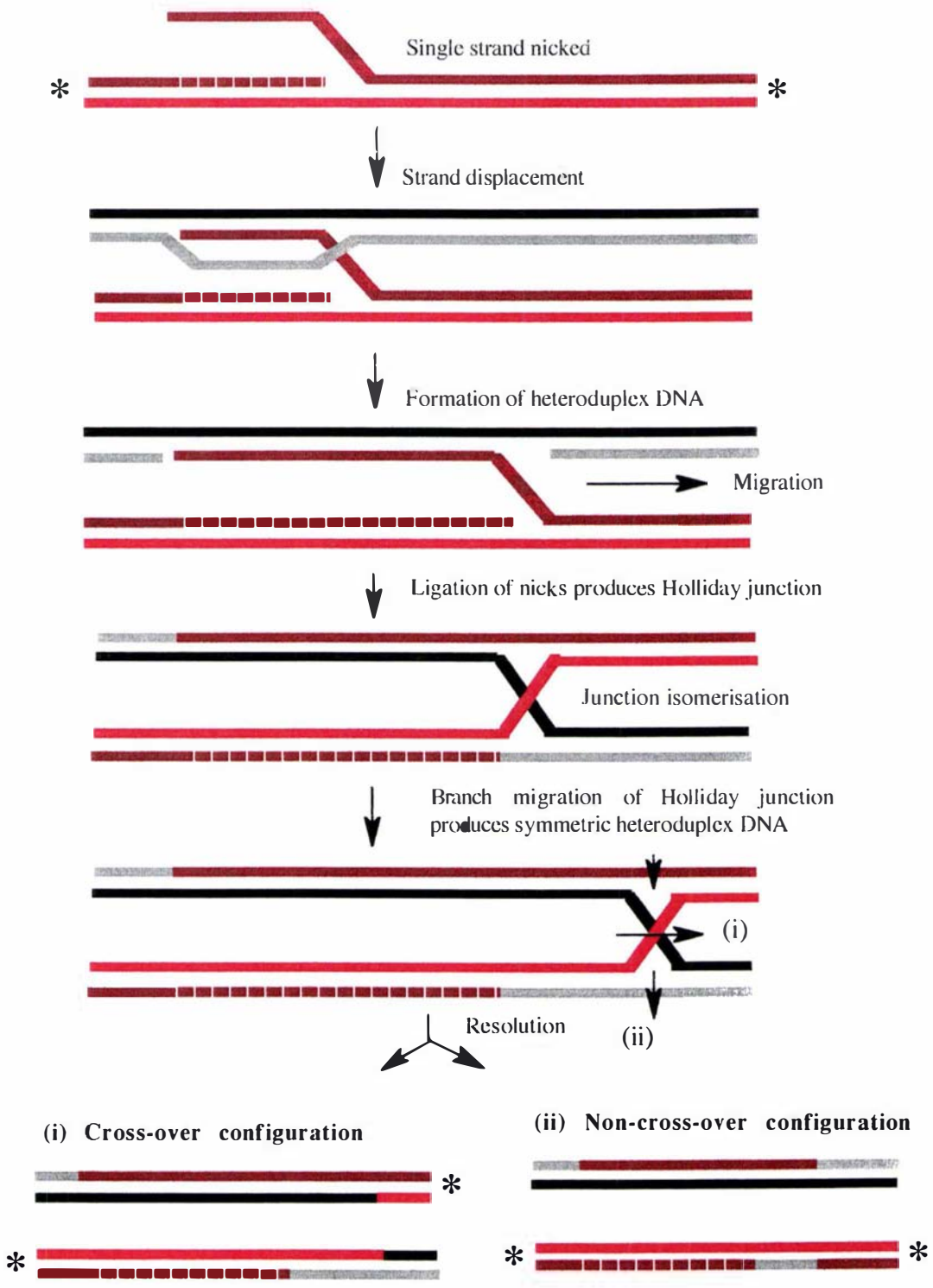


Fig 18 Meselson-Radding Model

Recombination is initiated by a single stranded nick which serves as a primer for DNA synthesis. This model accounts for the formation of symmetric and asymmetric heteroduplex DNA with (i), or without (ii), crossing over. Figure adapted from Orr-Weaver and Szostak (1985).

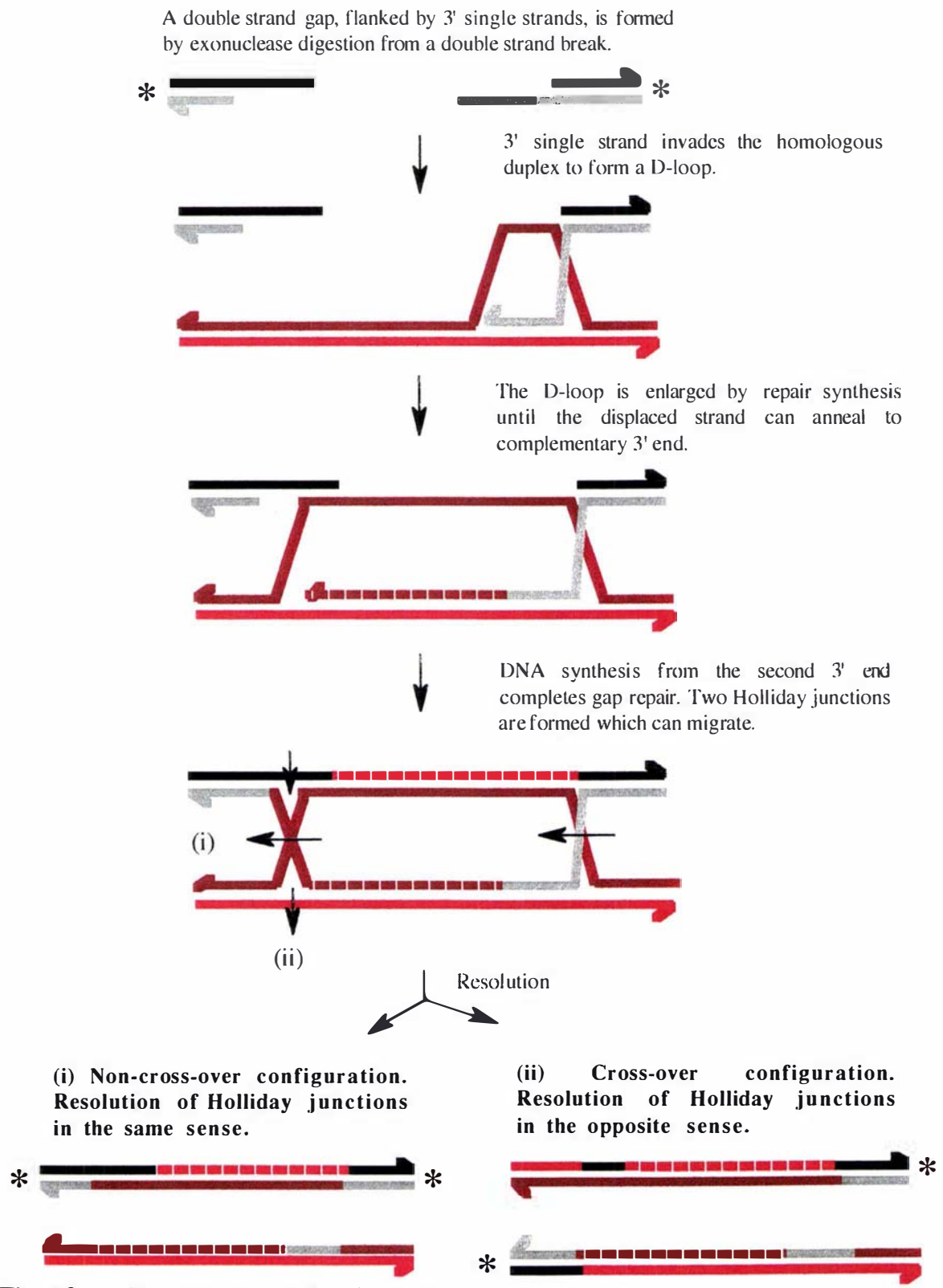


Fig 19 Double-Strand Break Repair Model

Initiation is by a double strand break in the recipient DNA which is extended by exonucleases leaving 3' single strand overhangs. Repair of the gap forms asymmetric heteroduplex DNA and Holliday junctions. Branch migration of the Holliday junctions forms symmetric heteroduplex DNA. Resolution of the Holliday junctions in the same (i), or opposite (ii), sense results in a non-cross-over, or cross-over, configuration. Figure adapted from Orr-Weaver and Szostak (1985).

more recent findings (Sun *et al.*, 1991; Jha *et al.*, 1993; Roitgrund *et al.*, 1993) and refinement (Sun *et al.*, 1991; Fishman-Lobell *et al.*, 1992), although it is difficult to totally exclude the Meselson-Radding model. Another model to come out of studies of the double strand break repair model is the single strand annealing model (SSA). This model was initially proposed after studies of recombination in mammalian cells (Lin *et al.*, 1990). The SSA model outlines a pathway where deletions are formed between directly repeated sequences. A double strand break is degraded by 5'-3' exonucleases to produce complementary single strands which can then anneal. This is not accompanied by the formation of a reciprocal recombination product. Double-strand breaks can therefore be 'repaired' by gap repair or single strand annealing (Fishman-Lobell *et al.*, 1992).

Mechanisms of recombination may involve a combination of models which will not be resolved until recombination can be studied directly at the molecular level. This is currently an active field of research investigating such ideas as the possibility of three-stranded DNA structures being involved in homologous recombination (Stasiak 1992).

As with recombination, both the Meselson-Radding model and the double-strand break repair model can explain homologous integration of circular DNA. They both provide the transfer of information with or without crossing over. The double-strand break repair model, however, is the only one to explain the integration of plasmids containing a gap in the homologous gene into *S. cerevisiae* with repair of the gap. If the plasmid is gapped outside the homologous region it is still integrated, but without repair of the gapped region.

5.2.1.2 Integration in Filamentous Fungi

Molecular analysis of *Neurospora* transformants has led to the identification, with various degrees of precision, of all three patterns of integration recognized by Hinnen *et al.* (1978). They have also been observed in *A. nidulans* since it was first transformed (Tilburn *et al.*, 1983). Over the last decade many fungal systems have been successfully transformed with a variety of homologous and heterologous markers. The resulting transformants have been analysed for the types of integration events.

In most filamentous fungi integration is at ectopic rather than homologous sites. The ratio of the two types of integration events has been observed to be affected by changing various parameters (Table 20). The length of homology was generally observed to influence the ratio of events with an increase in homologous events (gene targeting) observed with an increase in homology. The situation with vector linearisation was much more complicated. In filamentous fungi, as with yeast, the transformation frequency was generally observed to increase with

Table 20 Parameters Tested for an Effect on Gene Targeting

Parameter[a]	Fungus	Target locus	Effect	Reference
Linearisation (I, O)[b]	*Neurospora crassa*	*qa-2*	No	Dhawale and Marzluf 1985
Linearisation (I, O)[c]	*Neurospora crassa*	*trp-1*	Yes/No	Kim and Marzluf 1988
Linearisation (O)[d]	*Neurospora crassa*	*am*	No	Asch and Kinsey 1990
Linearisation (I)	*Aspergillus nidulans*	*trpC*	No	Yelton *et al.*, 1984
Linearisation (I)[e]	*Trichoderma reesei*	*cbhI*	Yes	Karhunen *et al.*, 1993
Linearisation[f]	*Tolypocladium niveum*	*simA*	No	Weber and Leitner 1994
Linearisation (I)[g]	*Phanerochaete chrysosporium*	*ura3*	No	Alic *et al.*, 1993
Linearisation (I)	*Alternaria alternata*	*BRM1*	Yes	Shiotani and Tsuge 1995
Linearisation[h]	*Acremonium chrysogenum*	*pcbC*	No	Walz and Kuck 1993
Linearisation (I)[i]	*Ascobolus immersus*	*met-2*	Yes	Goyon and Faugeron 1989
Linearisation (I)[j]	*Corprinus cinereus*	*TRP1*	No	Binninger *et al.*, 1991
Linearisation (I, O)[k]	*Ustilago maydis*	*leu*	Yes	Fotheringham&Holloman1989
Length of homology	*Neurospora crassa*	*am*	Yes	Asch and Kinsey 1990
Length of homology	*Aspergillus nidulans*	*tub*	Yes	May *et al.*, 1985
Length of homology	*Alternaria alternata*	*BRM1*	Yes	Shiotani and Tsuge 1995
Length of homology	*Corprinus cinereus*	*TRP1*	No	Binninger *et al.*, 1991
Length of homology[l]	*Trichoderma reesei*	*cbh1,cbh2, egl1,egl2*	Yes	Suomine *et al.*, 1993
Length of homology	*Tolypocladium niveum*	*simA*	Yes	Weber and Leitner 1994
Length of homology[m]	*Acremonium chrysogenum*	*pcbC*	Yes	Walz and Kuck 1993
Gene Activation	*Candida albicans*	*WH11,PEP1*	Yes	Srikantha *et al.*, 1995
Gene Activation	*Glomerella cingulata*	*gpdA*	No	Rikkerink *et al.*, 1994

[a] Parameter: Linearisation was within (I) or outside (O) the region of homology

[b] Transformed with circular vector and the vector linearised with a range of enzymes and enzyme combinations. Found an increase in transformation frequency with vector linearisation but not an increase in targeting.

[c] High level of targeting observed with circular and linear DNA where linearisation was within the selectable marker. The level of targeting decreased if linearisation was outside the selectable marker. This study found integration events depended on the host strain. Results complicated because of the RIP phenomenon.

[d] Found no difference in targeting using linear vs circular plasmids or plasmid vs lambda DNA.

[e] Linearisation produced a fragment with homologous ends interrupted by the amdS selectable marker.

[f] Can not determine if linearisation was within the region of homology

[g] Transformation was with single or double cut vector. No difference in the gene replacement frequency was observed with a vector with one or two homologous ends.

[h] Can not determine if linearisation was within the region of homology.

[i] Increase in targeting using single-stranded circular DNA and vector linearised within the region of homology.

[j] DNA conformation of circular single-stranded or double-stranded with a single-strand nick and linear DNA.

[k] Linearisation within or outside the region of homology greatly increased the frequency of transformation and the incidence of gene targeting.

[l] Although the level of targeting was high at all loci in *T. reesei*, targeting was relatively higher at the *cbh* locus, independent of the length of homology.

[m] Suggest that there is a minimum length of homology required for targeting.

vector linearisation. However, the level of targeting was often not altered. Very few studies have been done on the effect of the expression of the targeted locus on the level of gene targeting. The few, to date, suggest there may be an effect on the level of targeting. The vector the selectable marker is in (Faugeron *et al.*, 1989) and the genetic background of the strain used for transformation (Wernars *et al.*, 1985 and 1986; Kim and Marzluf, 1988) have both been shown to affect the ratio and type of integration events.

The length of DNA required for homologous integration shows considerable variation. A study of the length of homologous DNA required for homologous integration in *N. crassa* found that greater than 5.1 kb was needed in both plasmid and lambda vectors (Asch and Kinsey 1990). A similar study in *Cochliobolus heterostrophus* by Keller *et al.* (1991) suggested greater than 2 kb of homologous DNA is required for predominantly homologous integration. If 0.5-2 kb of homology was used a mixture of homologous and non-homologous integration events were observed. Transforming *A. nidulans* with the homologous β-tubulin genes (May *et al.*, 1985) showed 0.75 to 5.4 kb of homology resulted in the presence of homologous integrations.

There is also support for the presence of sequences promoting homologous integration. Transformation of *A. nidulans* with the *uaZ* gene resulted in transformants with exclusively homologous integration (Oestreicher *et al.*, 1993). Likewise when an *A. nidulans* (*uaZ11 argB2*) strain was transformed with a plasmid containing the complete homologous *uaZ* and *argB* genes, all 19 transformants showed homologous integration at either the *uaZ* site or *argB* site (Oestreicher *et al.*, 1993). The authors suggested there may be a sequence present in the vector which promotes homologous integration, but not necessarily at a specific site. However, for the *A. nidulans* homologous transformation systems *trpC*, *amdS*, *prnDBC*, *pkiA*, *argB* and *pyrG* (Yelton *et al.*, 1984; Wernars *et al.*, 1985; Durrens *et al.*, 1986; de Graff *et al.*, 1988, Upshall 1986; Oakley *et al.*, 1987) both homologous and heterologous integration events have been described, so this was unusual. Apart from the work by Oestreicher *et al.* (1993) only for *riboB* have all of the transformants analysed (10) arisen by homologous integration events (Oakley *et al.*, 1987).

The first report of a gene disruption by site specific, integrative transformation in *Aspergillus nidulans* was of the beta-tubulin genes (May *et al.*, 1985). For a list of the genes used in *A. nidulans* transformations refer to Table 3 (Chapter 3). Sometimes the disruption of the gene of interest causes a lethal mutation. One way to disrupt essential genes is to use diploids where the lethality of the disruption is readily observed upon haploidisation of the fungal diploid. An alternative is to exploit the presence of heterokaryons amongst transformants (Upshall 1986; Osmani *et al.*, 1988).

Transformants of filamentous fungi often contain transforming DNA in the form of tandem repeats at either ectopic or homologous sites. Transformants have been found which can be explained as resulting from successive rounds of integration into the homologous chromosomal locus, or into the plasmid copies already integrated. This can also result from non-homologous integration (ie non-homologous primary integration and successive rounds of homologous integration) (Wernars *et al.*, 1985). Wernars *et al.* (1985) found the number of copies of transforming DNA present was not proportional to the amount of DNA in the transformation mix. This suggests protoplasts receptive to DNA will assimilate and integrate multiple copies even from rather low external concentrations. It was suggested the larger number of protoplasts that take up DNA less avidly account for most of the single copy transformants that are found in most transformation experiments. This was not, however, observed by Walz *et al.* (1993) who transformed *Acremonium chrysogenum* with the homologous *pcbC* gene. They found that increasing the amount of plasmid DNA in the transformation mix resulted in a higher average number of integrated vector copies per transformant. The same was observed when transforming *S. cerevisiae* haploid and diploid strains where the results indicated plasmid-plasmid integration prior to chromosomal integration (Plessis and Dujon 1993).

It is important to develop efficient systems allowing optimal levels of homologous integration. The resulting gene replacement or disruptions can be used to make well defined alterations to the genetic make up of the fungus. The uses of the transformants are varied and plentiful: for example the essential nature of a particular gene, such as the *A. nidulans* cytochrome *c* (*cycA*) (Chapter 6), can be determined. Also important is the development and understanding of non-homologous integration systems. How random is integration in such systems? This becomes particularly important when looking at using heterologous plasmids to clone genes by insertional inactivation (plasmid 'tagging'). This is possible where selection for loss of function of the gene is available, but depends on random integration of the plasmid used. This method was used by Diallinas and Scazzocchio (1989) to clone the gene for uric acid-xanthine permease (*uapA*) of *A. nidulans*. However, they demonstrated non-random integration since their heterologous plasmid, carrying the *amdS* gene, integrated preferentially into the *uapA* gene. Their selection system detected integration events in any one of 9 loci so it was expected transformants would instead display disruptions of any of these 9 genes. The ORF of the targeted *uapA* gene was found to share a 10 bp, AT rich, sequence with the 3' region of the *amdS* gene, and this corresponded to the region of integration. A similar finding of preferential integration was described during the isolation of *wA* mutants in *A. nidulans* by transformation disruption (Tilburn *et al.*, 1990). Similarly Itoh *et al.* (1995), when using the hygromycin resistance plasmid pAN7-1 to transform *Penicillium paxilli*, observed brown spore colour mutants to arise at a frequency of 0.17% which was much higher than expected, although integration at different sites in the same locus was observed for the 4 transformants analysed.

Also observed were large deletions of the target site, and in one case a translocation, along with rearrangements of the plasmid sequences. These rearrangements have been observed in other systems involving heterologous integration (Durrens *et al.*, 1986; Wernars *et al.*, 1986; Razanamparany and Begueret 1988; Asch *et al.*, 1992; K istler and Benny 1992). Perkins *et al.* (1993) looked at chromosome rearrangements recovered following transformation of *N. crassa*. Transformation of *N. crassa* usually involves ectopic integration. At least 11% of stable transformants had new translocations. These were distributed in all seven linkage groups. There was an excess of translocations in strains having transforming DNA integrated at multiple ectopic sites, and translocations were completely absent in strains that had undergone only homologous transformation or conversion. It was shown these rearrangements were not attributed to protoplast formation and regeneration. The junction appeared to have resulted from the end joining of truncated vector sequences to breaks in chromosomes, without loss of chromosomal DNA. This mechanism did not appear to be driven by homology.

It is possible to recover integrated DNA from transformants by the technique of plasmid rescue. This involves the digestion, or partial digestion, of the genomic DNA with a restriction enzyme, then recircularisation of the fragments and transformation into *E. coli*. In this way it is possible to isolate the recipient DNA flanking the site of integration. It is also possible to recover the plasmids by transforming *E. coli* with untreated transformant DNA (Ballance and Turner 1985) even though the DNA has been shown to be integrated. It is assumed that there is a limited amount of free plasmid DNA that may have excised after integration. These are quite distinct from autonomously replicating plasmids.

Using this method the integration junction sequences of transformants of filamentous fungi have been studied. In many cases integration of transforming DNA has resulted in chromosomal and plasmid rearrangements (Razanamparany and Begueret 1988) from deletions to translocations (Asch *et al.*, 1992). This type of junction analysis is rare and therefore it is hard to draw many conclusions from the data, except that extensive matching of sequences is not required for ectopic integration, but a small amount of chance sequence may increase the probability of integration at a particular site.

Junction analysis of preferential integration into non-homologous sites in mammalian systems questions the existence of targeting (Sutherland *et al.*, 1993). Kotin *et al.* (1992) analysed the target sequence of the human parvovirus AAV in human chromosome 19 q13.3-qter. The sequence was analysed for possible recombination signals. They found the occurrence of direct repeats distributed non-uniformly, along with a CpG island containing transcription factor binding sites, and evidence of transcriptional activity.

These results stress the need to determine just how random 'ectopic' integration is. It is possible that there are hot-spots for integration throughout the genome, although these have not been characterised so how they may be recognised is not known. An interesting question is how the conformation of the recipient DNA effects the likelihood of integration. Does transforming DNA target actively transcribed regions more effectively than non-transcribed regions? How does the GC content of targeting and targeted DNA affect the chance of integration? By understanding more about ectopic integration we can improve our ability to design effective vectors for gene targeting.

5.2.2 Selection of Transformants

When selecting for fungal transformants the DNA treated cells are plated on agar medium which is selective for the desired type of transformants. The easiest way to select for transformants is directly, using a gene marker to complement an auxotrophic mutant. Some such markers in *A. nidulans* are orotidine-5'-monophosphate decarboxylase (*pyrG*) (Chapter 4), nitrate reductase (*niaD*) and acetamidase (*amdS*). The drawback in using selection markers for complementation is that there is a requirement for the appropriate mutation in the recipient strain.

Nitrate reductase is a particularly useful selective marker due to the availability of both positive and negative selection systems, as with OMPdecase. Transformants disrupted at the *niaD* locus can be identified by the reduced ability to utilise nitrate as a sole nitrogen source and by resistance to potassium chlorate. Chlorate is an analog of nitrate and is thought to be reduced by nitrate reductase to the toxic compound chlorite or hyperchlorite. However, Cove (1976) reports that chlorate resistance is a complex phenomenon and can arise from mutations in several different genes. These mutants map to 5 different linkage groups and are recessive to the wildtype allele in heterokaryons or heterozygous diploids (Debets *et al.*, 1990). Not every loss-of-function mutation at these loci confers chlorate resistance, suggesting factors other than loss of reduction may lead to chlorate resistance. Resistance of *A. nidulans* to chlorate is reported to occur when there are defects in the nitrate permease gene (*crnA*), nitrate reductase structural gene (*niaD*), two genes involved in regulation of nitrate metabolism (*nirA* and *areA*), or a gene (*cnx*) involved in the assembly of a molybdenum co-factor. Mutations in *niaD* can be distinguished from mutations at these other loci by growth on minimal salt medium supplemented with different nitrogen sources (Wu and Linz 1993). The *niaD* mutants are unable to use nitrate but can grow on medium containing each of the other nitrogen sources, whereas mutants with mutations in the other genes involved in nitrogen metabolism have different growth patterns on these nitrogen sources. For a summary of the growth patterns of *A. nidulans* mutants defective in nitrate assimilation refer to Table 21.

Table 21 Nitrogen Source Utilisation of *A. nidulans* Mutants

Gene mutation	Chlorate[a] resistance	Utilisation of sole nitrogen source[b, c]					
		nitrate	nitrite	ammonium	hypoxanthine	proline	glutamate
niaD$^-$	R	-	+	+	+	+	+
crnA$^-$	R	+	+	+	+	+	+
niiA$^-$	S	-	-	+	+	+	+
cnxA$^-$*J*$^-$	R	-	-	+	-	+	+
areA$^-$	R	-	-	+	-	-	-
nirA$^-$	R	-	-	+	+	+	+

[a] Resistance (R) or Sensitivity (S) to chlorate is as indicated.

[b] Table reproduced in part from Unkles *et al.* (1989).

[c] Growth equivalent to wildtype (+), or reduced growth (-), is as indicated.

Another useful feature of the nitrate assimilation pathway is that it is entirely dispensable, unlike purine biosynthesis. Manipulations of the nitrate pathway should not, therefore, alter growth or metabolic fluxes through biosynthetic pathways, which otherwise could result in altered product levels in industrial strains (Unkles *et al.*, 1989). Transformation using this marker is also observed to result in very little background growth in the form of abortive transformants (Unkles *et al.*, 1989).

The acetamidase gene is another useful selective marker as it also has the property of counter selection shown by *pyrG* and *niaD*. Mutations in the acetamidase gene are unable to grow on acetamide as a sole nitrogen source and can be selected by their resistance to fluoroacetamide (Hynes and Pateman 1970). Unfortunately transformation with this gene results in high background growth, presumably due to abortive transformants (Wernars *et al.*, 1985). This is also observed when the *amdS* gene of *A. nidulans* is used in heterologous systems (Penttila *et al.*, 1987). However, the *amdS* gene is particularly useful as it can be used as a dominant selectable marker in other systems as some fungi do not possess an *amdS* gene, therefore an appropriate mutant strain is not needed (Turgeon *et al.*, 1985; Penttila *et al.*, 1987; Yamashiro *et al.*, 1992). Transformants can be selected for by their ability to grow on acetamide media.

5.2.2.1 Disruption Vectors

Gene disruption vectors used in this study were constructed by cloning an internal fragment of the gene to be disrupted (X^+) into the multiple cloning site of the vector pGM32, containing the selectable marker *pyr-4*. If the gene disruption vector integrated into the homologous site (Type I integration event) then the gene would be disrupted as this would result in a duplication of the gene, separated by the vector, with one copy deleted in the 5' region and the other copy deleted in the 3' region. Both copies would therefore be inactive (X^-) and the transformant would be Pyr^+. If the gene disruption vector integrated into an ectopic site (Type II integration event) then the gene would remain intact (X^+) and the transformant would be Pyr^+.

The length of homology required for homologous integration at either the *niaD* or *amdS* locus in *A. nidulans* was not known. The total length of the homologous fragment was limited by the length, from start to stop codon, of the gene to be targeted due to the gene disruption vector containing an internal fragment of the gene.

5.2.3 Aims

In general, integration in yeast occurs mostly by homologous recombination (Orr-Weaver *et*

al., 1981) while in mammalian cells it occurs mostly by non-homologous integration at ectopic sites (Thomas and Capecchi 1987). Filamentous fungi tend to be intermediate to these extremes and therefore provide a useful system to study this process and thus, hopefully, to enable optimisation for specific integration events.

The ratio of the types of integration events varies considerably with such parameters as the genetic background of the fungal strain, selection marker, length of homologous DNA, degree of homology, type of integration vector etc. No one to date has done a systematic study using a base model system to thoroughly test these parameters, or to explore the many more that may also have an effect. An understanding of the mechanism of integration, and how to increase the specific types of integration events, would have benefits for both pure and applied research.

An investigation into altering parameters in the transformation procedure was commenced in order to provide a thorough and systematic study of their effects on gene targeting at two independent loci in a well characterised model organism. *A. nidulans* was chosen due to its ease of manipulation and the wealth of literature available (Martinelli and Kinghorn 1994). Gene targeting was investigated using the phenotypic markers of nitrate and acetamide utilisation. Disruption of the *niaD* or *amdS* genes resulted in reduced ability to grow on minimal media containing nitrate (Unkles *et al.*, 1989) or acetamide (Hynes and Pateman 1970) as sole nitrogen sources respectively. It is anticipated that these results will be of general relevance to targeted transformation of other fungi.

5.3 Results

5.3.1 Construction of Disruption Vectors

A series of gene disruption vectors were constructed targeting the *A. nidulans niaD* (Johnstone *et al.*, 1990)and *amdS* (Corrick *et al.*, 1987) loci. The genomic loci were studied to determine the largest fragment that could be isolated and still have sufficient 5' and 3' deletions to cause a disruption of gene function. This fragment was further digested to form two smaller fragments and then each was cloned into pGM32 to form a series of three vectors for each locus.

To construct pniaD1, 2 and 3 internal fragments of 0.90 kb, 1.24 kb and 2.15 kb respectively were isolated from pSTA8 (Table 1, Chapter 2) and cloned into pGM32 to produce pniaD1 and pniaD2 (Fig. 20A). For pamdS1, 2 and 3 internal fragments of 0.68 kb, 0.60 kb and 1.27 kb respectively were isolated from p3SR2 (Table 1, Chapter 2) and cloned into pGM32 to produce pamdS1, pamdS2 and pamdS3 (Fig. 20B).

The vector identities were thoroughly tested by comparing restriction digestion patterns with those expected for the correct constructs. Those showing the correct restriction pattern were prepared for transformation by large scale alkaline lysis (Section 2.6.2) and purified by PEG precipitation (Section 2.6.2.1). The identity of the vectors was then confirmed by sequencing from the forward and reverse primer sites in pGM32 over the cloning sites (Section 2.15). The sequencing results are recorded in Appendix 2.

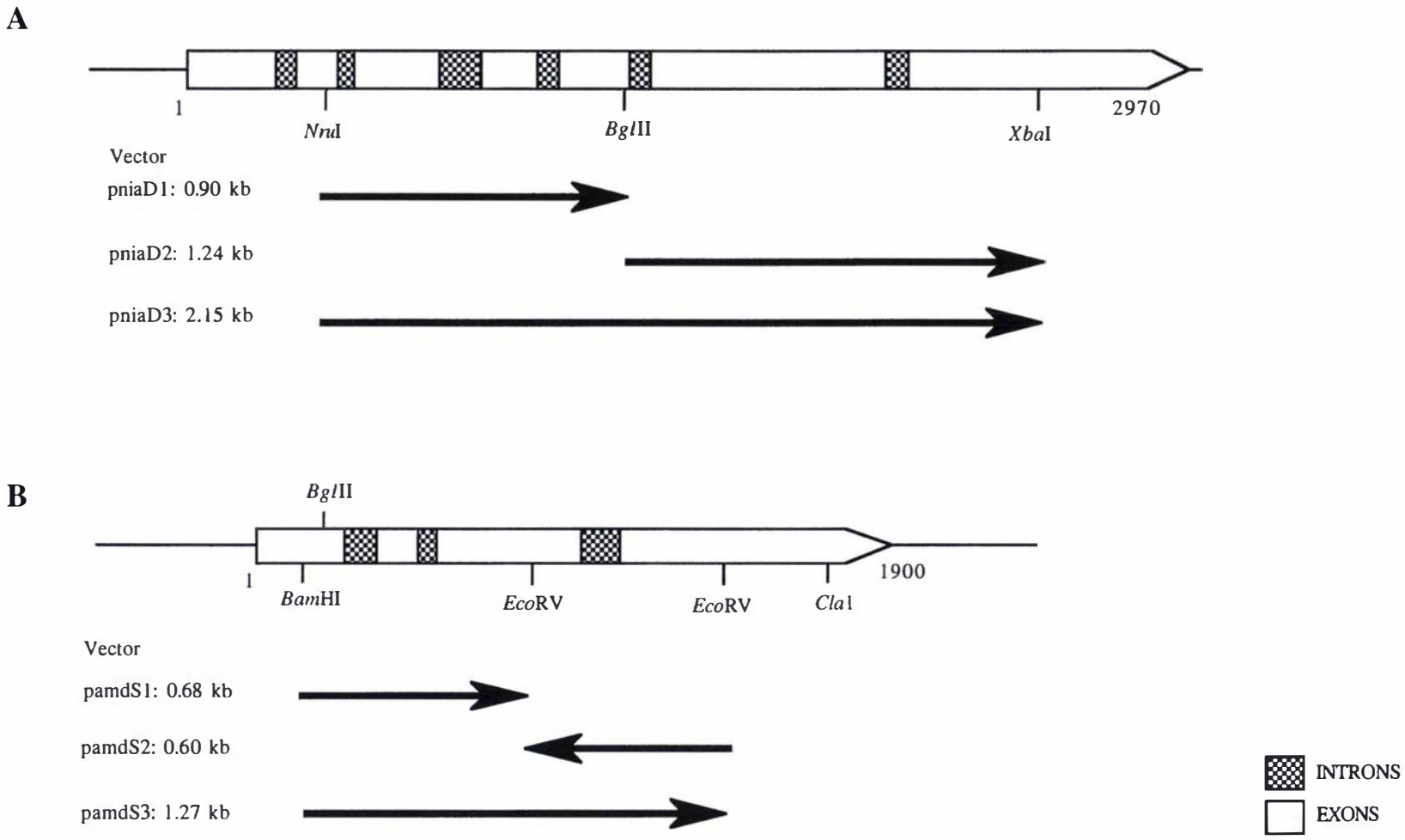
5.3.2 Transformation with Disruption Vectors and Screening of Transformants

The gene disruption vectors containing an internal portion of the gene to be disrupted, and the *N. crassa pyr-4* selectable marker, were used to transform the *Pyr*$^{-}$ *A. nidulans* haploid strain 1-85. The transformation procedure was based on the standard procedure outlined in Section 2.18 and the transformants were selected by uracil independence. N-type transformants (Chapter 4) were selected and purified by streaking for single spores on selective then non-selective media (Section 2.18.2). If the purity of the transformant was in doubt further rounds of streaking were performed. Those transformants belonging to the unstable sub-class of N-type transformants were excluded from further analysis, as were H-type transformants.

The ploidy of the transformants was determined as described in Section 2.19. It was necessary to determine the ploidy of the transformants as for both markers the disruption phenotype was recessive and would therefore be masked in a diploid transformant.

Fig 20 Maps of *niaD* and *amdS* and Construction of the pniaD and pamdS Vector Series

A Physical map of the *niaD* locus. The *niaD* transcription unit and its transcription direction are shown by the open arrow. The 2.15 kb *Xba*I/*Nru*I restriction fragment, internal to the transcription unit, was ligated into the pGM32 multiple cloning site (*Xba*I/*Sma*I), to produce pniaD3, and into pIC-19R (*Xba*I/*Nru*I) to produce pDW1. Internal *niaD* transcription unit fragments were isolated from pDW1 by digestion with *Bam*HI, *Bgl*II and *Xba*I, and inserted into the pGM32 multiple cloning site (*Bam*HI and *Xba*I) to produce pniaD1 and pniaD2. The relative direction of insertion of the segments into pGM32 is shown by the arrows. **B** Physical map of the *amdS* locus. The *amdS* transcription unit and its transcription direction are shown by the open arrow. Three restriction fragments within the transcription unit were isolated by digestion with *Bam*HI and *Eco*RV (partial) and inserted into the multiple cloning site of pGM32 (*Bam*HI and *Sma*I) to produce pamdS1, pamdS2 and pamdS3. The relative direction of insertion of the segments is shown by the arrows.


A
1
NruI
BglII
XbaI
2970
Vector
pniaD1: 0.90 kb
pniaD2: 1.24 kb
pniaD3: 2.15 kb
B
BglII
1
BamHI
EcoRV
EcoRV
ClaI
1900
Vector
pamdS1: 0.68 kb
pamdS2: 0.60 kb
pamdS3: 1.27 kb
INTRONS
EXONS

Diploid transformants from transformation with the pniaD series of vectors were screened for disruption of the *niaD* gene by PCR (Section 2.18.3.2) when the number of stable transformants was low. The nia1 primer and the reverse primer (Table 2) detect disruption of the *niaD* locus while the internal control primers, act1 and act2 (Table 2), amplify a section of the actin gene. PCR was performed on intact conidiospores. The optimal amount of spores per 25 µl reaction mix was determined to be 1×10^4 - 9×10^4. Separate reaction mixtures containing the primer pairs act1/act2 and nia1/reverse were required. Amplification of the actin gene resulted in a product of 425 bp. Disruption of the *niaD* locus with pniaD2 or pniaD3 resulted in a product of 2.4 kb, while disruption with pniaD1 resulted in a product of 1 kb. No product was obtained for ectopic integrants with the nia1 and reverse primers.

Diploids obtained from transformation with the pamdS series of vectors, and for the pniaD series when the number of transformants was high, were excluded from further analysis.

The stable, purified transformants were then tested for disruption of *niaD* and *amdS* on the basis of nitrogen source utilisation. Transformants of the pniaD series of vectors were grown on *Aspergillus* minimal media, supplemented as required, containing potassium nitrate, or sodium nitrite as the sole nitrogen source. Nitrite was chosen as the control nitrogen source as disruption of other genes in this pathway would result in impaired ability to grow on nitrite as well as nitrate, thereby allowing disruption of other genes in the pathway to be excluded (Unkles *et al.*, 1989).

The suitability of these media for screening transformants was confirmed using the control strains A691 (*niaD*$^-$) and wildtype 2-1 (*niaD*$^+$) (Table 1). All transformants grew on nitrate and nitrite media with a proportion showing severely retarded growth on the nitrate media. These were scored as Nia$^-$ and this was later confirmed by Southern analysis of a small selection.

Transformants of the pamdS series of vectors were grown on *Aspergillus* minimal media, supplemented as required, containing acetamide as the sole nitrogen source and caesium chloride. As determined by Tilburn *et al.* (1983) the addition of caesium chloride improved the distinction between homologous (Amd$^-$) and ectopic (Amd$^+$) integrants by reducing the growth of Amd$^-$ transformants. This was determined using the control strain MH1165 (*amdS*$^-$) and wildtype 2-1 (*amdS*$^+$) (Table 1). All transformants grew on *Aspergillus* complete media (or supplemented *Aspergillus* minimal media) and the acetamide/caesium chloride media with a proportion showing severely retarded growth on the acetamide/caesium chloride media. These were scored as Amd$^-$ and this was later confirmed by Southern analysis of a small selection.

5.3.3 Parameters Investigated for their Effect on Gene Targeting

5.3.3.1 Length of Homologous Fragments

The pamdS and pniaD series of plasmids (Fig. 20) were used to observe the size dependency of target DNA on the efficiency of gene targeting. The plasmids were used as circular molecules and the transformants were purified and assayed for nitrogen source utilisation.

The frequency of Nia^- and Amd^- transformants differed among the transforming plasmids. The difference depended on the locus targeted and also correlated with the size of the homologous fragment (Table 22). Transformation with pniaD2 and pamdS3, both containing a homologous fragment of approximately 1.25 kb in length, resulted in 27% Nia^- and 5.4% Amd^- transformants respectively (Table 22).

The size dependency of gene targeting was displayed clearly with the pniaD series of plasmids targeting the *niaD* locus. Plasmids containing 0.9 kb, 1.24 kb and 2.15 kb homologous segments resulted in targeting frequencies of 14%, 27% and 43% respectively (Table 22). These represent the combined results of three independent experiments using the same batch of frozen protoplasts. Chi-squared analysis comparing the three experiments indicated there was no significant difference between the levels of targeting observed between the experiments so the data was pooled. Chi-squared analysis of the pooled data from all three experiments showed a significant difference between targeting levels with the vectors pniaD1, pniaD2 and pniaD3. The trend of increasing targeting efficiency with increased length of homologous DNA in the vector was upheld by the pamdS series of vectors, although the number of targeted transformants was smaller and was therefore not statistically significant by chi-squared analysis.

To confirm that the Nia^- and Amd^- transformants were the result of integrative disruption of the *niaD* and *amdS* loci via homologous recombination of donor plasmids, Southern blots were prepared from a selection of transformants (Fig. 21). Total genomic DNA from transformants, and from the parental strain, was digested with *Eco*RI (pniaD transformants) and *Xba*I (pamdS transformants), which recognise one restriction site within the respective plasmids. The blots were then probed with the entire pniaD3 (Fig. 21A) and pamdS3 (Fig. 21B) plasmids respectively. DNA from Nia^+ and Amd^+ (ectopic/non-disrupted) transformants each contained a single band also present in the parental strain. These bands were not observed in the Nia^- and Amd^- transformants, indicating rearrangement at the homologous locus. Bands were observed that correspond to the pattern expected for integration via a single cross-over event within the

Table 22 *A. nidulans* Nia^+/Nia^- and Amd^+/Amd^- Transformants of 1-85

Series	Expt.	Plasmid	Size of homologous insert (kb)	No of transformants Ectopic	Targeted	Percentage Targeted Disruption
I[a, c]	1	pGM32	0.00	3	0	0%
		pniaD1	0.90	9	3	25%
		pniaD2	1.24	14	2	13%
		pniaD3	2.15	4	6	60%
	2	pGM32	0.00	6	0	0%
		pniaD1	0.90	10	2	17%
		pniaD2	1.24	9	5	36%
		pniaD3	2.15	23	15	39%
	3	pGM32	0.00	5	0	0%
		pniaD1	0.90	19	1	5%
		pniaD2	1.24	13	6	32%
		pniaD3	2.15	35	25	42%
	Combined[d] 1, 2 & 3	**pGM32**	**0.00**	**14**	**0**	**0%**
		pniaD1	**0.90**	**38**	**6**	**14%**
		pniaD2	**1.24**	**36**	**13**	**27%**
		pniaD3	**2.15**	**62**	**46**	**43%**
II[b]	1	pGM32	0.00	25	0	0%
		pamdS1	0.68	59	0	0%
		pamdS2	0.60	55	1	1.8%
		pamdS3	1.27	49	4	7.5%
	2	pGM32	0.00	0	0	0%
		pamdS1	0.68	25	0	0%
		pamdS2	0.60	45	0	0%
		pamdS3	1.27	56	2	3.4%
	Combined 1 & 2	**pGM32**	**0.00**	**25**	**0**	**0%**
		pamdS1	**0.68**	**84**	**0**	**0%**
		pamdS2	**0.60**	**100**	**1**	**1%**
		pamdS3	**1.27**	**105**	**6**	**5.4%**

[a] Series I experiments used a single protoplast batch that was stored at -70°C for 3 (Expt.1), 5 months (Expt. 2) and 6 months (Expt. 3).

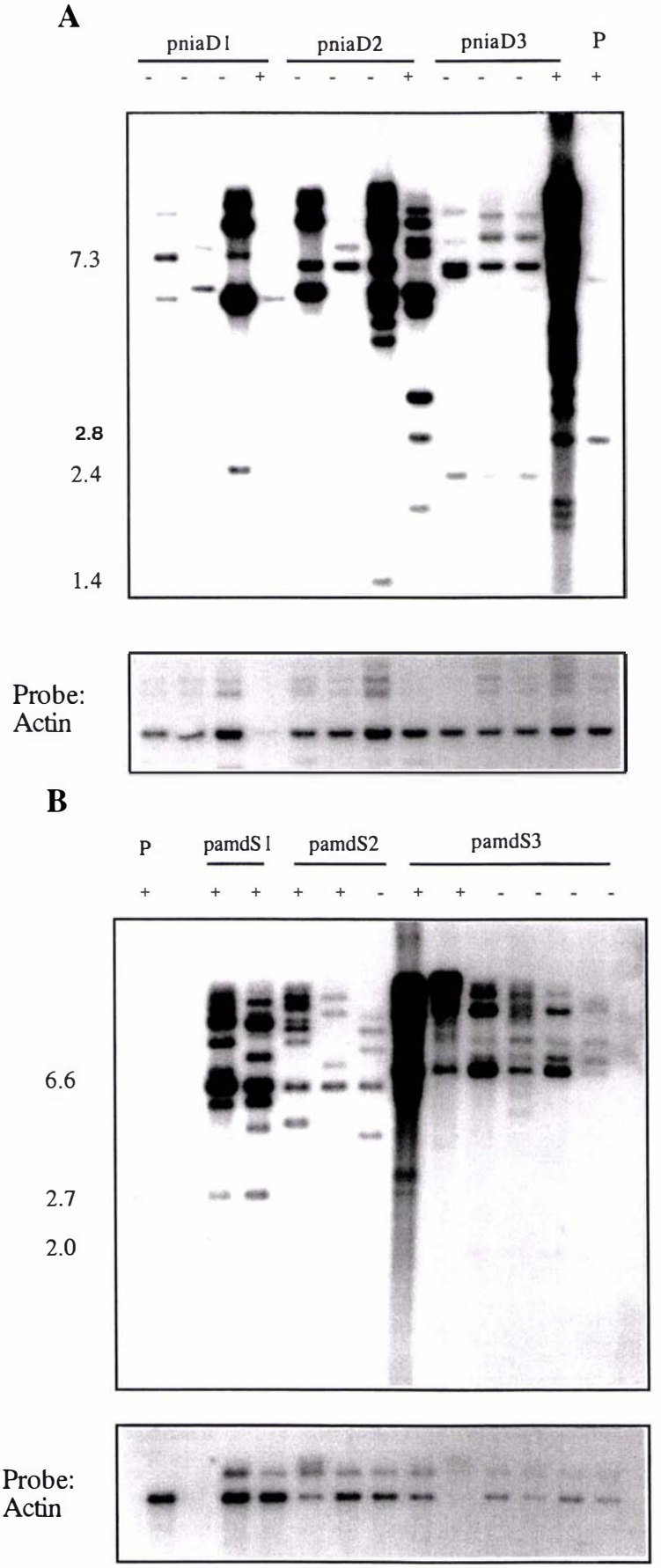
[b] Series II experiments used a single batch of protoplasts (different from I) fresh (Expt. 1) and stored frozen for 3 months (Expt. 2).

[c] Test of significance between experiments in Series I $\chi^2 = 0.32$; df = 2; $0.9 > P > 0.8$.

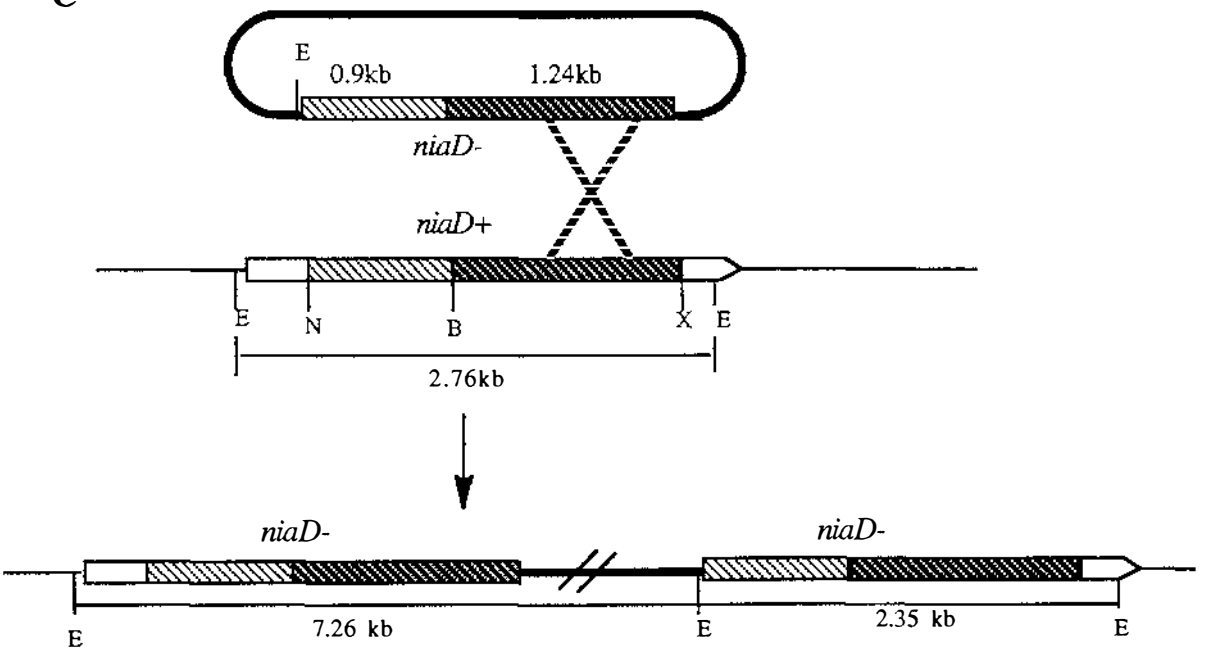
[d] Test of significance between pniaD1, pniaD2 and pniaD3 (pooled data from all three experiments) in Series I $\chi^2 = 12.94$; df = 2; $0.005 > P > 0.001$.

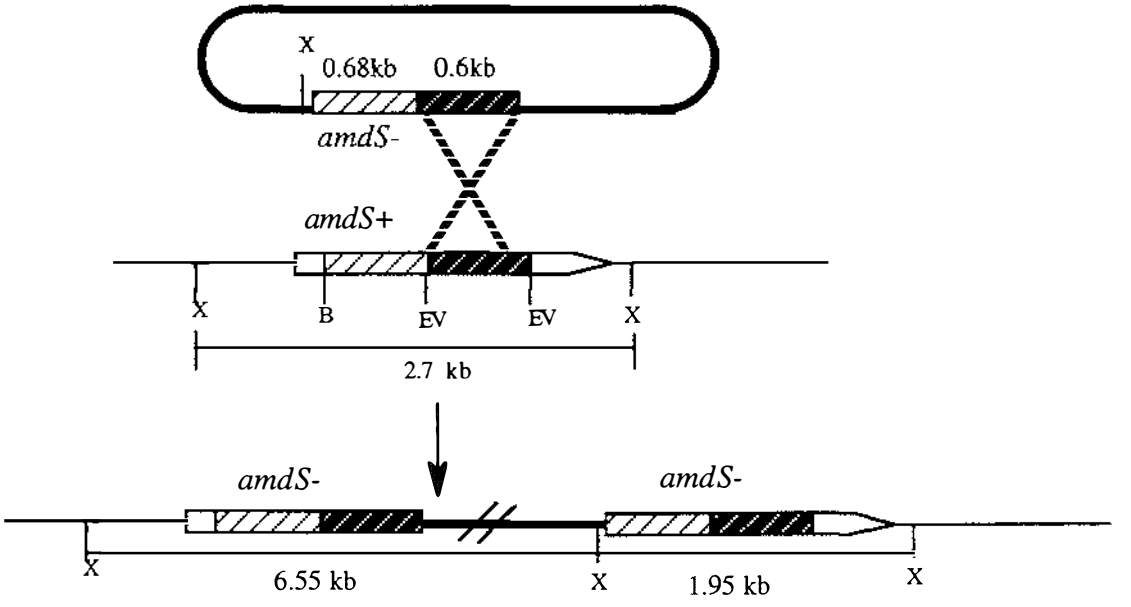
Fig 21 Southern Blot Analysis of Nia^+/Nia^- and Amd^+/Amd^- Transformants of 1-85

Total genomic DNA digested with *Eco*RI (*niaD* transformants) or *Xba*I (*amdS* transformants) was run on a 1% agarose gel, and the blot was probed with [α^{32}P] dCTP-labelled pniaD3 or pamdS3 respectively. Both blots were stripped and reprobed with [α^{32}P] dCTP-labelled actin. The size (in kb) of DNA fragments is indicated on the left. **A** Strain 1-85 (lane P) and Nia^+ (+) and Nia^- (-) transformants produced with the circular vectors pniaD1, pniaD2 and pniaD3 as indicated. **B** Strain 1-85 (lane P) and Amd^+ and Amd^- transformants produced with the circular vectors pamdS1, pamdS2 and pamdS3 as indicated. **C** Structure of the *A. nidulans* *niaD* and *amdS* loci before and after recombination with gene disruption vectors pniaD3 and pamdS3. The integration of 1 copy of each vector by homologous recombination as a single crossover event is shown as an example. Restriction site abbreviations: E, *Eco*RI; N, *Nru*I; B, *Bgl*II; X, *Xba*I; EV, *Eco*RV; B, *Bam*HI. The thick line represents the pGM32 vector containing the *pyr-4* selectable marker.


A
pniaD1
pniaD2
pniaD3
P
- - - + - - - + - - - + +
7.3
2.8
2.4
1.4
Probe:
Actin
B
P
pamdS1
pamdS2
pamdS3
+ + + + + - + + - - - -
6.6
2.7
2.0
Probe:
Actin

C


E
0.9kb
1.24kb
niaD-
niaD+
E
N
B
X
E
2.76kb
niaD-
niaD-
E
7.26 kb
E
2.35 kb
E


X
0.68kb
0.6kb
amdS-
amdS+
X
B
EV
EV
X
2.7 kb
amdS-
amdS-
X
6.55 kb
X
1.95 kb
X

region of homology (Table 23). In a selection of transformants a band the size of the transforming vector was also observed which indicated the vector had integrated in a head to tail tandem array. Some of the integration patterns observed were very complicated, particularly for transformants with ectopic integration. To determine if any of the bands present were due to partial digestion of the genomic DNA, and to compare loading of the DNA, the blots were re-probed with actin (Fig. 21). The results indicated partial digestion of genomic DNA had occurred, accounting for some of the extra bands observed. Despite the presence of partials the expected banding pattern for homologous integration was observed in all cases.

5.3.3.2 Effect of Double-Strand Breaks in Transforming DNA

Plasmids pniaD3, pamdS1 and pamdS3 were each linearised within the region of homology with *Bgl*II (Fig. 20) and used for transformation alongside the corresponding circular plasmids. The linear plasmids had sequence homology at both ends which was distributed evenly for the pniaD3 plasmid and unevenly for the pamdS1 and pamdS3 plasmids (Fig. 20). Linearisation of the vectors enhanced targeting to both the *niaD* and *amdS* loci by approximately two fold (Table 24). The targeting frequency continued to be locus dependent with greater targeting observed for the pniaD3 plasmid than either pamdS1 or pamdS3. An element of size dependency was again observed with the linear pamdS1 (0.68 kb) vector targeting at 1.7% while the pamdS3 (1.24 kb) vector targeted at 7.7%. Although the effect of vector linearisation at the *niaD* locus was statistically significant, the increase at the *amdS* locus was not, possibly due to the low number of Amd^- transformants. However, the pamdS3 results were from four independent transformations, of which three showed increases in targeting (1.55 fold, 2.2 fold and 2.4 fold) due to linearisation while one transformation resulted in 0% targeting with linear and circular DNA (Table 24).

Southern blot analysis of a selection of Nia^- and Nia^+ transformants obtained using linear and circular plasmids confirmed integration and disruption of the *niaD* locus (Fig. 22). The same pattern of integration was observed for all Nia^- targeted transformants tested regardless of the conformation of the vector used for the transformation.

5.3.3.3 Vector Concentration in the Reaction Mix

The circular pniaD2 vector, which targeted at a level intermediate to pniaD1 and pniaD3, was used to test the effect of the amount of DNA in the reaction mix on the targeting frequency. This was tested to ensure that the targeting frequency was not affected by small variations in the amount of DNA used in each reaction. The amount of DNA included in reaction mixes

Table 23 Hybridisation Patterns Expected for Homologous Integration at *niaD* and *amdS*

Vector	Vector Size[a, b]	Homologous integration Size of hybridising band[c]	
pniaD1	5.6 kb	6.02 kb	2.35 kb
pniaD2	5.94 kb	7.26 kb	1.44 kb
pniaD3	6.85 kb	7.26 kb	2.35 kb
pamdS1	5.2 kb	5.9 kb	1.95 kb
pamdS2	5.1 kb	5.8 kb	2.0 kb
pamdS3	5.75 kb	6.55 kb	1.95 kb

[a] The presence of a band the size of the vector indicates integration in a head to tail tandem repeat.

[b] Hybridising bands representing the non-disrupted loci are 2.76 kb for *niaD* and 2.7 kb for *amdS*.

[c] Hybridising bands expected for integration of the pniaD and pamdS series of vectors at the homologous site after digestion of genomic DNA with *Eco*RI and *Xba*I respectively.

Table 24 Effect of Vector Linearisation on the Frequency of Homologous Integration

Series	Expt.	Plasmid	Conformation[a]	No of transformants[b] Ectopic	Targeted	Percentage[c] Targeted Disruption
I	1	pniaD3	Circular	16	19	54%
		pniaD3	Linear	10	57	85%
	2	pniaD3	Circular	44	28	39%
		pniaD3	Linear	14	62	82%
	Combined 1 & 2	**pniaD3**	**Circular**	**60**	**47**	**44%**
		pniaD3	**Linear**	**24**	**119**	**83%**
II	1	pamdS1	Circular	61	0	0%
		pamdS1	Linear	57	1	1.7%
III	1	pamdS3	Circular	62	4	6.1%
		pamdS3	Linear	58	6	9.3%
	2	pamdS3	Circular	44	0	0%
		pamdS3	Linear	35	0	0%
	3	pamdS3	Circular	27	2	6.9%
		pamdS3	Linear	22	4	15.4%
	4	pamdS3	Circular	43	1	2.3%
		pamdS3	Linear	17	1	5.5%
	Combined 1, 2, 3 & 4	**pamdS3**	**Circular**	**176**	**7**	**3.8%**
		pamdS3	**Linear**	**132**	**11**	**7.7%**

[a] Linearisation of the vector was within the region of homology. The efficiency of vector linearisation was tested by transformation of *E. coli*. Bacterial transformation with the linear vector resulted in approximately 2% of the number of bacterial transformants observed for the circular vector. The vectors were treated identically, except for the addition of *Bgl*II to one restriction enzyme mix to form the linear vector.

[b] The values are the total number of transformants in each class from individual experiments for pniaD3 (series I), pamdS1 (series II) and pamdS3 (series III), and the combined values for pniaD3 and pamdS3 in series I and III.

[c] Test of significance between experiments in Series I: Expt. 1, $\chi^2 = 11.48$, df = 1, $P < 0.001$; Expt. 2, $\chi^2 = 28.26$, df = 1, $P < 0.001$.

[d] Test of significance for linearised and circular pamdS3 (pooled data from all four experiments) in Series III: $\chi^2 = 2.29$; df = 1; $0.2 > P > 0.1$.

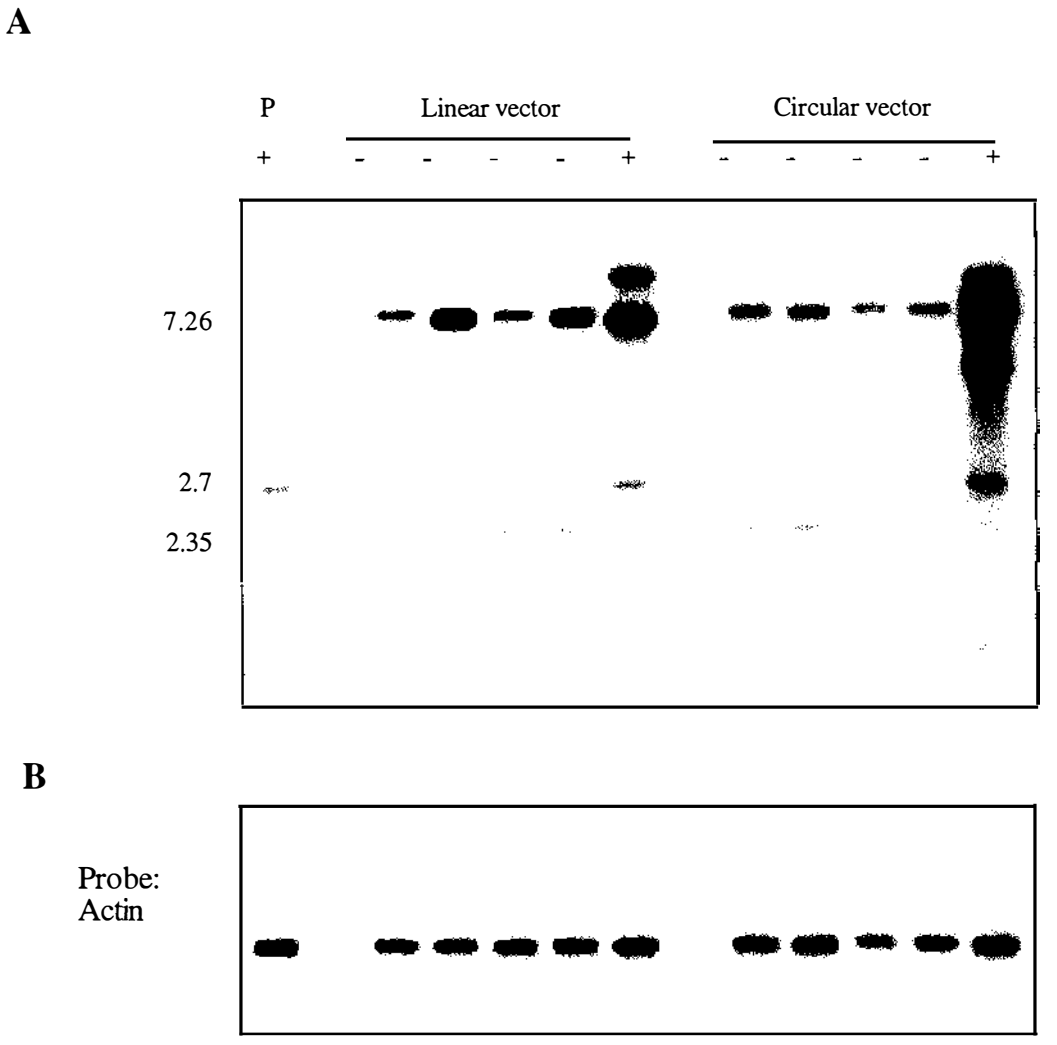


Fig 22 Southern Blot Analysis of Nia$^+$/Nia$^-$ Transformants of 1-85: Vector Conformation

Total genomic DNA digested with *Eco*RI was run on a 1% TBE agarose gel, and the blot was probed with [α^{32}P] dCTP-labelled pniaD3 (**A**) and, after stripping, [α^{32}P] dCTP-labelled actin (**B**). The size (in kb) of DNA fragments is indicated on the left. Strain 1-85 (lane P) and transformants produced with circular and linear pniaD3: - (targeted, Nia$^-$); +(ectopic, Nia$^+$).

containing 40 µl of protoplasts, and 10 µl of PEG solution, was 1 µg, 2 µg, 4 µg and 8 µg. The observed variation in the frequency of targeting for each amount of DNA was not statistically significant (Table 25).

A selection of eight Nia^+ and eight Nia^- transformants obtained using 1 µg and 8 µg of DNA (four for each) in the reaction mix were subjected to Southern blot analysis to study integration patterns. There appeared to be no difference in the number of copies integrated in transformants of 8 µg of DNA compared to 1 µg of DNA. However, due to the small sample size, any significant effect on copy number by increasing the amount of DNA in the reaction mix could not determined. As observed for other Southerns (Fig. 21 and 22) the integration patterns for the transformants with ectopic integration of the vector were more complicated than for those with homologous integration (Fig. 23).

5.3.3.4 Transcriptional Status (on/off) of the Targeted Loci

To observe the effect of the transcriptional status of the targeted loci on gene targeting efficiency, circular pniaD2 was used to target the *niaD* locus. Expression at this locus is induced on minimal media containing nitrate and nitrite, repressed by ammonia and not induced on glutamate. This was confirmed for strain 1-85 by Northern blot analysis (Fig. 24). *A. nidulans* strain 1-85 was grown on MU media and minimal media containing the nitrogen sources nitrate, ammonia, glutamate and a mixture of glutamate and nitrate. Induction was observed after growth on minimal media containing nitrate or nitrate plus glutamate, but not ammonia or glutamate. Slight induction was observed after growth on MU media, although not as strong as observed for media containing nitrate.

It is not known at which stage in the transformation process the vector DNA is integrated therefore the transcription inducing or neutral substrates were introduced at various stages during the transformation procedure. Selection of transformants on *Aspergillus* complete media and minimal media containing inducing or non-inducing nitrogen sources was compared but no obvious difference in the targeting efficiency was observed (Table 26). Addition of the inducing and neutral substrates to the reaction mix (Table 26, Series II), or to the reaction mix in addition to the selection media (Table 26, Series III), also resulted in no change in the targeting efficiency. However, an overall difference in the level of targeting was observed for selection on *Aspergillus* complete media (MYG), 24%, compared to minimal media (AMM) with an inducing or neutral nitrogen source, 18% (pooled results from Table 26). This observed trend is not, however, supported statistically (Appendix 3).

Table 25 Effect of Vector Concentration on the Frequency of Homologous Integration

Circular Plasmid	Amount of DNA per 50µl reaction mix[a]	No of transformants[b]		Percentage[c] Targeted Disruption
		Ectopic	Targeted	
pniaD2	1 µg	45	12	21%
pniaD2	2 µg	44	13	23%
pniaD2	4 µg	41	13	24%
pniaD2	8 µg	35	16	31%

[a] A standard 50 µl reaction mix (protoplasts and PEG buffer) would contain 2.5 µg of DNA.

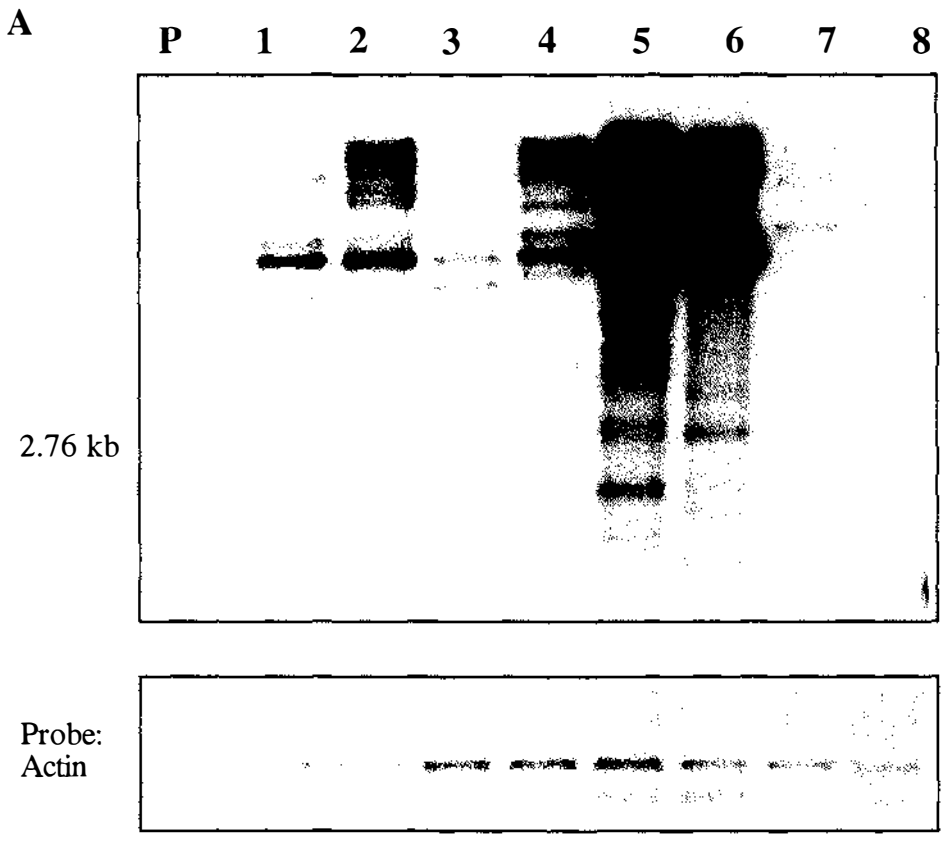
[b] The values were from a single experiment.

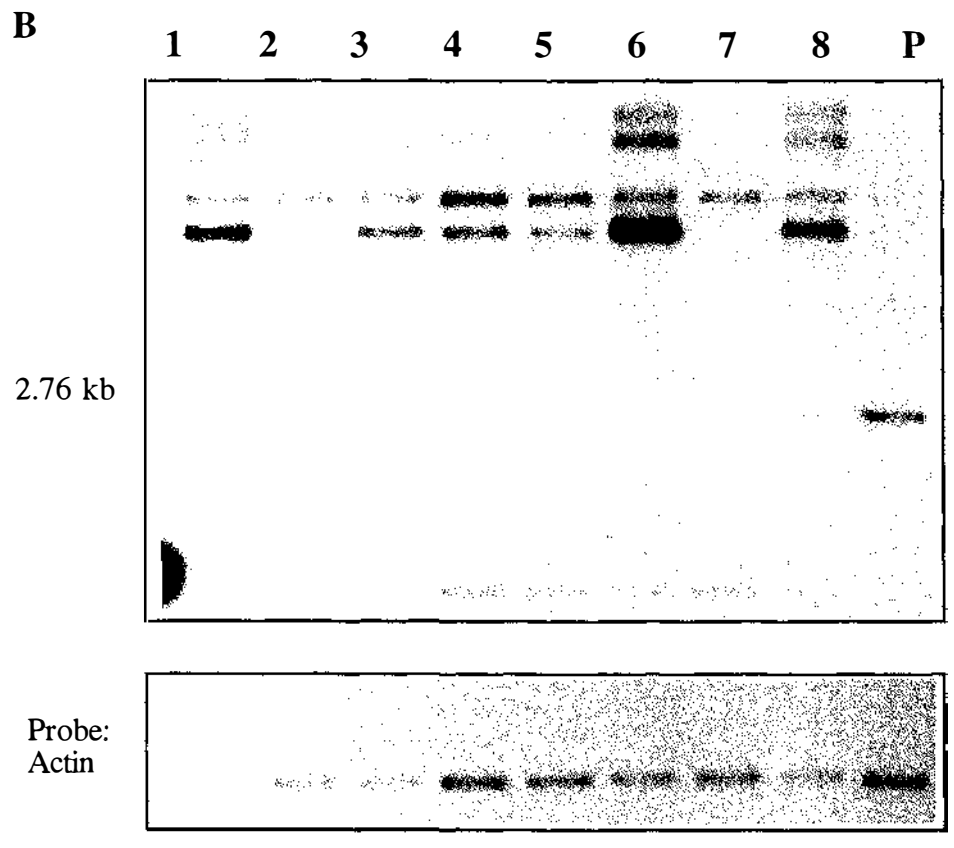
[c] Test of significance of data: $\chi^2 = 1.75$; df = 3, $0.7 > P > 0.5$.

Fig 23 Southern Blot Analysis of Nia^+/Nia^- Transformants of 1-85: Vector Concentration

Total genomic DNA digested with *Eco*RI was run on a 1% TBE agarose gel. The blot was probed with [α^{32}P] dCTP-labelled pniaD3 and then, after stripping, [α^{32}P] dCTP-labelled actin. The size (in kb) of DNA fragments is indicated on the left. **A** Strain 1-85 (lane P) and Nia^+ transformants produced with 1 μg (lanes 1 to 4) and 8 μg (lanes 5 to 8) circular pniaD2.

B Strain 1-85 (lane P) and Nia^- transformants produced with 1μg (lanes 1 to 4) and 8 μg (lanes 5 to 8) circular pniaD2.


A
P 1 2 3 4 5 6 7 8
2.76 kb
Probe:
Actin


B
1 2 3 4 5 6 7 8 P
2.76 kb
Probe:
Actin

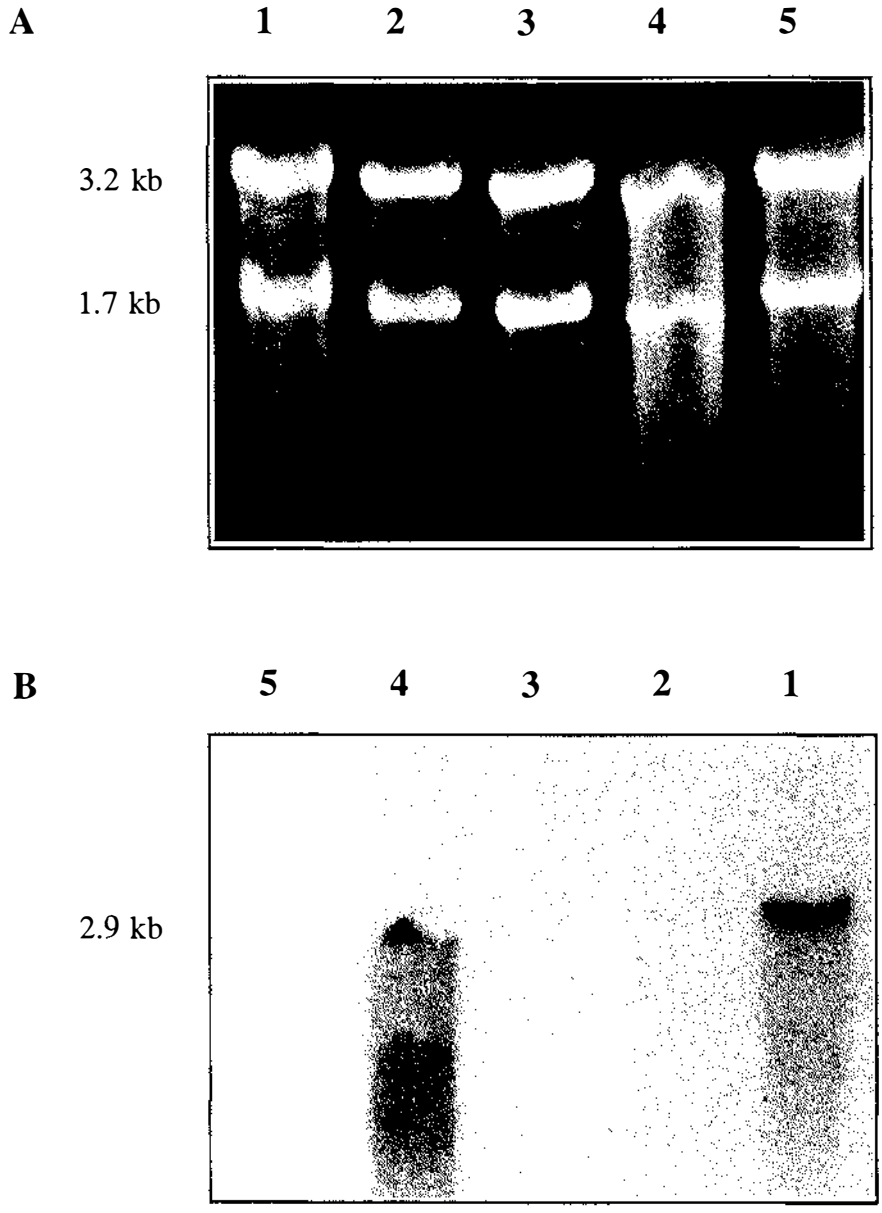


Fig 24 Northern Blot Analysis of Strain 1-85

Northern blot analysis of 1-85 grown on *Aspergillus* complete media supplemented with uracil (MU) and *Aspergillus* minimal media (AMM) supplemented with uracil and different nitrogen sources. **A** Total glyoxylated RNA was electrophoresed in 1% agarose MOPS gel. **B** Autoradiograph of gel in **A** probed with [α^{32}P] dCTP-labelled *niaD* fragment. The approximate size (in kb) of the RNA is indicated on the left. RNA from 1-85 grown on AMM + 5 mM glutamate + 5 mM nitrate (lane 1), AMM + 10 mM glutamate (lane 2), AMM + 10 mM ammonia (lane 3), AMM + 10 mM nitrate (lane 4) and MU (lane 5).

Table 26 Effect of Transcriptional Status of the Target Loci on the Frequency of Homologous Integration

Series Expt.	Reaction[a, b, c]	Selection media[b]	No. of transformants Ectopic	Targeted	Percentage Targeted Disruption
I[d] 1	std	MYG	20	7	26%
	std	MM+glutamate	21	6	22%
	std	MM+nitrate	30	5	14%
2	std	MYG	54	18	25%
	std	MM+glutamate	62	13	17%
	std	MM+nitrate	79	17	18%
3	std	MYG	13	1	7.1%
	std	MM+glutamate	16	5	24%
	std	MM+nitrate	14	4	22%
	std	MM+nitrite	16	1	5.9%
Combined	std	**MYG**	87	26	**23%**
1, 2, & 3	std	**MM+glutamate**	99	24	**20%**
	std	**MM+nitrate**	123	26	**17%**
	std	**MM+nitrite**	16	1	**5.9%**
II 1	**water**	MYG	27	10	**27%**
	glutamate	MYG	30	10	**25%**
	nitrate	MYG	30	11	**27%**
	nitrite	MYG	38	11	**22%**
III 1	**glutamate**	**MM+glutamate**	53	11	**16%**
	nitrate	**MM+nitrate**	49	12	**19%**
	nitrite	**MM+nitrite**	39	11	**22%**

[a] Std refers to the standard reaction mix (Section 2.18).

[b] Supplementation of reaction mixes or selection media (Complete media, MYG or Minimal media, MM) with a nitrogen source was at a total final concentration of 10 mM. If the nitrogen source in the selection media was nitrate or nitrite then glutamate was also added so the *niaD*$^-$ transformants were able to grow.

[c] Transformation of *A. nidulans* was with the circular vector pniaD2

[d] The three experiments for Series I utilise the same batch of frozen protoplasts (-70^{o}C).

5.3.3.5 Temperature of the Reaction Mix Incubation and Transformant Regeneration

Circular pniaD2 was used to test the effect of changing the temperature of incubation of the reaction mix, and the temperature of transformant regeneration, on gene targeting efficiency. After the addition of the second volume of PEG solution to the reaction mix (Section 2.18) it was incubated at 22°C, 37°C or 42°C and on ice for 20 minutes. No obvious trend in gene targeting efficiency was observed by altering this parameter (Appendix 3). Regeneration of the transformants at 37°C or 25°C resulted in targeting efficiencies of 23% and 32% respectively which were statistically significant (Table 27, series I) (Appendix 3). When repeated with pniaD2 for 37°C, 25°C and 18°C the trend was again observed with respective targeting efficiencies of 12%, 27% and 16% (Table 27, series II). For the total of six experiments using the pniaD2 plasmid (four combined in series I and two combined in series II) an increase in targeting was observed with a lowering of the selection temperature (from 37°C to 25°C) for five experiments, and no effect was observed for one experiment. The probability of this occurring by chance is approximately 3%, given that there is an equal probability of the targeting frequency increasing or decreasing. When subsequently targeting the *amdS* locus with pamdS2 and pamdS3, the total number of Amd$^-$ transformants obtained was not high enough to lend support or otherwise to this trend (Table 27, series III and IV).

5.4 Discussion

A. nidulans was used as a model organism to systematically investigate ways of enhancing gene targeting in filamentous fungi. The *niaD* and *amdS* genes, both involved in nitrogen source utilisation, were selected as target loci as disruptants were easily distinguishable from the parental phenotype. Integrative disruption of the loci was via a single cross-over event between an internal segment of the gene in the vector and the homologous genomic region.

Vectors were constructed containing different sized internal fragments of the *niaD* and *amdS* genes. Each series of three plasmids consisted of one plasmid containing a relatively large segment of the gene, and two plasmids containing smaller segments which, if joined, would form the larger segment. The frequency of homologous integration in filamentous fungi is extremely variable between species and loci (Fincham, 1989). Using circular DNA both the *niaD* and *amdS* loci were successfully targeted to produce Nia$^-$ and Amd$^-$ transformants. Using circular plasmids the frequency of gene targeting was proportional to the length of homologous sequence. This was more evident with the pniaD plasmid series as the *niaD* locus was targeted much more efficiently than the *amdS* locus. The percentage of targeted transformants obtained

Table 27 Effect of the Incubation and Selection Temperature on the Frequency of Homologous Integration

Series	Expt.	Incubation Temp.	Circular Plasmid	Selection Temp.	No of transformants Ectopic	Targeted	Percentage Targeted Disruption
I[a]	1	ice	pniaD2	37°C	36	9	20%
		ice	pniaD2	25°C	50	24	32%
	2	22°C	pniaD2	37°C	34	13	28%
		22°C	pniaD2	25°C	50	23	32%
	3	37°C	pniaD2	37°C	39	9	19%
		37°C	pniaD2	25°C	48	21	30%
	4	42°C	pniaD2	37°C	32	11	26%
		42°C	pniaD2	25°C	30	17	36%
	Combined		**pniaD2**	**37°C**	**141**	**42**	**23%**
	1, 2, 3 & 4		**pniaD2**	**25°C**	**178**	**85**	**32%**
II[a]	1	22°C	pniaD2	37°C	39	6	13%
		22°C	pniaD2	25°C	24	16	40%
		22°C	pniaD2	18°C	32	10	24%
	2	42°C	pniaD2	37°C	40	5	11%
		42°C	pniaD2	25°C	34	5	13%
		42°C	pniaD2	18°C	39	4	9.3%
	Combined		**pniaD2**	**37°C**	**79**	**11**	**12%**
	1 & 2		**pniaD2**	**25°C**	**58**	**21**	**27%**
			pniaD2	**18°C**	**71**	**14**	**16%**
III	**Combined**		**pamdS2**	**37°C**	**52**	**0**	**0%**
	1 & 2		**pamdS2**	**25°C**	**71**	**0**	**0%**
IV	**Combined**		**pamdS3**	**37°C**	**69**	**1**	**1.4%**
	1, 2, 3 & 4		**pamdS3**	**25°C**	**68**	**0**	**0%**

[a] Series I and II each represent one transformation mix incubated at the indicated temperature after dividing the transformation mix and adding of 9 volumes of PEG (Section 2.18).

by the pniaD3 vector (43%) was approximately additive of vectors pniaD1 (14%) and pniaD2 (27%). The targeting efficiency did not appear to be determined by the inclusion of a specific region of the gene in the targeting vector. This indicates that homologous integration of circular molecules is dependent on the length of homology for these loci, not the introduction of a hot spot for recombination, over the loci and lengths tested.

The effect of introducing a double stranded break within the region of homology was investigated. One vector targeting the *niaD* locus, pniaD3, and two vectors targeting the *amdS* locus, pamdS1 and pamdS3, were linearised with the same restriction enzyme. For each vector an increase in the level of targeting of approximately two-fold was observed following the linearisation of the vectors. There was again an element of homology length dependency as the larger pamdS3 vector targeted more efficiently than the pamdS1 vector for both circular and linear molecules. The pamdS plasmids continued to target at a much lower frequency than the pniaD plasmids. The distribution of homology between the two ends of the linearised vectors differed considerably between linearised pniaD3 and pamdS1 and pamdS3. The pniaD3 vector had a relatively even distribution while the pamdS1 and pamdS3 plasmids had an uneven distribution of homology. This was found to have a marked effect on the frequency of integration in mammalian systems where an even distribution of homology resulted in increased targeting efficiency at the *Cftr* locus (Dickinson *et al.*, 1993). This is in contrast to work targeting the *Hprt* locus where, although targeting was shown to increase when a linearised vector was used, this was independent of the distribution of homology (Hasty *et al.*, 1991). Increases in targeting by vector linearisation have been reported for other eukaryotic systems, including filamentous fungi (Capecchi 1989; Fincham 1989; Hynes 1986; Szostak *et al.*, 1983; Zimmer 1992). For some the increases have been particularly dramatic with vector linearisation resulting in 99% targeting regardless of the length of homology (Shiotani and Tsuge 1995). One group reported an increase in targeting in *Ustilago maydis* with vector linearisation within or outside the region of homology (Fotheringham and Holloman 1989).

Southern analysis of Nia$^-$ transformants obtained from circular and linear pniaD3 indicates that integration in both is by a single cross-over event as the integration patterns were identical. This suggests that the linearised vector either recircularised prior to integration or integrated at the point of the double strand break, as predicted by the double strand break repair model (Orr-Weaver and Szostak 1985).

The difference in targeting efficiency at the two loci, using vectors with segments the same length, raises the question of why different loci are targeted with different efficiencies. One possibility is the relative levels of expression of different genes. There is evidence to suggest that in some eukaryotic systems this plays an important role in the efficiency of gene targeting (Schroppel and Soll 1995; Thomas and Rothstein 1989; Thyagarajan *et al.*, 1995). This was

therefore investigated using the circular pniaD2 plasmid targeting the *niaD* locus which is induced by nitrate and nitrite (Hawker *et al.*, 1992). No significant change in the targeting frequency for the *A. nidulans niaD* locus was observed when the transformation mix was incubated with inducing or neutral substrates at various stages in the transformation procedure. This would suggest that, contrary to the situation in the yeast *Candida albicans* (Schroppel and Soll 1995) and mammalian systems (Thyagarajan *et al.*, 1995), induction of the target locus has no effect on targeting efficiency at the *A. nidulans niaD* locus. The observed difference in targeting frequency at the *niaD* and *amdS* loci may therefore be due to some factor other than the level of gene expression, such as the chromosomal position or the level of methylation.

The effect of altering the temperature at which the transformation mix and the selection plates were incubated was tested using the circular pniaD2 plasmid. The targeting frequencies observed when the transformation mix was incubated at different temperatures were variable and did not show any general trend. However, when transformants were grown on plates incubated at 25°C instead of 37°C a higher level of targeting was observed in five out of six transformations. This suggests that integration occurs as the protoplasts start to regenerate. This is supported by the observation of many groups of the appearance of abortive transformants which are believed to be due to the transient expression of the selectable gene (Ballance and Turner 1985; Hartingsveldt *et al.*, 1987; Tilburn *et al.*, 1983; Wernars *et al.*, 1985) and which may be stabilised by growing on non-selective media (Tilburn *et al.*, 1983; Wernars *et al.*, 1985). Slowing the regeneration of the protoplasts by lowering the temperature of incubation may help stabilise the DNA interactions and therefore allow more time for targeted integration.

The effect the amount of DNA in the reaction mix on the level of targeting was considered. This was in part to ensure that any increase in targeting was due to the parameter being investigated, and not some subtle difference in the amount of DNA used in the transformation reaction. The circular pniaD2 plasmid was used at concentrations of 0.4, 0.8, 1.6 and 3.2 times the standard amount used in a transformation reaction mix. No significant difference in the targeting frequency was observed for any of these concentrations.

The aim of many groups working in molecular biology is to genetically modify organisms of choice in a defined manner. The well characterised filamentous fungus *A. nidulans* was chosen to systematically investigate the effect of altering a number of parameters on the level of gene targeting. During this work it was noted that the transformation protocol results in a great level of variation in both transformation frequency and level of gene targeting. It was also noted that transformant strains showing homologous integration had simpler integration patterns than those showing non-homologous integration. This is in line with the observation in *Neurospora*

crassa that protoplasts form a heterogeneous population that differ in their ability to be transformed, and to integrate DNA at homologous or heterologous sites (Grotelueschen and Metzenberg 1995; Miao *et al.*, 1995).

5.5 Conclusion

This study has shown which of the tested parameters have influences on gene targeting in this model system. It is hoped that this work will benefit other groups using transformation as a tool for targeted gene replacement/modification and the following recommendations are made.

Gene targeting is very locus dependent, with some loci targeting at much higher frequencies than others in the same organism. When designing a disruption vector the internal portion of the targeted gene, contained within the vector, should be the maximum length, while still forming a non-functional gene once integrated. Ideally there will be a unique restriction site near the centre of the internal fragment which will enable linearisation of the vector within the region of homology. While transformation may be under the standard conditions, selection at a lower temperature may be beneficial. The temperature of incubation needs to be weighed against the growth rate of the fungus being transformed, therefore lowering the temperature is more suited to fast growing fungal strains.

Using a disruption vector integration is by a single cross-over event within the homologous region resulting in two non-functional copies of the gene interrupted by the vector containing the selectable marker. Alternatively a gene replacement vector may be used. The gene is replaced by a selectable marker flanked by the 5' and 3' regions of the locus to be targeted. Integration is by a double cross-over event in the homologous regions thereby replacing the gene with the selectable marker. Using this system there are less restrictions on the length of homology and the vector is transformed as a linear molecule (Refer to gene disruption of the *cyc* locus outlined in Chapter 6). As there is no duplication of the targeted locus, as with the gene disruption vectors, the resulting construct may also be more stable.

Chapter 6

Disruption of the Cytochrome *c* (*cycA*) Gene in *Aspergillus nidulans*

6.1 Summary

The effect of inactivating the *cycA* gene in *Aspergillus nidulans* was investigated. Using an *A. nidulans* diploid strain one copy of the *cycA* gene was inactivated by means of a gene replacement strategy. Haploidisation of the diploid transformant localised the chromosomal position of *cycA* to chromosome I. Segregation of sexual crosses allowed the isolation of slow growing and poorly conidiating *cycA* mutants. Thus *cycA* mutants were shown to be viable in *A. nidulans* which was unexpected, and the power of reverse genetics using precise targeting of genes was illustrated.

6.2 Introduction

Filamentous fungi form a diverse group of micro-organisms which impact on our lives in both a positive and negative sense. They are frequently exploited for the biosynthesis of products or for use as biocontrol agents. They are also known to produce a number of toxins and form a large group of pathogens of humans, animals and plants. Many of these aspects are affected by the respiration and energy production of the fungal systems. For example oxygen is required for penicillin biosynthesis from *Penicillium chrysogenum* (Bainbridge *et al.*, 1992), and the asexual sporulation of the biocontrol agent *Colletotrichum truncatum* (Slinger *et al.*, 1993). Consequently fungal metabolism is an important area of research.

Research into this and many other areas has been facilitated by reverse genetics. The mutant phenotype of genes that have been cloned can be studied by precise gene disruption strategies.

6.2.1 Cellular Energy Sources

The following brief overview was summarised from the biochemistry text by Stryer (1981).

6.2.1.1 Presence of Oxygen

There are three stages in the extraction of energy from foodstuffs (Fig. 25). Stage one involves the breakdown of large molecules into smaller units which generates no useful energy. Stage

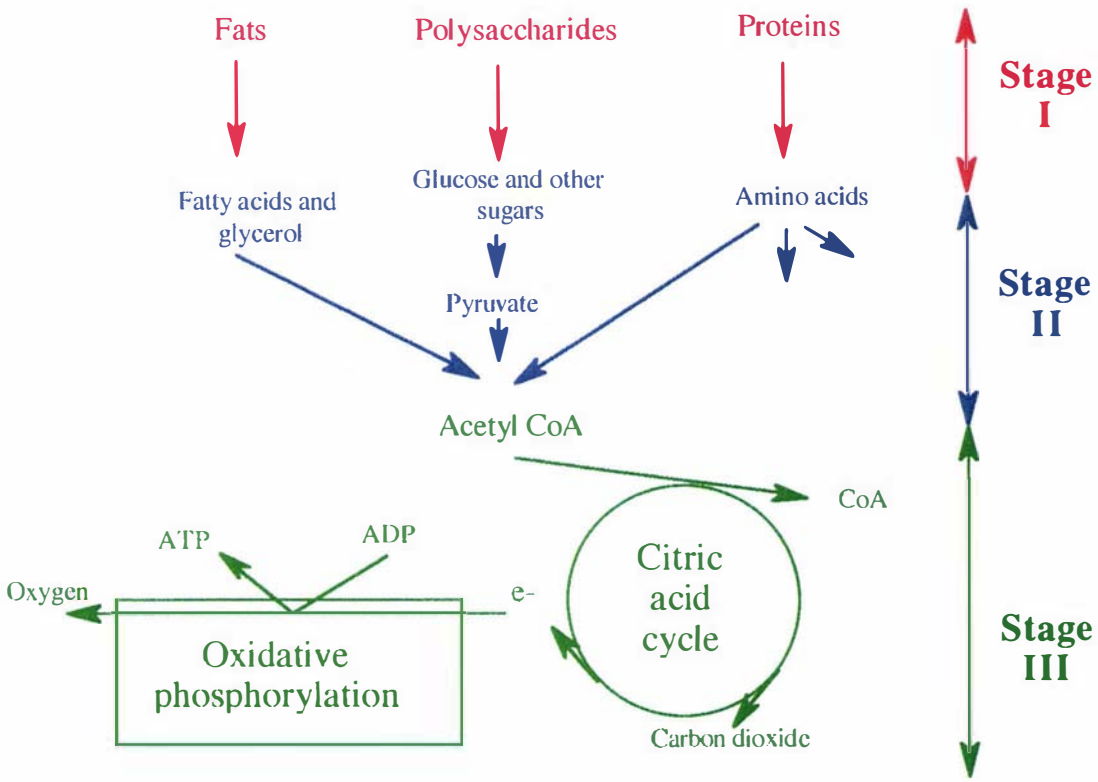


Fig 25 Stages (I, II and III) in Energy Extraction From Food

two involves the degradation of small molecules into a few simple units which generates a small amount of useful energy. For carbohydrate catabolism this involves the Embden-Meyerhof glycolytic pathway which degrades 6-carbon compounds to pyruvate. Stage three involves the citric acid cycle and oxidative phosphorylation, generating most of the useful energy. Oxidative phosphorylation is the process in which adenosine triphosphate (ATP) is formed as electrons are transferred from nicotinamide adenine dinucleotide phosphate (NADH) and flavin adenine dinucleotide dihydrogen ($FADH_2$), which are produced during the citric acid cycle and the Embden-Meyerhof glycolytic pathway, to oxygen by a series of electron carriers. Transfer of electrons is by a series of cytochrome complexes which pump protons out of the mitochondrial matrix resulting in a membrane potential (Fig. 26). The flow of protons back generates ATP. This pathway can be blocked at various stages by certain chemicals (Fig. 26). The complete oxidation of one mol of glucose to CO_2 and H_2O by the respiratory pathway produces 36 mol of ATP.

ATP is the universal currency of free energy in biological systems. ATP (or GTP, UTP or CTP in some cases) is an energy-rich molecule because its triphosphate unit contains two phosphoanhydride bonds. The ATP-ADP (adenosine diphosphate) cycle is the fundamental mode of energy exchange in biological systems (Fig. 27).

Metabolic control must be flexible as the external environment of cells is not constant. The complex network of reactions needs to be regulated. This is achieved by a variety of mechanisms such as: controlling the amount of certain enzymes by altering their rate of synthesis or degradation; controlling the catalytic activities of certain enzymes by reversible allosteric control or feedback inhibition and covalent modifications of enzymes, such as phosphorylation.

The energy status of the cell is an important determinant of regulation. ATP-generating pathways are inhibited by a high energy charge, whereas ATP-utilising pathways are stimulated by a high energy charge (which is buffered).

Energy charge (0-1) = [ATP] + 1/2[ADP] / [ATP] + [ADP] + [AMP]

To facilitate the regulation and control of metabolism, biosynthetic and degradative pathways are almost always distinct. In eukaryotes this is often enhanced by compartmentalisation.

6.2.1.2 Absence of Oxygen

In order to meet the cellular ATP requirements in the absence of oxygen, anaerobic glycolysis may be activated. The total amount of ATP produced by the fermentation pathway (Fig. 28) is considerably reduced compared to the respiration pathway. The yield from converting one mol

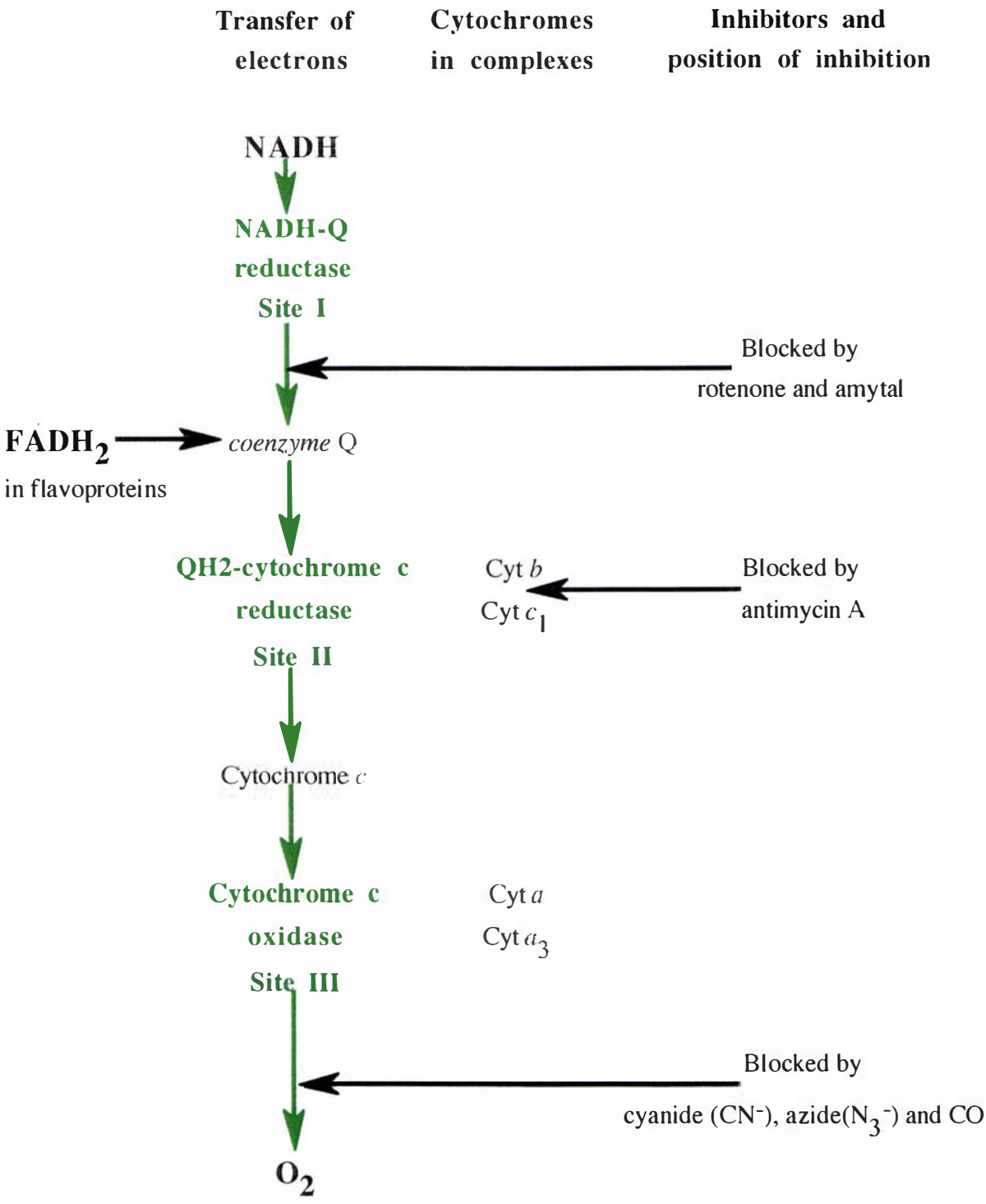


Fig 26 Electron Transfer During Oxidative Phosphorylation

The sequence of electron carriers by which electrons are transferred from NADH, or $FADH_2$, to O_2, and the position of action of inhibitors of electron transport. The protons are pumped across the membrane by the three complexes shown in **green** and ATP is generated (**phosphorylation sites**).

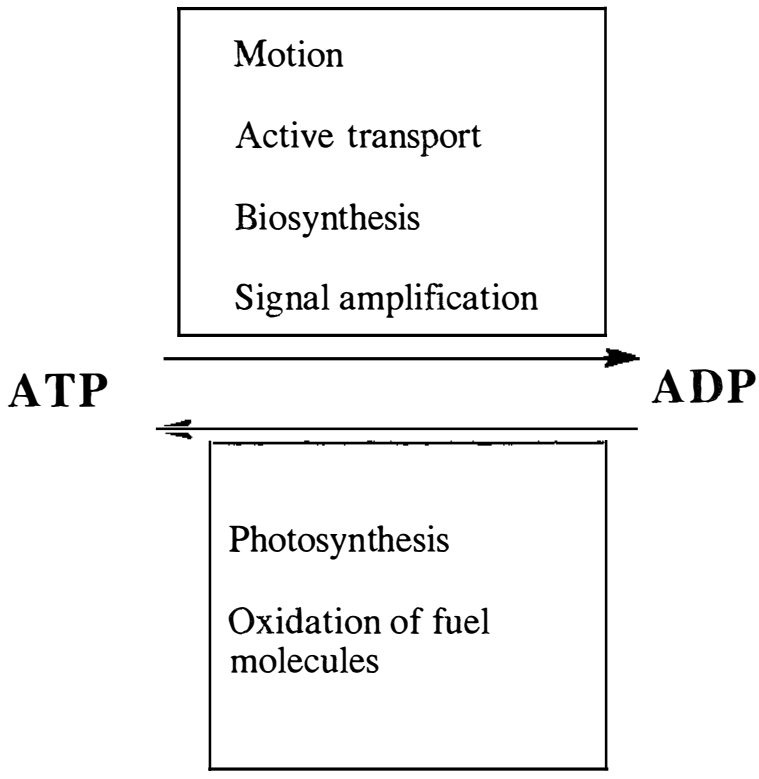


Fig 27 The ATP-ADP Cycle

The fundamental mode of energy exchange in biological systems.

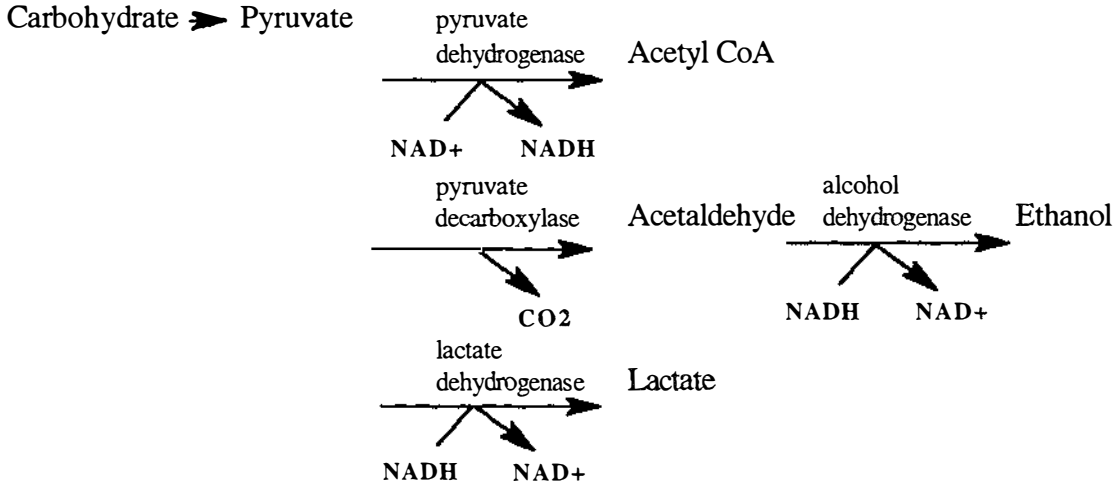


Fig 28 The Fate of Pyruvate

Pyruvate can be converted to ethanol (formed in yeast and other microorganisms), lactate (formed in a variety if microorganisms) and Acetyl Coenzyme A (formed in the mitochondrial matrix).

of glucose to two mol of lactate or ethanol, is 2 mol of ATP (compared to 36 for complete oxidation) and is produced during the section of the pathway shared by both the fermentation and respiration pathways (Embden-Meyerhof glycolytic pathway). The conversion of pyruvate to ethanol, or lactate, does not yield any ATP but recycles the NADH to NAD.

Fermentation may be inhibited by the presence of oxygen, this is termed the Pasteur effect (Storey 1985). When oxygen is available the cellular energy requirements are met by the respiratory pathway as this is the more economical method of cellular energy production. When oxygen is lacking the rate of fermentative metabolism rises to meet cellular energy demands.

An alternative response to oxygen limitation is to depress the metabolic rate. Many facultative anaerobes facing exposure to natural environmental anoxia do not show the Pasture effect but rather use this response mechanism (Storey 1985).

6.2.2 Fungal Cytochrome *c* Genes and Respiratory Mutants

Cytochrome *c* is an integral part of the electron transport chain (Fig. 26) and, although encoded by a nuclear gene, functions in the mitochondria. The *cycA* gene, coding for cytochrome *c*, for the obligate aerobe *A. nidulans* has been cloned and shown to be present at a single locus by DNA hybridisation (Raitt *et al.*, 1994).

For the filamentous fungus *Neurospora crassa* mutants defective in the cytochrome *c* gene have been isolated and the defective phenotype mapped to a single locus per haploid genome [*cyc1-1* (Stuart *et al.*, 1987)]. Similarly cytochrome *c* mutants have been isolated for the yeasts *Kluyveromyces lactis* [*9cyc1* (Chen and Clark-Walker 1993)] and *Schizosaccharomyces pombe* (Russell and Hall 1982) and mapped to a single locus per haploid genome. In contrast, the facultatively anaerobic yeast *Saccharomyces cerevisiae* has two cytochrome *c* proteins, encoded by the genes *CYC1* and *CYC7*, with amino acid sequences that are 80% identical. The two isoforms display different levels of stability which is believed to be involved in regulation under different physiological conditions, such as the concentration of available oxygen, carbon source, growth phase etc (Dumont *et al.*, 1990). In an aerobic exponentially growing culture, iso-1-cytochrome *c* (*CYC1*) comprises approximately 95% of the total cellular cytochrome *c* and iso-2-cytochrome *c* (*CYC7*) makes up the remaining 5% (Creusot *et al.*, 1988).

To study respiratory pathways mutants in the cytochrome *c* loci have been sought. Mutants of *S. cerevisiae CYC1* have 5% of the normal levels of cytochrome *c*, due to expression of *CYC7*, and are able to grow normally on the nonfermentable carbon sources glycerol and ethanol, but only very slowly on nonfermentable lactate. Mutants with no cytochrome *c* grow

very slowly on all nonfermentable substrates (Sherman *et al.*, 1974). Mutants of cytochrome *c* have been obtained for *N. crassa*. These were characterised by slow growth and a reduction in the spectrophotometrically-detectable levels of cytochrome *c*, but not a complete absence of cytochrome *c* (Bottorff *et al.*, 1994). Complete absence of cytochrome *c* has been obtained for the yeast *K. lactis,* where mutants were observed to be small and only able to grow on fermentable carbon sources (Chen and Clark-Walker 1993).

6.2.2.1 Alternative Respiration

Alternative respiratory pathways are known to be present in filamentous fungi as they have been identified in *Podospora* (Marbach 1994), *Aspergillus* (Turner and Rowlands 1976) and *Neurospora* (Bridge and Bertrand 1984), and in the yeast *Schwanniomyces castellii* (Segueilha *et al.*, 1992).

Alternative respiratory pathways have been studied in *S. castellii,* which possesses three respiratory pathways (Dubreucq *et al.*, 1990). The first, which is sensitive to antimycin and azide, is the cytochromic pathway. The two other pathways are antimycin-insensitive; one is sensitive to azide and not sensitive to salicylhydroxamic acid (SHAM) (azide sensitive pathway) and the other is sensitive to SHAM and not sensitive to azide (SHAM sensitive pathway). The SHAM sensitive pathway separates from the respiratory pathway at coenzyme Q. These two alternative pathways can form up to 50% of the total respiration and are repressed when the glucose concentration is very high (Dubreucq *et al.*, 1990). All three respiratory pathways involve phosphorylation site I (Fig. 26), as demonstrated by inhibition by rotenone, which allows the alternative pathways to produce ATP (Sequeilha *et al.*, 1992).

Work with *N. crassa* suggests the alternative respiratory pathway is fully activated when the cytochrome pathway is reduced to 40% (Bridge and Bertrand 1984) and involves an alternative terminal oxidase (Lambowitz *et al.*, 1972). Oxidative phosphorylation in mitochondria from wild type and poky (mitochondrial mutant) strains of *N. crassa* were studied. The alternative oxidase is resistant to antimycin A and cyanide and in the poky mutants was shown to accommodate any surplus electron flux (Lambowitz *et al.*, 1972). This alternative oxidase of *N. crassa* mitochondria has been identified immunologically and shows similarities to plant alternative oxidases (Lambowitz *et al.*, 1989).

Under conditions of citric acid fermentation *Aspergillus niger* was shown to possess at least two respiratory systems. One was sensitive to cyanide (CN) (involving the cytochrome chain) and the other to SHAM (Fig. 29). Mycelial respiration was partially inhibited by CN or SHAM and completely inhibited in the presence of both. As the length of the culture time increased,

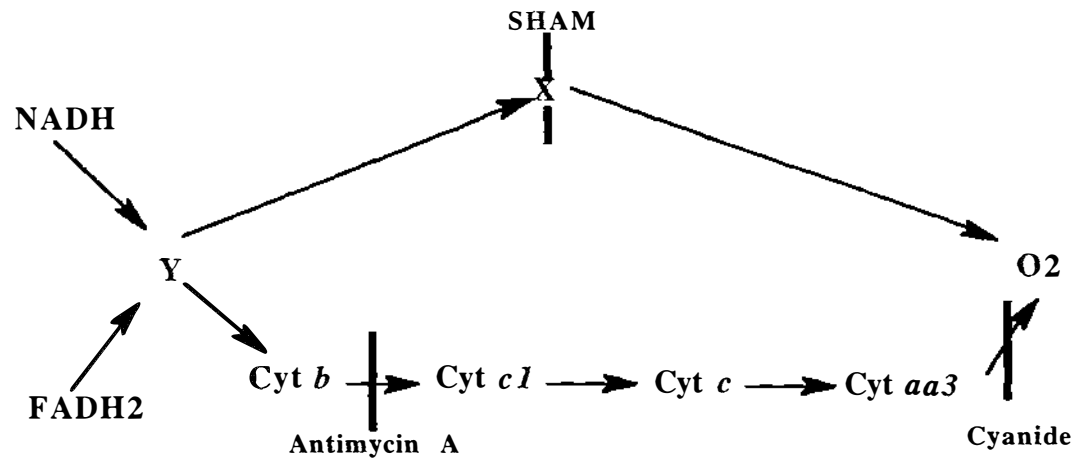


Fig 29 Branched Electron Transport System in *A. niger*

X denotes the antimycin A- and CN-insensitive oxidase system, and Y the hypothetical branch point (Kirimura *et al.*, 1987).

CN sensitivity decreased while SHAM sensitivity increased. The SHAM sensitive respiration was localised to the mitochondria (Kirimura *et al.*, 1987).

It appears that alternative pathway respiration branches from the cytochrome pathway at ubiquinone (coenzyme Q) and donates electrons to oxygen to form water. This does not contribute to the transmembrane potential as the electrons that flow down this pathway lose two of three potential coupling sites for proton transport, and thus ATP production (Fig. 26 and Fig. 29). There is much speculation as to the function and regulation of alternative oxidases in fungi and plants (McIntosh 1994).

6.2.3 Construction of Mutants by Transformation

Introduction of cloned DNA sequences into chromosomes by homologous recombination has allowed the development of direct one-step (Rothstein 1983) and indirect two-step (Scherer and Davis 1979) procedures for the precise replacement of chromosomal sequences in fungi. Both direct and indirect procedures have been used in *A. nidulans* (Miller *et al.*, 1985). This is a practical method of introducing well defined mutations of specific genes.

Often mutation of the gene of interest will have an unknown phenotype. If it is suspected the mutation may be lethal, or the effect on cell viability severe, then consideration has to be given to the parental strain used for the transformation, and the selection criteria for the transformants. If the mutant gene can be rescued by the presence of the wildtype gene, then heterokaryons or diploid transformants can overcome this problem. A technique called 'Sheltered RIP' was used to inactivate the essential gene for a mitochondrial protein import receptor in *N. crassa.* The mutant nuclei was sheltered in a heterokaryon which could be grown on specific media to alter the nuclear ratios so that the mutant nuclei predominated, thereby allowing the mutant phenotype to be studied (Harknes *et al.*, 1994). A diploid parental strain (D10) was used to isolate a mutant of the *pgk* gene of *A. nidulans,* and then haploidisation of the transformants successfully identified the mutant phenotype (Streatfield and Roberts 1993). Likewise a diploid parental strain was used to isolate a mutation of the actin gene of *S. cerevisiae.* The mutation segregated as a recessive lethal tightly linked to the selectable genetic marker on the integrated plasmid (Shortle *et al.*, 1982).

6.2.4 Aim

No mutants of the cytochrome *c* gene in *A. nidulans* have been isolated. As the cytochrome *c* gene of *A. nidulans* (*cycA*) has been cloned (Raitt *et al.*, 1994) the aim of this study was to isolate a Cyc^- strain of *A. nidulans* using a gene replacement strategy. This would allow the

phenotype of a Cyc^- strain to be determined, and the locus to be mapped. The role of cytochrome *c* in the alternative respiratory pathway, as well as the cyanide-sensitive pathway, could then be addressed.

6.3 Results

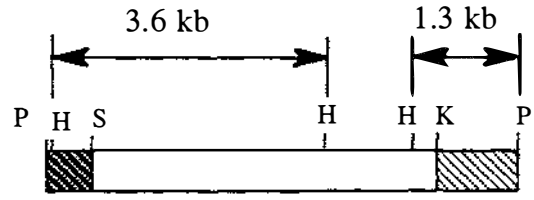
6.3.1 Construction of a *cycA* Gene Replacement Vector

The *cycA* gene was inactivated using a gene replacement strategy. To construct the *cyc* gene replacement vector a 1 kb *Kpn*I-*Pst*I fragment containing the sequence 3' of the *cyc* coding region was isolated. This was subcloned into the *Pst*I and *Kpn*I sites of the multiple cloning site of pGM32, which contains the *N. crassa pyr-4* gene. A 0.6 kb *Hin*dIII-*Sph*I fragment containing the sequence 5' of the *cyc* coding region was subcloned into pIC-20R. After deletion of the resident *Pst*I site the 0.6 kb fragment was removed by digestion with *Bam*HI and *Eco*RV, and cloned into the *Bam*H1 and *Sma*I sites of pGM32 containing the 3' 1 kb fragment. The resulting vector, pR54, when linearised with *Pst*I, contained the 5' and 3' regions of the *cyc* gene in the same orientation, separated by the pGM32 vector (Fig. 30). This vector was prepared by R. Bradshaw.

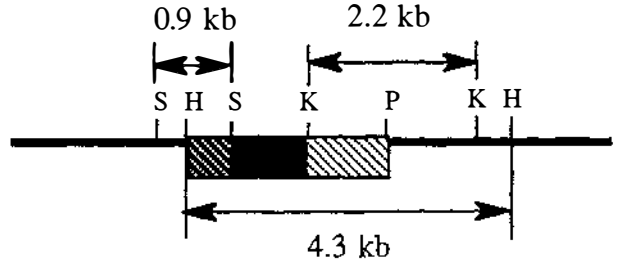
6.3.2 Transformation of the Diploid *A. nidulans* Strain, D10, with the Gene Replacement Vector and Southern Analysis of Transformants

To inactivate the *cycA* gene of *A. nidulans* a direct gene replacement strategy was employed which involved a double cross-over event (Fig. 30). As it was anticipated that a cytochrome *c* deficient strain may be inviable, a diploid was initially chosen as the recipient strain. It was assumed that disruption of the one copy of the *cycA* gene could be rescued by the presence of a wild type copy. A homologous *pyrG*$^{-}$/*pyrG*$^{-}$ diploid strain of *A. nidulans*, D10, which is uracil-dependent, was transformed with the gene replacement construct, pR54, containing the orotidine 5' monophosphate decarboxylase (*pyr-4*) gene of *Neurospora crassa*, which complements the *pyrG* mutation. Transformants were initially selected for uracil independence (Pyr^{+}), then screened for disruption of the *cycA* gene by Southern analysis. A large number of diploid transformants had already been isolated, by R. Bradshaw and co-workers, and the partial characterisation of these transformants commenced. A range of transformants were selected from this work and their status reassessed by Southern analysis as described. Genomic DNA was isolated and subjected to double digestion with the enzymes *Pst*I and *Hin*dIII, and single digestion with the enzymes *Sph*I and *Kpn*I. Southern analysis of the *Pst*I and *Hin*dIII digests using the 2.3 kb *cycA* fragment as a probe confirmed integration of the vector DNA (Fig. 31A). Using *Pst*I and *Hin*dIII the same pattern of integration, 2.3 kb band representing the intact *cyc* gene and bands of 1.3 kb and 3.6 kb representing the integrated vector, was observed for homologous and ectopic integration of the vector. To distinguish between the two types of integration events DNA was digested separately with *Kpn*I and *Sph*I for Southern analysis. *Kpn*I digests were initially probed with the 1 kb 3' fragment. Integration

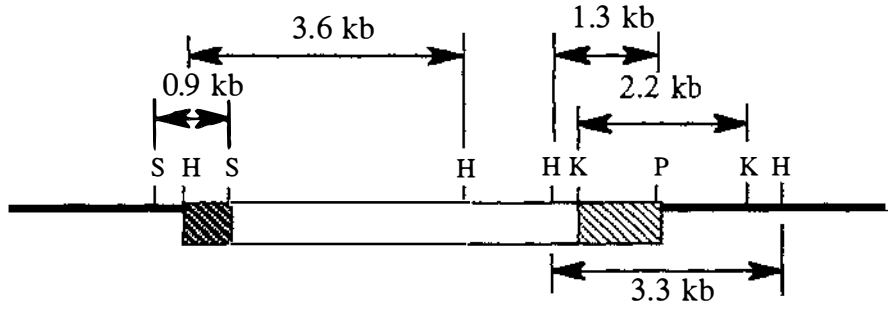
pR54- linearised with *Pst*I



Genomic region



Gene replacement



5' *cyc* (0.6 kb)

cyc coding region (0.9 kb)

3' *cyc* (1.0 kb)

pGM32 vector

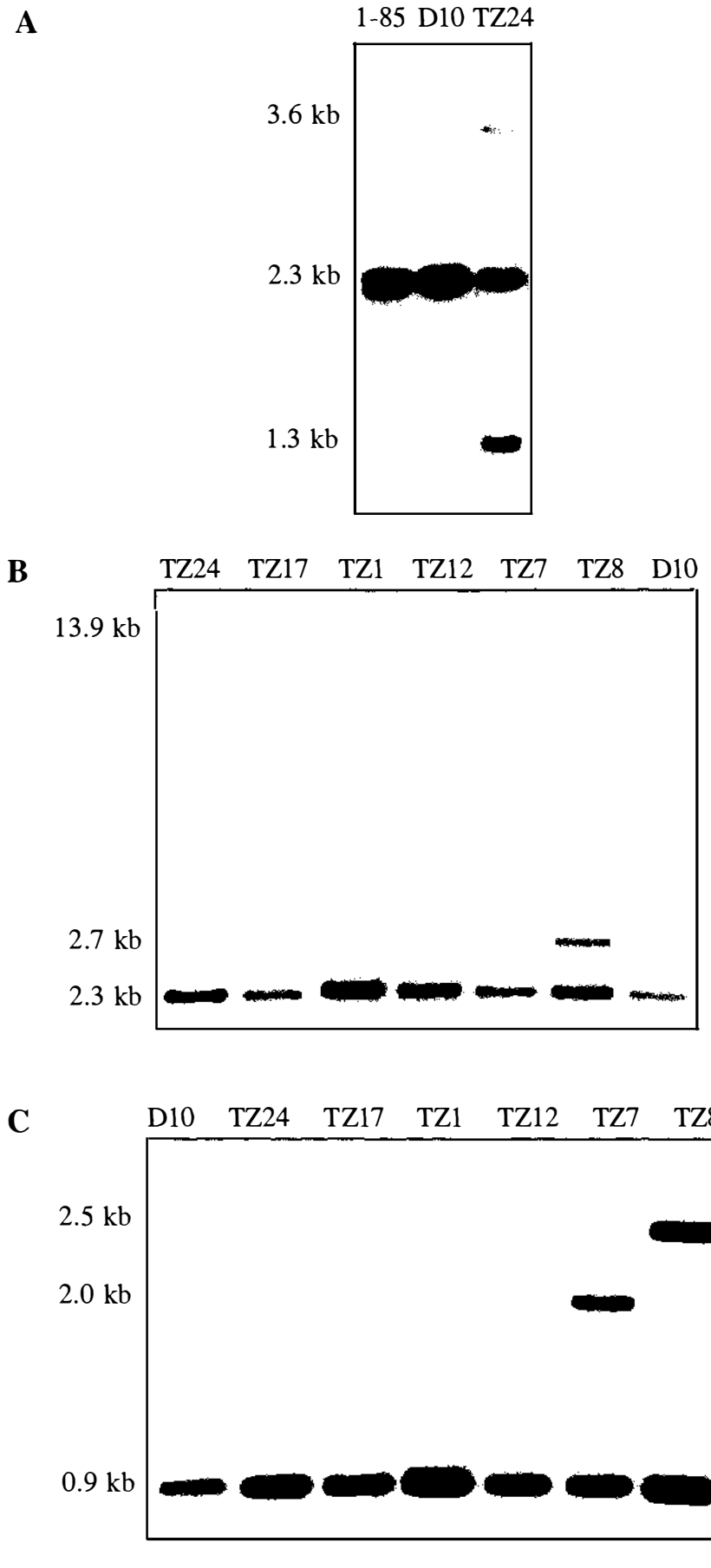
genomic DNA

Fig 30 The *A. nidulans cycA* Locus

Structure of the *A. nidulans cycA* locus before and after integration of the hybrid plasmid pR54. Positions of restriction sites are indicated and distances between relevant sites shown in kilobases (kb). Restriction site abbreviations: H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sph*I.

Fig 31 Southern Analysis of Parental Strains and pR54 Transformants

Southern analysis of the parental strains 1-85 and D10 and the transformants TZ1, TZ7, TZ8, TZ12, TZ17 and TZ24. The fragment sizes in kb are indicated on the left. **A** Total genomic DNA digested with *Pst*I and *Hind*III was run on a 1% TBE agarose gel and the blot probed with the [α^{32}P] dCTP labelled *Pst*I-*Hin*dIII 2.3 kb *cycA* fragment (the same pattern observed for TZ24 was observed for TZ1, TZ8, TZ12 and TZ17, while for TZ7 the 1.3 kb was replaced by a 1.5 kb band indicating rearrangement at the 3' end). **B** Total genomic DNA digested with *Kpn*I was run on a 1% TBE agarose gel and the blot probed with the [α^{32}P] dCTP labelled *Kpn*I-*Pst*I 1 kb 3' *cyc* fragment (the two extra bands, very faint, observed in TZ7 lane are due to partial digestion of the genomic DNA). **C** Total genomic DNA digested with *Sph*I was run on a 1% TBE agarose gel and the blot probed with the [α^{32}P] dCTP labelled *Hin*dIII-*Sph*I 0.6 kb 5' *cyc* fragment. Blots in **B** and **C** were stripped and probed with actin which confirmed the partial digestion of the TZ7 DNA.


A
1-85 D10 TZ24
3.6 kb
2.3 kb
1.3 kb
B
TZ24 TZ17 TZ1 TZ12 TZ7 TZ8 D10
13.9 kb
2.7 kb
2.3 kb
C
D10 TZ24 TZ17 TZ1 TZ12 TZ7 TZ8
2.5 kb
2.0 kb
0.9 kb

at the homologous site resulted in a single band of 2.2 kb representing the 3' region. Integration at an ectopic site resulted in two bands, one of 2.2 kb from the intact *cycA* genes, and one from integration of the vector at an ectopic site (Fig. 31B). The blot was then probed with the entire plasmid giving information on the 5' region. The *Sph*I digests were probed with the 0.6 kb 5' fragment to independently check the 5' integration sites and thereby confirm the status of the transformants as ectopic or homologous integrants (Fig. 31C). The strains indicating homologous integration, and chosen for further study, were TZ1, TZ12, TZ17 and TZ24 (Fig. 31). Controls used for further study were the parental strain, D10, and transformants with ectopic integration; TZ7 and TZ8 (Fig. 31).

6.3.3 Analysis of Haploid Segregants from Diploid Transformants

A gene replacement event was expected to generate a transformed diploid strain which would give rise to two phenotypic classes of haploid mitotic segregants: Cyc^+, Pyr^- and Cyc^-, Pyr^+ (*cycA::pyr-4*$^+$). The segregation of *pyr-4*$^+$ and other genetic markers was tested by inducing haploidisation in the six transformed strains and the parental strain (D10) on complete media containing benomyl. It was not known if the Cyc^-, Pyr^+ segregants would be able to grow on this media, or what their phenotype would be. The haploid segregants isolated in this way were classified for their genetic markers (Table 28).

The diploid transformants containing a disruption of the *cycA* gene (TZ1, TZ12, TZ17 and TZ24) by integration of the pR54 vector gave rise to only Pyr^- haploid segregants indicating that only haploids with an intact *cycA* gene (Cyc^+, Pyr^-) were recovered. The segregation patterns of the other genetic markers with the Pyr^- phenotype, from the disrupted transformants, further suggested that the integration of pR54, and therefore the *cycA* gene, was on chromosome(χ) I. Strains TZ7 and TZ8, which contain the pR54 vector integrated at an ectopic site, gave rise to a mixture of Pyr^+ and Pyr^- haploid segregants as expected. With TZ7 the Pyr^+ marker segregated with Qut^+ indicating it had integrated into chromosome VIII, while with TZ8 it segregated with the *pyroA4* mutation, indicating integration into chromosome IV (Fig. 32). Although both Pyr^+ and Pyr^- segregants were observed for the ectopic controls, TZ7 and TZ8, the numbers of each type selected were not equal. This is probably due to the size of the Pyr^+ haploids being greater than that of the Pyr^- haploids. With benomyl-induced haploidisation the segregants were formed from a single inoculation point, so segregation products were crowded. As the Pyr^+ segregants cover a greater surface area the probability of selecting a Pyr^+ haploid from the segregating culture was greater. It was therefore possible that

Table 28 Haploid Segregant Analysis

Analysis of haploid segregants obtained by benomyl-induced haploidisation of the parental diploid strain, D10, and the transformed diploid strains: TZ1; TZ7; TZ8; TZ12 and TZ17.

Diploid	Uracil dependence[a]		Number of haploid sectors in each class with respect to chromosome markers									
			Chromosome									
			I		I		II		IV		VIII	
			pabaA1/+		*yA/+*[b]		*wA3/+*		*pyroA4/+*		*qutE208/+*	
D10	Pyr^+	0	0	0	0	0	0	0	0	0	0	0
	Pyr^-	53	27	26	16	11	26	27	23	30	27	26
TZ24	Pyr^+	0	0	0	0	0	0	0	0	0	0	0
	Pyr^-	34[c]	32	0	20	0	14	20	16	16	9	10
TZ1	Pyr^+	0	0	0	0	0	0	0	0	0	0	0
	Pyr^-	43	43	0	21	0	22	21	24	19	23	20
TZ12	Pyr^+	0	0	0	0	0	0	0	0	0	0	0
	Pyr^-	22	22	0	12	0	10	12	9	13	12	10
TZ17	Pyr^+	0	0	0	0	0	0	0	0	0	0	0
	Pyr^-	21	0	21	0	7	14	7	9	12	8	14
TZ7	Pyr^-	5	3	2	3	2	0	5	3	2	5	0
	Pyr^+	34	21	13	7	9	18	16	16	18	0	34
TZ8	Pyr^-	4	3	1	1	1	2	2	0	4	3	1
	Pyr^+	35	17	18	10	7	18	17	35	0	19	16

[a] Uracil-independence (Pyr^+) demonstrates the presence of the *N. crassa pyr-4* gene.

[b] The segregation of the *yA* marker was only recorded for wA^+ segregants.

[c] Transformant TZ24 was analysed twice for haploid segregants. The marker qutE was not scored for the first set.

A Chromosome I

pyrG89	*pabaA1*	*yA*	*cycA*$^+$
+	+	+	*cycA::**pyr-4***

B Chromosome I

pyrG89	*pabaA1*	*yA*	*cycA::**pyr-4***
+	+	+	*cycA*$^+$

C Chromosome I Chromosome VII

pyrG89	*pabaA1*	*yA*	*cycA*$^+$	*qutE208*	
+	+	+	*cycA*$^+$	+	***pyr-4***

D Chromosome I Chromosome IV

pyrG89	*pabaA1*	*yA*	*cycA*$^+$	+	
+	+	+	*cycA*$^+$	*pyroA4*	***pyr-4***

Fig 32 Genetic Maps of Transformed Diploid Strains

A TZ24, TZ1 and TZ12; **B** TZ17; **C** TZ7; **D** TZ8. The maps are based on the parental haploid genotypes and the analysis of the haploid segregants (Table 28). The position of chromosomal integration of the *pyr-4* marker is shown although the relative position, with respect to the other markers on the chromosome, is not known.

Cyc^-, Pyr^+ haploid strains were present amongst the segregating TZ24, TZ1, TZ12 and TZ1 colonies, but were obscured due to much slower growth compared to the Cyc^+, Pyr^- segregants.

6.3.4 Isolation and Analysis of Haploid *A. nidulans* Progeny from Diploid Transformants

In a further attempt to isolate haploid Cyc^- strains from the diploid transformants, known to have one copy of *cycA* disrupted, the sexual cycle was utilised. The aim was to isolate independent haploid progeny without the crowding/selection effect experienced with benomyl-induced haploidisation. However, in *A. nidulans*, the sexual cycle only occurs between haploid parents, with a transient diploid phase, hence meiosis could not be induced in diploid transformants. The approach taken was to induce mitotic haploidisation and then to induce sexual crossing of the resultant haploids. The transformed diploid strain TZ24, in which one of the two *cycA* genes was disrupted, was haploidised on benomyl media and the plates sealed and incubated until cleistothecia were observed to have formed (Section 2.4.2.2). This was also done with transformant TZ7, in which the vector had integrated ectopically into chromosome VIII, to serve as a control. Individual cleistothecia were harvested from both transformants. In total three of the harvested cleistothecia produced colonies of differing colours indicating a cross between different haploid strains, as opposed to homothallic self-fertilisation of the haploids. One cleistothecium was from TZ24 (cTZ24) and two were from TZ7 (cTZ7a and cTZ7b). Ascospores were plated and progeny were tested for segregation of genetic markers (Table 29). With the cleistothecia from the ectopic transformant TZ7, cTZ7a and cTZ7b , both Pyr^+ and Pyr^- colonies were isolated. The haploid parental strains were not purified prior to the sexual cross from which the cleistothecium were obtained, therefore the parental genotypes were unknown. As the parental strains arose from haploidisation of the diploid transformants they could contain any combination of the genetic markers. Segregation of the markers within the haploid progeny allowed the determination of the parental genotypes (Fig. 33). Examination of intrachromosomal segregation, where individual chromosomes contained more than one marker (ie χI and χVIII for cTZ7a and cTZ7b), showed that the progeny were predominantly non-recombinants (81%) with a few recombinants (19%) as expected, indicating meiotic crossing over (Fig. 33).

For cleistothecium cTZ24 two populations of colonies were observed on media containing uracil (Fig. 34). One population grew at the same rate as Pyr^- colonies and segregation of the colour markers was observed. The second population was much slower growing and sporulation was poor. Colonies from both types were randomly selected and analysed for

Table 29 Haploid Progeny Analysis

Analysis of haploid progeny derived from cleistothecia harvested from haploidised transformed diploid strains TZ7 and TZ24.

Cleistothecium	Uracil dependence	Number of haploid sectors in each class with respect to chromosome markers									
		Chromosome									
		I		I		II		IV		VIII	
		pabaA1/+		*yA*/+(a)		*wA3*/+[a]		*pyroA4*/+		*qutE208*/+	
cTZ24	Pyr^-	14	6	25	15	36	40	6	14	8	12
	Pyr^+	8	10	6	4	10	10	13	5	18	0
cTZ7a	Pyr^-	8	15	23	34	0	57	23	0	16	7
	Pyr^+	15	8	26	27	0	53	23	0	2	21
cTZ7b	Pyr^-	11	15	12	13	28	25	23	0	20	6
	Pyr^+	13	13	7	9	22	16	23	0	6	20

[a] Progeny were plated on complete media containing uracil and the number of Pyr^+ and Pyr^-, yellow, white and green colonies were counted.

Fig 33 Genetic Maps of Haploid Parental Strains

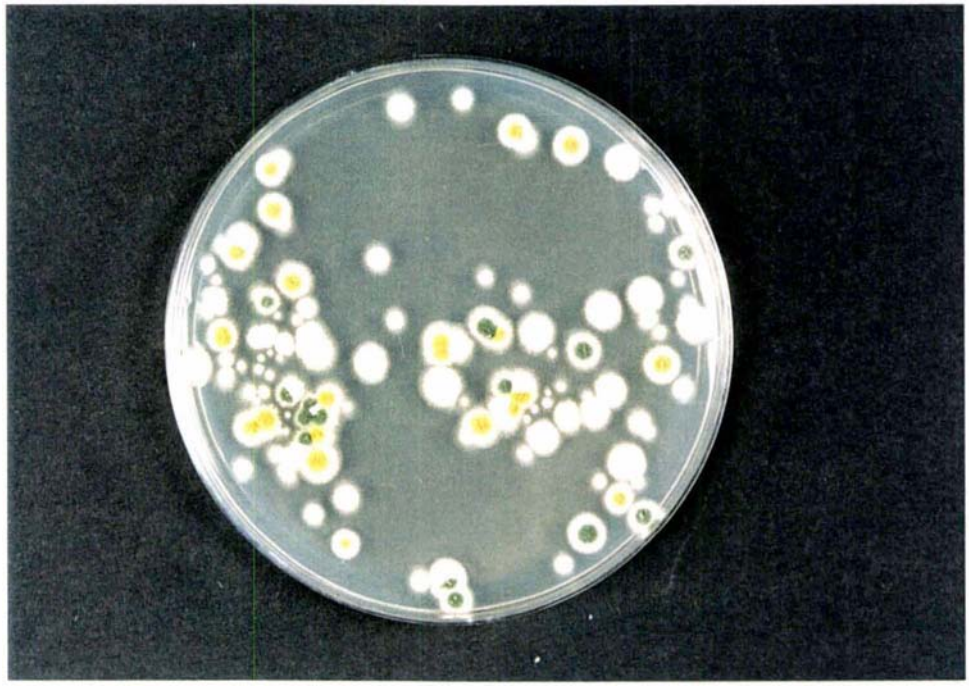
Putative genetic maps of the haploid parental strains of cleistothecium cTZ7a, cTZ7b and cTZ24, and the non-recombinant and recombinant classes of progeny. The number of progeny within each class is shown (number of progeny in class/total number of progeny analysed).

Chromosome (χ) Parental Strains	Progeny: Non-recombinants	Recombinants
TZ7a		
χI		
pyrG89 ***pabaA1*** ***yA*** *cycA*+	***pabaA1*** ***yA***(20/24)	***pabaA1*** *yA*+(3/22)
pyrG89 *paba*+ *yA*+ *cycA*+	*paba*+ *yA*+(19/22)	*paba*+ ***yA***(4/24)
χII		
wA+		
wA+		
χIV		
pyroA4		
pyroA4		
χVII		
qutE208	*qutE208* (16/23)	*qutE208* ***pyr-4*** **(2/23)**
qut+ ***pyr-4***	***qut***+ ***pyr-4*** **(21/23)**	***qut***+ **(7/23)**
TZ7b		
χI		
pyrG89 ***pabaA1*** ***yA*** *cycA*+	***pabaA1*** ***yA*** (10/13)	***pabaA1*** *yA*+ (1/14)
pyrG89 *paba*+ *yA*+ *cycA*+	*paba*+ *yA*+ (13/14)	*paba*+ ***yA*** (3/13)
χII		
wA3		
wA+		
χIV		
pyroA4		
pyroA4		
χVII		
qutE208	*qutE208* (20/26)	*qutE208* ***pyr-4*** **(6/26)**
qut+ ***pyr-4***	***qut***+ ***pyr-4*** **(20/26)**	***qut***+ **(6/26)**

Fig 33 continued

Chromosome (χ) **Parental Strains**				**Progeny:** **Non-recombinants**		**Recombinants**	
TZ24a							
χI							
pyrG89	***pabaA1***	***yA***	***cycA*+**	***pabaA1***	***cycA*+** (14/20)	***pabaA1***	*cycA::pyr-4* (8/18)
pyrG89	*paba*+	*yA*+	*cycA::pyr-4*	*paba*+	*cycA::pyr4* (10/18)	*paba*+	***cycA*+** (6/20)
				pabaA1	***yA*** (10/10)	***pabaA1***	*yA*+ (3/12)
				paba+	*yA*+ (9/12)	*paba*+	***yA*** (0/10)
				yA	***cycA*+** (6/10)	***yA***	*cycA::pyr-4* (4/12)
				yA+	*cycA::pyr4* (8/12)	*yA*+	***cycA*+** (4/10)
χII							
wA+							
wA+							
χIV							
pyroA4							
pyroA4							
χVII							
qutE208							
***qut*+**							

A



B

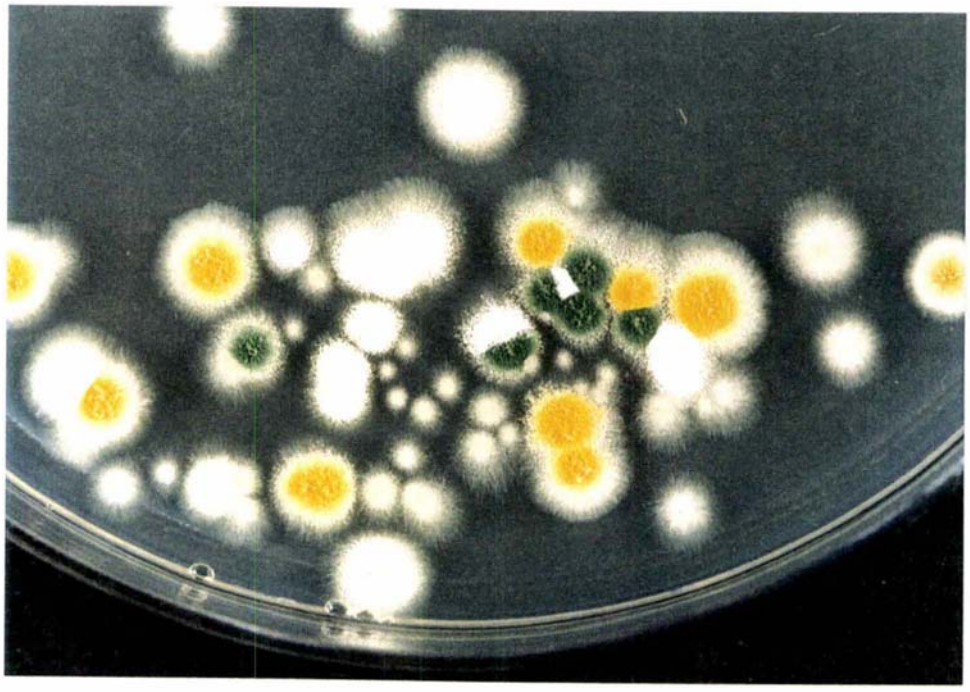


Fig 34 Growth of cTZ24 Progeny

A Growth of progeny from cleistothecia cTZ24 on complete media containing uracil. **B** Enlargement of a section of **A**.

segregation of genetic markers (Table 29). The larger of the two types were Pyr^- while the smaller were Pyr^+ indicating they contained a disrupted *cycA* locus. If the Pyr^+ progeny were grown for longer, so that spores were more abundant, then a colour could be identified. Due to the low level of sporulation this was very faint. The genotypes of the haploid parents could be determined (Fig. 33). Whilst the *qutE208* marker segregated both Qut^+ and Qut^- from this cross, no Qut^+ were seen amongst the Pyr^+ progeny. This is possibly due to the inability of the Cyc^- strains to utilise quinic acid as a sole carbon source. As with the progeny of the TZ7 crosses, analysis of the intrachromosomal segregation patterns of the genetic markers on chromosome I (*pabaA1, yA and cycA::pyr-4*+), showed there were more non-recombinants (70%) than recombinants (30%) amongst the cTZ24 progeny. The number of progeny analysed was too low to map the position of the *cycA* gene with respect to *pabaA1* or *yA*.

To confirm that the Pyr^+ haploid progeny from cleistothecium cTZ24 contained a disrupted *cycA* gene, genomic DNA was isolated from three independent Pyr^+ and Pyr^- progeny. The DNA was digested with both *Hin*dIII and *Pst*I and also with *Hin*dIII alone. Probing of the *Hin*dIII/*Pst*I Southern blot with the 2.3 kb *cyc* fragment resulted in a single band of 2.3 kb for the Pyr^- strains, indicating the presence of an intact *cyc* gene. The Pyr^+ strains did not contain the 2.3 kb band, instead the 1.3 kb and 3.6 kb bands expected for disruption of the *cyc* gene were observed (Fig. 35A). The *Hin*dIII Southern blot was probed with the 1 kb 3' region and resulted in the 4.2 kb wildtype band in the Pyr^- strains, while a smaller band of 3.3 kb, indicating disruption of the *cyc* gene, was present in the Pyr^+ strains (Fig. 35B).

The isolation of viable *A. nidulans* haploid strains without a functional *cycA* gene was surprising. To investigate alternative methods of obtaining energy requirements growth of the strains on different carbon sources was investigated (Table 30). Although growth of the Pyr^+/Cyc^- strains was poor compared to the Cyc^+/Pyr^- and Cyc^+/Pyr^+ strains, they grew best on glucose and sucrose but only very weakly on galactose, glycerol and sodium acetate. No growth was observed on lactic acid or quinic acid (Table 30).

6.3.5 Transformation of the Haploid *A. nidulans* Strain 1-85 with the Gene Replacement Vector pR54

Having determined that *A. nidulans cycA* mutant strains were viable under certain growth conditions, the *pyrG* mutant haploid strain of *A. nidulans*, 1-85, was transformed with linearised pR54 in an attempt to obtain *cycA* mutants directly. Of the eight stable uracil independent transformants obtained, one displayed slow growth with poor sporulation (R546).

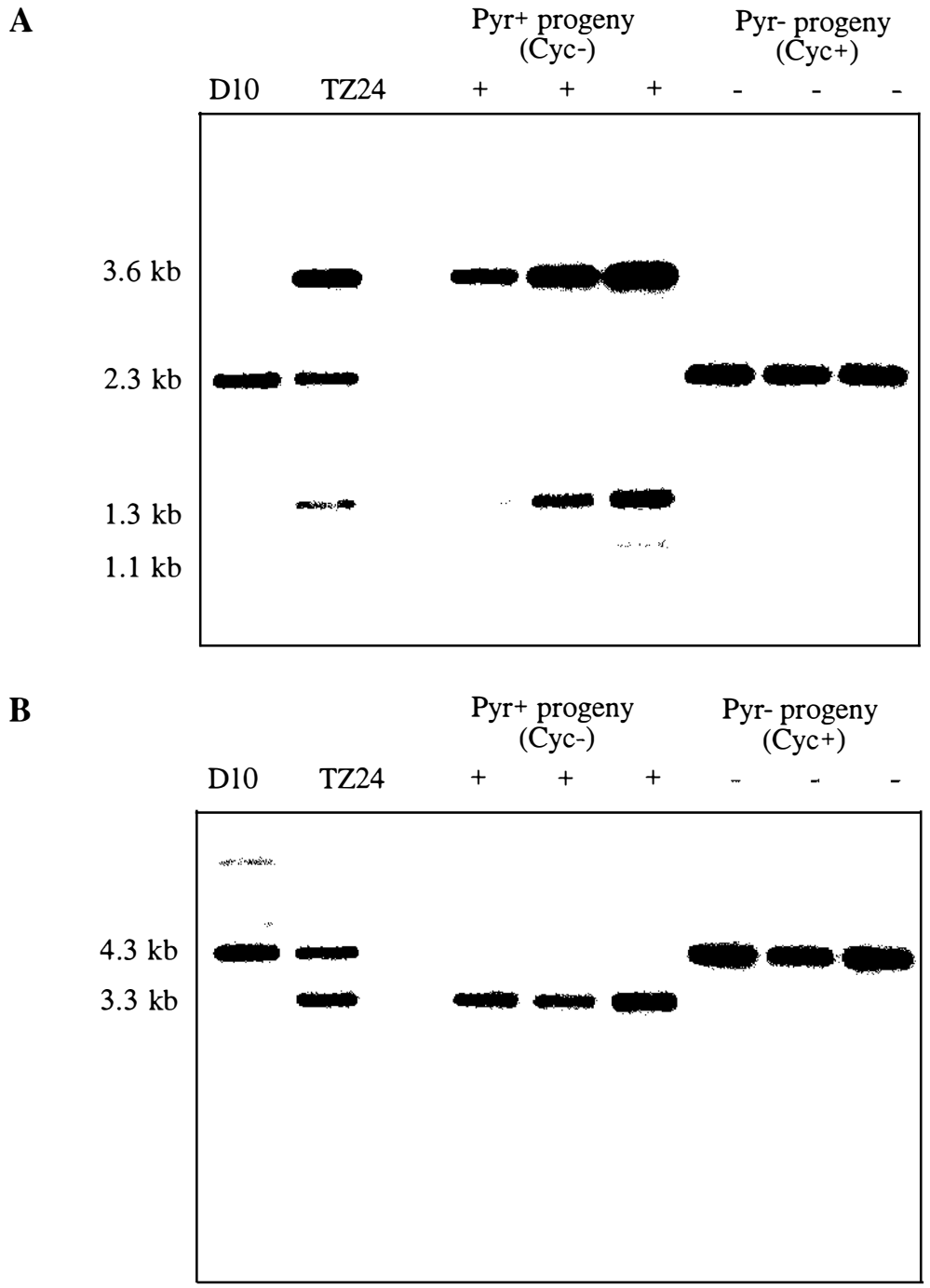


Fig 35 Southern Analysis of the Progeny of cTZ24

Southern analysis of the parental strain D10, transformant TZ24 and progeny of cleistothecium cTZ24. **A** Total genomic DNA digested with *Pst*I/*Hin*dIII was run on a 1% TBE agarose gel, and the blot was probed with the [α^{32}P] dCTP-labelled pR54. **B** Southern analysis of the strains in **A** after digestion with *Hin*dIII and probing with the 1 kb 3' fragment. The blots in **A** and **B** were stripped and probed with actin which confirmed the extra bands were due to partial digestion of the DNA.

Table 30 Growth of Meiotic Segregants on Different Carbon Sources

Carbon Source in agar medium[a]	Fermentable	Pyr^+, Cyc^{+c}	Pyr^+, Cyc^{-d}	Pyr^-, Cyc^{+d}
Glucose	Yes	+++	++	+++
Diameter on glucose[b]		40mm	15mm	27mm
Sucrose	Yes	+++	++	+++
Galactose	Yes	+++	+	+++
Sodium acetate	No	++	+/-[e]	++
Glycerol	Yes[f]	++	+	++
Lactic acid	No	+	-	+

[a] Carbon sources were at a final concentration of 1%.

[b] Measurement taken after four days incubation at 37°C.

[c] Progeny from cleistothecium harvested from transformant TZ7.

[d] Progeny from cleistothecium harvested from transformant TZ24.

[e] Growth at inoculation point only.

[f] Requires a functional mitochondrial enzyme, glycerol-3-phosphate dehydrogenase (Hondmann *et al.*, 1991)

Southern analysis of a selection of these transformants confirmed that the slow growing, poorly sporulating, transformant contained the pR54 construct integrated at a homologous site, replacing the *cycA* gene. The faster growing transformants all contained an intact *cyc* gene (Fig. 36).

6.4 Discussion

By means of a gene replacement strategy we have isolated Cyc$^-$ haploid strains of *A. nidulans*, by using a diploid strain to circumvent anticipated difficulties in isolating Cyc$^-$ transformants directly. Replacement of the *cycA* locus by the pGM32 vector in the mutant strain was verified by Southern blot analysis, and the mutant phenotype cosegregated mitotically and meiotically with genetic markers on chromosome I (*pabaA1* and *yA*), demonstrating that the *cycA* locus is on this chromosome.

Although the *cycA* gene appears to be present in a single copy, from Southern analysis, a cytochrome spectra is required for absolute confirmation that no cytochrome *c* remains in the mutants. Provided no cytochrome *c* is detected by cytochrome spectra this is the first filamentous fungus to be shown to be viable after complete replacement of a functional cytochrome *c* gene.

Cytochrome *c* mutants have been described in the filamentous fungus *N. crassa* (Bottorf *et al.*, 1994). Three mutants were described which have severely low levels of cytochrome *c* but can be distinguished on the basis of their cytochrome aa_3 content. These mutants were characterised by slow growth and low levels of spectrophotometrically detectable cytochrome *c*. They were therefore not completely lacking of cytochrome *c*.

The yeast *K. lactis* has a unique *cyc1* gene (Clark-Walker, 1991) which has been successfully disrupted using gene targeting (Chen and Clark-Walker, 1993). Strains lacking cytochrome *c* were able to grow on glucose but not on non-fermentable substrates. Therefore disruption of the cytochrome *c* gene was not lethal and *K. lactis* can grow fermentatively in the absence of cytochrome *c* mediated respiration.

Studies of *Aspergillus* species at different oxygen concentrations demonstrated *Aspergillus* was able to grow at low oxygen concentrations but not if there was no oxygen presence, therefore there was an oxygen requirement for growth (Hall and Denning 1994). *Aspergillus* species are known to contain an alternative respiratory pathway which is cyanide resistant and salicylhydroxamic acid (SHAM) sensitive. The function of this pathway may allow *A. nidulans*

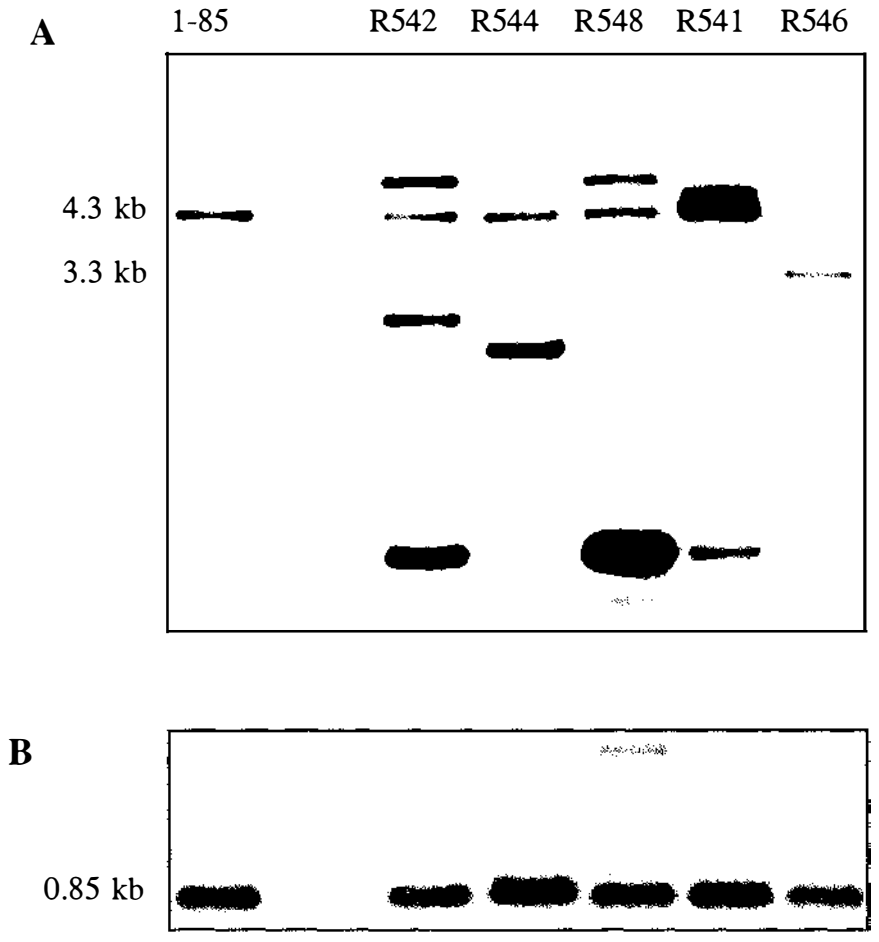


Fig 36 Southern Analysis of 1-85 Transformants of pR54

Southern analysis of the parental strain 1-85 and the transformants R541, R542, R544, R546 and R548 (With the exception of the diploid transformant R541, the remaining transformants are haploids). **A** Total genomic DNA digested with *Hin*dIII was run on a 1% TBE agarose gel and the blot was probed with the [α^{32}P] dCTP-labelled 1 kb 3' *cyc* fragment. **B** The blot was stripped and probed with actin. The extra bands observed for R542 and R548 are due to incomplete stripping of the blot.

to survive in the absence of cytochrome *c*. These pathways were demonstrated to be affected by citric acid fermentation in *A. niger*. During citric acid fermentation the cyanide sensitive respiration decreased and SHAM sensitive respiration increased (Kirimura *et al.*, 1987). An alternative terminal oxidase, which shows similarities to plant alternative oxidases, has been identified immunologically in *N. crassa* (Lambowitz *et al.*, 1989). A similar oxidase may function in *A. nidulans*. However, oxygen is usually the terminal electron acceptor for these pathways. It is not known what involvement, if any, cytochrome *c* has in these alternative pathways.

Although required for growth oxygen is not required for survival as *Aspergillus* conidia can survive during oxygen deprivation and then germinate when transferred to permissive conditions (Hall and Denning 1994). Alcohol dehydrogenases are involved in the pathway whereby sugars are fermented to ethanol to provide energy. These are induced under anaerobic conditions and are believed to be important in allowing *A. nidulans* to survive during periods of anaerobic stress (Kelly *et al.*, 1990).

The Cyc$^-$ strains were able to grow on a variety of fermentable carbon sources but were unable to grow on the strictly non-fermentable carbon source, lactate. This indicates that fermentation may play a key role in the providing the energy requirements of the fungi in the absence of oxidative phosphorylation, due to disruption of the *cycA* gene.

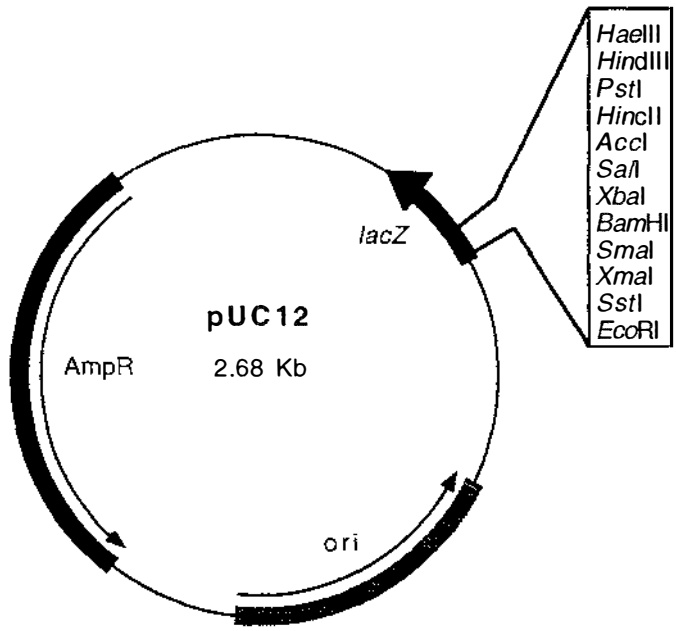
6.5 Conclusion

The construction of a gene replacement vector for the *cycA* locus, and the subsequent isolation of haploid Cyc$^-$ strains, has confirmed that cytochrome *c* is not essential for survival or growth of *A. nidulans* and that the gene is located on chromosome I. The preliminary results indicate that cytochrome *c* deficient strains survive using fermentation and/or an alternative respiratory pathway.

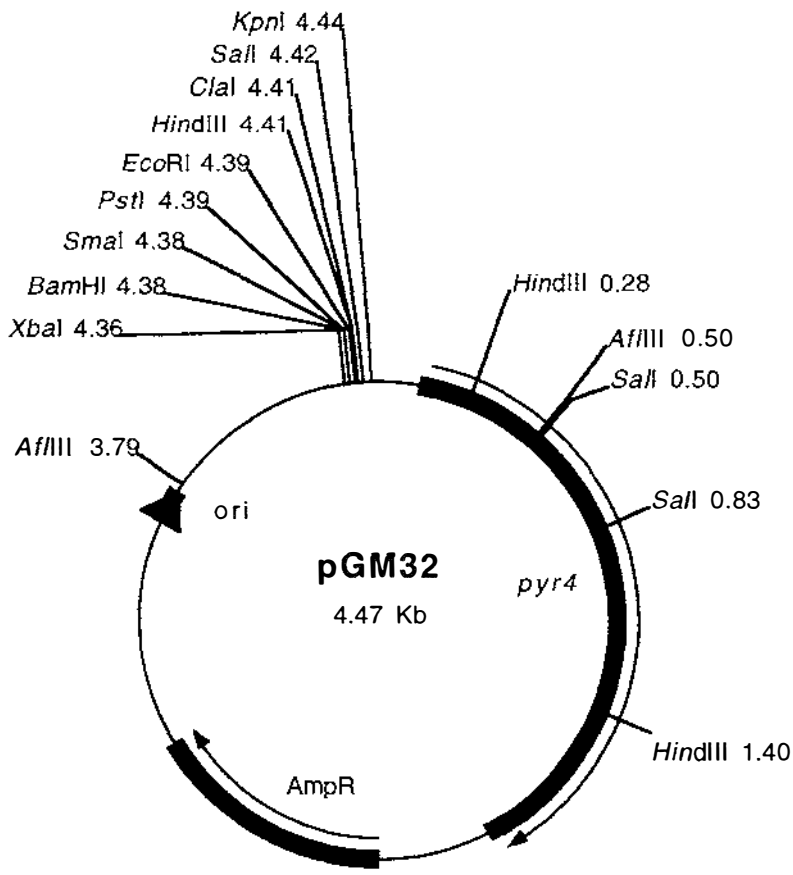
Although the *cycA* gene appears to be present in a single copy, from Southern analysis, a cytochrome spectra is required for absolute confirmation that no cytochrome *c* remains in the mutants.

Appendix 1: Vector Maps

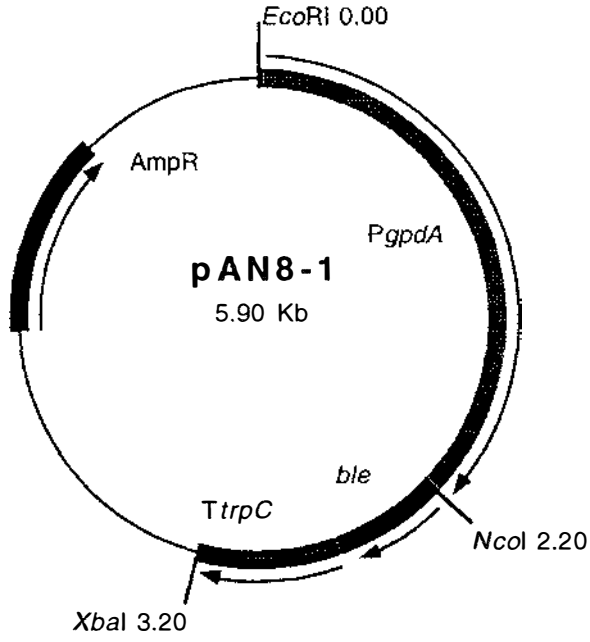
A.1 Restriction map of pUC12 showing resistriction enzymes in the polycloning site.



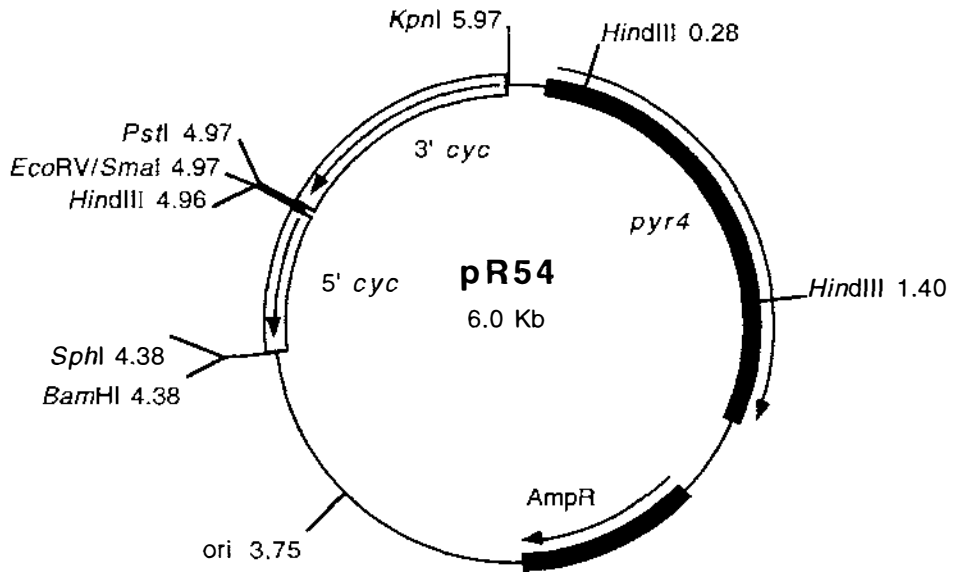
A.2 Restriction map of pGM32 showing sites for *Afl*III, *Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sal*I, *Sma*I and *Xba*I



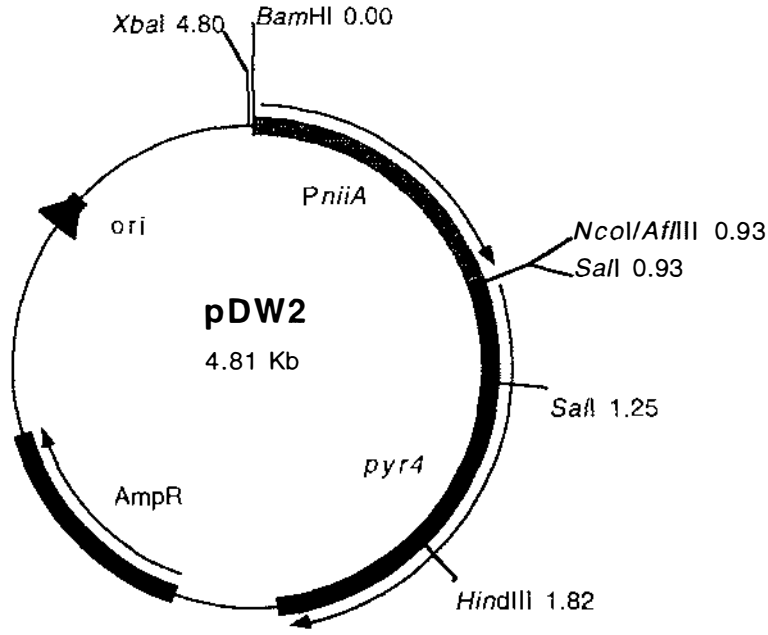
A.3 Restriction map of pAN8-1 showing restriction sites for *Eco*RI, *Nco*I and *Xba*I.



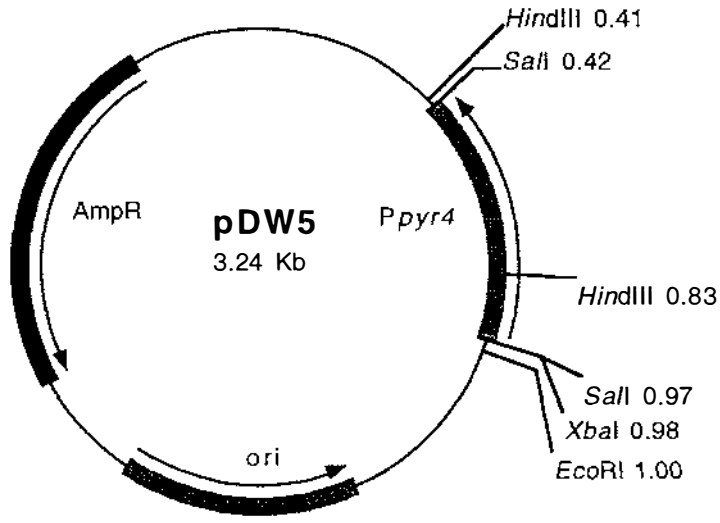
A.4 Restriction map of pR54 showing restriction sites for *Bam*HI, (*Eco*RV), *Hin*dIII, *Kpn*I, *Pst*I, (*Sma*I) and *Sph*I. Sites in brackets are the original sites which are disrupted in the vector.



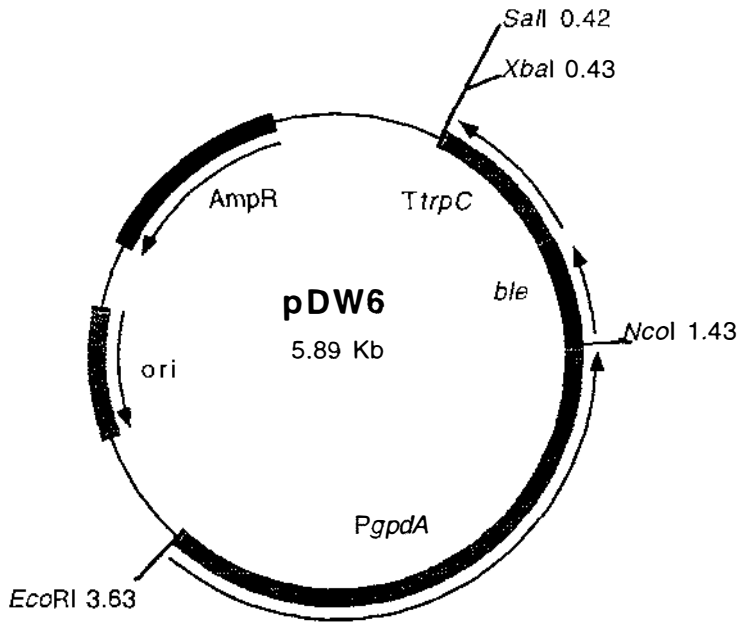
A1.5 Restriction map of pDW2 showing restriction sites for (*Afl*III), *Bam*HI, *Hin*dIII, (*Nco*I), *Sal*I and *Xba*I. Sites in brackets are the original sites which are disrupted in the vector.



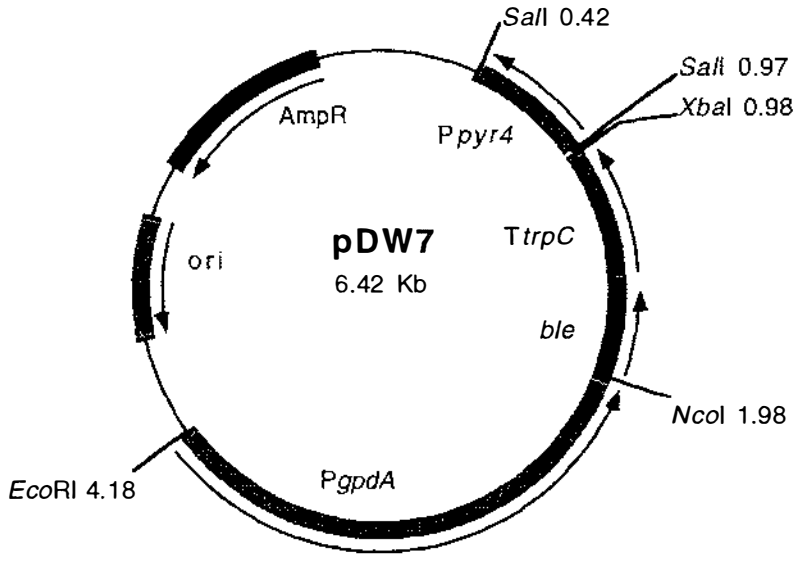
A1.6 Restriction map of pDW5 showing restriction sites for *Eco*RI, *Hin*dIII, *Sal*I and *Xba*I.



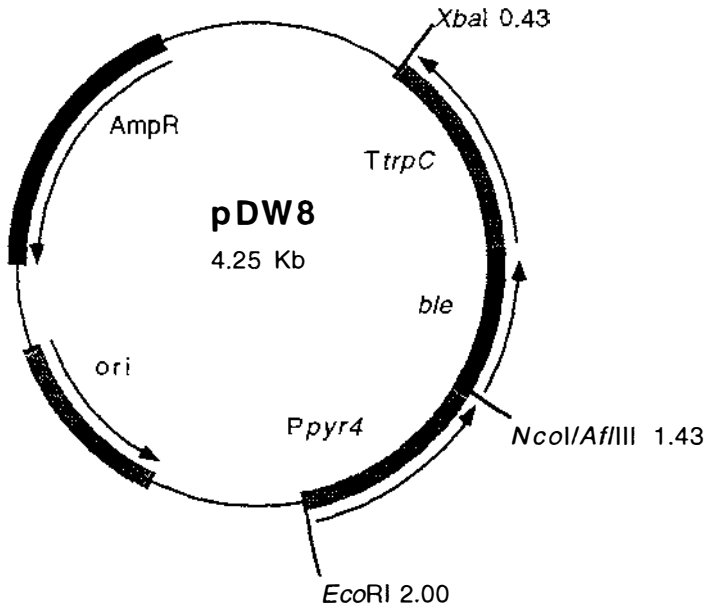
A1.7 Restriction map of pDW6 showing restriction sites for *Eco*RI, *Nco*I, *Sal*I and *Xba*I.



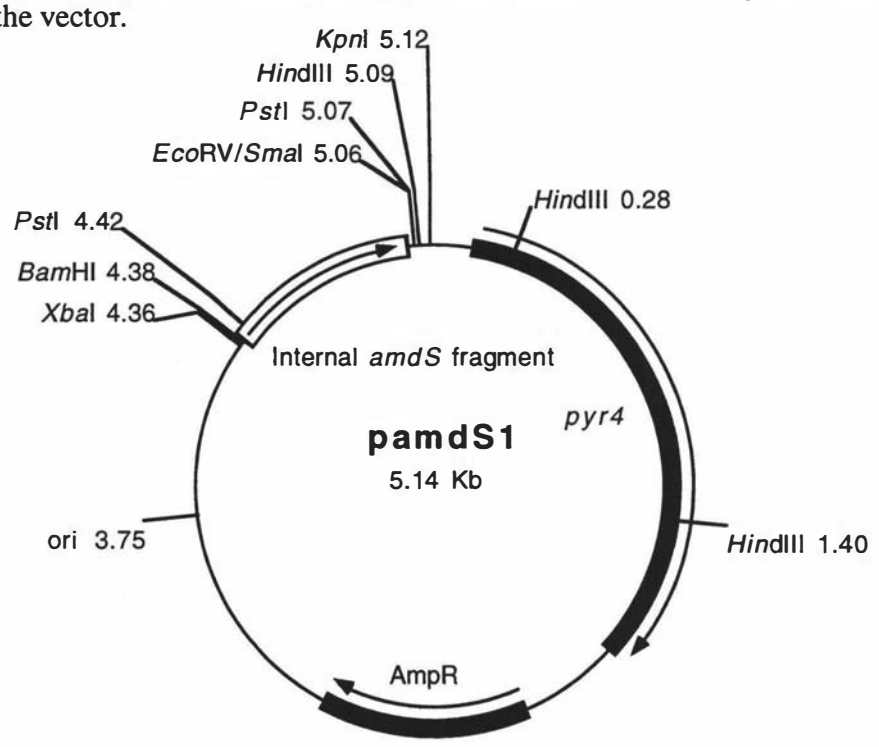
A1.8 Restriction map of pDW7 showing restriction sites for *Eco*RI, *Nco*I, *Sal*I and *Xba*I.



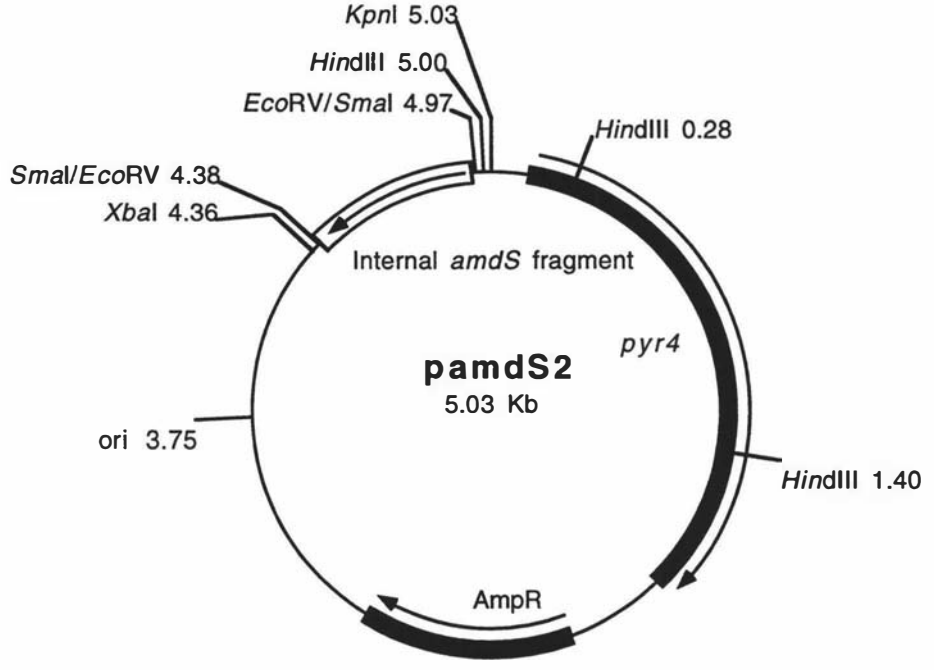
A1.9 Restriction map of pDW8 showing restriction sites for (*Afl*III), *Eco*RI, (*Nco*I), *Sal*I and *Xba*I. Sites in brackets are the original sites which are disrupted in the vector.



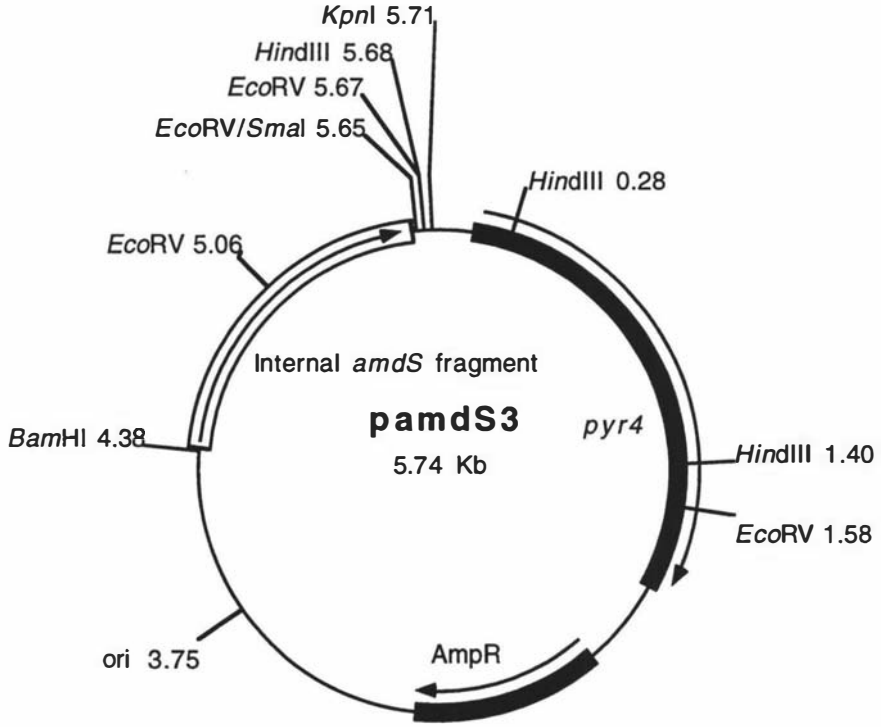
A1.10 Restriction map of pamdS1 showing restriction sites for *Bam*HI, (*Eco*RV), *Hin*dIII, *Kpn*I, *Pst*I, (*Sma*I) and *Xba*I. Sites in brackets are the original sites which were disrupted in the vector.



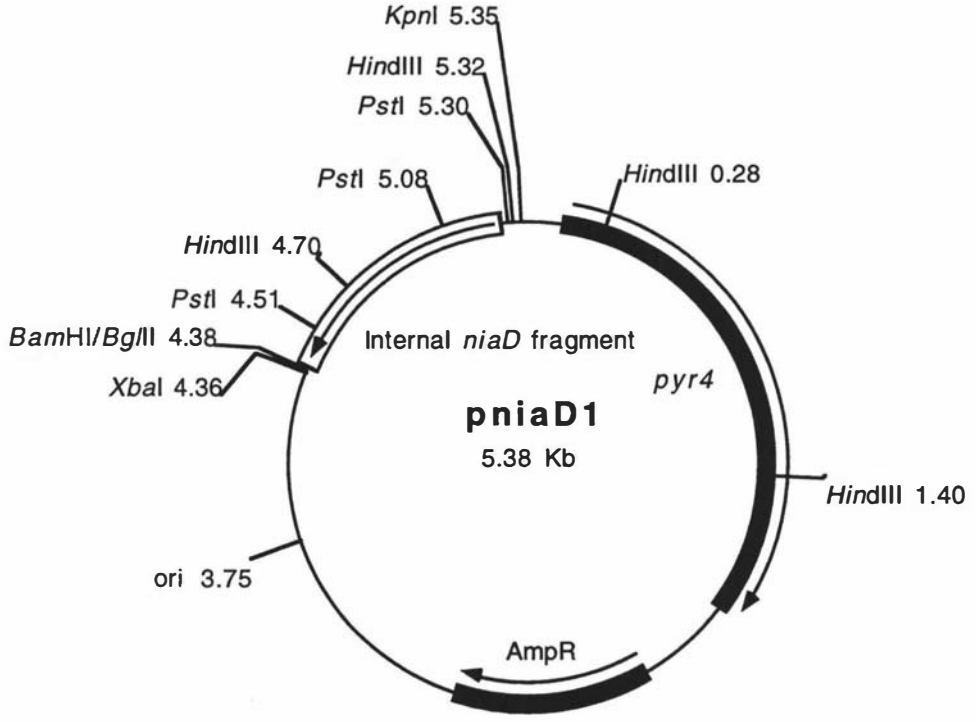
A1.11 Restriction map of pamdS2 showing restriction sites for *Bam*HI, (*Eco*RV), *Hin*dIII, *Kpn*I, *Pst*I, (*Sma*I) and *Xba*I. Sites in brackets are the original sites which were disrupted in the vector.



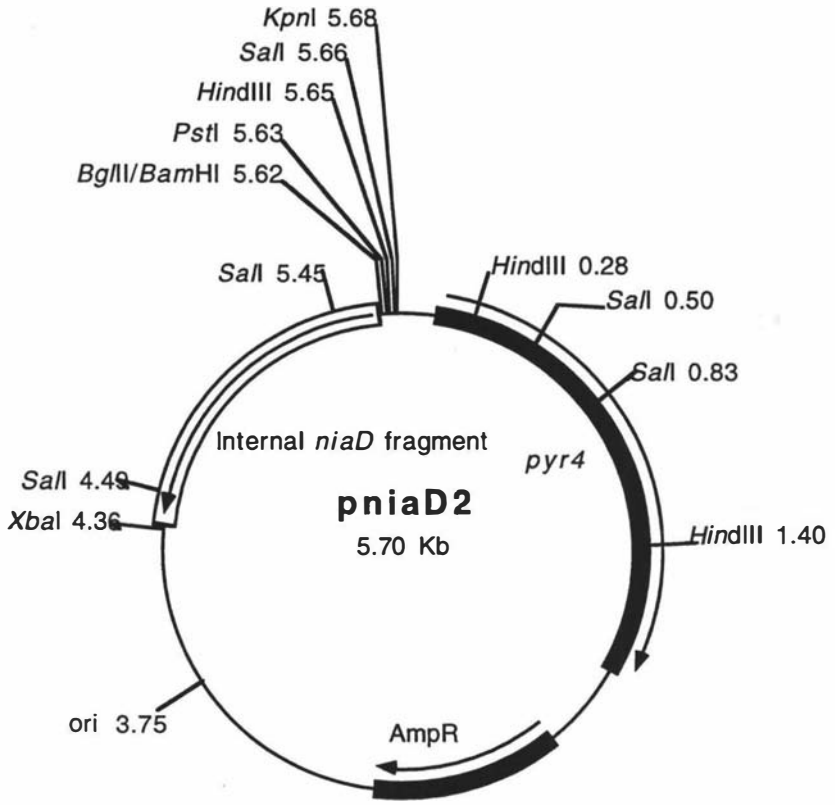
A1.12 Restriction map of pamdS3 showing restriction sites for *Bam*HI, *Eco*RV, (*Eco*RV), *Hin*dIII, *Kpn*I, *Pst*I and (*Sma*I). Sites in brackets are the original sites which were disrupted in the vector.



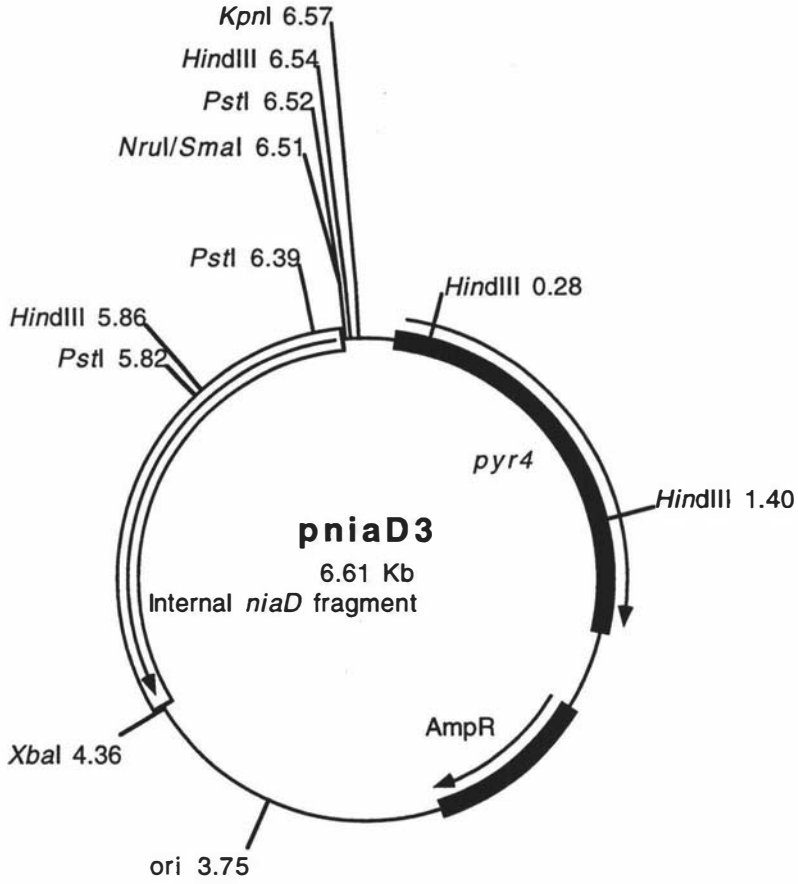
A1.13 Restriction map of pniaD1 showing restriction sites for (*Bgl*II), *Bam*HI, (*Bam*HI), *Hin*dIII, *Kpn*I, *Pst*I and *Xba*I. Sites in brackets are the original sites which were disrupted in the vector.



A1.14 Restriction map of pniaD2 showing restriction sites for (*Bgl*II), (*Bam*HI), *Hin*dIII, *Kpn*I, *Pst*I, *Sal*I and *Xba*I. Sites in brackets are the original sites which were disrupted in the vector.



A1.15 Restriction map of pniaD3 showing restriction sites for *Hin*dIII, *Kpn*I, (*Nru*I), *Pst*I, (*Sma*I) and *Xba*I. Sites in brackets are the original sites which were disrupted in the vector.



Appendix 2: Sequencing Results

Disruption vectors pniaD1-3 and pamdS1-3 were sequenced from the forward and reverse primer sites. The sequence boundary between vector and vector, or vector and gene, are indicated in bold with the details in brackets. The restriction enzymes used to isolate the fragment for cloning are recorded above the boundary site: bold, restriction site retained in the construct; plain, restriction site disrupted in the construct. Vector sequence is underlined. Discrepancies in the sequence are indicated in brackets: plain, nucleotide missing; italics, nucleotide incorrect. These were most abundant in pniaD3 (reverse primer) where relatively poor sequence was obtained.

A2.1 Vector: pniaD1

Primer-forward

GTAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATAT

*Bam*HI *Bam*HI *Nru*I

CGAATTCCTGCAGCCCGGG**GGA**(pGM32/pIC19R)**TCC**GTCGACCTGCAGCCAAGCTT**TCG**(pIC19R

*Nru*I

/*niaD*)**CGA**CGAGGACATTCCCAACTGGGAACTCCGCATTGAAGGGTCCGTCAGTCTCGTTTC

Primer-reverse

*Bam*HI

CCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGT**GGA**(pGM32/

*Bgl*II

niaD)**TCT**CGACACGGGTTATTCTTCGTCCGCCTCCTGCATAGGCATACCCTTTTGCTGTGTAGAACGGCC

A2.2 Vector pniaD2

Primer-forward

GTAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATAT

*Bam*HI *Bgl*II

CGAATTCCTGCAGCCCGGG**GGA**(pGM32/*niaD*)**TCT**CGCTGGATAAGGGCAAATGTACGCTTCCTAACTAC

CAAGCCCATAAATCTGCATCCCGTTGAATCCACTGCTAACATCTTACAGCATGGCGCCTTGCGCGGATCGAATAC

Primer-reverse

*Xba*I *Xba*I

CCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGC**TCT**(pGM32/niaD)**AGA**TACTCG

AAACGCCCAGTAGGACCCTTGCATTCGATGACG

A2.3 Vector pniaD3

Primer-forward

*Sma*I *Nru*I

GGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAG**CCC**(pGM32/*niaD*)**CGA**

CGAGGACATTCCCAACTGGGAACTCCGCATTGAAGGGTCCGTCAGTCTCGTTTCAGCTTGTGAACTACTCCCTGA

CAAGTGCTAGACTCGTTGAAAAGCCAATCACCCTCAGCTTCAAGCAGATCCTGCAGAACTAT

Primer-reverse

*Xba*I

CTCACTAAAGGGAACAN(A)AAG(C)TGGAGCTCCAC(C)GCGGTGGCGGC(C)GC**TCT**(pGM32/*niaD*)**A**

*Xba*I

GA(T)ACTCGAAACGCCCAGTAGGACC(C)TTGCATTCGATGA

A2.4 Vector pamdS1

Primer-forward

GTAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATAT

*Sma*I *Eco*RV

CGAATTCCTGCAG**CCC**(pGM32/*amdS*)**ATC**CGTTCCTACACCGATGACGCCACCACGAATCCCAACGATCG

CACCCTCACCACCAGAACTGTC

Primer-reverse

*Bam*HI

CCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGT**GGA**(pGM32/

*Bam*HI

amdS)**TCC**TTTCAGAGGCCGAACTGAAGATCACAGAGGCCTCCGCTGCAGATCTTGTGTCCAAGCTGGCGGCC

GGAGAGTTGACCTCGGTGGAAGTT

A2.5 Vector pamdS2

Primer-forward

*Sma*I

GAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAG**CCC**(pGM

*Eco*RV

32/*amdS*)**ATC**GGTGGCTCGATTCGAGTGCCGGCCGCGTTCAACTTCCTGTACGGTCTAAGGCCGAGTCATGG

GCGGCTGCCGTATGCAAAGATGGCGAACAGCATGGAGGGTCAGGAGACGGTGCACAGCGTTG

Primer-reverse

*Sma*I

CCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGG (*G*) CGCTCTAGAACTAGTGGATCC**CCC** (**pG**

*Eco*RV

M32/*amdS*) ATCGCGCATTACGTCGGCGCTGCCGTCAGCCGCGTAGATATGGGAGATGAGATCGTGG

A2.6 Vector pamdS3

Primer-forward

*Sma*I

GAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAG**CCC** (**pGM**

*Eco*RV

32/*amdS*) ATCGCGCATTACGTCGGCGCTGCCGTCAGCCGCGTAGATATGGGAGATGAGATCGTGGCCGAAA

Primer-reverse

***Bam*HI**

CCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGT**GGA** (**pGM32/am**

***Bam*HI**

***dS*) TCC**TTTCAGAGGCCGAACTGAAGATCACAGAGGCCTCCGCTGCAGATCTTGTGTCCAAGCTGGCGGCCGGA

GAGTTGACCTCGGTGGAAGTTA

Appendix 3: Statistical Analysis of Data

Statistical Analysis

The experimental data obtained by comparing the effect of changing parameters on transformant types or gene targeting is in the form of proportions: N-type or H-type and 'targeted' (-) or 'not targeted' (+), respectively. Statistically this can be analysed by chi-squared which is used for discontinuous data that approaches continuous data as long as the expected (E) values were greater than 5 (E > 5).

The E values were calculated based on the Null Hypothesis (NH) that there was no effect on targeting or transformant type by the parameter investigated. Chi-square was then calculated.

$$\chi^2 = \Sigma (O - E)^2 / E$$

When the parameter invested was tested more than once, the difference between experiments was tested using chi-squared. If no significant difference was found between experiments then the data was pooled and the effect of the parameter being investigated was tested by chi-squared.

If the values for E were less than 5 (E < 5) then Yates' correction was applied. This involves reducing the value of each difference (O-E) by 0.5 before squaring. This has the effect of lowering the value of χ^2 and thereby reducing the chance of false significance.

$$\chi^2 = \Sigma \{[+(O - E)] - 0.5\}^2 / E$$

The degrees of freedom (df) were calculated from the number of rows and columns:
df = (rows - 1) x (columns - 1) or (r - 1) x (c - 1).

The probability values were obtained from abridged tables in Statistical and Experimental Design by GM Clark (1994) and Statistical Analysis in Biology by K Mather (1964).

A3.1 Types of Transformants

A3.1.1 Vector Conformation: Vector pGM32

Repetition of experiments = 3

Parameters tested (linear vs circular) = 2

Test difference between experiments

Observed results:

	N-type	H-type	Total
Exp1	52	224	276
Exp2	36	188	224
Exp3	25	262	287
Total	113	674	787

Expected results:

	N-type	H-type	Total
Exp1	39.6	236	276
Exp2	32.2	192	224
Exp3	41.2	246	287
Total	113	674	787

$\chi^2 = 12.43$

df = (3-1) x (2-1) = 2

Probability between 0.01 and 0.001. Therefore significant; reject NH.

Test difference between circular and linear vectors

As there was a significant difference between the experiments they need to be considered separately.

Experiment 1:

Observed results:

	N-type	H-type	Total
Circular	18	180	198
Linear	34	44	78
Total	52	224	276

Expected results:

	N-type	H-type	Total
Circular	37	161	198
Linear	15	63	78
Total	52	224	276

$\chi^2 = 43.6$

df = (2-1) x (2-1) = 1

Probability less than 0.001. Therefore significant; reject NH.

Experiment 2:

Observed results:

	N-type	H-type	Total
Circular	18	135	153
Linear	18	53	71
Total	36	188	224

Expected results:

	N-type	H-type	Total
Circular	25	128	153
Linear	11	60	71
Total	36	188	224

$\chi^2 = 14.3$

df = (2-1) x (2-1) = 1

Probability less than 0.001. Therefore significant; reject NH.

Experiment 3:

Observed results:

	N-type	H-type	Total
Circular	10	202	212
Linear	15	60	75
Total	25	262	287

Expected results:

	N-type	H-type	Total
Circular	18	194	212
Linear	7	68	75
Total	25	262	287

$\chi^2 = 17.4$

df = (2-1) x (2-1) = 1

Probability less than 0.001. Therefore significant; reject NH.

A3.1.2 Amount of Vector: pGM32

Repetition of experiments = 2

Parameters tested (amount of DNA) = 2

Test difference between experiments

Observed results:

	N-type	H-type	Total
Expl	33	483	516
Exp2	23	278	301
Total	56	761	817

Expected results:

	N-type	H-type	Total
Expl	35.4	481	516
Exp2	20.6	280	301
Total	56	761	817

$\chi^2 = 0.46$

df = (2-1) x (2-1) = 1

Probability equals 0.5. Therefore not significant; accept NH.

Test difference between amount of vector DNA in the tranformation mix

As there was no significant difference between the experiments data was combined for comparison of the effect of the different amounts of vector DNA included in the reaction mix.

0.25 to 0.5 µg of DNA:

Observed results:

µg DNA	N-type	H-type	Total
0.25	4	30	34
0.50	9	86	95
0.50	14	104	118
Total	27	220	247

Expected results:

µg DNA	N-type	H-type	Total
0.25	3.7	30.3	34
0.50	10.4	84.6	95
0.50	12.9	105.1	118
Total	27	220	247

As some of the expected values (E) were below 5 (E<5) Yates' correction was applied to all (O - E) values.

$\chi^2 = 0.131$

df = (3-1) x (2-1) = 2

Probability greater than 0.9. Therefore not significant; accept NH.

0.75 to 2.5 µg of DNA:

Observed results:

µg DNA	N-type	H-type	Total
0.75	6	85	91
1.00	4	77	81
1.00	8	138	146
1.50	5	106	111
2.00	3	48	51
2.50	3	87	90
Total	29	541	570

Expected results:

µg DNA	N-type	H-type	Total
0.75	4.6	86.4	91
1.00	4.1	76.9	81
1.00	7.4	138.6	146
1.50	5.6	105.4	111
2.00	2.6	48.4	51
2.50	4.6	85.4	90
Total	29	541	570

As some of the expected values (E) were below 5 (E<5) Yates' correction was applied to all (O - E) values.

$\chi^2 = 0.511$

df = (6-1) x (2-1) = 5

Probability greater than 0.9. Therefore not significant; accept NH.

As there was no significance between the values for 0.25 to 0.5 µg of DNA and 0.75 to 2.5 µg of DNA the data was pooled.

Combined results

Observed results:

µg DNA	N-type	H-type	Total
0.25-0.5	27	220	247
0.75-2.5	29	541	570
Total	56	761	817

Expected results:

µg DNA	N-type	H-type	Total
0.25-0.5	17	230	247
0.75-2.5	39	531	570
Total	56	761	817

$\chi^2 = 9.2$

df = (2-1) x (2-1) = 1

Probability between 0.01 and 0.001. Therefore significant; reject NH.

A3.2 Length of Homologous DNA

A3.2.1 Targeting the *niaD* Locus

Repetition of experiments = 3

Parameters tested (number of vectors) = 3

Test difference between experiments

Observed results:				Expected results:			
	+	-	Total		+	-	Total
Exp1	27	11	38	Exp1	25.7	12.3	38
Exp2	42	22	64	Exp2	43.3	20.7	64
Exp3	67	32	99	Exp3	66.99	32.0	99
Total	136	65	201	Total	136	65	201

$\chi^2 = 0.3238$

df = (3-1) x (2-1) = 2

Probability between 0.9 and 0.8. Therefore not significant; accept NH.

Test difference between vectors

As there was no significant difference between the experiments data was pooled for comparison of the effect of the different vectors.

Observed results:				Expected results:			
	+	-	Total		+	-	Total
pniaD1	38	6	44	pniaD1	29.8	14.2	44
pniaD2	36	13	49	pniaD2	33.2	15.8	49
pniaD3	62	46	108	pniaD3	73.1	34.9	108
Total	136	65	201	Total	136	65	201

$\chi^2 = 12.9398$

df = (3-1) x (2-1) = 2

Probability between 0.005 and 0.001. Therefore significant; reject NH.

A3.2.2 Targeting the *amdS* locus

Repetition of experiments = 2

Parameters tested (number of vectors) = 3

Test difference between experiments

Observed results:				Expected results:			
	+	-	Total		+	-	Total
Exp1	163	5	168	Exp1	164.03	3.97	168
Exp2	126	2	128	Exp2	124.97	3.03	128
Total	289	7	296	Total	289	7	296

As some of the expected values (E) were below 5 (E<5) Yates' correction was applied to all (O - E) values.

$\chi^2 = 0.1674$

df = (2-1) x (2-1) = 1

Probability between 0.7 and 0.5. Therefore not significant, accept NH.

Test difference between vectors

As there was no significant difference between the experiments data was pooled for comparison of the effect of the different vectors.

Observed results:

	+	-	Total
pamdS1	84	0	84
pamdS2	100	1	101
pamdS3	105	6	111
Total	289	7	296

Expected results:

	+	-	Total
pamdS1	82.01	1.99	84
pamdS2	98.61	2.39	101
pamdS3	108.38	2.62	111
Total	289	7	296

$\chi^2 = 4.7245$

df = (3-1) x (2-1) = 2

Probability between 0.1 and 0.05. Therefore not significant; accept NH.

A3.3 Conformation of the Transforming Vector

A3.3.1 Targeting the *niaD* Locus: Vector pniaD3

Repetition of experiments = 2

Parameters tested (circular or linear) = 2

Test difference between experiments

Observed results:

	+	-	Total
Exp1	26	76	102
Exp2	58	90	148
Total	84	166	250

Expected results:

	+	-	Total
Exp1	34.27	67.73	102
Exp2	49.73	98.27	148
Total	84	166	250

$\chi^2 = 5.076$

df = (2-1) x (2-1) = 1

Probability between 0.025 and 0.01. Therefore significant; reject NH.

Test difference between vector conformations

As there appears to be a significant difference between the targeting observed in each of these two experiments it was necessary to consider each experiment separately.

Experiment 1:

Observed results:

	+	-	Total
Circular	16	19	35
Linear	10	57	67
Total	26	76	102

Expected results:

	+	-	Total
Circular	8.92	26.08	35
Linear	17.08	49.92	67
Total	26	76	102

$\chi^2 = 11.4805$

df = (2-1) x (2-1) = 1

Probability less than 0.001. Therefore significant; reject NH.

Experiment 2:

Observed results:

	+	-	Total
Circular	44	28	72
Linear	14	62	76
Total	58	90	148

Expected results:

	+	-	Total
Circular	28.22	43.78	72
Linear	29.78	46.22	76
Total	58	90	148

$\chi^2 = 28.2606$

df = (2-1) x (2-1) = 1

Probability less than 0.001. Therefore significant; reject NH.

A3.3.2 Targeting the *amdS* Locus: Vector pamdS3

Repetition of experiments = 4

Parameters (circular or linear) = 2

Test difference between experiments

Observed results:

	+	-	Total
Exp1	120	10	130
Exp2	79	0	79
Exp3	49	6	55
Exp4	60	2	62
Total	308	18	326

Expected results:

	+	-	Total
Exp1	122.82	7.18	130
Exp2	74.64	4.36	79
Exp3	51.96	3.04	55
Exp4	58.58	3.42	62
Total	308	18	326

As some of the expected values (E) were below 5 (E<5) Yates' correction was applied to all (O - E) values.

$\chi^2 = 6.7795$

df = (4-1) x (2-1) = 3

Probability between 0.1 and 0.05. Therefore not significant; accept NH.

Test difference between vector conformations

As there was no significant difference between the experiments the data was pooled.

Observed results:

	+	-	Total
Circular	176	7	183
Linear	132	11	143
Total	308	18	326

Expected results:

	+	-	Total
Circular	172.90	10.10	183
Linear	135.10	7.90	143
Total	308	18	326

$\chi^2 = 2.2947$

df = (2-1) x (2-1) = 1

Probability between 0.2 and 0.1. Therefore not significant; accept NH.

A3.4 Amount of DNA in the Transformation Mix

A3.4.1 Targeting the *niaD* Locus: Vector pniaD2

Parameter tested (different amounts of DNA) = 4

Test difference between amounts of transforming vector

Observed results:				**Expected results:**			
	+	-	Total		+	-	Total
1µg	45	12	57	1µg	42.95	14.05	57
2µg	44	13	57	2µg	42.95	14.05	57
4µg	41	13	54	4µg	40.68	13.32	54
8µg	35	16	51	8µg	38.42	12.58	51
Total	165	54	219	Total	165	54	219

$\chi^2 = 1.7455$

df = (4-1) x (2-1) = 3

Probability between 0.7 and 0.5. Therefore not significant; accept NH.

A3.5 Selective Media

A3.5.1 Targeting the *niaD* Locus: Vector pniaD2

Repetition of experiments = 3

Parameters tested (types of selective media) = 3

Test difference between experiments

Observed results:				**Expected results:**			
	+	-	Total		+	-	Total
Exp1	71	18	89	Exp1	71.43	17.57	89
Exp2	195	48	243	Exp2	195.03	47.97	243
Exp3	43	10	53	Exp3	42.54	10.46	53
Total	309	76	385	Total	309	76	385

$\chi^2 = 0.0383$

df = (3-1) x (2-1) = 2

Probability greater than 0.9. Therefore not significant; accept NH.

Test difference between selection media

As there was no significant difference between the experiments the data was pooled for comparison of selection on different media.

Observed results:

	+	-	Total
MYG	87	26	113
Glut.	99	24	123
Nitrate	123	26	149
Total	309	76	385

Expected results:

	+	-	Total
MYG	90.69	22.31	113
Glut.	98.72	24.28	123
Nitrate	119.59	29.41	149
Total	309	76	385

$\chi^2 = 1.2571$

df = (3-1) x (2-1) = 2

Probability between 0.7 and 0.5. Therefore not significant; accept NH.

Test difference between selection media: minimal media vs complete media

As this was not tested directly data was pooled from all relevant experiments.

Parameters (types of media) = 2

Observed results:

	+	-	Total
AMM	363	84	447
MYG	212	68	280
Total	575	152	727

Expected results:

	+	-	Total
AMM	353.54	93.46	447
MYG	221.46	58.54	280
Total	575	152	727

$\chi^2 = 3.1435$

df = (2-1) x (2-1) = 1

Probability between 0.1 and 0.05. Therefore not significant; accept NH.

A3.6 Additions to Selective Media and/or Reaction Mix

A3.6.1 Targeting the *niaD* Locus: Vector pniaD2

Test difference between additives to reaction mix and the selective media

Repetition of experiments = 1

Parameters tested (additives) = 3

Observed results:

	+	-	Total
Glut.	53	11	64
Nitrate	49	12	61
Nitrite	39	11	50
Total	141	34	175

Expected results:

	+	-	Total
Glut.	51.57	12.43	64
Nitrate	49.15	11.85	61
Nitrite	40.29	9.71	50
Total	141	34	175

$\chi^2 = 0.4192$

df = (3-1) x (2-1) = 2

Probability between 0.9 and 0.8. Therefore not significant; accept NH.

Test difference between additions of additives directly to the reaction mix

Repetition of experiments = 1

Parameters tested (additives) = 4

Observed results:

	+	-	Total
Water	27	10	37
Glut.	30	10	40
Nitrate	30	11	41
Nitrite	38	11	49
Total	125	42	167

Expected results:

	+	-	Total
Water	27.69	9.31	37
Glut.	29.94	10.06	40
Nitrate	30.69	10.31	41
Nitrite	36.68	12.32	49
Total	125	42	167

$\chi^2 = 0.2826$

df = (4-1) x (2-1) = 3

Probability greater than 0.9. Therefore not significant; accept NH.

A3.7 Temperature of incubation of the transformation mix.

A3.7.1 Targeting the *niaD* Locus: Vector pniaD2

Repetition of experiments = 2

Parameter tested (different temperature) = 4

Test difference between incubation temperatures: Selected at 25°C

Observed results:

	+	-	Total
ice	50	24	74
22°C	50	23	73
37°C	48	21	69
42°C	30	17	47
Total	178	85	263

Expected results:

	+	-	Total
ice	50.08	23.92	74
22°C	49.41	23.59	73
37°C	46.70	22.30	69
42°C	31.81	15.19	47
Total	178	85	263

$\chi^2 = 0.4528$

df = (4-1) x (2-1) = 3

Probability greater than 0.9. Therefore not significant; accept NH.

Test difference between incubation temperatures: Selected at 37°C

Observed results:				Expected results:			
	+	-	Total		+	-	Total
ice	36	9	45	ice	34.67	10.33	45
22oC	34	13	47	22oC	36.21	10.79	47
37oC	39	9	48	37oC	36.98	11.02	48
42oC	32	11	43	42oC	33.13	9.87	43
Total	141	42	183	Total	141	42	183

χ^2 = 1.4583

df = (4-1) x (2-1) = 3

Probability between 0.7 and 0.5. Therefore not significant; accept NH.

A3.8 Temperature of Incubation of Selective Plates

A3.8.1 Targeting the *niaD* Locus: Vector pniaD2

Parameter tested (different temperatures) = 2

Test difference between selection temperatures

Observed results:				Expected results:			
	+	-	Total		+	-	Total
37oC	141	42	183	37oC	130.89	52.11	183
25oC	178	85	263	25oC	188.11	74.89	263
Total	319	127	446	Total	319	127	446

χ^2 = 4.6506

df = (2-1) x (2-1) = 1

Probability between 0.05 and 0.02. Therefore significant; reject NH

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