CONSTRUCTION OF A NOVEL FUNGAL GUS EXPRESSION PLASMID, AND ITS EVALUATION IN *ASPERGILLUS NIDULANS*.

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ABSTRACT

A GUS expression plasmid, pFunGus, was constructed containing a multi-cloning site for the insertion of gene regulatory elements, to be used in fungal reporter gene studies. A derivative of pFunGus (pFG-gpd) was constructed by the insertion of the *gpdA* promoter (glyceradehyde-3-phosphate dehydrogenase) into the multi-cloning site of pFunGus for the assessment of the plasmid's transformation and expression properties in *Aspergillus niduans*. The correct construction of pFunGus and pFG-gpd was verified by analytical restriction digests and by its property of GUS expression in *A. nidulans*.

The plasmid was integrated into the *A. nidulans* genome via cotransformation with the phleomycin resistance plasmid, pAN8-1. Transformation frequencies of between 3 and 250 transformants per μg of pAN8-1 DNA were obtained. Initial screening for cotransformation yielded no pFG-gpd transformants. Attempts to improve cotransformation frequencies by optimisation of cotransformation conditions were unsuccessful. However, large scale screenings of transformants lead to cotransformants being isolated at a very low cotransformation frequency. Approximately 0.45% of pAN8-1 transformants possessed the GUS phenotype.

The eight pFG-gpd transformants obtained were analysed by Southern hybridisation. Six out of the eight transformants had a single copy integration. Of the remaining two transformants, one had three copies integrated at separate locations, one of which was disrupted, and the other had four copies integrated as tandem repeats, one of which was disrupted. All the transforming DNA appeared to be integrated ectopically.

The physiology of the transformants was assessed by dry weight increase, colony extension and total protein content. These showed that the transformants biology was not significantly compromised by the transforming DNA.

Finally, high levels of GUS expression were observed in all pFG-gpd transformants and the GUS expression per copy of the GUS expression cassette integrated into the genome was constant. These results showed that the transformed gene copy number determined the levels of gene activity rather than the position of integration in the genome.

Overall these results demonstrate the potential application of the versatile GUS expression plasmid, pFunGus for reporter gene studies in filamentous fungi.

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1. INTRODUCTION.

1.1 THE BIOLOGICAL IMPORTANCE OF FUNGI.

Fungi are eukaryotic organisms that have provided model systems for studying cell processes, as stated by Watson *et al.*, 1987 in a text on molecular biology: "If we can solve the same problem equally well with yeast or with human cells, common sense tells us to stick with the simpler less expensive system". This thinking led to a wealth of research using fungi, and headed an understanding of basic processes in eukaryotic cells, in particular of meiosis and mitosis. New research developments have enabled studies in animal and plant systems to become increasingly viable, therefore research of fungi has turned to studying them in there own right as unique life forms, for their contribution to the ecosystem, and importance medically and economically (Wessels, 1991).

Fungi obtain organic nutrients by either infecting other living organisms as parasites, or by breaking down dead organic matter as saprophytes (Alexopoulos and Mims, 1979). They are responsible for the majority of known plant diseases as well as many diseases of human and animals (Wessels, 1991). They also provide the basis for many industrial processes, including commercial fermentations for organic acid production (e.g. citric acid) and in the food industry for the preparation of cheeses. The health industry depends on fungi for the production of drugs, for example corgmentrine and cortisone, vitamin preparations, and finally the manufacture of a number of antibiotics, e.g. penicillin and cephalosporin (reviewed by Davies, 1991; Martin, 1991).

1.2 THE BIOLOGY AND GENETICS OF *ASPERGILLUS NIDULANS.*

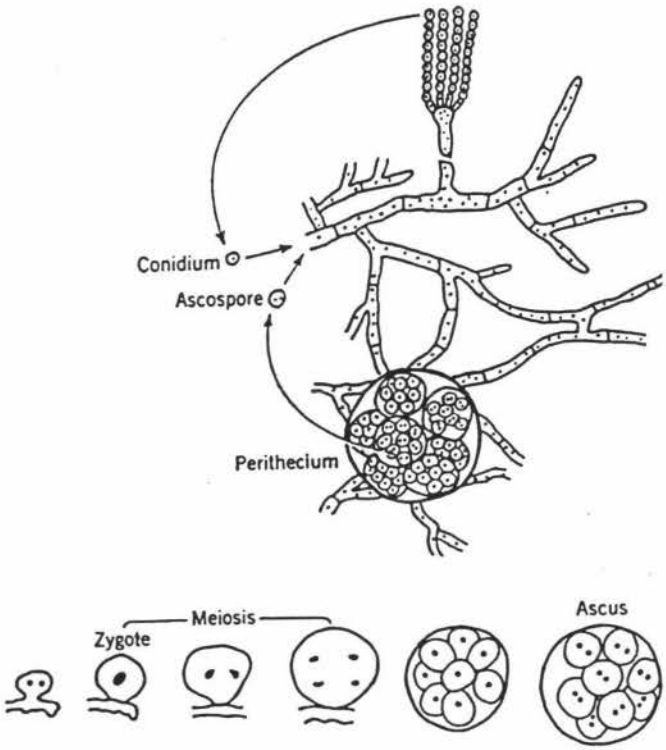
Aspergillus is a widely distributed genus that plays a major role in the natural world as well as in human processes, positively in industry for the production of secondary metabolites and negatively in causing disease in plants, animals and spoilage of foods (Martinelli, 1994). The species *A. nidulans* has been utilised as a model system for the

study of biological and genetic processes in filamentous fungi. Several features of *A. nidulans* make it suitable for study as a model organism (Pontecovo, 1953), and the following discussion will describe *A. nidulans* from this perspective.

Aspergillus nidulans is a filamentous fungi which develops as hyphae which are collectively known as mycelia. Hyphae have cell walls enclosing a cytoplasmic mass which moves inside the tubes. As the cell wall is built at the tip the cytoplasmic mass moves forwards and ramifies into branches (Wessels, 1991). In *A. nidulans* hyphae are septate with multi-nucleate compartments. Undifferentiated hyphae may form structures that produce asexual spores that can then germinate and produce more hyphae. The asexual stage is prevalent in *A. nidulans* and produces spores, (conidia) that contain a single haploid nucleus. The sexual stage can occur by the fusing of two hyphae, either from different or the same mycelia making *A. nidulans* is a homothallic species. Sexual recombination results in meiosis and the production of a multi-cellular structure, an ascus, containing four diploid ascospores. This defines *A. nidulans* as a member of the fungal class, Ascomycetes (Martinelli, 1994). This life cycle is shown in Figure 1.

Aspergillus nidulans has minimal nutritional requirements, and prefers to grow at temperatures around 37-40°C. At 37°C it grows relatively rapidly with a rate of hyphal extension of approximately 4μm per minute on complete or minimal media (Pontecorvo, 1953). Conidia lend themselves to plating techniques used for yeast and bacteria. Rapid growth generally means conidia are available for harvest after 48 hours, and colonies are classifiable by morphology, colour of conidia, etc., thus allowing quick analysis of many generations. Tetrad analysis can be applied to the ascospores produced by the sexual stage and these ascospore have been utilised for the long term storage of strains (Pontecorvo, 1953).

Figure 1 Life cycle of *Aspergillus nidulans,* (reproduced from Pontecorvo, 1953).



The genetics of filamentous fungi has been well studied, especially in *A. nidulans*. This species, relative to other fungi, has a small genome of approximately 20,000kb, about 7 times the size of *E. coli* and 1% the size of a human haploid genome. The genome is organised on 8 chromosomes per haploid genome (reviewed by Turner, 1993). Unique sequences make-up 97-98% of the total genome, with the remaining 2-3% repeated sequences being assigned to the ribosomal RNA genes. The 5′ region of a typical gene carries sequences responsible for the binding of regulatory proteins and general transcription factors. Many genes are strongly regulated by activation and/or repression at the level of transcription (reviewed by Caddick and Turner, 1993). A feature of fungal genome organisation seen in *A. nidulans* is functionally related genes occurring in clusters. These contain two or more loci that code for related functions, examples of these are the loci involved in; alcohol utilisation (Lockington *et al.*, 1985), penicillin biosynthesis, (McCabe *et al.*, 1990), and nitrate utilisation (Johnston *et al.*, 1990). Of the 82+ genes isolated by cloning, many have been sequenced permitting studies of their structure and function (reviewed by Matinelli *et al.*, 1994). For these reasons *A. nidulans* provides a parallel system for the study of processes in more complex and less well defined fungi.

Methods for transforming sequences into *A. nidulans* are well established (Tilburn *et al.*, 1983; Ballance *et al.*, 1983; Yelton *et al.*, 1984). The DNA is integrated into the genome and produces mitotically stable transformants. The multi-nucleate nature of hyphae means balanced heterkaroytes can be created (Pontecorvo, 1953). These contain a mixture of two parental nuclei that differ at one or more genetic loci, and provide the opportunity create and investigate lethal mutations. Transformation has provided an opportunity to study gene expression and regulation at the molecular level. This is discussed in more detail in Section 1.3.

In summary *A. nidulans* provides a good model system for the study of biological and genetic phenomena in filamentous fungi. It is (i) rapidly growing, (ii) its genome organisation is relatively simple with little repeating DNA and (iii) regulation of gene expression predominantly occurring a the level of transcription, (iv) many control and structural genes have been cloned and sequenced, and (v) transformation systems have been established.

1.3 TRANSFORMATION IN FILAMENTOUS FUNGI.

1.3.1 Overview.

Transformation is an experimental method for introducing DNA sequences into genomes. It was first reported in filamentous fungi (*Neurospora crassa*) in 1973 (Mishra and Tatum, 1973), and now is widely established in many types of fungi. Development of transformation systems has not only allowed the introduction of endogenous sequences into fungi, but the transfer of genes between fungal species and from prokaryotes, animal, and plants into fungi. This is termed 'heterologous expression', and predominantly these genes are transcribed, translated, and give functional products. Many gene products of bacteria, plant and animal origin have been produced under the control of fungal regulatory systems, and provide many applications (reviewed by Saunders *et al.*, 1989; Davies, 1994). Also, regulatory systems are transferable between Ascomycetes. This shows conservation of regulatory mechanisms within this class of filamentous fungi (Austin *et al.*, 1990; Couteaudier *et al*, 1993).

There is extreme flexibility in the genes that can be expressed in *A. nidulans* (reviewed by Davies, 1994; Goosen *et al.*, 1992). These include; vertebrate proteins for pharmaceuticals, other fungal proteins for industrial and research purposes, bacterial proteins for selection systems (Section 1.3.2.1) and insect proteins, as reporter genes for the study of gene expression (reporter genes are discussed in Section 1.3.7).

1.3.2 Components of Fungal Expression Vectors used in Transformations.

Vectors used for transformation in filamentous fungi contain, (i) the expression cassette containing the DNA sequence to be expressed attached to fungal regulatory elements, (ii) a bacterial origin of replication, (iii) bacterial selectable marker for cloning purposes e.g. ampicillin resistance, and may also contain (iv) a fungal selectable marker. (The latter can also be supplied on a separate vector though the process of cotransformation; Section 1.3.7) In the following section some of the vector components will be described (reviewed by Turner, 1994).

1.3.2.1 Selectable Markers in A. nidulans.

Several types of selectable markers are available for fungi. These are either auxotrophic selectable markers, or dominant selectable markers (reviewed by Goosen *et al.*, 1992; Turner, 1994). Auxotrophic markers require the complementation of a phenotype in a mutant strain. For example, an *A. nidulans* strain with a mutation in the orotidine-5'-phosphate decarboxylase (*pyrG*) gene will only grow when media is supplemented with uracil or uridine. When transformed with the *pyrG* gene, transformants can be selected on media lacking uracil (Oakley *et al.*, 1987). The drawbacks in using auxotropic markers are the requirement for the appropriate mutation in the recipient strain, and that the mutant strains are often compromised even when supplied with the required substrate (Turner, 1994). Therefore these transformants do not truly represent the wild type genetic background and it is often more desirable to select on the basis of a dominant selectable marker.

Fungal antibiotic resistance markers have been difficult to obtain due to the variable sensitivity of filamentous fungi to antibiotics commonly used in yeast and higher eukaryotes (Goosen *et al.*, 1992). Therefore dominant selectable markers have been created by fusing bacterial antibiotic genes to fungal expression signals. These hybrid-dominant selectable markers have been constructed to confer resistance to hygromycin B (Punt *et al.*,1987), and phleomycin (Mattern *et al*, 1988) for example. Phleomycin resistance is becoming the dominant selectable marker of choice due to *A. nidulans'* sensitivity to it. Little or no growth is seen in the wild type strain at antibiotic concentrations of greater than 10μg per ml of growth medium (Punt *et al.*, 1992). Phleomycin is a metalloglucopeptide antibiotic causing DNA strand scission, and the resistance gene from *Streptomyces hygroscopicus* produces a product that binds and inactivates the antibiotic (Malpartida *et al.*, 1983).

1.3.2.2 The Expression Cassette.

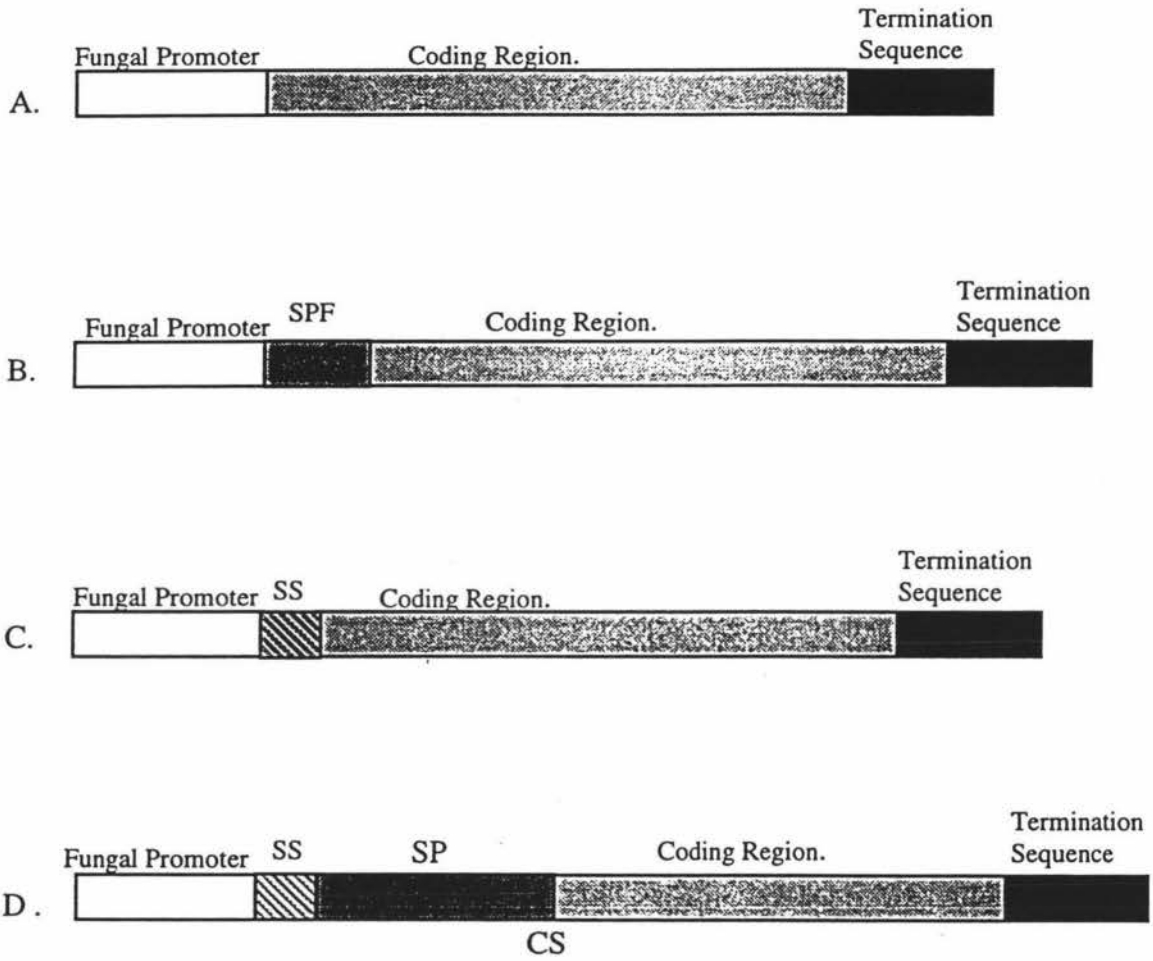
A simple expression cassette contains; a fungal promoter (or regulatory element), the coding region, and the fungal transcription stop sequence. For the expression of heterologous genes the expression cassette may also contain a sequence for a stabilising

protein, a signal sequence e.g. for secretion, or other processing sequences such as a cleavage sequence (Davies, 1994). The types of expression cassette constructs are shown in Figure 2.

In *A. nidulans* several promoters have been used in expression cassettes. The choice is generally based on whether a constitutive or inducible promoter is required. Most promoters used originate from genes involved in carbon-source utilisation pathways, as their expression tends to be high (reviewed by Davies, 1994). Constitutive promoters are generally more convenient because inducible conditions are not required and the product is obtained faster. Examples of constitutive promoters are glyceraldehyde-3-phosphate dehydrogenase (*gpdA*; Punt, 1990), and triose phosphate isomerase (*tpi*; McKnight *et al.*, 1986). However, if the protein to be produced is not stable, is toxic to the cell, or the product needs to be produced at a certain developmental stage, then inducible promoters can be used. Under the control of these promoters gene expression is turned on by a chemical or environmental trigger. Commonly used examples of these are the promoters of acetamidase (*amdS;* Turnbull *et al.*, 1989), and alcohol dehydrogenase I (*alcA;*Gwynne *et al.*, 1987). Finally, parts of promoters and regulatory elements have been used for the assessment of these elements in driving gene expression. For example, the promoter of the *A. nidulans* isopenicillin N synthase gene has been dissected by this method (Pérez-Esteban *et al.*,1993) .

All eukaroytic genes contain a conserved sequence in the region immediately surrounding the translational start codon, called the Kozak sequence (Kozak, 1987). This is involved in the recognition and binding of the gene expression machinery. If heterologous genes being expressed are not of eukaroytic origin then they are often modified to contain this sequence (Ballance, 1991).

Figure 2 Schematic representation of types of expression cassettes.



A and B are cassettes designed for the expression of intracellular gene products, while C and D are cassettes designed for secreted gene products (Section 1.3.2.2). SPF, stabilising protein fusion; SS, signal sequence for secretion; SP, secreted protein coding sequence; CS, endogenous site for enzymatic cleavage of unrequired sequences. This diagram presents constructs described by Davies (1994).

Finally, transcriptional terminators are required for the correct termination and processing of the mRNA product (Devchand and Gwynne, 1991).

1.3.3 DNA Uptake.

Transformation procedures are well established in *A. nidulans* (Tilburn *et al.*, 1983; Ballance *et al.*, 1983; Yelton *et al.*, 1984). The most commonly used and most successful transformation techniques involve preparation of osmotically sensitive cells by the removal of the fungal wall by cell wall-digesting enzymes. These protoplasts are subsequently treated with $CaCl_2$, polyethylene glycol (PEG), and exogenous DNA. After an incubation period the mixture is diluted and aliquots spread on stabilised selective media, either directly or in a soft agar overlay (methods reviewed by Goosen *et al.*,1992; Fincham, 1989). Filamentous fungi do not have non-chromosomal replicons with high copy numbers, such as those used in yeast and bacteria, therefore transformation relies on integration of the DNA into the genome (Davies, 1991). Three types of integration events are observed (Hinnen *et al.*, 1978), and these are discussed in detail in Section 1.3.4.

A model of DNA uptake during transformation is the 'competent cells' model that predicts that not all cells are capable of DNA uptake, but that those that are will take up multiple DNA molecules (Grotelueschen and Metzenberg, 1995). Evidence from cotransformation experiments (Section 1.3.4) heavily favours the 'competent cells' model as cotransformation with two separate vectors occurs at a high frequency (Austin and Tyler, 1990; Wernars *et al.*, 1987).

If only a certain number of cells are competent, what keeps the rest incompetent? Grotelueschen and Metzenberg (1995) hypothesised that this could be a feature of either permeability of the cell membrane or the characteristics of the nuclei. They showed that the requirement for competence is at the level of the nucleus. They then hypothesised that the limiting event in transformation is the availability of suitable sites for integration. The nuclei in a protoplast population divide asynchronously, and therefore there may be only a narrow window in the nuclear cycle in which the genome is available to be transformed. It was shown by Contour-Clamped Homogeneous Electric

Field (CHEF) gel electrophoresis and Southern hybridisation analysis that there are competent chromosome sites for ectopic integration (Grotelueschen and Metzenberg, 1995). This followed hypothetical models proposed in mammalian and yeast cells that ectopic integration may occur at pre-existing breaks in the chromosome (Roth and Wilson, 1988; Schiestl *et al.*, 1993).

The hypothesis that the limiting event in transformation is DNA integration is corroborated by transient expression studies (Mönke and Schäfer, 1993). Research showed that the frequency of transformation resulting in transient expression levels in protoplasts far exceeded the frequency of stable transformation. Also that at least 99% of transformants lose the transforming DNA. This implies that the limiting factor in transformation is DNA integration rather than DNA uptake (Mönke and Schäfer, 1993).

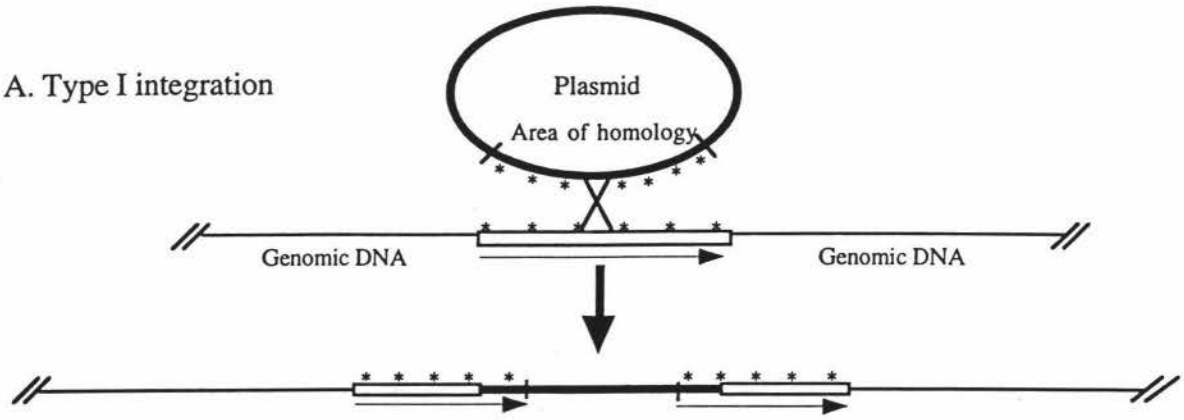
1.3.4 Fate of Transforming DNA: Integration Events.

Stable transformation of *A. nidulans* requires the integration of the exogenous DNA into the genome. Three types of integration were first defined in yeast by Hinnen *et al.* (1978), and those observed in filamentous fungi are very similar (reviewed by Goosen *et al.*, 1992; summarised in Figure 3). Type I integration occurs at a homologous site resulting from a single crossover event between homologous sequences present in the plasmid and the genome. This results in a linked duplication of the sequences separated by the plasmid DNA. Type II integration occurs at a heterologous sites in the genome. It is called 'ectopic integration' and results from a single cross-over event between two non-homologous sequences. With both these types of integration the transformation can contain one or more copies arranged in a tandem array. Finally, type III integration occurs a homologous site resulting in a double cross-over event between the homologous sequences. This is termed gene conversion or replacement and results in the plasmid sequence replacing the sequence in the genome. Methods have been developed using type III integration for the deletion, mutation, and cloning of genes (e.g. Miller *et al*, 1985; Wernars *et al*, 1987). All these integration types have been shown to occur simultaneously in a single fungal transformant (Yelton *et al.*, 1984).

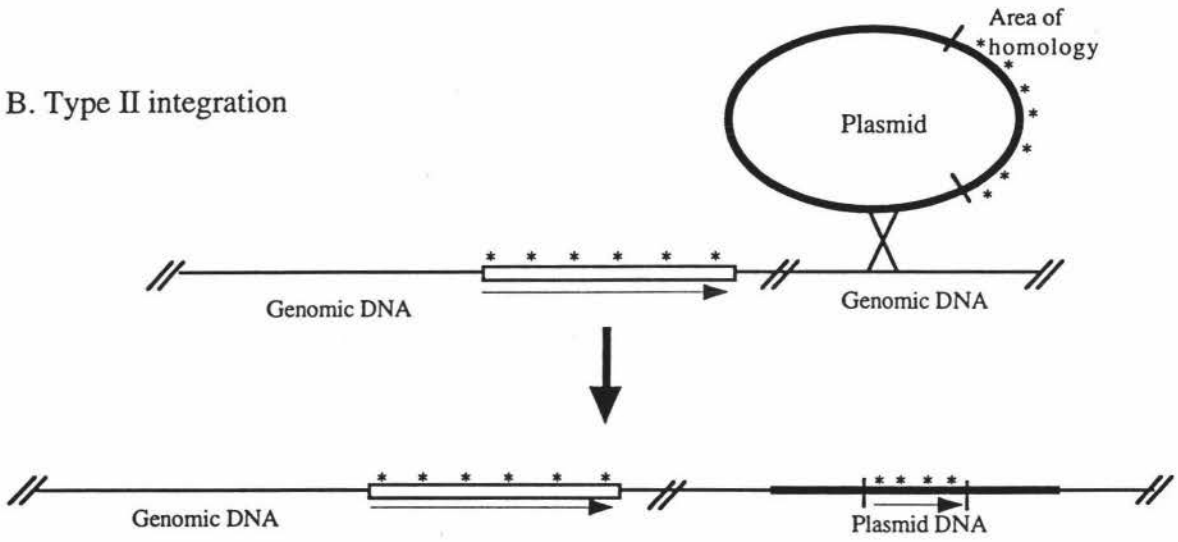
Figure 3 Diagram of the intergration events observed in fungal transformation.

Type I integration results in integration at a homologous site, type II results in ectopic integration and type III results in sequence replacment (Section 1.3.4). The diagram represents the types of integration events described by Hinnen et al, (1978).

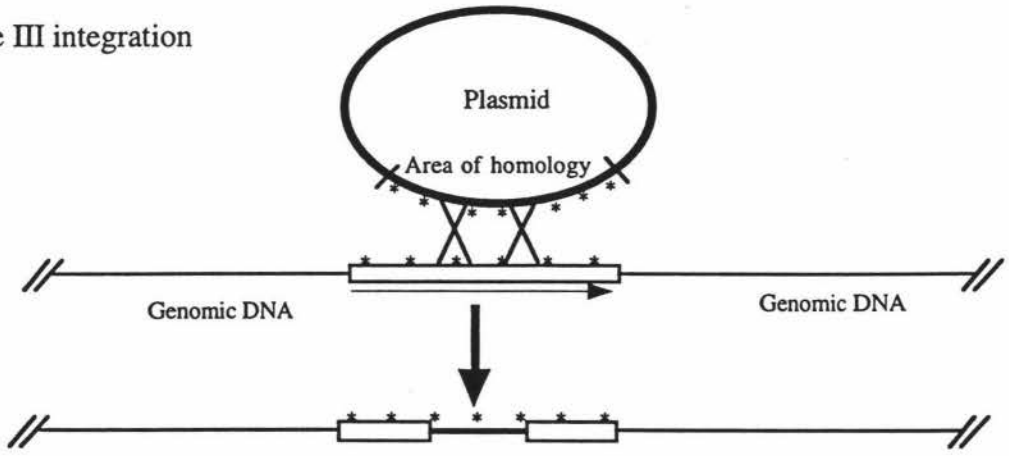
A. Type I integration



B. Type II integration



C. Type III integration



The frequencies with which the different types of integration will occur in a given transformant are difficult to predict as they vary depending on the composition of the vector, the nature of the selective marker, the form in which the vector is used (circular or linear), and the genetic background of the recipient strain. However one general rule is that the number of multi-copy transformants can be increased by increasing the total amount of DNA (reviewed by Goosen *et al.*, 1992).

1.3.5 Fate of Transforming DNA: Transient Transformants.

In transformation experiments two types of transformants are commonly observed. The first type grow strongly and spore well on selective media, the second type present weaker growth and fail to grow when transferred to fresh selective media. The latter type has been classified as abortive transformants (Tilburn *et al.*, 1983). It is hypothesised that the molecular basis of this is transient expression of the transformed vector. The vector is not integrated into the genome and is lost during cell division. The product of the transient expression may confer the transformed phenotype until it is titrated out by mitosis. Transient expression can be used to investigate gene expression without the need for stable integration. This method saves time, circumvents the problems of multi-copy integration and position effects (Section 1.3.6), and is useful for establishing transformation techniques and for rapid evaluation of expression signals (Mönke and Schäfer, 1993; Judelson *et al.*, 1993).

Stable expression requires integration into the genome and once this has occurred stability remains through many rounds of sub-culturing on non-selective media (Yelton *et al*, 1984). However meiotic instability has been observed in some filamentous fungi, and has been extensively studied in *N. crassa*. It has been shown to be due to gene inactivation by methylation, and is termed RIP - Repeat Induced Point Mutation (Selker, 1990). RIP seems limited to a subset of filamentous Ascomycetes, and has not been reported in *A. nidulans*, although other forms meiotic instability, for example unequal recombination, may still occur (Goosen *et al*, 1992).

1.3.6 Position Effects and Gene Copy Number in Determining Levels of Transformed Gene Expression.

Once an expression cassette has integrated into the genome levels of transformed gene expression may still vary depending on the number of copies of the cassette introduced and the area of the genome that the cassette is introduced into (position effects; Turner, 1994). There are two sources of position effects. Firstly, the transformed gene may integrate downstream of promoter elements. These elements may be positively or negatively regulated, and therefore will have an additive effect with the transformed regulatory elements to alter the expression of the transformed gene . Secondly, the region of the genome that the DNA integrates into may be transcriptionally active or inactive, and may vary in activity depending on the developmental stage or on external stimuli (Couteaudier *et al.*, 1993). It has been clearly shown that position effects play a large role in the expression of transformed genes in animals and plants, while they play a very small role in bacteria (Martinelli, 1994). The significance of position effects seems to climb as the complexity of the genome increases and the genome contains larger amounts of repeated DNA sequences.

In keeping with this is the observation that in fungi the strength of position effects is connected with the genome complexity. For example in the position effects are significant in the fungal class, Oomycetes, although to a lesser extent than plants and animals (Judelson *et al.*, 1993). The genome of Oomycetes' is more complex than the genome of the Ascomycetes', also, *A. nidulans* has a relativity simple genome with only 2-3% consisting of repeated sequences (Mao and Tyler, 1991; Section 1.2). Therefore compared to Oomycetes, animals and plants, position effects may be expected to play a less significant role in determining the levels of expression of a transformed gene. This has been demonstrated by a correlation between an increase in copy number and an increase in hygromycin resistance (Punt *et al.*, 1987) in *Aspergillus* species. In the production of heterogeneous gene products, e.g. human interferon, high copy number also gave increased yields of the product (Gwynne *et al.*, 1987). A comprehensive study of the activity of interferon α-2 under the control of *alcA* (alcohol dehydrogenase) expression signals was carried out for transformants containing different copy numbers

of the expression cassette. The importance of the copy number of the *alcR* (alcohol dehydrogenase regulatory gene) was also analysed. It was concluded that expression increased in a roughly linear fashion with the number of copies present up to a number of 7-10 copies. It was hypothesised that when the *alcA* copy number reached 10 copies the *alcR* product was titrated out. This was corroborated by experimental evidence showing that the multi-copy *alcR* transformants continued the linear relationship up to 20 copies. Therefore, in this case copy number showed a linear relationship with yield (Davies, 1991). However, in another Ascomycetes, *Fusarium oxysporum*, it was concluded that position effects were more important than gene copy number. This was based on the comparison of two single copy transformants, one single copy transformant showed enzyme activity 80-fold higher than another single copy transformant. Plasmid rescue and sequence analysis indicated that the highly expressing single copy transformant had integrated downstream of a recognisable enhancer element (Couteaudier *et al.*, 1993). Studies on the relative effects of copy number and position are complicated due to the difficulty in determining the number of active copies of the cassette accurately.

1.3.7 Cotransformation.

When a sequence is introduced into an organism, it may be a disadvantage to need to physically link the sequence to a selectable marker. However, introduction of a vector containing a non-selectable sequence can be achieved by dual transformation with a vector containing a selectable gene, without the need for prior covalent linkage of the two vectors. This process is called cotransformation, and it has been established in *A. nidulans* (Wernars *et al.*, 1987). Cotransformation occurs since not all protoplasts in a preparation are available to be transformed, however a single protoplast that is competent can transform more than one plasmid simultaneously (Grotelueschen and Metzenberg, 1995; discussed in Section 1.3.2).

This principle was first demonstrated by Wernars *et al.* (1987), *A. nidulans* was cotransformed with vectors containing the *amdS* gene and *trpC* gene. Each vector was initially selected for, and transformants screened for the presence of the second vector. Results showed that the cotransformation frequencies varied depending on which vector

was initially selected for; 95% of the *amdS*$^+$ transformants were also *trpC*$^+$, while only 62% of the *trpC*$^+$ transformants were *amdS*$^+$. This was hypothesised to reflect a relatively large number of *amdS* genes becoming inactivated during the act of integration, since similar results were obtained with a plasmid containing both the genes together. This shows that the frequency of cotransformation is dependent on the nature of the selection system. It was also observed that cotransformation frequencies increased with increases in the total amount of cotransforming vectors, and increases in the ratio of cotransforming vector to transforming vector (containing the selectable marker).

Since these initial experiments cotransformation has been extensively used to introduce a variety of genes for the production of heterologous gene products in industry, e.g. *E. coli* enterotoxin subunit B (Turnbull *et al.*, 1989) and human interferon α-2 (Gwynne *et al*,1987) and reviewed by Saunders *et al.*, 1989. Also for the study of gene expression by the introduction of the reporter genes e.g. β-galactosidase (*lacZ*; Van Gorcom *et al.*, 1986) and β-glucuronidase (*gusA*; Roberts, *et al.*, 1989) (discussed in Section 1.3.7). Most papers report cotransformation as a percentage of the transformants, of above 60%. In a study by Roberts *et al.*, (1989) it was reported that when cotransforming with vector containing the *gusA* gene and the selectable marker *argB*, they reported cotransformation frequencies "up to 50%".

1.4 THE USE OF REPORTER GENES IN THE STUDY OF FUNGAL PROCESSES.

1.4.1 Overview of Methods to Study Fungal Cell Processes.

Through microscopy, with or without chemical staining procedures, it is possible to see cells and the organelles within them, but this gives only limited information about the metabolic processes that are occurring. A classical path for the elucidation of fungal cell metabolic processes has used a combination of classical genetic techniques for the creation of mutants, and the characterisation of these mutants by biochemical analysis. In *A. nidulans*, examples of metabolic processes that have been studied this way are; carbon

metabolism, alcohol metabolism, inorganic nitrogen assimilation, sulphur metabolism etc (reviewed in Martinelli and Kinghorn, 1994). A question not answered by this type of biochemical analysis is how these processes are spatially organised in the fungal cells. Recent staining techniques utilise fluorochromes, dyes that are absorbed by cell organelles or bind to specific residues on or inside the cells (reviewed by Butt *et al.*, 1989). Upon binding to their target these produce a fluorescence that is detectable through a microscope. In mycology, this has revealed much information about cell processes, and the differentiation and growth of the hyphae. However, these dyes have disadvantages. Their cytological use is rather limited, they are only available for certain cell types under certain conditions, and it is not known what damage they or the cell fixing process required inflicts on the cell (Yuan and Heath, 1990). Recent molecular genetic techniques have been developed that allow the study of specific cell processes by both visual observation and biochemical assay. This involves the linking the promoter of a gene involved in a process of interest to a reporter gene (Bardonnet, 1988).

A reporter gene is a gene whose product is easy to detect and assay. When fused to expression signals from a gene of interest, the reporter gene expression reflects the expression characteristics determined by those signals in the original gene (Jefferson, 1987). There are several considerations when using reporter genes in fungi. For analysis of expression signals downstream from the promoter the reporter gene may need to be fused in frame with the protein-coding region of the gene of interest, and some processing will not occur as for the endogenous protein. The reporter gene product will not be transported in the same manner as the endogenous protein, although the fusing of a signal peptide may alleviate this. Because fungal vectors must integrate into the genome, the level of reporter gene activity may not only reflect the activity conferred by the expression signals, but also will be influenced by position effects and gene copy number (Section 1.3.6). Another consideration is the stability of the reporter gene product. A stable gene product may be necessary for some applications, but for quantitative measurements, the levels of reporter gene activity may not represent the levels of promoter activity at that time but the accumulation of the reporter gene product over development. This problem can be circumvented by using inducible promoters (Section 1.3.2).

The first reporter gene used in filamentous fungi was the *E. coli* β-galactosidase (*lacZ*). This system is commonly used in bacteria and plants (reviewed by Silhavy and Beckwith, 1985), and was experimented with in *A. nidulans* (van Gorcom *et al.*, 1985; 1986). In *A. nidulans, lacZ* fusions have facilitated the molecular characterisation of promoters and their individual elements, such as the promoters of 3'-phosphoglycerate kinase (Streatfield *et al.*, 1992) and isopenicillin-N-synthetase (Pérez-Esteban *et al.*, 1993). However, the sensitivity of this system is low due to the endogenous β-galactosidase enzyme activity that is present in many fungi (Roberts *et al*, 1989).

1.4.2 The β-glucuronidase (*gusA*) Reporter Gene System.

Recently attention has focused on an alternative reporter gene, the *E.coli uidA* (or *gusA*) gene. Encoding β-glucuronidase, a hydrolase enzyme (Jefferson, 1987). It has been developed as a powerful tool for the assessment of gene activity in transgenic plants and for the development of molecular genetic analysis of plants. This system is effective in plants as there is no endogenous enzyme activity, and both histochemical and fluorometric substrates are available (Jefferson, 1987). These substrates are cleaved by the GUS enzyme to give a product that can be qualitatively or quantitatively detected. Another attractive feature of the *gusA* gene system is that its product is able to tolerate large amino-terminal additions, therefore construction of translational fusions are feasible and provide a valuable method for assaying the behaviour of transit or signal peptides (Jefferson, 1987). Also, with the appropriate signals the GUS enzyme can be translocated across chloroplast membranes with high efficiency indicating an ability to be translocated across all cell membranes (Jefferson, 1987).

The use of *gusA* as a potential reporter gene in fungi was initially studied by Roberts *et al.* (1989). In this study, *A. niger, A. nidulans*, and *Fulvia fulva* were transformed with the *gusA* gene driven by the *A. nidulans* promoter, *gpdA*. Histochemical staining of mycelia and assays of cell extracts with a fluorometric substrate showed that the *gusA* gene had been expressed. Unlike *lacZ* fusions in fungi, no significant endogenous GUS activity was observed. Since these initial studies *gusA* systems have been set up in other fungi, e.g. the plant pathogens *Pseudocercosporella herpotrichoides* (Bunkers, 1991), and *Fusarium*

oxysporum (Couteaudier *et al.*, 1993). The *gusA* reporter gene system has been used in several applications. GUS activity was demonstrated in perennial ryegrass infected with *gusA* transformed *Acremonium* endophyte. This demonstrated the use of a surrogate host for the introduction of foreign genes into perennial ryegrass (Murray *et al.*, 1992). Transient expression of transformed genes has been demonstrated in *Cochliobolus heterosrophus* using *gusA* (Mönke and Schäfer, 1993). The GUS reporter gene system has been used to demonstrate lack of conservation of gene expression machinery between the classes of fungi, Ascomycetes and Oomycetes (Judelson, 1992; Pieterse *et al.*, 1995). Finally, *gusA* fusions to the *adh1* (alcohol dehydrogenase) and *ver1* (aflatoxin biosynthesis gene) promoters of *A. flavus* have allowed the evaluation of transcriptional control and induction of aflatoxin production *in situ* (Woloshuk and Payne, 1994; Flaherty *et al.*, 1995).

1.4.3 GUS Expression Plasmids.

The components of GUS expression vectors are essentially the same as those for the expression of other heterologous genes (see Section 1.3.2). The GUS expression cassette is the most important component in the expression vector. It includes the *gusA* gene, transcriptional regulatory sequences, and sequences required for the termination and processing of the RNA transcript (Devchand and Gwynne, 1991). Because of the application GUS expression vectors to studying and comparing many promoter elements, a basic GUS vector without the expression sequences, and with a multi-cloning site for ease in cloning in these sequences can be initially constructed (this study).

A popular promoter for driving *gusA* expression when systems are being developed is the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (e.g. Roberts *et al.*, 1989; Bunkers, 1991). This is a gene from the glycolytic pathway, therefore it is strongly expressed in all cell types at all times in development. Other attractive features of this gene are that it is expressed in the cytosol, and that regulation is at the transcription level (Punt et al, 1988). This promoter has previously been used to drive the expression of *lacZ* (Punt et al, 1990), and other heterologous genes (e.g. phleomycin resistance; [Punt, 1992]).

1.5 AIMS AND OBJECTIVES OF THIS STUDY.

The aim of this project was to develop a new fungal GUS expression vector with a multi-cloning site and to assess its properties relevant to uses in reporter gene studies.

The steps to be taken in the project were:

- Construction of the GUS expression vector containing a multi-cloning site,
- Testing of the vector by insertion of the constitutive promoter, *gpdA*,
- Evaluation of its performance in cotransformation using the dominant selectable marker, phleomycin resistance,
- Testing the physiological impact of the presence of the vector DNA in transformation,
- Assessing the relationship between transformed gene copy number and GUS expression levels in the transformants.

2. MATERIALS AND METHODS.

2.1 FUNGAL STRAINS, BACTERIAL STRAINS AND PLASMIDS

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

2.2 GROWTH MEDIA.

2.2.1 Liquid Media.

All media were prepared using deionized water (prepared by the Milli-Q system). After preparation the media was sterilised by autoclaving at 121°C for 20 minutes. Media were cooled to room temperature before the addition of antibiotics, supplements and/or inoculation. Media were stored at 4°C until use.

Luria Broth (LB) (Miller,1972), contained 10g/l typtone (Difco), 5g/l yeast extract (Difco), and 5g/l NaCl. The pH was adjusted to 7.0.

SOC Media (Dower *et al.*, 1988) was prepared with 20g/l typtone (Difco), 5g/l yeast extract (Difco), 0.6g/l NaCl, 0.2g/l KCl, 0.95g/l $MgCl_2$, 2.5g/l $MgSO_4.7H_2O$ and 3.6g/l glucose.

Potato Dextrose Broth (PD) was prepared using 24g/l potato dextrose (Difco). The pH was adjusted to 6.5.

Aspergillus Complete Media (ACM) (Bird *et al.*, Unpublished) was prepared with 5.0 g/l malt extract (Difco), 2.5 g/l yeast extract (Difco), 0.01g/l $CuSO_4.5H_2O$, and 10g/l D-glucose.

Aspergillus Complete Media (osmotically stabilised) was prepared as above with the addition of 274g/l sucrose prior to autoclaving.

Table 1 Strains and Plasmids.

Strain or Plasmid	Relevant Characteristics	Source or Reference
Fungal strains		
Aspergillus nidulans		
R21	*paba*(A1), *y*(A1)	Waldron and Roberts, 1973.
Glasgow WT	-	FGSC (NRRL194)[a]
Bacterial strains		
Escherichia coli		
XL-1	*sup*E *hsd*R *rec*A *end*A *gyr*A *thi rel*A lac⁻F' [*pro*AB⁺ *lac*I^{q}Δ (lacZ) M15 Tn10(tet^r)]	Bullock *et al.*, 1987.
DH1	F' *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 (r^-m^+) *sup*E44 *rel*A1 λ-	Hanahan, 1983; Low, 1968.
JM101	Δ(*lac-pro*AB *sup*E *thi*-1 F' [*lac*I^q ZΔM15 *tra*D36 *pro*AB$^+$]	Yanisch-Perron, 1985.
Plasmids		
pGEM®-1	OriR, Amp^R, (2.9kb).	Promega.
pRAJ279	Amp^r, containing 1.9kb GUS gene fragment, (4.6kb).	Jefferson, 1987.
pAN8-1	Phleo^r, Amp^r, (5.9kb).	Mattern and Punt, 1988.
pNom102	Amp^r, containing a 1.9kb GUS gene fragment, *A. nidulans* 0.6kb TtrpC fragment and 2.2kb *gpd*A promoter, (7.4kb).	Roberts *et al.*, 1989.
pBR322	Amp^r, Tet^r, (4.4kb).	Boehringer Mannheim.
pUC118	rep$_{pMB1}$ Amp^r lacZ', (3.2kb).	Messing, 1983.
pFunGus	pGEM-1 containing a 1.9kb GUS fragment from pRAJ275 and 0.6kb TtrpC fragment from pNOM102, (5.6kb).	This study.
pFG-gpd	pFunGus containing a 2.2kb *A. nidulans gpd*A promoter fragment from pNOM102, (7.7kb).	This study.

[a] Catalogue of Strains - Fungal Genetics Stock Centre. **Supplement to Genetics Newsletter 39**. (1992). Dept. Microbiology, University of Kansas Medical Centre, Kansas City.

Punt Complete Media, (PCM), (Punt and van del Hondel, 1992) was prepared with: 70mM $NaNO_3$, 7mM KCl, 11mM KH_2PO_4, 2mM Mg_2SO_4, 1% (w/v) glucose, 0.5% (w/v) yeast extract, 0.2% (w/v) casamino acids, trace elements; 0.076mM $ZnSO_4$, 0.178mM H_3BO_3, 0.025mM $MnCl_2$, 0.018mM $FeSO_4$, 0.0071mM $CoCl_2$, 0.0064mM $CuSO_4$, 0.0062 mM Na_2MoO_4, 0.174mM ethylenediaminetetraacetic acid (EDTA) (from 1000× stock solution) and vitamins; 0.1mg/l thiamin, 0.1mg/l ribofavin, 0.1mg/l nicotinamide, 0.05mg/l pyridoxine, 0.01mg/l pantothenic acid, 0.2µg/l biotin] (from 1000× stock solution).

Punt Complete Media (osmotically stabilised) was prepared as above with the addition of 1.2M sorbitol.

2.2.2 Solid Media.

Solid media were prepared by the addition of agar (Davis) prior to autoclaving. After sterilisation, media was cooled to 50°C before the addition of antibiotics or supplements, and pouring into petri dishes or bijou bottles for slopes. Plates and slopes were stored at 4°C until use.

Media for plates and slopes was prepared by adding 15g/l agar (Davis) to liquid media.

Media for top agar overlay was prepared by adding 8g/l agar (Davis) to liquid media.

2.2.3 Media Antibiotics and Supplements.

For *E. coli* selection LB was supplemented with Ampicillin (Boehringer Mannheim).was used at a final concentration of 100µg/ml (Amp^{100}) or 150µg/ml (Amp^{150}) from a stock solution of 1mg/ml of deionised sterile water. This was stored at -20°C.

For *A. nidulans* selection the antibiotic Phleomycin (Sigma) was used at a final concentration of 10µg/ml from a stock of 25mg/ml, stored at 4°C (Punt and van del Hondel, 1992).

ACM was supplemented for *A. nidulans* strain R21 with 1g/l ρ-aminobenzoic acid (PABA), from a stock of 1g/ml, (stored at 4°C), prior to autoclaving (Waldron and Roberts, 1973).

2.3 MAINTENANCE OF CULTURES.

E. coli cultures were maintained on LB plates supplemented with the appropriate selective antibiotics (Section 2.2). Cultures were grown at 37°C overnight and then stored at 4°C. They were regularly restreaked onto fresh LB plates.

E. coli cultures were stored as glycerol stocks. A 1ml aliquot of glycerol solution (65% glycerol, 0.1M $MgSO_4$, 0.025M Tris-HCl pH 8.0) was mixed with 1ml of overnight LB broth culture. These were stored at -70°C (Ausubel *et al.*, 1994).

A. nidulans cultures were maintained on ACM slopes with appropriate supplements (Section 2.2). These were grown at 37°C until conidia were produced and then stored at 4°C. They were transferred onto fresh ACM slopes every 2 months.

2.4 COMMON BUFFERS AND SOLUTIONS.

All solutions were prepared using deionised water, and autoclaved at 121°C for 20 minutes, unless otherwise stated. All recipes were obtained from Ausubel *et al..*, (1994) unless otherwise stated.

2.4.1 TE buffer.

Contained 10mM Tris-HCl, 1mM Na_2EDTA and was prepared from 1M Tris-HCl (pH 8.0) and 0.5M Na_2EDTA (pH 8.0) stock solutions.

2.4.2 1 × TBE Buffer.

Contained 89mM Tris-HCl, 2.5mM Na_2EDTA and 89mM boric acid, pH 8.3.

2.4.3 1 × TAE Buffer.

Contained 40mM Tris-HCl, 20mM acetic acid and 2mM Na_2EDTA.

2.4.4 Tris-Equilibrated Phenol.

The phenol was melted at 50°C, and an equal volume of 1M Tris-HCl (pH 8.0) added, this was mixed and allowed to separate. The phenolic phase was removed and the washes continued until pH of the phenol was 7.5-8.0. The equilibrated phenol was stored under 100mM Tris-HCl (pH 8.0), in a brown bottle, at -20°C until required (NB. Phenol was not autoclaved).

2.4.5 DNase free RNaseA.

Contained 10mg/l RNaseA in sterile deionised water, which was heated to 100°C for 15 minutes, and stored in aliquots at -20°C (NB. RNaseA was not autoclaved).

2.4.6 Lysis Buffer.

Contained 25mM Tris-HCl, 10mM Na_2EDTA, 15% (w/v) sucrose, it was filter sterilised and stored at 4°C. When required 2mg/ml of powdered lysozyme (Sigma) was added (Kreg and Melton, Harvard University, unpublished).

2.4.7 Gel Loading Buffer (10 ×).

Contained 20% (w/v) Ficoll 4000, 0.1M Na_2EDTA, pH 8.0, 1% (w/v) soduim dodecyl sulphate, 0.25% (w/v) bromophenol blue, and 0.25% (w/v) xylene cyanol.

2.4.8 OM Buffer.

Contained 1.2M $MgSO_4.7H_2O$, 10mM Na_2HPO_4, and 100mM $NaH_2PO_4.2H_2O$ which was added until the pH was 5.8, (Itoh *et al.*, 1994).

2.4.9 STC Buffer.

Contained 1M sorbitol, 50mM Tris-HCl, pH 8.0, 50mM $CaCl_2$ (Yelton *et al.*, 1984).

2.4.10 40% PEG Solution.

Contained 40% (w/v) PEG 4000, 50mM $CaCl_2.2H_2O$, 50mM Tris-HCl pH 8.0, 1M sorbitol (Yelton *et al.*, 1984).

2.4.11 GUS Extraction Buffer.

Contained 50mM $NaPO_4$, pH7.0, 10mM Na_2EDTA, 0.1% (w/v) sodium lauryl sarcosine, 0.1% (w/v) Triton x-100. This was autoclaved and 10mM β-mercaptoethanol was added prior to use (Jefferson, 1987).

2.4.12 10 × SSPE.

Contained 1.8M NaCl, 0.1M Na_2HPO_4/NaH_2PO_4, (either salt can be used), 10mM Na_2EDTA, pH 7.0 (Sambrook *et al.*, 1989).

2.4.13 1000× Ethidium Bromide Stock (for staining of agarose gels).

Contained 0.5mg/ml of sterile deionised water and stored in the dark at 4°C.

2.5 DNA PREPARATIONS.

2.5.1 Phenol/Chloroform Purification of DNA.

An equal volume of phenol/chloroform (equal volumes of Tris-equilbrated phenol and chloroform) was added to the DNA solution and the mixture was vortexed for at least 1 minute. This was micro-centrifuged for 10 minutes. The upper aqueous phase was transferred to a fresh tube, if there was visible material at the interface then the phenol/chloroform purification was repeated. An equal volume of chloroform /isoamyl alcohol (24 volumes chloroform and 1 volume isoamyl alcohol) was added to upper phase and vortexed and centrifuged as above. The upper phase was collected in a fresh tube and concentrated by ethanol precipitation (Section 2.5.2; Ausubel *et al.*, 1994).

2.5.2 Concentration of DNA by Ethanol Precipitation.

To concentrate the DNA solutions, 0.1 volume of 3M sodium acetate pH 4.6 (pH adjusted with glacial acetic acid) was added to the preparation. This was followed by 2 to 3 volumes of absolute ethanol, this was mixed, and placed at -20°C for at least 30 minutes. The DNA was pelleted by centrifugation for 15 minutes, and washed with an

equal volume of 70% ethanol. The DNA was vacuum dried and resuspended in sterile TE buffer (Section 2.4.1; Ausubel *et al.*, 1994).

2.5.3 Boiling DNA Mini-preparation.

Small scale preparations of bacterial plasmids were carried out according to the method in Ausubel *et al.*, (1994). Five millilitres of sterile liquid LB medium with the appropriate supplements (Section 2.2) was inoculated with a single bacterial culture and the culture was grown to saturation overnight. A 1.5ml aliquot of this culture was spun for 30 seconds in a micro-centrifuge to pellet the cells, and the supernatant was removed. The pellet was resuspended in 300µl of STET solution (8% (w/v) sucrose, 5% (w/v) triton x-100, 50mM EDTA, 50mM Tris-HCl pH 8.0, filter sterilised and stored at 4°C) with 200µg lysozyme added immediately prior to use, and the pellet was resuspended by vortexing. This was placed on ice for 10 minutes, then in a boiling water bath for 1 to 2 minutes. There after the tube was immediately spun in a micro-centrifuge for 15 to 30 minutes and the pellet removed with a toothpick. An equal volume of cold isopropanol was added to the supernatant and the tube was placed at -20°C for 15-30 minutes, then spun for 5 minutes in micro-centrifuge. The supernatant was removed, the pellet washed with 1ml of 70% ethanol and then harvested as before. The supernatant was discarded and the washed pellet vacuum dried. Finally the pellet was resuspended in 50µl TE buffer (Section 2.4.1). For plasmid digests 5µl of this preparation was used with the addition of RNase A (Sections 2.7.1 and 2.4.5).

2.5.4 Large Scale Plasmid Preparation with Purification by PEG Precipitation.

This protocol was developed by Kreg and Melton. *E. coli* strains were grown overnight on a shaker in 250ml of LB selective media with good aeration. Cells were centrifuged at 5000 × g (5800 rpm, GSA rotor) at 4°C for 15 minutes, and resuspended in 6ml of freshly prepared ice cold lysis buffer (Section 2.4.6). Cells were placed in ice cold water for 20 minutes, following this 12mls of freshly prepared 0.2N NaOH, 1% (w/v) SDS was added then mixed by inversion. This was placed in ice cold water for 10 minutes then a 7.5ml aliquot of 3M sodium acetate, pH4.6, was added and mixed by inversion.

This was placed in ice water for 20 minutes then centrifuged at 10,000 × g (9300 rpm, SS34 rotor) for 15 minutes. The supernatant was removed and 50µl of 1mg/ml RNaseA (Section 2.4.5) was added and the mixture incubated at 37°C for 20 minutes. DNA was purified with phenol/chloroform (Section 2.5.1). Two volumes of absolute ethanol were added to the aqueous phase and it was mixed and left for 30 minutes at -20°C. The preparation was then centrifuged at 10,000 × g (9300 rpm SS34 rotor) for 20 minutes. The resulting pellet was dissolved in 1.6ml sterile water, 0.4ml of 4M NaCl was added, then 2ml of 13% polyethylene glycol (13% (w/v) PEG 4000 in sterile deionised water), this was mixed and placed in ice cold water for 60 minutes. The preparation was centrifuged at 10,000 × g (9300 rpm, SS34 rotor) of 10 minutes, the supernatant removed and the pellet washed with 70% ethanol. This was spun for 5 minutes, the ethanol poured off and the pellet dried. Finally the pellet was resuspended in sterile TE buffer (Section 2.4.1). The DNA solution was quantitated as in Section 2.6.1.

2.5.5 Large Scale Plasmid Preparation with Purification by CsCl/EtBr Centrifugation.

This method was based on that of Ish-Horowitz and Burke (1981). Cells containing the plasmid were grown in 500ml of liquid LB + Amp (see Section 2.2), overnight on a shaker at 37°C with good aeration. The cells were harvested by centrifuging at 10400 × g (8000 rpm; GSA rotor) for 10 minutes. The pellet suspended in 100ml of TE buffer (Section 2.4.1) and cells centrifuged at 10400 × g (8000 rpm; GSA rotor) for 10 minutes as before and suspended in 30ml Solution I, (50mM glucose, 25mM Tris-HCl pH8.0, 10mM Na_2EDTA). A 3ml aliquot of lysozyme solution was added, (50mg/ml lysozyme dissolved in Solution I), and the preparation was left at room temperature for 10 minutes. Then, 60ml of freshly prepared Solution II (0.2N NaOH, 1% (w/v) sarkosyl) was added, the preparation was mixed by inversion and placed on ice for 10 minutes. Next, 45ml of Solution III (60ml of 5M potassium acetate, 11.5ml glacial acetic acid, 28.5ml H_2O), was added, the preparation mixed by inversion, placed on ice for 10 minutes, and then centrifuged at 10400 × g (8000 rpm; GSA rotor) for 10 minutes. The DNA was precipitated by adding 0.6 volumes of cold isopropanol to the supernatant, and the mixture was left at room temperature for 20 minutes. DNA was pelleted by

centrifuging at 17300 × g (12000 rpm; GSA rotor) for 10 minutes and the pellet was washed with 70% ethanol. The DNA pellet was dried and resuspended in 7.5ml TE buffer (Section 2.4.1). CsCl was added at a concentration of 1.05g/ml of DNA solution, and 75µl/ml of ethidium bromide stock (10mg/ml) was added, mixed well, and placed at 4°C overnight. The next day the preparation was centrifuged at 17300 × g (12000 rpm; SS34 rotor) for 10 minutes and the supernatant retained. The refractive index (Refractometer, Waston Victor) of the supernatant was checked to be between n=1.3860 and 1.3920, if it was below 1.3860 a small amount of CsCl was added until the correct refractive index was reached. The preparation was centrifuged in Beckman TLV-100 at 70000 rpm for 5 hours. The band containing the plasmid DNA was visualised as a fluorescent band under long-wave light and removed with a 18 gauge hypodermic needle and syringe. Ethidium bromide was removed by extraction with equal volumes of isopropanol saturated with 20× SSC buffer (3m NaCl, 0.3M sodium citrate), until the pink colour disappeared. Finally, the DNA was dialysed against 1 litre of TES buffer (10mM Tris-HCl, 1mM EDTA, 100mM sucrose) at 4°C with 4 changes of buffer. The DNA solution was quantitated as in Section 2.6.1.

2.5.6 Mini Plasmid Preparation for Checking Presence of Insert DNA.

This method was developed by Malone, (personal communication). Transformed bacteria were transferred by toothpick into 3ml liquid LB + Amp (see Section 2.2) and grown overnight on a shaker at 37°C. A 1ml aliquot of each culture to be analysed was micro-centrifuged for 45 seconds and the supernatant was removed. Followed by the addition of 50µl of freshly prepared Cracking solution (50mM Tris-HCl pH 7.0, 1% (w/v) SDS, 2mM Na_2EDTA, 0.4M sucrose, 0.01% (w/v) bromophenol blue dye, stored at room temperature, with 1mg/ml lysozyme, and 25µg/ml RNaseA (from stock, Section 2.4.5) added prior to use), and vortexing of the mixture. The preparations were micro-centrifuged for 30 minutes and the supernatant was loaded directly onto a 0.9% agarose electrophoresis gel (see Section 2.7.2).

2.5.7 Mini-preparation for Isolation of DNA from Fungal Cultures.

The method for small scale preparations of genomic DNA from *A. nidulans* was based on the method of Yoder (1988). *A. nidulans* liquid cultures were grown to mid log phase, mycelia were removed by filtration and freeze dried overnight. Approximately 30mg (dry weight) of mycelia were ground in liquid nitrogen and suspended in 500µl extraction buffer, (100mM LiCl, 10mM EDTA, 10mM Tris-HCl pH 7.4, 0.5% (w/v) SDS), this was vortexed for 10 seconds. Phenol/chloroform purification was carried out (Section 2.5.1), RNaseA (Section 2.4.5) was added and the preparation was incubated at 37°C for 20 minutes. Two further rounds of phenol/chloroform purification were carried out, the DNA was concentrated by ethanol precipitation (Section 2.5.2) and resuspended in TE buffer (Section 2.4.1). The DNA solution was quantified as in Section 2.6.1.

2.5.8 Extraction of DNA Fragments from Agarose Gel.

DNA fragment to be extracted was purified by electrophoresis on 0.7-1% agarose which was dissolved in 1× TAE buffer (Section 2.4.3) in 1× TAE electrophoresis buffer at 25-35 volts overnight. The gel was stained for 30 minutes in 1× ethidium bromide solution (Section 2.4.13) in 1× TAE buffer. The bands were visualised by long-wave UV light, and the molecular weight standards were used to cut out the correct band. The DNA was extracted from the agarose using 'The Geneclean® Kit' (BIO 101 Inc.). To the excised band, 2.5 to 3 volumes of NaI sock solution was added, and the mixture was incubated for 5 minutes at 55°C. A volume of Glassmilk® (depending on the amount of DNA, according to the manufactures instructions) was added and the solution was vortexed. The DNA/NaI/Glassmilk® solution was incubated at room temperature for between 5 minutes and 2 hours (depending on the volume) with intermittent mixing. The Glassmilk®/DNA complex was pelleted for 5 seconds, and the supernatant removed and set aside. The pellet was then washed 3 times with NEW buffer. The DNA was eluted into TE buffer (Section 2.4.1) by incubation at 55°C for 5 minutes. The DNA fragment was quantified (Section 2.6.2). If recovery was low, the procedure was repeated with the supernatant retained from the pelleting step.

2.6 DETERMINATION OF DNA CONCENTRATION.

Quantification of DNA was performed using two methods, spectrophotometrically for DNA prepared by CsCl purification, and fluorometrically for all other DNA samples

2.6.1 Determination of DNA concentration by spectrophotometric assay.

Concentrated DNA solutions were diluted appropriately and the absorbance of the solutions in quartz cuvettes with a 1 cm light path was determined at both 260nm and 280nm (Shimdzu, UV-160A). The reading at 260nm allowed calculation of the concentration of nucleic acid present in the sample since an OD of 1 corresponds to approximately 50µg/ml double stranded DNA. The ratio of readings at 260nm and 280nm (OD_{260}/OD_{280}) was used as an estimate of the purity of the DNA. Pure DNA has an OD_{260}/OD_{280} value of 1.8 (Sambrook *et al.*, 1989).

2.6.2 Determination of DNA concentration by fluorometric assay.

Concentration of DNA in solution was determined by fluorometric assay in a Hoefer Scientific TKO 100 Fluorometer according to the manufactures instructions. DNA was quantitated in 1× TNE Buffer (10mM Tris-HCl, 1mM Na_2EDTA and 100mM NaCl, pH 7.4) containing either (a) 0.1 µl/ml (for DNA concentrations of 0-500µg/ml) or (b) 1µl/ml (for DNA concentrations 500-1500µg/ml) of 1mg/ml Hoechst 33258 dye (dissolved in deionised, filtered water and stored in the dark at 4°C). The scale was set to (a)'100' or (b) '1000' using 20µl of (a) 10µg/ml or (b) 100µg/ml calf thymus DNA (from 1mg/ml stock, stored at -20°C), in 2ml of TNE/Dye solution to give final concentrations of (a) 100ng in 2ml and (b) 1000ng in 2ml. A 2µl aliquot of sample DNA was added to 2ml TNE/Dye solution and reading recorded as the concentration of DNA in µg/ml [ie. Reading(z) = zng/2ml. Initial DNA solution concentration (µg/ml) = z × dilution factor(1000)]

2.7 DNA MANIPULATIONS.

2.7.1 Restriction Enzyme Digests.

2.7.1.1 Plasmid digests.

Plasmid DNA restriction enzyme digests were carried out in 1× restriction buffer (10× buffer supplied by the manufacturers with the enzyme). DNA to be used was quantified (Section 2.6.2) and digestion was performed with 1-10 units of enzyme per µg DNA. The enzyme volume did not exceed one tenth the reaction volume. Unless stated otherwise the digests were performed for 2 hours at 37°C, *Sma*I digests were performed at 30°C. When necessary, RNA was removed by adding RNaseA (Section 2.4.5) to the digest mixture to a final concentration of 1µg/ml. An aliquot of the digest was run on a mini agarose gel, (Section 2.7.2), to check whether digestion of DNA was complete. Digestion was stopped by either heat inactivating the restriction enzyme (*Hind*III 90°C, *Eco*RI 65°C, *Sal.*I 80°C and *Nco*I 80°C; Ausubel *et al.*, 1994), or the addition of 10× gel loading buffer (Section 2.4.7).

2.7.1.2 Genomic digests.

An aliquot containing 4-6µg of *A. nidulans* genomic DNA was digested in 50µl reaction volume. Buffer supplied by the manufacture was used with 30 units of restriction enzyme, and 50ng acetylated BSA (Sigma). Digestion was performed at 37°C for 4 hours. An aliquot of the digest was run on a mini agarose gel (Section 2.7.2) to check that digestion was complete. If digestion was not complete the volume was increased to 100µl and a further 30 units of restriction enzyme was added. This mixture was incubated for a further 4 hours and rechecked on a mini agarose gel. The digestion was stopped by the same methods as above (Section 2.7.1.1).

2.7.2 Agarose-gel Electrophoresis of DNA.

2.7.2.1 Mini Agarose Gels.

Digested DNA was size fractionated through 0.7% agarose dissolved in 1× TBE buffer (Section 2.4.2) mini-gel. 10× gel loading buffer (Section 2.4.7) was added to DNA samples to give a final concentration of 10% and samples were loaded into gel wells. Gel was run in 1× TBE electrophoresis buffer at 80-100V for 1-2 hours. After electrophoresis the gels were stained in 1× ethidium bromide solution (Section 2.4.13) for 30 minutes, and de-stained in deionised H_2O for 5-10 minutes. The gels were visualised on a UV transilluminator and photographed using polaroid film type 667.

2.7.2.2 Large Agarose Gels.

Digested DNA was size fractionated through 0.7-1% agarose in 1× TBE buffer (Section 2.4.2) or 1× TAE buffer (Section 2.4.3). Gel loading buffer (Section 2.4.7) was added to samples to give a final concentration of 10%. Samples were loaded into wells a of large gel (20.5 cm × 14 cm, Hoefer Submarine Large Gel Unit) and the gels were run at 25-35V for 16-20 hours. Gels were stained a photographed the same as above (Section 2.7.2.1).

2.7.3 Determination of Fragment Sizes.

Samples of BRL 1kb ladder standard markers were run alongside the DNA samples on agarose gels. The mobility of the standard markers from the wells was measured and used to determine the molecular weight of the unknown fragments by comparing the relative mobility between the lanes.

On analytical gels, digested plasmids with known band sizes and similar DNA quality, were used as well as 1kb ladder. Unknown fragment sizes were determined by relative mobility in the same way as above.

2.7.4 Repairing 3′ or 5′ Overhanging DNA Ends to Generate Bunt Ends using the Klenow Fragment.

This method was a modification of that described in Ausubel *et al.*, (1994). The Klenow fragment of *E. coli* DNA polymerase I was used to repair overhanging ends created by restriction enzyme digestion in order to generate blunt ends. After restriction enzyme digestion, (Section 2.7.1.1), the DNA was phenol/chloroform purified, concentrated by ethanol precipitation (Sections 2.5.1 and 2.5.2) and suspended in TE buffer (Section 2.4.1) to a concentration of approximately 100ng/µl. To each 20µl of DNA, 0.5µl of 1M 4NTP's (Boehringer Mannheim), and 5 units of Klenow fragment (Boehringer Mannheim) were added. The mixture was incubated for 15 minutes at 30°C, and then the reaction was stopped by heating to 75°C for 15 minutes.

2.7.5 Dephosporalytion of Digested DNA 5′ Ends.

This method was based on that of Sambrook *et al..*, (1989). DNA was digested with the appropriate restriction enzyme, and once the digest was completed the restriction enzyme was heat inactivated (Section 2.7.1.1). Then, 1 unit of CAP (Calf intestinal alkaline phosphatase; Boehringer Mannheim) per 10µg DNA, was added and the mixture incubated for 30 minutes at 37°C. After incubation Na_2EDTA was added to give a final concentration of 5mM and SDS to give a final concentration of 0.5% (w/v). This was mixed and then proteinase K was added to give a final concentration of 50µg/ml. The mixture was incubated at 56°C for 30 minutes to ensure the inactivation of CAP. Finally, the DNA was purified by phenol/chloroform extraction and concentrated by ethanol precipitation (Sections 2.5.1 and 2.5.2) .

2.7.6 Ligation Reactions.

Blunt and overhanging end ligations were carried out using a combination of manufacturers instruction (BRL) and method from Ausubel *et al.*, (1994). Ligation mixtures contained a 3:3:1 molar ratio of insert1:insert2:vector for three way ligations and 3:1 molar ratio of insert:vector for two way ligations. A total amount of DNA of

300µg/60µl ligation mix was used. Vector DNA was capped (Section 2.7.5) to prevent any self-ligation and therefore reducing background in transformations (Section 2.8.2). A 2µl aliquot of ligation mix was removed prior to addition of T4 ligase, and 1/10 volume of 10× gel loading buffer added. To the DNA the following solutions were added: 1µl of 3-fold dilution of T4-DNA ligase (New England Biolabs), 12µl of manufactures (New England Biolabs) 5× ligation buffer, 3µl of 10mM ATP's, and sterile deionised H_2O to make mixture up to 60µl. This was incubated at 26°C overnight (for overhanging ends to ligate), and then a further 3µl T4-DNA ligase was added and the mixture was incubated a further 24 hours at 14°C (for blunt ends to ligate). Finally, 2µl was removed after incubations and 10× gel loading buffer (Section 2.4.7) added. Samples of ligation mixtures prior to, and after ligation, were examined by running along side each other on a mini-gel to observe the presence of extra bands, indicating that ligation had occurred (Section 2.7.2.1; ligation gel). The remainder of the ligation mix was stored at 4°C until transformation (Section 2.8.2).

2.8 TRANSFORMATION OF *E. COLI.*

2.8.1 Transformation using Calcium Chloride.

Plasmid DNA was introduced into calcium chloride competent *E. coli* cells. Preparation of calcium chloride competent cells and transformation of plasmid DNA was performed according to Ausubel *et al.*, (1994).

2.8.1.1 Preparation of Calcium Chloride Competent Cells.

A single colony of *E. coli* DHI (Section 2.1) was inoculated into 50ml LB media (Section 2.2.1), and grown overnight with moderate shaking (250 rpm; Lab-line Orbit Shaker). A 4ml aliquot of this culture was inoculated into 400ml LB media in a 2 litre flask, and this was grown at 37°C (250 rpm) until the culture reached an OD_{590} of 0.375 (Nova Tech). Then 50ml aliquots of culture were placed into eight 50ml chilled polypropylene tubes, and left on ice for 5 to 10 minutes. Cells were centrifuged for 7 minutes at 1600 × g (3000 rpm, SS34 rotor) at 4°C, and allowed to decelerate without

the brake. The supernatant was removed and the pellets resuspended in 10ml ice-cold $CaCl_2$ solution (60mM $CaCl_2$, 15% glycerol, 10mM PIPES pH 7.0). Cells were centrifuged for 5 minutes at 1100 × g (2500 rpm, SS34 rotor) at 4°C, the supernatant discarded, and the pellet resuspended in 2ml ice-cold $CaCl_2$ solution and kept on ice for 30 minutes. This last centrifugation, and resuspension step was repeated. Competent cells were stored in 500µl aliquots at -70°C.

The competency of the cells for DNA uptake was assessed by transforming (Section 2.8.1.2) with 10ng pBR322 (Section 2.1) and plating aliquots onto LB Amp^{100} plates (Section 2.2) and incubating at 37°C overnight. The number of transformed colonies per aliquoted volume (µl) × 10^5 is equal to the number of transformants per µg DNA. Transformation efficiencies of 10^6-10^7 are expected for *E. coli* strain DHI (Section 2.1).

2.8.1.2 Transformation of $CaCl_2$ Competent Cells.

A volume containing 10ng of DNA (in a volume of 10 to 25 µl) was placed in a 15ml sterile, round-bottom test tube and put on ice. A 100µl aliquot of rapidly thawed competent cells (Section 2.8.1.1) was added to the DNA and the mixture was kept on ice for 10 minutes. The cells were heat-shocked by placing tubes into a 42°C water bath for 2 minutes, then 1ml of LB medium (Section 2.2.1) was added to the tube and the mixture was incubated for 1 hour at 37°C. Aliquots were plated on LB Amp^{100} plates (Section 2.2) and incubated at 37°C overnight.

2.8.2 Transformation using Electroporation.

Plasmid DNA was transformed into electroporation competent *E. coli* cells by high voltage electroporation with a Gene Pulser™ Apparatus (Biorad). The method used was described by Dower *et al.* (1988).

2.8.2.1 Preparation of Electro-competent E. coli *Cells.*

One litre of LB broth (Section 2.2.1) was inoculated (1/100) with *E. coli* strain XL-1 and grown on a shaker at 37°C for 3 hours, (OD_{600} 0.5-1.0; Nova Tech). The cultures

were chilled on ice for 20 minutes and then harvested by centrifugation at 4000 × g (5000 rpm, SS34 rotor) for 10 minutes at 4°C. The cells were washed by suspension followed by pelleting by centrifugation (as above) first in 1000ml ice cold water, then 500ml ice cold water, then 20ml ice cold 10% glycerol, and then finally resuspended in 4ml ice cold-glycerol. Electro-competent cells were stored at -70°C in 200µl aliquots.

2.8.2.2 Transformation of DNA by Electroporation.

Electro-competent cells prepared as above were thawed on ice. A 40µl aliquot of the suspension was dispensed into cold eppendorf tubes and 1-2µl of ligation mix added (Section 2.7.6), this mixture was left on ice for 1 minute. The Gene Pulser™ apparatus (BioRad) was set to 25µF and 2.5kV and the pulse controller was set to 200 Ω resistance, in parallel with the sample chamber. The contents of the eppendorf tubes were transferred to the bottom of a cold 0.2cm electroporation cuvette, which was placed into the equipment and pulsed until a time constant of 4-5 milli second was obtained. The cells were transferred to 1ml SOC media (Section 2.2.1) and incubated at 37°C for 1 hour. Suitable positive control plasmids and a negative control containing only water were also transformed. Cells were plated in various dilution onto LB Amp^{100} plates (Sections 2.2) and incubated overnight at 37°C..

2.9 COTRANSFORMATION OF *A. NIDULANS.*

2.9.1 Preparation of Spore Suspensions from *A. nidulans.*

ACM or PD plates were inoculated with *A. nidulans* spores, and incubated at 37°C for at least 2 days. Approximately 3ml of sterile Tween 80/Saline solution (0.25ml/l Tween 80, 0.14M NaCl), was poured onto each plate and the surface was scraped with a flamed glass spreader. The resulting spore suspension was drawn off the plates and placed in a centrifuge tube. The suspension was washed twice with sterile saline (0.14M NaCl) by resuspension and centrifugation at 1600 × g (3000 rpm, SS34 rotor) and then resuspended in saline solution. An aliquot of spores were diluted and counted with the haemocytometer. The volume of liquid held over the centre square of the grid is 0.1 cm

$\times$ 0.1 cm $\times$ 0.01 cm = 10^{-4} cm^3. Since 1 ml = 1 cm^3, then formula for the calculation of cell density (in units of cells/ml) = the average number of cells over the centre square of the grid (n) $\times$ 10^4. The remaining conidia were diluted to 10^{10} spores/ml and stored in 100µl aliquots at -20°C.

2.9.2 Preparation of Fungal Protoplasts.

Protoplasts were prepared using a modification of Murray *et al.*, (1992). Cellophane discs were placed on the surface of six ACM plates (Section 2.2.2). These plates were inoculated by spreading 2µl of a suspension of 10^{10} spores/ml (Section 2.9.1) and incubated at 37°C for 18-20 hours. Mycelia covered discs were stacked face down in a petri dish containing Novozyme solution (Novozyme 234 10mg/ml in OM buffer; Section 2.4.8). These were incubated at 37°C with gentle shaking (10 rpm; Lab-line Orbit Shaker) for 1.5 hours and then a 50µl aliquot of the Novozyme solution was checked under a light microscope for the presence of protoplasts. The protoplast-containing Novozyme solution was placed in corex tubes (5ml/tube) together with extra OM buffer used to wash excess protoplasts off the cellophane discs. The preparation was overlaid with 1ml ST buffer (0.6M sorbitol, 100mM Tris-HCl, pH 8.0), and centrifuged for 5 minutes at 1600 $\times$ g (3000 rpm, SS34 rotor). The protoplasts formed a white layer between the ST buffer and Novozyme solution; this was removed and placed in a fresh corex tube with 5ml of STC buffer (Section 2.4.9) added. The preparation was centrifuged at 1600 $\times$ g (3000 rpm, SS34 rotor) for 5 minutes, the supernatant was discarded and protoplasts were resuspended in 5ml STC buffer. The suspension was then centrifuged again at 1600 $\times$ g (3000 rpm, SS34 rotor) for 5minutes, the supernatant was discarded and protoplasts resuspended in 0.5ml STC buffer. The number of protoplasts was estimated using the haemocytometer (Section 2.9.1) and the preparation was diluted to a concentration of 1.25 $\times$ 10^8 protoplasts/ml in STC buffer. These were kept on ice and used immediately or 0.2 volumes of 40% PEG solution (Section 2.4.10), were added and protoplasts were stored in aliquots at -70°C. Prior to use in transformation the viability of the protoplasts was determined by plating on osmotically stabilised ACM plates (Section 2.2).

2.9.3 Cotransformation of *A. nidulans* (Method 1).

Cotransformation was performed using a modification of method in Murray *et al.*, (1992). To transform *A. nidulans* between 5 and 30μg DNA in a ratio of 1:1 to 1:20 of pAN8-1 (Section 2.1) to non-selected plasmid DNA, was added to 80μl fresh protoplasts or 100μl thawed protoplasts (Section 2.9.2) and 2μl 50mM Spermidine (Sigma). This was mixed and left on ice for 30 minutes. A 900μl aliquot of 40% PEG solution (Section 2.4.9) was added, the transformation was mixed and left at room temperature for 15-20 minutes. The mixture was then diluted 10 fold in STC buffer (see Section 2.4.9). Aliquots of 100-500μl were added to 3ml molten overlay, osmotically stabilised, phleomycin containing, ACM at 50°C, and these were poured onto osmotically stabilised, phleomycin containing, ACM plates (see Section 2.2). Plates were incubated at 37°C for 3 days.

2.9.4 Cotransformation of *A. nidulans* (Method 2).

This transformation method is a modification of the method described by Punt and van del Hondel(1992). To transform *A. nidulans* between 5 and 30μg DNA in a ratio of 1:1 to 1:20 of pAN8-1 (Section 2.1) to non-selected plasmid DNA, was added to 100μl fresh protoplasts (Section 2.9.2). This was incubated at 20-25°C for 30 minutes. In three steps 250, 250, and 850μl of 60% PEG solution (60% (w/v) PEG4000, 10mM Tris-HCl, 50mM $CaCl_2$) were added with gentle mixing. The suspension was incubated for 20 minutes at 20-25°C. Then the suspension was diluted with 10ml of STC1700 (1.2M sorbitol, 10mM Tris-HCl pH7.5, 50mM $CaCl_2$, 35mM NaCl), and protoplasts were collected by centrifugation at 1600 × g (3000 rpm, SS34 rotor), 0°C for 10 minutes. The protoplasts were resuspended in 500μl of STC1700 and 100μl aliquots were spread onto osmotically stabilised, phleomycin, PCM plates (Section 2.2). Plates were incubated at 37°C for 3 days.

2.9.5 Selection of Cotransformants.

Cotransformants were tested for the presence of GUS activity by dotting 1µl X-Gluc (ProGen) stock (20mg/ml in dimethylformamide) onto the transformed colonies. The plates were incubated in the dark at 37°C for 15 to 30 minutes and GUS activity was shown by the presence of a blue colouration.

2.9.6 Single Spore Purification of Transformants.

A. nidulans GUS positive transformants were single spore purified by streaking conidia with a glass needle on ACM plates (Section 2.2). The plates were grown for two days at 37°C. A single GUS positive colony was picked and the procedure was repeated. Finally, the purified transformants were stored on ACM slopes (Section 2.3).

2.10 MEASUREMENT OF TRANSIENT EXPRESSION OF TRANSFORMED DNA.

A modification of the method used in Mönke and Schäfer (1993) was used to measure transient expression in the fungal protoplasts. Protoplasts were prepared and transformed with test and control DNA (Section 2.9.2 and 2.9.3). The transformed protoplasts were incubated in the STC buffer (Section 2.4.9) at 37°C with gentle shaking (20 rpm; Lab-line Orbit Shaker) for 17-24 hours. The protoplasts were then collected by centrifugation and resuspended in GUS extraction buffer (Section 2.4.11). An aliquot of the transformation mixture was added to 1mM MUG (Sigma) in GUS extraction buffer and the GUS assay carried out and fluorometer data were collected (Section 2.13.2).

Flurometer readings give the concentration of MU in the assay samples. Linear regression was performed on these fluorometer readings to give the increase in MU concentration over time which was then used to calculate the rate of enzyme activity in pmols MU produced per min.

$$\text{Reaction rate (pmolsMU / min)} = \frac{\text{Reaction rate (nMMU in 200}\mu\text{l / min)}}{1000} \times 2 \times 10^{-10}\,\text{mols} \times 10^{12}$$

Finally the specific activity of the enzyme in pmols MU per min per $1x10^6$ protoplasts was determined. In each assay were 3 x 10^5 protoplasts, assuming 100% viability from the original protoplast count, then;

$$\text{Specific enzyme activity (pmolsMU / min / } 1 \times 10^6 \text{ protoplasts)} = \frac{\text{Reaction rate (pmolsMU / min)}}{3 \times 10^5} \times 1 \times 1$$

The coefficient of linear correlation (correlation coefficient) was determined to shown that the GUS enzyme converts MUG to MU in a linear fashion over time (Section 2.14.1).

2.11 DNA HYBRIDISATIONS.

2.11.1 Southern Blotting.

This method was performed according to Schmid *et al.*, (1990) as a modification of a method developed by Malone (personnal correspondence). A volume containing 1.5µg of DNA was loaded into each well of a large (19.5 × 14.5 cm) 1× TBE agarose gel and the gel was run at 30V overnight. The gel was stained with EtBr for 30 minutes and photographed under UV light with a ruler along side it (Section 2.7.2). To the container holding the gel, 250ml of 0.2M HCl was added and this was shaken gently for 15 minutes. The HCl solution was discarded and 250ml 0.5M NaOH/1.5M NaCl was added to the gel and this was shaken gently for 15 minutes to denature the DNA. The wash solution was removed and this step was repeated. Neutralisation was then performed by adding 500ml of 1.5M NaCl/0.5M Tris-HCl (pH 7.5) and shaking the gel for 30-40 minutes.

The blotting apparatus was then assembled. A glass plate was placed on a baking dish containing 10× SSPE (Section 2.4.12). A piece of 3mmm Whatman filter paper soaked in 10× SSPE was placed as a wick over the glass plate with both ends in the baking dish in contact with the liquid. Care taken to ensure that there was no bubbles under the

wick. Three pieces of Whatman filter paper were cut the same size as the gel, and using a clean razor blade, a piece of BA 85 / 0.45 µm Schleicher and Schuell nitrocellulose was also cut the same size. The gel was placed on top of the wick on the glass plate, and then the nitrocellulose was moistened in deionised water and placed to top of the gel, with care taken to remove all bubbles. The three pieces of Whatman filter paper were pre- moistened in 10× SSPE and placed on top of the nitrocellulose. The entire set-up was covered by Gladwrap and a window was cut, using a razor blade, the size of the gel. Paper towels were placed evenly on top of the apparatus for a height of 5-8cm, and on top of these a weight of 500g was evenly distributed. The assembled apparatus was then left overnight for the DNA transfer to occur.

The following day the apparatus was disassembled, and the nitrocellulose was place in 2× SSPE (Section 2.4.12) so that the gel side of the nitrocellulose was facing down in the liquid. This was shaken gently for 10 minutes. The nitrocellulose was removed and placed onto a piece of 3mmm Whatman filter paper so that the gel side was facing upward. The blot was immediately exposed to UV light for 3.5 minutes (in UV box, 260nm), allowed to dry and then stored on filter paper at 4°C.

2.11.2 Radioactive (^{32}P) Labelling of DNA Probe.

The DNA fragment for the probe was prepared by the method described in Section 2.5.8. The probe was prepared by nick translation according to the manufacturer (GIBCO BRL) using 0.4µg of DNA and 2.5µl of [α-^{32}P]dCTP (3000Ci/mmol). In a micro-centrifuge tube the DNA and 2.5µl [α-^{32}P]dCTP was mixed with 2.5µl solution A (dATP, dTTP, and dGTP) and water to make up 22.5µl. Then 2.5µl of solution C (DNA polymeraseI/DNaseI) was added and the solutions were mixed by careful pipetting up and down. After incubation for 60 minutes at 15°C the reaction was stopped by the addition of 2.5µl solution D (Na_2EDTA).

The unincorporated nucleotides were separated from labelled DNA using a ProbeQuant™ G-50 micro column as in manufacturers instructions (Pharmacia Biotech). The column was prepared by vortexing to resuspend the resin, loosing the cap ¼ turn and snapping off the bottom closure. The column was pre-spun for 1 minute at 3000

rpm. The prepared column was placed in a 1.5ml tube and 50µl of prepared probe was added to the top centre of the resin. The column was spun for 2 minutes and the probe was collected at the bottom of the support tube. The volume was increased to 500µl with TE buffer (Section 2.4.1). A scintillation counter was used to determine the counts per minute (cpm) of the probe by adding 5µl to 3ml of scintillation cocktail (using full energy spectrum setting). The result given enabled the calculation of total number of counts, (which should be about 5×10^7). The probe was stored at -20°C.

2.11.3 Southern Hybridisation.

This method was performed according to Schmid *et al.,* (1990) as a modification of a method developed by Malone (personnal correspondence). A 30ml portion of hybridisation buffer (5× SSPE (Section 2.4.12) with 5% (w/v) dextrane sulphate, 0.3% (w/v) SDS, stored at -20°C), was warmed to 65°C. While buffer was warming the calf thymus DNA stock solution (10mg/ml CT-DNA dissolved in deionised water, stored at -20°C), was thawed and sonicated for 2 × 20 seconds (Soniprep 150 MSE). The CT-DNA was then heated to 97-100°C in a dry bath for 10 minutes. 300µl of the heated CT-DNA was added to the warmed hybridisation buffer, and the mixture was placed back into the 65°C water bath until use.

The blot (Section 2.11.1), was placed into a hybridisation tube with the DNA side facing inward. The prepared 30ml of hybridisation buffer was added to the tube, the tube was incubated, rotating at 65°C for ≥ 60 minutes. A volume of probe containing at least 1 $\times 10^7$ cpm was heat-denatured at 97 to 100°C for 10 minutes and added to the hybridisation tube. The hybridisation tube containing the blot and probe was incubated, rotating at 65°C overnight.

The next day the blot wash solution (2× SSPE, 0.2% (w/v) SDS) was prepared and prewarmed to 65°C. The hybridisation solution was removed from the tube and the tube half filled with the wash solution. This was incubated, rotating at 65°C for 20 minutes, and was followed by 2 more washes. After the washes the blot was removed from the

tube and wrapped in gladwrap, with caution to remove wrinkles on the DNA side of the blot.

2.11.4 Autoradiography of Southern Blots.

The blot (Section 2.11.5) was placed in a exposure cassette, with X-ray film placed over it (in the dark), and then a Cronex intensifying screen. The cassette was stored at -70°C for development.

After a set length of exposure time the cassette was bought to room temperature. In the darkroom, the film was removed and placed in developing solution (D19 developer) for 5 minutes, and then fixer solution for 5 minutes. Finally, the film was washed in water and dried at room temperture.

2.12 GROWTH RATE DETERMINATION OF FUNGAL TRANSFORMANTS.

2.12.1 Colony Growth.

The centre of ACM plates (Section 2.2) were inoculated with the fungal spore suspensions using a toothpick. The plates were incubated at 37°C and the diameter of the colony on each plate was measured every 24 hours for 7 days using callipers. Growth rates were determined by linear regession analysis on data showing the increase in colony diameter over time (Section 2.14.1).

2.12.2 Dry Weight Determination.

Fresh spore suspensions were made and the spore concentration was estimated using a haemocytometer (Section 2.9.1). The spore viability was determined by plating dilutions of the spore suspension on ACM plates and incubating at 37°C. Flasks (500ml) containing 150ml liquid ACM (Section 2.2.1) were inoculated with 5×10^6 viable spores/ml. These were placed on a shaker (250 rpm; Controlled Environment Incubator Shaker) and 5ml samples were taken at 1 or 2 hour intervals for 24 hours. The mycelia were separated from the broth in the samples by filtration through a

filtration manifold, they were collected on a pre-weighed filter and this was placed in a pre-weighed eppendorf tube. The sample tubes were stored at -20°C until completion of the sample collection. Tubes were then placed in the freeze drier overnight, weighed the next day and the data were recorded. The log of the growth data was plotted aginst time and the linear portion of this growth curve produced represented the log phase of the growth. Exponential regression analysis on this portion gives the culture doubling time (Section 2.14.1).

2.13 MEASUREMENT OF GUS EXPRESSION.

2.13.1 Protein Quantification.

Protein was quantified in fungal extracts using the method described in Bradford (1976) and adapted by Ausubel *et al.*, (1994). Aliquots of 50µl GUS extract (Section 2.13.2) + 50µl GUS extraction buffer (Section 2.4.11) and 100µl GUS extract of the unknowns were placed in tubes. A 1ml aliquot of Coomassie Brilliant Blue solution (100mg Coomassie Brilliant Blue G-250, 50ml of 95% ethanol, 100ml of 85% phosphoric acid) was added to each tube, these were vortexed and allowed to stand at room temperature for at least 2 minutes. To create a protein standard curve different volumes of a 0.5mg/ml BSA protein standard were added to microcentrifuge tubes to give standards containing 2.5 to 10µg of protein. A tube containing no protein was also set arise as the zero control. The volume of assay tubes was made up to 100µl with GUS extraction buffer (Section 2.4.11), and 1ml of Coomassie Brilliant Blue solution added and incubated as above. The absorbance at 595nm of the all the samples was measured by spectrophotometry, using a 1-cm path length micro-cuvette (Nova Tech). The sample without protein was used to zero the spectrophotometer and a standard curve was constructed by plotting absorbance at 595nm versus protein concentration. The protein concentration of the unknown GUS extracts was determined extrapolating from the linear portion of the standard curve.

2.13.2 Quantification of GUS Expression.

The GUS expression was quantified using a variation of a method described by Jefferson, (1987). The mycelia were harvested by filtration from mid-log phase cultures of the pFG-gpd transformant and an untransformed control (mid-log phase was determined by Section 2.12.2), and these samples were freeze dried. Duplicates of 10mg of the freeze dried mycelia from each sample were weighed out into micro-centrifuge tubes and these were frozen with liquid nitrogen and ground using eppendorf grinders. The ground mycelia were suspended in 1.5ml GUS extraction buffer (Section 2.4.11), vortexed, micro-centrifuged for 30 seconds to remove debris and placed on ice. The GUS extract was diluted 10 fold and used for the GUS and protein assays (Section 2.13.1).

For the GUS assays 20µl of each of the GUS extracts was added to 1ml assay buffer (1mM 4-methyl umbelliferyl β-D-glucuronide (MUG, Sigma) in GUS extraction buffer) and tubes were placed at 37°C in the dark. Samples of 10µl were taken at 0, 15, 30, and 60 minutes, and added to 10ml STOP buffer (0.2M Na_2CO_3).

The STOP buffer was used to zero the fluorometer, 100µl of 1µM 4-methyl umbelliferone (MU, Sigma), (in STOP buffer) was added to 1.9ml of STOP buffer and the fluorometer scale set to '1000', this setting was checked twice more to ensure it remained constant. A 100µl aliquot of 100nM MU (in STOP buffer) was added to 1.9ml of STOP buffer and the fluorometer scale checked to ensure it read '100'. Finally, 2ml (or dilutions of) assay samples were placed in the fluorometer and the data collected. The data were plotted aginst the time samples were taken and linear regression performed (Section 2.14.1). The following calculations were carried out for the determination of GUS specfic activity in the GUS extracts.

For the calculation of concentration of MU in assay samples;

100µl of 1µM MU in 2ml = a reading of '1000' = 50nM.

Therefore, undiluted reading(x) / 20 = x nM MU

Calculation of reaction rate from linear regression on the raw GUS data;

100µl of 1µM MU into 1.9ml GUS STOP = a reading of '1000' = concentration in the cuvette of 50nM MU

50nM = 5 x 10^{-8} mols/l = '1000' , therefore all reading from the fluorometer were divided by 20

There are 1 x 10^{-10} mols MU in the cuvette (2ml), therefore;

$$\text{Rate (molsMU / min)} = \frac{\text{rate (nMMU / min)}}{1000} \times 1 \times 10^{-10}$$

For the calculation of GUS specfic acitivies;

There was 26ng of dry weight assayed in the cuvette, therefore;

$$\text{Specfic enzyme activity (mol MU/ min /ng dry weight)} = \frac{\text{Rate (molsMU/ min)}}{26\text{ng}}$$

In 2ml assayed there was 0.04µl of diluted GUS extract, therefore;

$$\text{Amount of protein in assay (xng)} = \frac{0.04}{1000} \times \text{protein concerntration (mg / ml)}$$

$$\text{Specfic enzyme activity (molsMU / min / ng protein)} = \frac{\text{Rate (molsMU / min)}}{\text{Amt protein (xng)}}$$

2.14 STATISTICAL ANALYSIS.

2.14.1 Linear Regression, Correlation Coefficient, and Exponential Regression.

When data seems to follow a linear relationship with another variant then this relationship can be described by the linear regression equation. This equation expresses one variant, the dependent variant (y), in terms of the other, independent variant (x). The coefficient of linear correlation (r^2) measures how close the relation between (x) and (y) is to linearity, (Clarke, 1994). A r^2 value of 1 is equal to a perfect correlation. Exponential regression expresses the relationship between data that follows an exponential relationship. Linear regression, correlation coefficient and exponential

regression analysis were carried out using the CA-Cricket Graph III package for the Macintosh (Computer Associates®).

2.14.2 Z-test.

To test whether a difference between two proportions is statistically significant the z-test was used. One-tail tests were performed if the alternative hypotheses stated that one proportion was greater than the other. The z-test was performed using the following equations (Clarke, 1994).

Null hypothesis: $p_1=p_2$

Alternative hypothesis: $p_1>p_2$

n_1 = no. tested $\qquad$ n_2 = no. tested

p_1 = no. events $\qquad$ p_2 = no. events

$$P = \frac{\text{no. of events}}{\text{total no. tested}}$$

$$se(p_1 - p_2) = \sqrt{P(1-P)\left(\frac{1}{n_1} + \frac{1}{n_2}\right)} \qquad t = \frac{p_1 - p_2}{se(p_1 - p_2)}$$

From a table of critical t values, at 99% confidence if t is less than $t_{\sim}$ the null hypothesis is accepted, if t is greater than $t_{\sim}$ then the null hypothesis is not accepted.

3. RESULTS.

3.1 CONSTRUCTION OF A GUS EXPRESSION PLASMID, pFUNGUS.

Observation of gene expression can be achieved by attaching a promoter of interest to a reporter gene in order to drive the expression of that particular gene. The timing, position and levels of expression of the reporter gene in the promoters' endogenous environment closely reflect that of the endogenous gene (Jefferson, 1986).

A plasmid containing a reporter gene was constructed to enable the observation of gene expression in filamentous fungi. The modified *uidA* gene, or *gusA* gene (Jefferson, 1987) as it has come to be known, was the reporter gene chosen for this work. Its' properties as a reporter gene have been intensively demonstrated in plants (reviewed in Gallagher, 1991), and more recently its' potential for similar use in fungi has been shown (Roberts *et al*, 1989). Previously constructed fungal GUS expression plasmids did not contain a multi-cloning site for insertion of different promoters. Therefore it was necessary to construct a new GUS expression plasmid for this function. The plasmid constructed, pFunGus, has the following features; (i) the *gusA* gene for GUS expression, (ii) T*trpC* transcriptional termination sequence, (iii) multi-cloning site for the insert of promoters, (iv) Amp^r for selection in *E. coli*, (v) bacterial origin of replication for amplification in *E. coli* and (vi) small size (5.5 kb) for flexibility when inserting promoters (sources of sequences are provided in Section 2.1).

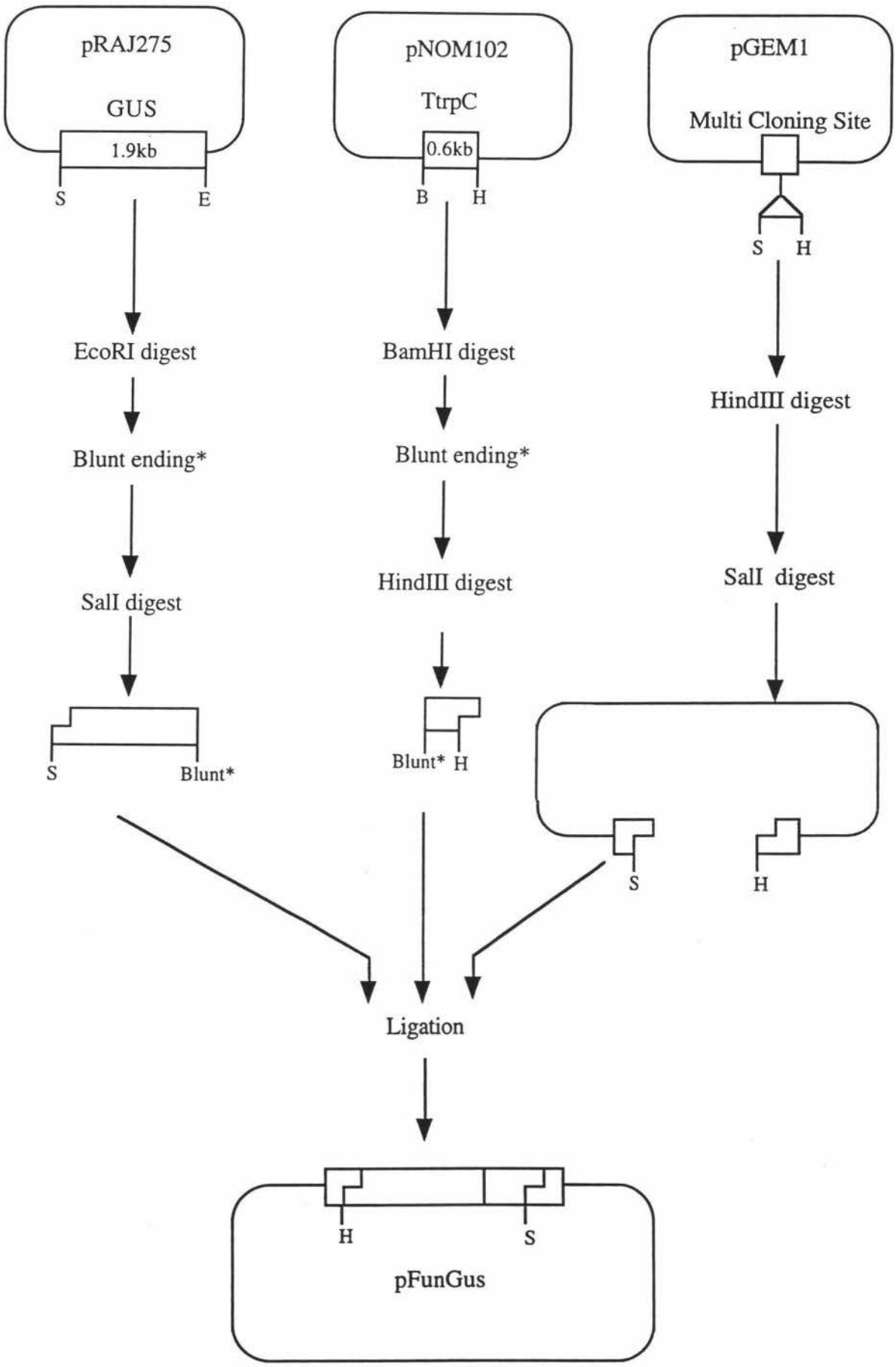
3.1.1 Preparation of DNA Fragments Required for pFunGus Construction.

Three plasmids were used to construct pFunGus; pGEM-1 was used as the source of the multi-cloning site, Amp^r gene and origin of replication, pRAJ275 as the source of the *gusA* gene, and finally, pNOM102 as the source of the T*trpC* sequence (Section 2.1; Appendix 1). Fragments of the plasmids were prepared and quantified (Sections 2.5.4 and 2.6.2). The steps required to construct pFunGus are summarised in Figure 4.

Figure 4 Summary of pFunGus Construction.

The construction of pFunGus required the ligation of 3 components: the *Hin*dIII-Blunt* 1.9 kb *gusA* fragment, the Blunt*-*Sal*I 0.6 kb T*trpC* fragment, and the *Sal*I-*Hin*dIII 2.8 kb pGEM-1 backbone containing the multiple cloning site. The resulting plasmid pFunGus is 5.5 kb. A complete restriction map is shown in Figure 6. Restriction sites shown are: H; *Hin*dIII, B; *Bam*HI, E; *Eco*RI, S; *Sal*I. (Figure 4 is not drawn to scale).

* Blunt indicates digested ends that have had their overhanging ends removed by the Klenow Fragment (Section 2.7.4).


pRAJ275
GUS
1.9kb
S
E
pNOM102
TtrpC
0.6kb
B
H
pGEM1
Multi Cloning Site
S
H
EcoRI digest
BamHI digest
HindIII digest
Blunt ending*
Blunt ending*
SalI digest
HindIII digest
SalI digest
S
Blunt*
Blunt*
H
S
H
Ligation
H
S
pFunGus

The pGEM-1 DNA was digested with *Hin*dIII and *Sal*I (Section 2.7.1.1). After the *Hin*dIII reaction the digest was checked to ensure that digestion was complete. It was phenol/chloroform purified, and ethanol precipitated (Section 2.5.1 and 2.5.2) to guarantee the first restriction enzyme was removed before the second digest commenced. After the second digest *Sal*I was inactivated at 80°C for 15 minutes, and the digested plasmid ends were dephosphorylated (Section 2.7.5). This would prevent any self-ligation of digested pGEM-1, or reformation of pGEM-1 by ligation of the small fragment removed by the double digest (previous experiments showed a problem with background in the transformation of ligation mixtures, and therefore every precaution was required in order to reduce this background). The digested pGEM-1 was purified by agarose gel electrophoresis (Section 2.7.2.2), and the fragment extracted from the agarose gel (Section 2.5.8) to further reduce background. The digested pGEM-1 was quantified and stored at 4°C for ligations (Section 3.1.2).

The pRAJ275 DNA was digested with *Eco*RI and *Sal*I (Section 2.7.1.1) to remove the *gusA* gene (1.9 kb). After the first digest with *Eco*RI the reaction was phenol /chloroform purified and ethanol precipitated (Section 2.5.1 and 2.5.2). This was important to ensure the purity of the DNA for the optimisation of the removal of the overhanging ends by the Klenow Fragment. The overhanging ends of the digested DNA were removed with the Klenow Fragment (Section 2.7.4), creating blunt ends. The second digest with *Sal*I was carried out and the DNA size fractionated by agarose gel electrophoresis (Section 2.7.2.2). The desired band of 1.9 kb containing the *gusA* gene was extracted from the agarose gel (Section 2.5.8). The *gusA* gene DNA was quantified (Section 2.6.2) and stored at 4°C for ligations.

The pNOM102 DNA was digested with *Bam*HI and *Hin*dIII (Section 2.7.1.1) to remove the T*trpC* transcriptional termination sequence (0.6 kb). After the first digest with *Bam*HI the restriction enzyme and enzyme buffer was removed by phenol/chloroform purification and ethanol precipitated (Section 2.5.1 and 2.5.2). The overhanging ends of the digested DNA were removed with the Klenow Fragment (Section 2.7.4). A second digest with *Hin*dIII was carried out and the DNA size fractionated by agarose gel electrophoresis (Section 2.7.2.2). The desired band of 0.6 kb was extracted from the

agarose gel (Section 2.5.8) and the amount of T*trpC* DNA sequence obtained was quantified (Section 2.6.2) and stored at 4°C for ligations.

3.1.2 Ligation of DNA Fragments for pFunGus Construction.

A three way ligation was carried out between, (i) linearised pGEM-1, (ii) *gusA* and (iii) T*trpC*, (Section 3.1.1); involving both blunt and cohesive end ligations. A combination of different DNA ratios, temperatures and T4 Ligase concentrations were used (Section 2.7.6). Controls of phosphorylated and dephosphorylated linearised pGEM-1 were also ligated. All ligation reactions were size fractionated on ligation agarose gels to observe the presence of additional ligated bands. On the ligation gel there were two visible bands that were not present in the unligated mixture (data not shown). It was concluded that these represented ligated products and therefore the ligated mixture might contain the pFunGus plasmid. The ligation mix was stored at 4°C for transformation into *E. coli.*

3.1.3 *E. coli* transformation of Ligated Fragments and Preliminary Analyses of Transformants.

A volume containing 7ng of the ligation reaction (Section 3.1.2) was transformed by electroporation (Section 2.8.2) into competent XL-1 cells (Section 2.1). Also transformed were the following controls; a negative control without DNA, 7ng undigested pGEM-1 (positive control), 7ng of pGEM-1 that was digested and dephosphorylated and ligated (Section 3.1.1), (a control to show the expected background). The transformed cells were plated on LB Amp plates (Section 2.2) and were incubated at 37°C overnight. The transformation frequencies were determined from the number of transformed colonies (results not shown). The ligation control showed that the transformation frequency of digested, dephosphorylated and ligated pGEM-1 was at the same level as the three component ligation reaction, indicating that only a small fraction of transformants would contain the pFunGus construct.

3.1.4 Identification and Confirmation of Potential pFunGus Containing *E. coli* Transformants.

Although the transformation frequencies indicated a low probability of obtaining pFunGus (Section 3.1.2), 8 clones were assessed by running quick mini-plasmid preparations (Section 2.5.6) on an analytical 0.7% agarose mini-gel (Section 2.7.2.1). As a control, pGEM-1 DNA was prepared in the same fashion from an *E. coli* transformant and was run on the same gel. Seven out of 8 of the transformants showed the same plasmid band size as pGEM-1. One transformant did not migrate as far down the gel indicating a larger plasmid size; this clone was further analysed to determine if it contained the plasmid pFunGus.

A plasmid mini-preparation (Section 2.5.3) was made from the potential pFunGus clone and subjected to various digests. Plasmids of similar DNA quality were used as size determination markers as well as the 1 kb ladder (Section 2.7.3). Digests of size marker plasmids were chosen in order to produce bands with a range of sizes through the pFunGus band sizes. Resolution of the bands was enhanced by size fractionating on a large 0.7% agarose overnight gel (Section 2.7.2.2). Figure 5 shows these results.

The presence of the multi-cloning site and the size of the potential pFunGus plasmid was tested by digesting with *Eco*RI. This enzyme cuts once within the multi-cloning site. As Figure 5, lane 3 shows, in addition to the undigested DNA, there was a 5.5 kb band as expected for the correct construct. Integrity of the Kozak sequence (a consensus sequence around the translational start codon ATG of the *gusA* gene; Kozak, 1981) was confirmed by the presence of the *Nco*I site. This site is a few nucleotides from where the *gusA* gene was cloned into pGEM-1. The potential pFunGus DNA was digested with *Nco*I, and a 5.5 kb band was produced (lane 4[a] and 14) confirming the presence of the *Nco*I site. Digestion with *Nae*I confirmed the presence and orientation of the T*trpC*

[a]A gel shift occurred in lanes 4 and 8 due to a contaminating factor in the *Nco*I restriction enzyme reaction mixture, therefore the pFunGus *Nco*I digest result was confirmed on a second gel, shown in Figure 5 as lane 14.

Figure 5. Agarose gel showing the profile of bands produced for analysis of the potential pFunGus clone.

Lanes 1 and 12 contain the BRL 1 kb size markers. The potential pFunGus clone was run undigested (lane 2), digested with *Eco*RI (lane 3), digested with *Nco*I (lanes 4 and 14), digested with *Nae*I (lane 5), and digested with *Ssp*I (lane 6). Size markers and digest controls were run of: *Bam*HI-*Hin*dIII digested pNOM102 giving 7 kb and 0.6 kb bands (lane 7), *Nco*I digested pNOM102 giving 1.9 kb and 5.7 kb bands (lane 8), *Eco*RI digested pRAJ275 giving a 4.6 kb band (lanes 9 and 13), *Eco*RI-*Sal*I digested pRAJ275 giving 1.9 kb and 2.7 kb bands (lane 10), and *Eco*RI digested pGEM-1 giving a 2.9 kb band (lane 11). In lanes 4 and 8 a gel shift has occurred, therefore the pFunGus, *Nco*I digest was repeated and is shown in lane 14.

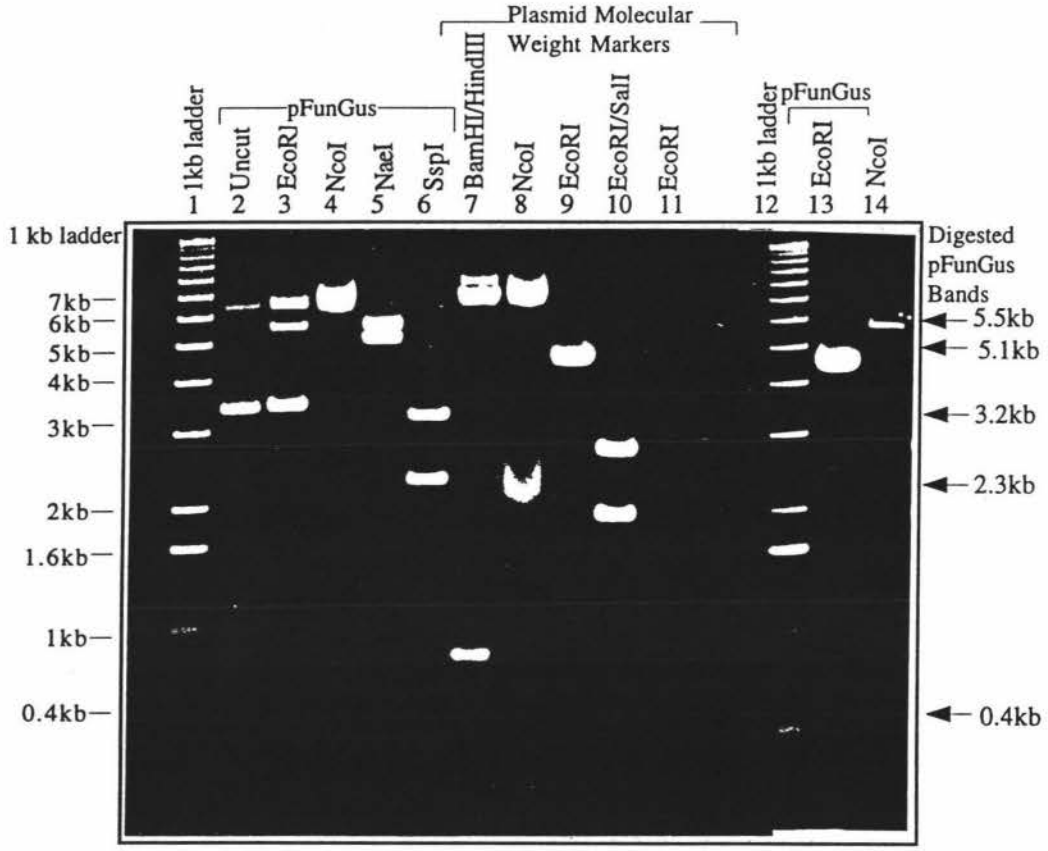

Plasmid Molecular Weight Markers
1kb ladder
pFunGus
Uncut
EcoRJ
NcoI
NaeI
SspI
BamHI/HindIII
NcoI
EcoRI
EcoRI/SalI
EcoRI
1kb ladder
pFunGus
EcoRI
NcoI
1 2 3 4 5 6 7 8 9 10 11 12 13 14
1 kb ladder
7kb
6kb
5kb
4kb
3kb
2kb
1.6kb
1kb
0.4kb
Digested pFunGus Bands
5.5kb
5.1kb
3.2kb
2.3kb
0.4kb

Table 2. Summary of band sizes produced from digests for the analysis of the potential pFunGus clone.

Plasmid	*Eco*RI (kb)	*Ssp*I (kb)	*Nae*I (kb)	*Nco*I (kb)
Expected pFunGus	5.5	3.2, 2.3	5.1, 0.4	5.5
Potential pFunGus	5.5 (4.6 - 5.7)[a]	3.2 (2.7 - 4.6)[a], 2.3 (1.9 - 2.7)[a]	5.1 (4.6 - 5.7)[a], 0.4 (<0.6)[a]	5.5 (4.6 - 5.7)[a]

[a] The range of plasmid molecular weight markers that the pFunGus bands fall between.

termination sequence by cutting once within the T*trpC* and once within pGEM-1 sequence. Figure 5 shows the potential pFunGus DNA produces the expected bands of 5.1 kb and 0.4 kb (lane 5). An addition 5.5 kb band produced corresponded to a partial digest of the potential pFunGus DNA. Digestion with *Ssp*I tested the presence and orientation of the *gusA* gene by cutting once within the *gusA* gene and once within pGEM-1 sequence. Figure 5 shows the potential pFunGus DNA produced the expected bands of 3.2 kb and 2.3 kb (lane 6). Undigested potential pFunGus DNA was run on the analytical gel (lane 2) for the correct interpretation of partial digests (as seen in lane 3). Finally, digests were also carried out to produce a range of bands as size markers for the interpretation of the analytical gel. In the potential pFunGus digests, the bands fell between the appropriate size marker bands. These size markers are seen in lanes 7, 8[a], 9, 10, 11 and 13.

The production of the correct bands by digestion of the potential pFunGus clone permitted the assumption that it contained the desired construct. A complete restriction map of this construct is shown in Figure 6.

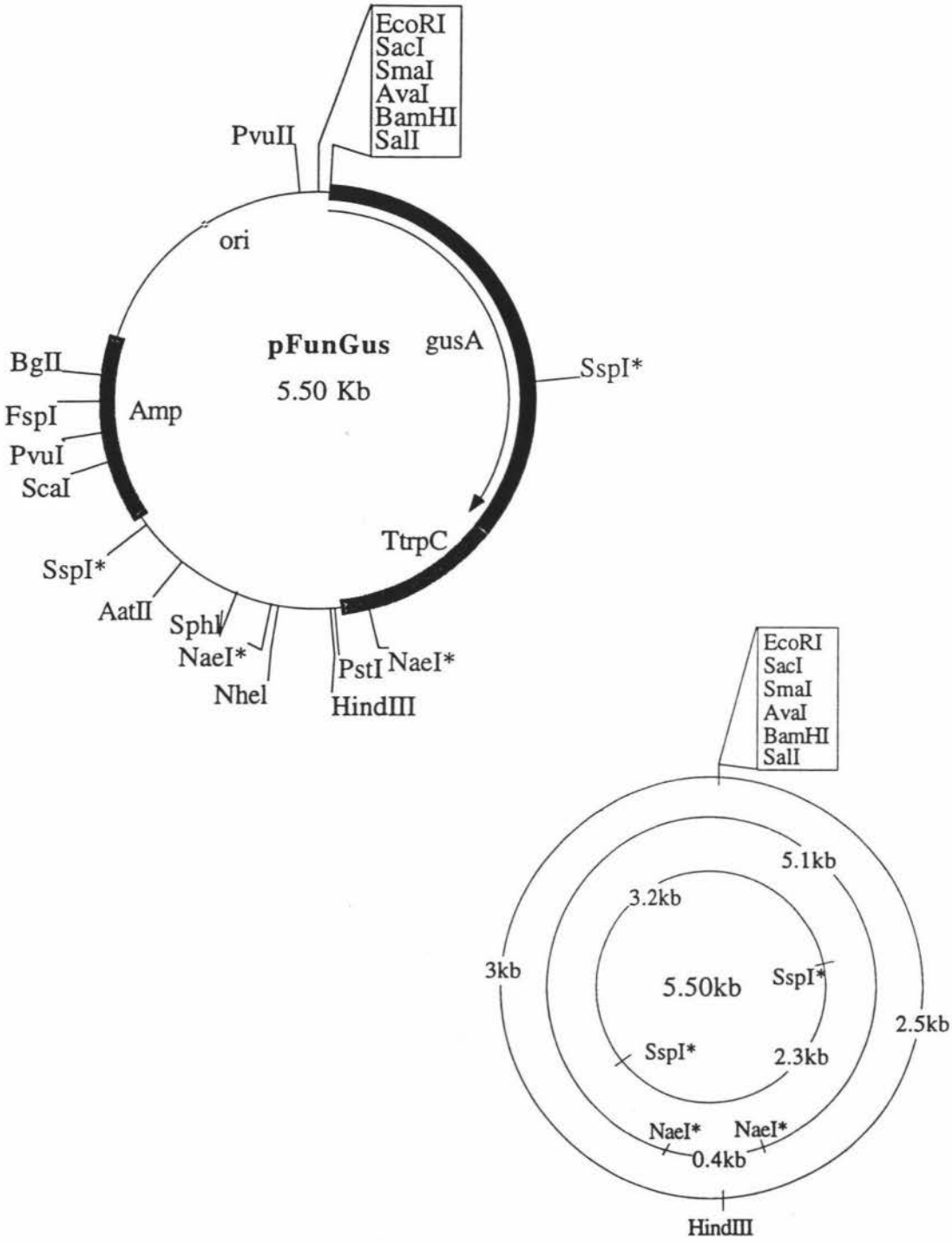
3.2 CONSTRUCTION OF pFG-GPD, A PROMOTER CONTAINING DERIVATIVE OF pFUNGUS.

The promoter for the glyceraldehyde-3-phosphate dehydrogenase gene (p*gpdA)* from *A. nidulans* is a constitutive, highly expressed promoter (Punt *et al*, 1988). This promoter has been used in studies for the assessment of GUS expression plamids in fungal hosts (Roberts *et al*, 1989). A constitutive promoter was required initially to assess whether the plasmid expressed the *gusA* gene. Therefore the *gpdA* promoter was cloned into pFunGus in order to drive GUS expression in the *A. nidulans* transformants. By inserting the promoter of *gpdA* an identical GUS expression cassette to that in pNOM102 was created.

3.2.1 Preparation of DNA Fragments Required for pFG-gpd Construction.

The pFunGus DNA prepared and quantified as described in Sections 2.5.4 and 2.6.2 was digested with *Sma*I and *Nco*I (Section 2.7.1.1), restriction enzymes that cut within the

Figure 6 Restriction map of pFunGus.



Restriction maps of pFunGus showing sites for: *Eco*RI, *Sac*I, *Sma*I, *Ava*I, *Bam*HI, *Sal*I, *Nco*I*, *Ssp*I*, *Nae*I*, *Pst*I, *Hin*dIII, *Nhe*I, *Sph*I, *Aat*II, *Sca*I, *Pvu*I, *Fsp*I, *Bgl*I, and *Pvu*II. Also showing sizes of bands produced by digestion with *Nae*I, *Ssp*I and *Eco*RI-*Hin*dIII.

* Restriction enzymes that cut pFunGus more than once.

multi-cloning site of pFunGus. After the first reaction the digest was checked on a mini-agarose gel (Section 2.7.2.1) to ensure that digestion was complete. The digested DNA was phenol/chloroform purified, and ethanol precipitated (Section 2.5.1 and 2.5.2) to guarantee the first restriction enzyme was removed before the second digest commenced. After the second digest *Nco*I was inactivated at 80°C for 15 minutes. The digested plasmid was dephosphorylated (Section 2.7.5) to prevent any self-ligation of digested pFunGus, or reformation of pFunGus by ligation of the small fragment that was removed by the double digest (as in Section 3.1.1, previous experiments showed high background in the transformation of ligation mixtures, and therefore every precaution was required in order to reduce this background). The linear pFunGus fragment was purified on, and exacted from, a 0.7% agarose gel (Sections 2.7.2.2 and 2.5.8). This isolates the digested from undigested plasmid, thus reducing background. The amount of digested pFunGus DNA obtained was quantified and the preparation stored at 4°C for ligations (Section 3.1.3).

The pNOM102 DNA was digested with *Nco*I and *Eco*RI (Section 2.7.1.1) to remove the 2.2 kb *gpd*A promoter sequence (see Appendix 1). After the first digest with *Nco*I the reaction was phenol/chloroform purified and ethanol precipitated (Sections 2.5.1 and 2.5.2). The cohesive ends of the digested DNA were removed with the Klenow Fragment (Section 2.7.4). The second digest with *Eco*RI was carried out and the DNA size fractionated by agarose gel electrophoresis (Section 2.7.2.2). The desired band of 2.2 kb was separated from the rest of pNOM102 and this band was extracted from the agarose gel (Section 2.5.8). The amount of p*gpdA* DNA obtained was quantified (Section 2.6.2) and the preparation stored at 4°C for ligations.

3.2.2 Ligation of DNA Fragments for pFG-gpd Construction.

A two way ligation was carried out between linearised pFunGus and p*gpdA* (Section 3.2.1). This involved both blunt and cohesive end ligations, therefore a combination of temperatures and T4 Ligase concentrations were used (Section 2.7.6). Controls of dephosphorylated pFunGus and phosphorylated pFunGus were also ligated. All ligation reactions were run on an agarose gel to observe the presence of concatamers (Section 2.7.2.1). Bands in addition to the fragments bands were present indicating that ligation

had occurred (results not shown). The ligation mixture was stored at 4°C for transformation into *E. coli*.

3.2.3 *E. coli* Transformation of Ligated Plasmid and Preliminary Analysis of Transformants.

Volumes containing 12ng and 24ng of the ligation reaction (Section 3.1.2) were transformed by electroporation (Section 2.8.2) into competent XL-1 cells (Section 2.1). Also transformed were the following controls; negative control containing no DNA, 10ng undigested pPUC118 (positive control), and 10ng of undigested pFunGus (positive control). The transformed cells were plated on LB Amp^{100} plates (Section 2.2), and these were incubated at 37°C overnight. The transformation frequencies were determined from the numbers of transformed colonies. These showed frequencies for the component mixture higher than that of the background control, indicating the presence of the pFG-gpd construct in the ligation mixture.

3.2.4 Identification and Confirmation of Potential pFG-gpd Containing *E. coli* Transformants.

Following the transformation (Section 3.2.3) 21 clones were assessed by running quick mini plasmid preparations (Section 2.5.6) on an analytical 0.7% agarose mini-gel (Section 2.7.2.1). As a control, pFunGus DNA prepared in the same fashion was run on the same gel. Four of 21 transformants tested (potential pFG-gpd 3, 5, 8, and 19) contained plasmids larger than pFunGus, as expected for pFunGus derivatives containing the promoter (results not shown).

Boiling plasmid mini-preps (Section 2.5.3) were made from the four transformants and subjected to various digests, together with digests of pNOM102 and pFunGus to identify transformants carrying a pFunGus derivative containing the promoter in the correct orientation. Figure 7 shows these results.

Figure 7 Agarose gel showing the profile of bands produced for analysis of the potential pFG-gpd clones.

Lane 1 contains the BRL 1 kb size markers. The potential pFG-gpd clones (numbered 3, 5, 8, and 19) were digested with *Sal*I; 19 (lane 3), 8 (lane 4), 3 (lane 5), and 5 (lane 6) and *Eco*RI-*Nco*I; 19 (lane 9), 8 (lane 10), 5 (lane 11) and 3 (lane 12). Size markers and digest controls were run of *Sal*I digested pFunGus giving a 5.5 kb band (lane 2), *Eco*RI digested pNOM102 giving a 7.5 kb band (lane 7), and *Eco*RI-*Nco*I digested pNOM102 giving 3.3 kb, 2.2 kb, and 1.9 kb bands (lane 8). Undigested controls were run of pNOM102 (lane 13), and pFunGus (lane 14), so partial digests could be interpreted accurately .

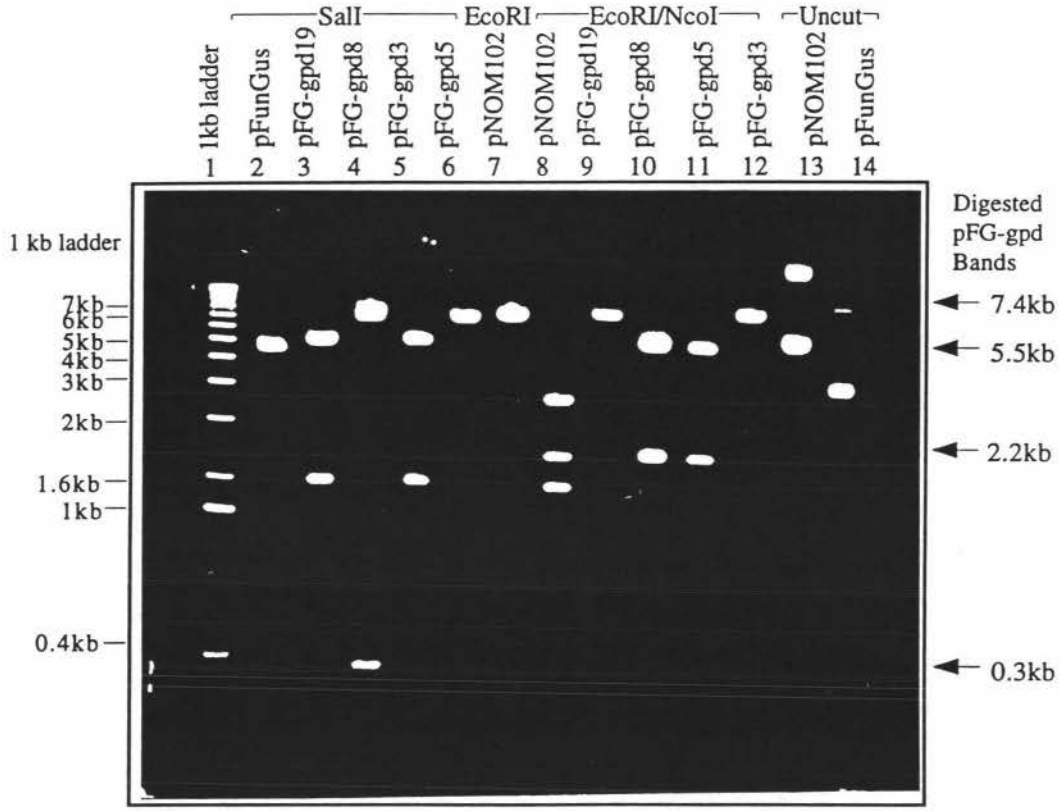

SalI
EcoRI
EcoRI/NcoI
Uncut
1kb ladder
pFunGus
pFG-gpd19
pFG-gpd8
pFG-gpd3
pFG-gpd5
pNOM102
pNOM102
pFG-gpd19
pFG-gpd8
pFG-gpd5
pFG-gpd3
pNOM102
pFunGus
1 2 3 4 5 6 7 8 9 10 11 12 13 14
1 kb ladder
7kb
6kb
5kb
4kb
3kb
2kb
1.6kb
1kb
0.4kb
Digested pFG-gpd Bands
7.4kb
5.5kb
2.2kb
0.3kb

Table 3 Summary of band sizes produced from digests for the analysis of the potential pFG-gpd clones.

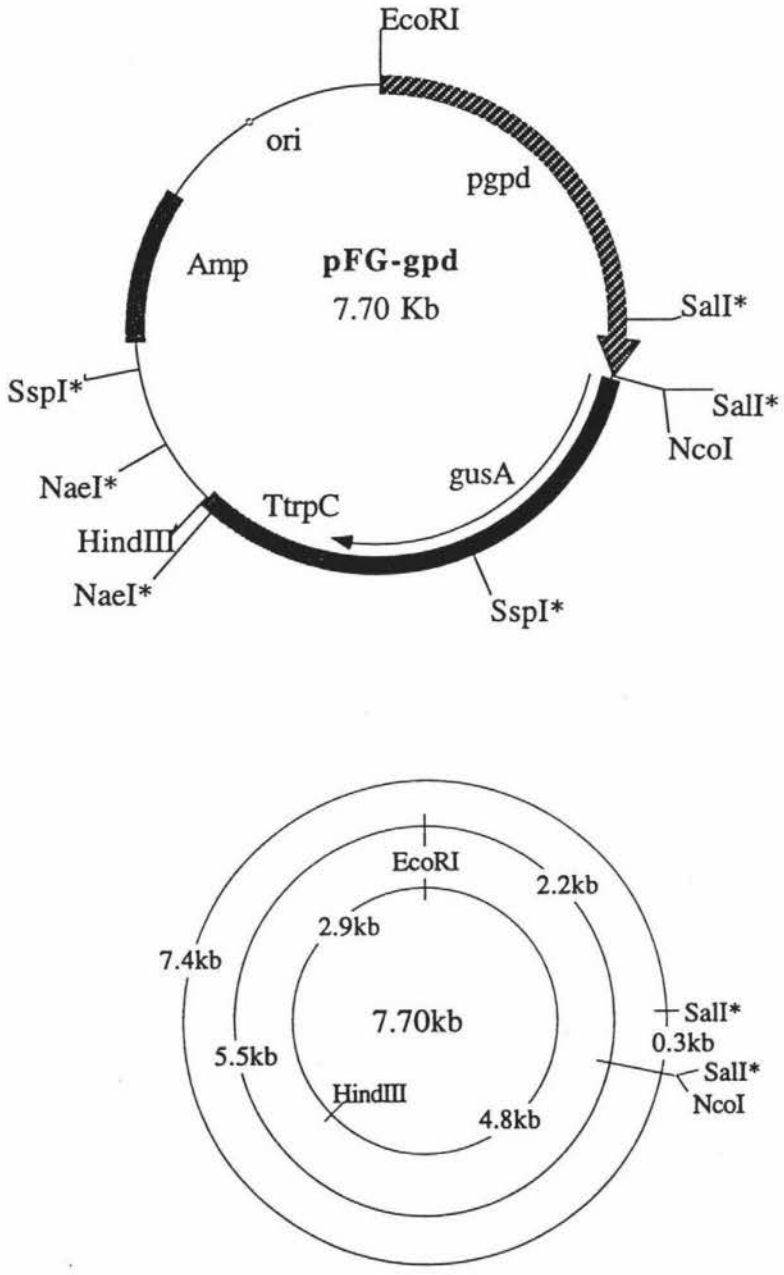
Plasmid	*Sal*I (kb)	*Eco*RI/*Nco*I (kb)
Expected pFG-gpd	7.4, 0.3	5.5, 2.2
Potential pFG.gpd 19	5.8, 1.9	7.7
Potential pFG.gpd 8	7.4, 0.3	5.5, 2.2
Potential pFG.gpd 3	5.8, 1.9	7.7
Potential pFG.gpd 5	7.4, 0.3	5.5, 2.2

Double digests with *Nco*I and *Eco*RI were used to test for the presence of the *gpdA* promoter fragment in the constructs. The promoter should be removed from the pFG-gpd, yielding a band of 2.2 kb, corresponding in size to the *gpdA* promoter fragment obtainable from digestion with the same enzymes of pNOM102 (see plasmid maps in Figure 8 and Appendix 1). As Figure 7 shows, *Eco*RI/*Nco*I double digests of transformants potential pFG-gpd 5 and potential pFG-gpd 8 (lanes 10 and 11) produced the expected 2.2 kb band, equal in size to the promoter fragment obtained by digestion of pNOM102 with the same enzymes (lane 8).

An additional analytical digest was carried out to assess the orientation of the promoter: *Sal*I digestion of pFG-gpd DNA should yield a fragment of 300 bp and a second fragment of 7.4 kb if the promoter is inserted in the correct orientation. The 7.4 kb band should be approximately equal size to a single cut digest of pNOM102 with *Eco*RI (7.5 kb; lane 7). If the promoter was inserted in the wrong orientation, two bands of 1.9 kb and 5.8 kb should result (see plasmid map in Figure 8). Again, *Sal*I digests of DNA from transformants potential pFG-gpd 5 and potential pFG-gpd 8 yielded the 7.4 kb and 0.3 kb bands expected for the correct construct (lanes 4 and 6) with the 7.4 kb band running marginally quicker than the 7.5 kb band obtained by single digestion of pNOM102 (lane 7). The clones containing potential pFG-gpd 3 and 19 gave bands in both sets of digests that did not correspond to the correct construction of the plasmid. In these clones the bands indicated that the promoter of *gpdA* had been inserted in the wrong orientation (lanes 3, 5, 10 and 13).

Additional information about these two clones can be inferred from digests with restriction enzymes *Sal*I and *Nco*I (in digests with *Eco*RI). These sites overlap with the consensus sequence surrounding the translational initiation codon (Kozak sequence; see Section 3.1.4) and their presence confirms that this sequence has been retained undamaged. As described above, potential pFG-gpd clones 5 and 8 gave the results expected for the correct constructs, namely that the Kozak sequence had been retained undamaged. Clones potential pFG-gpd 5 and 8 were therefore considered to contain the desired constructs. A complete restriction map of pFG-gpd is shown in Figure 8.

Figure 8 Restriction map of pFG-gpd.



Restriction maps of pFG-gpd showing sites for: *Eco*RI, *SalI**, *NcoI**, *SspI**, *NaeI**, and *Hin*dIII. Also showing sizes of bands produced by digestion with *SalI*, *Eco*I-*Nco*I and *Eco*RI-*Hin*dIII.

* Restriction enzymes that cut pFG-gpd more than once.

3.3 TRANSIENT EXPRESSION OF THE PFG-GPD PLASMID.

Having constructed the GUS expression plasmid, pFG-gpd, it was planned to integrate this plasmid by cotransformation into the *A. nidulans* genome. The pFG-gpd analytical digests indicated that the plasmid was constructed correctly (Section 3.2.4). Nevertheless intial experiments called into question whether the plasmid could express the *gusA* gene in the *A. nidulans* environment. As will be discussed later (Section 3.4), cotransformation experiments initially failed to give *A. nidulans* transformants expressing the *gusA* gene within the plasmid, pFG-gpd. Therefore to study whether the *gusA* gene capable of being expressed, the transient expression of pFG-gpd in *A. nidulans* protoplasts was measured.

Transient expression of a plasmid can occur without integration into the genome (the limiting event in transformation; Section 1.3.3; Mönke and Schäfer, 1993). Although transient expression is not stable as the plasmid cannot replicate itself, it is a fast and convenient way to determine if a plasmid can be expressed. The method used for this investigation was based on experiments performed to measure transient expression in *Cochliobolus heterostrophus* (Section 2.10; Mönke and Schäfer, 1993).

3.3.1 Transient GUS Expression Levels in *A. nidulans* Transformants.

The following experiment was conducted to determine whether there was transient GUS expression in transformed *A. nidulans* protoplasts and to show that this was due to the transforming DNA rather than an endogenous phenomenon (eg. a GUS-like enzyme cleaving the substrate). The correlation between the transforming DNA and GUS activity can be shown by measuring the conversion of a substrate to a detectable product in transformed but not untransformed protoplasts.

A. nidulans protoplasts were transformed with 3µg and 30µg of pFG-gpd DNA and a control transformation of 30µg of pFunGus DNA. The promoter-less pFunGus plasmid was transformed to show that any GUS activity observed was a feature of expression driven by the *gpdA* promoter in pFG-gpd and not another phenomenon eg. insertion of the *gusA* gene downstream of a promoter element. Also subjected to the transformation

procedure were controls of untransformed *A. nidulans* Y21 protoplasts, 100 fold dilution of *A. nidulans* Y21 transformed with pNOM102, (a GUS expression plasmid; Section 3.4; referred to as 'GUS positive protoplasts' forthwith). GUS positive protoplasts were diluted 100 fold in untransformed protoplasts. In all cases duplicate transformations were made, with an equal volume TE buffer replacing DNA solutions in nil DNA controls (Section 2.9.3). Transformation mixtures were incubated in STC buffer, slowly shaking at 37°C. Observations have shown that these incubation conditions allowed cell metabolism and gene expression to take place, but formation of cell walls was delayed, and cell division didn't occur (Mönke and Schäfer, 1993). After 21 hours protoplasts were harvested (Section 2.10) and resuspended in 1mM MUG (in GUS extraction buffer; Section 2.4.11). The GUS assay was carried out as in Section 2.13.2.

The results of the assays are shown in Table 4. These show that negative controls of untransformed and pFunGus transformed protoplasts had no significant GUS activity, while the positive control protoplasts that were stability transformed with pNOM102 showed significant GUS activity. Protoplasts transformed with pFG-gpd showed GUS activity 50 to 80 fold higher than untransformed protoplasts. However, transient expression by pFG-gpd transformants was notably lower than that observed for the stable transformant. This result was expected as stable expression has been shown to be significantly higher than transient expression (Mönke and Schäfer, 1993). From these results it was concluded that the plasmid pFG-gpd was expressing the GUS gene.

Table 4 GUS enzyme specific activity in transformed protoplasts[a].

	Controls						Transient Expression			
	GUS positive protoplasts from an integrative pNOM102 transformant[b].		Untransformed protoplasts		Protoplasts transformed with 30µg of promoter-less pFunGus DNA.		Protoplasts transformed with 3µg pFG-gpd.		Protoplasts transformed with 30µg pFG-gpd.	
	a	b	a	b	a	b	a	b	a	b
Specific activity of GUS enzyme (pmol MU/min/ $1x10^6$ protoplasts)[cd]	224	116	0.10	0.13	0.20	0.27	4.5	4.8	6.6	7.7

[a] Two transformations and assays per transformant.

[b] Included as a GUS expression control rather than a transient expression control, thus GUS expression levels are higher than those seen for transient expression.

[c] Calculations for the determination of GUS specific activity are shown in Section 2.10.

[d] All assays gave correlation coefficients of between 0.99 and 1.0, with the exception of the untransformed protoplasts and the promoter-less pFunGus transformed protoplasts.

3.4 COTRANSFORMATIONS OF *A. NIDULANS.*

Cotransformation allows filamentous fungi to be transformed with a plasmid without the need for the DNA of interest to be covalently linked to the vector containing the selectable marker. (see introduction Section 1.3.7 for further explanation). Previous experiments by others have shown that cotransformation in *A. nidulans* can be a very efficient process, with an upper limit of 95% (Wernars *et al*, 1987) and lowest report of "up to 50%" (Robert *et al*, 1989). In this study problems were experienced in obtaining the cotransformation frequencies reported in the literature, therefore the following includes an investigation of the parameters affecting cotransformation.

For simplicity of presentation the cotransformation experiments performed are split into three sections; initial cotransformations, optimisation of cotransformations with pNOM102, and final cotransformations in which cotransformants were obtained.

3.4.1 Preparation of Transforming DNA for all Cotransformations.

As a vector with a selectable marker for cotransformation the plasmid pAN8-1 containing the phleomycin resistance gene (*ble*) was chosen. *A. nidulans* transformed with pAN8-1 shows resistance to phleomycin at concentrations above 10μg/ml of media, while this concentration is lethal to untransformed *A. nidulans* (Punt *et al*, 1992). The pAN8-1 DNA was transformed into *E. coli* (Section 2.8.1). Plasmid DNA was prepared and either PEG purified (Section 2.5.4) or purified on a cesium chloride gradient (Section 2.5.5).

The pNOM102, pFunGus, and pFG-gpd DNA was prepared and PEG purified (Section 2.5.4). Later it was prepared and purified on a cesium chloride gradient. (Section 2.5.5).

3.4.2 Initial Cotransformations of *A. nidulans* R21.

3.4.2.1 Experiments for the Classification of Colony Types Observed on Transformation Plates.

As a prerequisite for the accurate determination of cotransformation frequencies colonies of various sizes on transformation plates were analysed as to whether the transformed phleomycin resistance marker was maintained as a stable phenotype. From observation of the transformation plates after 3 days at 37°C three classes of colonies were defined. Class I, were >10mm in diameter, class II were between 5-10mm in diameter, and class III were <5mm in diameter. Twenty colonies of each class were tested by simultaneously transferring onto selective and non-selective plates and incubating at 37°C for 2 days. Only some of the colonies grew on the selective plates, the duplicate colonies to these growing on the non-selective plates were transferred back onto selective media to demonstrate the stability of the resistant phenotype.

Colonies of classes I and II were viable through the sub-culturing, while class III transformants were abortive. Because of the absence of any colony growth in control transformations without DNA (Section 3.4.2.3), these abortive colonies were hypothesised to grow due to transient expression of pAN8-1. The plasmid is lost during the initial cell divisions but the residue gene product confers resistance until sufficient cell divisions have occurred for the product to be titrated out (Section 1.3.5). Based on these experiments only colonies of class I and II were scored as pAN8-1 transformants.

3.4.2.2 Rationale for Initial Cotransformations.

Initial cotransformations were carried out using *A. nidulans* R21 protoplasts, and the plasmids pFG-gpd and pAN8-1. In all transformations controls without DNA were carried out. Other controls were; pAN8-1, to test transformation frequency without a cotransforming plasmid, and pAN8-1 + pNOM102, cotransformation positive control with a GUS expression plasmid. This control allowed the determination of cotransformation frequencies with an established plasmid that had previously given high frequencies in cotransformations (Roberts *et al.*, 1989), permitting assessment of pFG-

gpd performance relative to the pNOM102 plasmid. Finally, pAN8-1 + pNOM102 + pFG-gpd controls were carried out to verify that the presence of pFG-gpd did not interfere with the cotransformation process.

The cotransformation procedure (Murray *et al*, 1992; Section 2.9.3), features PEG mediated DNA uptake into fungal protoplasts. All transformations used a concentration of protoplasts of $1x10^7$ protoplasts/ml. Following transformation various volumes of the protoplasts were spread on solid media containing 10μg/ml phleomycin. Protoplast viability was also tested by spreading diluted aliquots of the transformation mix on non-selective media.

3.4.2.3 Results of Initial Cotransformations.

Three transformations were initially carried out using the method described by Murray *et al*, (1992), with controls as described in Section 3.4.2. In all transformations controls without DNA gave no colonies on selective media. Parameters tested in initial transformations were; total amount of DNA and ratio of pAN8-1 to pFG-gpd or/and pNOM102. Protoplast viability, transformants per μg pAN8-1, and % of pAN8-1 transformants that were also transformed with pNOM102 and/or pFG-gpd (referred to as cotransformation frequency) were determined. Results are shown in Table 5.

Transformation frequencies ranged between 3 and 411 transformants/ μg pAN8-1. In all three transformations a total of 117 transformants were tested for pFG-gpd + pAN8-1 cotransformation. No cotransformants were identified translating to a cotransformation frequency of less than 0.85%. For transformations containing pNOM102 + pAN8-1 a total of 108 transformants were tested with 3 cotransformants identified from a single transformation. This result translated to a cotransformation frequency of 7% for that transformation or 2.8% overall. The plasmid pNOM102 appeared to show the highest cotransformation frequency when the total amount of pNOM102 DNA exceeded that of pAN8-1 DNA by 10 times. From these results we could conclude that pNOM102 and pFG-gpd were not cotransformed at the same frequency with 99% confidence (shown by the z-test; Section 2.14.2). Providing that there is no alternative explanation such as DNA quality, we can accept that the plasmid pNOM102 is cotransformed at a higher frequency than the plasmid pFG-gpd (discussed further in Section 4.1.2).

Table 5 Results of initial cotransformation experiments.

Transfor-mation no.[a]	Transforming DNA	Ratio of plasmids[b]	% protoplasts/ viable[c]	Transformants /µg pAN8-1	% Cotransformants[d]
1[e]	pAN8-1	N/A	2.3%	3	N/A[f]
	pAN8-1 pNOM102	1:1	not tested	6.3	<8% (0/13)
	pAN8-1 pNOM102 pFG-gpd	2:1:1	not tested	12	<17% (0/6)
	pAN8-1 pFG-gpd	1:3	not tested	19.3	<3.7% (0/27)
2[g]	pAN8-1	-	not tested	200	N/A
	pAN8-1 pNOM102	1:1	2.1%	60	<0.2% (0/50)
	pAN8-1 pNOM102 pFG-gpd	1:1:1	not tested	110	not tested
	pAN8-1 pFG-gpd	1:1	not tested	38	<2.5% (0/40)
3[h]	pAN8-1 pNOM102	1:6	0.42%	8	not tested
	pAN8-1 pNOM102	1:10	4.5%	250	7% (3/45)
	pAN8-1 pFG-gpd	1:6	4.8%	240	not tested
	pAN8-1 pFG-gpd	1:10	3.0%	411	<2% (0/50)
	pAN8-1 pFG-gpd pNOM102	1:2:2	2.7%	212	<1.5% (0/66)

[a] Negative controls without DNA were carried out for all three transformations and gave less than 1.5 colonies per µg pAN8-1.

[b] Ratios of plasmid in order seen in column 2.

[c] Calculated by comparing the number of colonies obtained by plating aliquots of dilutions of the transformation mix onto selective media, with the concentration of protoplasts in the mix measured with a haemocytometer.

[d] The % cotransformation shown was the percentage of pAN8-1 transformants that were GUS positive. The number of GUS positive colonies and the number of colonies tested are shown in the brackets.

[e] A total of 5μg added of appropriate plasmids.

[f] Not applicable.

[g] 0.625μg of each plasmid.

[h] 3μg total added of plasmids.

For transformations containing pFG-gpd + pNOM102 + pAN8-1 no cotransformants were identified out of 72 tested, translating to a transformation frequency of less than 1.4%. From these results we could conclude, with 99% confidence, that pNOM102 when combined in a transformation mix with pFG-gpd was not cotransformed at the same frequency as pNOM102 alone (shown by the z-test; Section 2.14.2). Therefore we could hypothesise that an aspect of plasmid pFG-gpd was causing inhibition of the pNOM102 cotransformation process (discussed further in Section 4.1.2).

From previously described experiments, cotransformation frequencies were expected to be much higher. Two initial hypothesises were proposed to explain the inability to obtain a single pFG-gpd cotransformant. Either; the vector was unable to express the *gusA* gene, or the cotransformation process was failing. These two hypotheses were tested; the ability of pFG-gpd to express the *gusA* gene was examined by measuring the transient expression of transformed protoplasts (Section 3.3) and optimisation of the cotransformation conditions was attempted with the GUS expression plasmid, pNOM102 (Section 3.4.3).

3.4.3 Attempt at Optimisation of Cotransformation Procedure using pNOM102.

It was attempted to optimise the reactions with pNOM102 + pAN8-1 cotransformations. The transformations attempted thus far were analysed, and other parameters of transformation were tested in further cotransformations with the aim to increase the incidence of cotransformation. The parameters of the cotransformation system tested were; (i) total amount of DNA (transformations 1 to 5) (ii) the method by which the DNA was obtained (transformations 4 and 5), (iii) the use of protoplasts fresh or thawed from previously prepared frozen stocks (transformations 4 and 5), and (iv) the ratios of cotransforming plasmids (transformation 3). Table 6 shows the summary of cotransformation results with pNOM102 and pAN8-1. These results show that cotransformation frequencies were not increased beyond 7% and gave an average frequency of 2.2%. Increasing the total amount of DNA, the method by which the DNA was obtained, or whether the protoplasts used were prepared fresh or thawed from previously prepared frozen stocks did not increase cotransformation frequencies.

Table 6 Summary of cotransformations with pNOM102 and pAN8-1.

Trans-forma-tion no.	Proto-plasts prepa-ration	Type of DNA prepara-tion	Ratio of pNOM102 : pAN8-1	Transfor-mants/ µg pAN8-1	Transformants /viable protoplast[a]	% co-transfor-mants
1[b]	fresh	PEG	1:1	61	$7x10^{-4}$	<14% (0/7)
2[b]	frozen	PEG	1:1	60	$3x10^{-3}$	<2% (0/40)
3[c]	frozen	PEG	5:1	8	$4x10^{-4}$	<25% (0/4)
	frozen	PEG	10:1	250	$1x10^{-3}$	7% (3/45)
4[d]	frozen	PEG	1:1	36	$4x10^{-3}$	3% (1/26)
	frozen	CsCl	1:1	50	$6x10^{-3}$	2.5% (1/40)
5[d]	fresh	CsCl	1:1	52	$4x10^{-3}$	2.5% (1/41)
	frozen	CsCl	1:1	102	$9x10^{-3}$	<1.5% (0/60)
	fresh	CsCl[e]	1:1	94	$6x10^{-3}$	4.8% (2/42)

[a] Protoplast viability was determined from plating dilutions of the transformed protoplasts on non-selective media.

[b] Total DNA in transformation of 1.25µg.

[c] Total DNA in transformation of 3µg.

[d] Total DNA in transformation of 2µg.

[e] DNA obtained from Dr. Rosie Bradshaw, Department of Microbiology and Genetics, Massey University.

Increasing the total DNA ratio of pNOM102 to pAN8-1 from 1:1 to 10:1 increased the cotransformation frequency. This was shown by the z-test, with 99% confidence, that the cotransformation frequency of pNOM102 in the experiment with a 10:1 ratio was higher than the cotransformation frequencies of experiments where the amounts of pNOM102 and pAN8-1 DNA were in equal (Section 2.14.2). Despite this, the highest cotransformation frequency of 7% was still significantly lower than the levels expected, (discussed further in Section 4.1.1).

3.4.4 Large Scale Cotransformations of *A. nidulans* Yielding Cotransformants with pFG-gpd + pAN8-1.

Several parameters were varied to ensure that cotransformants were obtained from the transformation procedure. Ratios of plasmids were varied to between experiments to give an excess of pAN8-1 or pFG-gpd in separate experiments. An excess of the non-selectable plasmid in cotransformation had previously been shown to increase cotransformation frequencies (Wernars *et al.*, 1985), and this appeared to occur with pNOM102 (Section 3.4.2.3). However it was also possible that a high copy number of pFG-gpd would prove lethal. Transformations were also carried out with a different strain of *A. nidulans*, 'Glasgow WT', also using a different transformation method, and different media. These changes were carried out to reproduce published conditions, with the only variance being the pFG-gpd plasmid (Punt and van del Hondel, 1992). Differences between in the transformation method from that previously used were minor (Section 2.9.4.). In the final two transformations plasmids pAN8-1 and pFG-gpd were cotransformed into *A. nidulans* in equal molar ratios and 9:1 ratio of pFG-gpd to pAN8-1. The transformation mixtures were plated at a very high concentration so a maximum amount of transformants could be tested for cotransformation.

Results are shown in Table 7. In the final two transformations, cotransformants were finally obtained from the screening of approximately 2000 transformants at a cotransformation frequency of approximately 0.45%. These results showed with 99% confidence that the change of method and strain did not significantly raise the cotransformation frequency (Section 2.14.2). Instead, the cotransformants obtained were the consequence of large scale screening of pAN8-1 transformants.

Table 7 Summary of results for the final cotransformation experiments.

Trans-forma-tion no.	Plasmids trans-formed	Total DNA (μg)	Ratio of pFG-gpd : pAN8-1	Transfor-mants/ μg pAN8-1[a]	Transformants /μg pAN8-1 / viable protoplast	% Cotrans-formation
1[b]	pAN8-1	1	NA	61	not available	NA
	pFG-gpd pAN8-1	11	10:1	199	$1.2x10^{-3}$	<0.7% (0/140)
	pFG-gpd pAN8-1	21	20:1	74	$4x10^{-4}$	<1.5% (0/70)
2[b]	pFG-gpd pAN8-1	22	1:1	83	$6x10^{-3}$	<1.2% (0/83)
	pFG-gpd pAN8-1	4	1:3	26	$1.4x10^{-3}$	<3% (0/26)
3[c]	pAN8-1	5	NA	~100	not available	NA
	pFG-gpd pAN8-1	10	1:1	~100	not available	~0.6% (3/~ 500)
	pFG-gpd pAN8-1	20	9:1	~250	not available	~0.4% (2/~ 500)
4[c]	pFG-gpd pAN8-1	10	1:1	~100	not available	~0.6% (6/ ~1000)

[a] Only approximate transformation frequencies are available for transformations 3 and 4, as the number of colonies on the plates were uncountable.

[b] Transformation method 1 (Section 2.9.3) and *A. nidulans* R21 strain.

[c] Transformation method 2 (Section 2.9.4) and *A. nidulans* WT strain.

Analysis of all cotransformation experiments with pFG-gpd and pNOM102 showed that the cotransformation frequencies of pNOM102 were significantly higher than those for pFG-gpd (99% confidence), even when the DNA preparations were of equal quality (determined by OD_{260}/OD_{280} ratio readings measured in the spectrophotometer; Section 2.6.1).

3.4.5 Selection and Purification of Cotransformants.

Cotransformants were obtained by screening transformation plates as described in Section 2.9.5. Eleven GUS positive cotransformants were obtained from the final two transformations (Section 3.4.4). Of these 11 GUS positive transformants, 9 remained stable through 3 sets of single spore isolations (Section 2.9.5). These stable transformants were stored on ACM slopes (Section 2.3).

3.5 ANALYSIS OF PFG-GPD *A. NIDULANS* TRANSFORMANTS.

3.5.1 Analysis of Integration of pFG-gpd into the *A. nidulans* Genome.

The number of copies integrated intact of the GUS [*gpdA* promoter - *gusA* - T*trpC* termination] cassette (within the plasmid pFG-gpd) and whether the GUS cassettes were integrated into the *A. nidulans* genome singularly, or in tandem repeats, was studied by genomic digestion and Southern hybridisation. By digesting with a restriction enzyme that cuts once within the plasmid and probing with the *gusA* gene, the number of copies, and whether these copies are integrated separately or in tandem repeats, can be determined. By cutting with two restriction enzymes which cut either side of the GUS cassette and probing with the *gusA* gene, it can be determined whether the cassette has integrated as an intact unit, or whether the integration event has disrupted it.

Genomic DNA was prepared (Section 2.5.7) from the *A. nidulans* cotransformants (Section 3.5.1). The genomic DNAs were then digested with *Hin*dIII, and *Eco*RI /*Hin*dIII (Section 2.7.1.2). *Hin*dIII cuts once within the plasmid at the end of the T*trpC* termination sequence (see plasmid map in Figure 8). When probed the Southern autoradiograph shows bands produced by cutting once in the plasmid and in the flanking DNA. The number of copies that are integrated separately into the genome, and the number of copies that have integrated as tandem repeats are observed. The number of tandem repeats can be estimated by comparing the intensity of the band with the concentration standards. *Eco*RI cuts within the multi-cloning site, at the start of the *gpdA* promoter. Therefore a double digest with *Eco*RI and *Hin*dIII removes the GUS cassette, (see plasmid map in Figure 8). When probed the Southern autoradiograph shows a band of 4.8 kb represents the intact GUS expression cassette. The number of copies of the intact cassette can be again estimated by comparing the band intensity with the concentration standards. A band of a larger or smaller size represents an interrupted cassette, with adjoining flanking DNA.

The *gus*A gene fragment was chosen to be used as the probe for the Southern blots as it was part of the functional pFG-gpd GUS expression cassette, but unlike the *gpdA* promoter, and the T*trpC* termination sequence it is not endogenous to *A. nidulans*,

therefore it will not hybridise to *A. nidulans* genomic sequences. DNA for the probe was obtained by digesting pRAJ275 DNA with *Eco*RI and *Sal*I, (Section 2.7.1.1) to remove a 1.9 kb fragment containing the *gusA* gene, (see plasmid map in Appendix 1). The 1.9 kb fragment was separated from the rest of the plasmid by agarose gel electrophoresis and the fragment extracted from the agarose gel (Section 2.5.8). The 1.9 kb fragment was then labelled with [α-^{32}P]dCTP by nick translation (Section 2.11.2).

DNA from untransformed *A. nidulans* was prepared for the negative control, and pFG-gpd concentration standards. These concentration standards consisted of untransformed genomic DNA with 0.35ng, 0.78ng 1.56ng and 3.12ng of pFG-gpd plasmid DNA (Section 2.5.5) per μg of genomic DNA to give 1, 2, 4, and 8 copies of the pFG-gpd plasmid per *A. nidulans* genome.

All the digests were size fractionated by overnight electrophoresis on 0.7% large agarose gels (Section 2.7.2.2) and the gels were then Southern blotted (Section 2.11.1). Agarose gels are shown in Figure 9 to demonstrate consistency in amount of DNA, and equal degrees of digestion between lanes. Southerns blots A and B were probed with the ^{32}P-labelled DNA (Section 2.11.3), and autoradiographs were produced of each blot (Section 2.11.4).

For the purposes of this study the pFG-gpd transformants have been categorised into 3 types based on the type of integration event observed. 'Type I' is single copy integration, 'type II' is multi-copy integration in tandem repeats, and 'type III' is multi-copy integration at separate sites. The integration events seen for each transformant are summarised in Table 8, and the arrangements are diagrammatically represented in Figure 11.

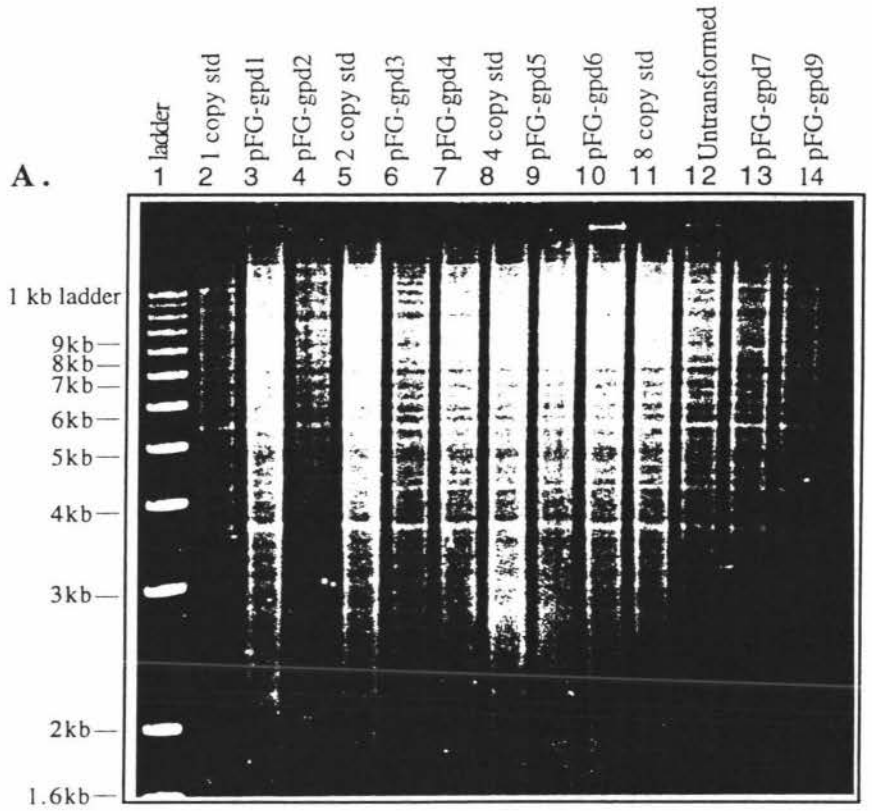
Figure 9 Agarose gels showing the profile of bands produced by digesting *A. nidulans* DNA to demonstrate consistency in the DNA concentration and equal degree of digestion between lanes.

The gel lanes show 1.5µg of digested DNA from transformants; pFG-gpd 1 (lane 3), pFG-gpd 2 (lane 4), pFG-gpd 3 (lane 6), pFG-gpd 4 (lane 7), pFG-gpd 5 (lane 9), pFG-gpd 6 (lane 10), pFG-gpd 7 (lane 13), and pFG-gpd 9 (lane 14). The BRL 1 kb ladder for size determination is seen in lane 1. Also run were controls of untransformed digested *A. nidulans* DNA (lane 12) and concentration standards of 1 copy of pFG-gpd/genome (lane 2), 2 copies of pFG-gpd/genome (lane 5), 4 copies of pFG-gpd/genome (lane 8), and 8 copies of pFG-gpd/genome (lane 11). [The amount of pFG-gpd DNA in these concentration standard was calculated from a haploid genome size 20,000 kb (Bennet and Klich, 1992) and plasmid size of 7.7 kb. Therefore for 0.39ng of pFG-gpd DNA per µg of genomic DNA is required for each copy.]

The DNA is digested with:

A. *Hin*dIII.

B. *Hin*dIII and *Eco*RI.


A.
1 ladder
2 1 copy std
3 pFG-gpd1
4 pFG-gpd2
5 2 copy std
6 pFG-gpd3
7 pFG-gpd4
8 4 copy std
9 pFG-gpd5
10 pFG-gpd6
11 8 copy std
12 Untransformed
13 pFG-gpd7
14 pFG-gpd9
1 kb ladder
9kb
8kb
7kb
6kb
5kb
4kb
3kb
2kb
1.6kb

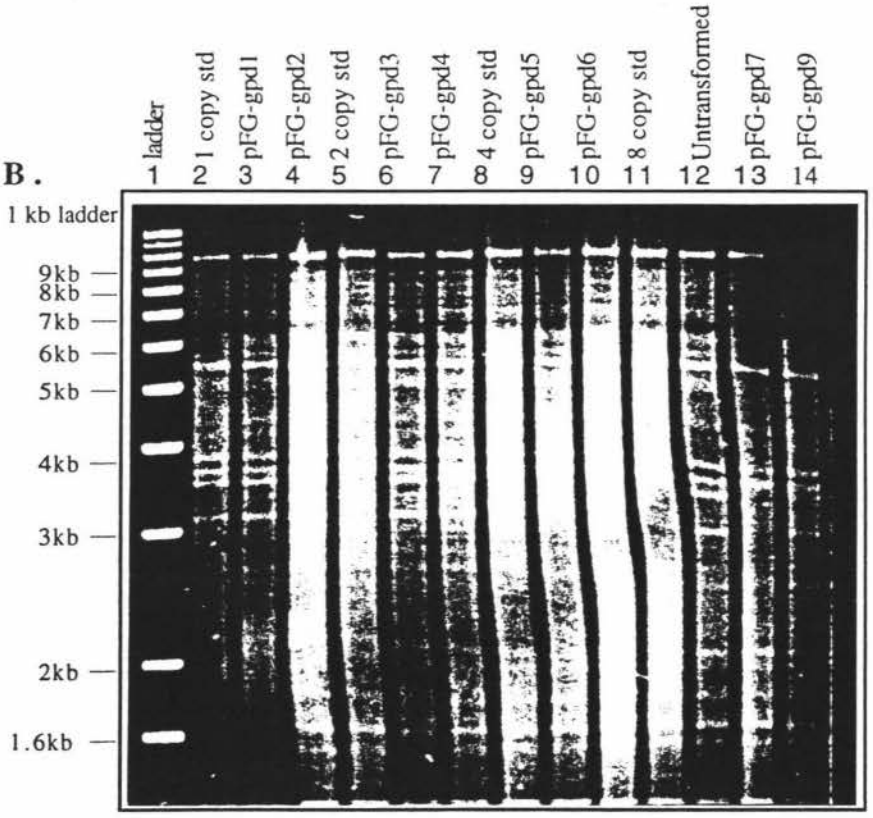

B.
1 ladder
2 1 copy std
3 pFG-gpd1
4 pFG-gpd2
5 2 copy std
6 pFG-gpd3
7 pFG-gpd4
8 4 copy std
9 pFG-gpd5
10 pFG-gpd6
11 8 copy std
12 Untransformed
13 pFG-gpd7
14 pFG-gpd9
1 kb ladder
9kb
8kb
7kb
6kb
5kb
4kb
3kb
2kb
1.6kb

Figure 10 Profiles of the integration of the GUS cassette in pFG-gpd transformants.

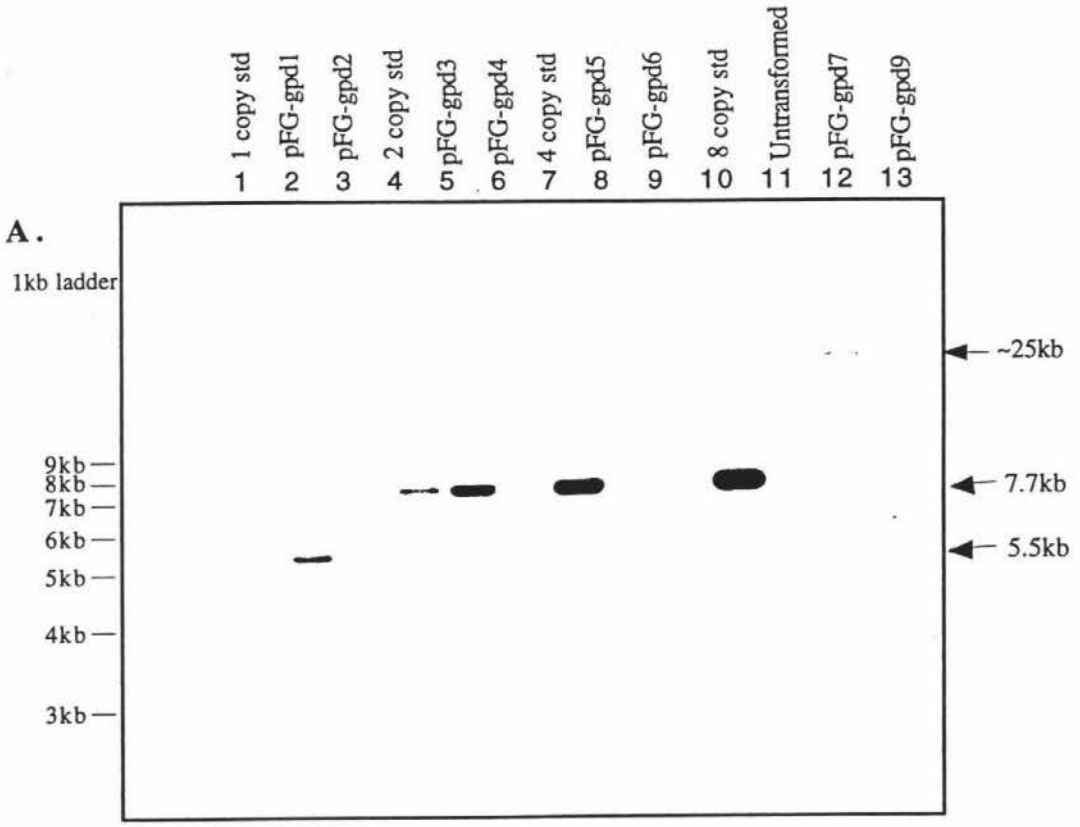
Shown are autoradiographs of Southern blots of gels shown in Figure 9 hybridised to [α-^{32}P]CTP labelled 1.9 kb *gusA* gene fragment.

The digested DNAs are from transformants; pFG-gpd 1 (lane 2), pFG-gpd 2 (lane3), pFG-gpd 3 (lane 5), pFG-gpd 4 (lane 6), pFG-gpd 5 (lane 8), pFG-gpd 6 (lane 9), pFG-gpd 7 (lane 12), and pFG-gpd 9 (lane 13). Also run were controls of untransformed digested *A. nidulans* DNA (lane 11) and concentration standards of 1 copy of pFG-gpd/genome (lane1), 2 copies of pFG-gpd/genome (lane 4), 4 copies of pFG-gpd/genome (lane 7), and 8 copies of pFG-gpd/genome (lane 10). [The amount of pFG-gpd DNA in these concentration standard was calculated from a haploid genome size 20,000 kb (Bennet and Klich, 1992) and plasmid size of 7.7 kb. Therefore for 0.39ng of pFG-gpd DNA per µg of genomic DNA is required for each copy.]

The DNA is digested with:

A. *Hin*dIII.

B. *Hin*dIII and *Eco*RI.


A.
1kb ladder
1 1 copy std
2 pFG-gpd1
3 pFG-gpd2
4 2 copy std
5 pFG-gpd3
6 pFG-gpd4
7 4 copy std
8 pFG-gpd5
9 pFG-gpd6
10 8 copy std
11 Untransformed
12 pFG-gpd7
13 pFG-gpd9
9kb
8kb
7kb
6kb
5kb
4kb
3kb
~25kb
7.7kb
5.5kb

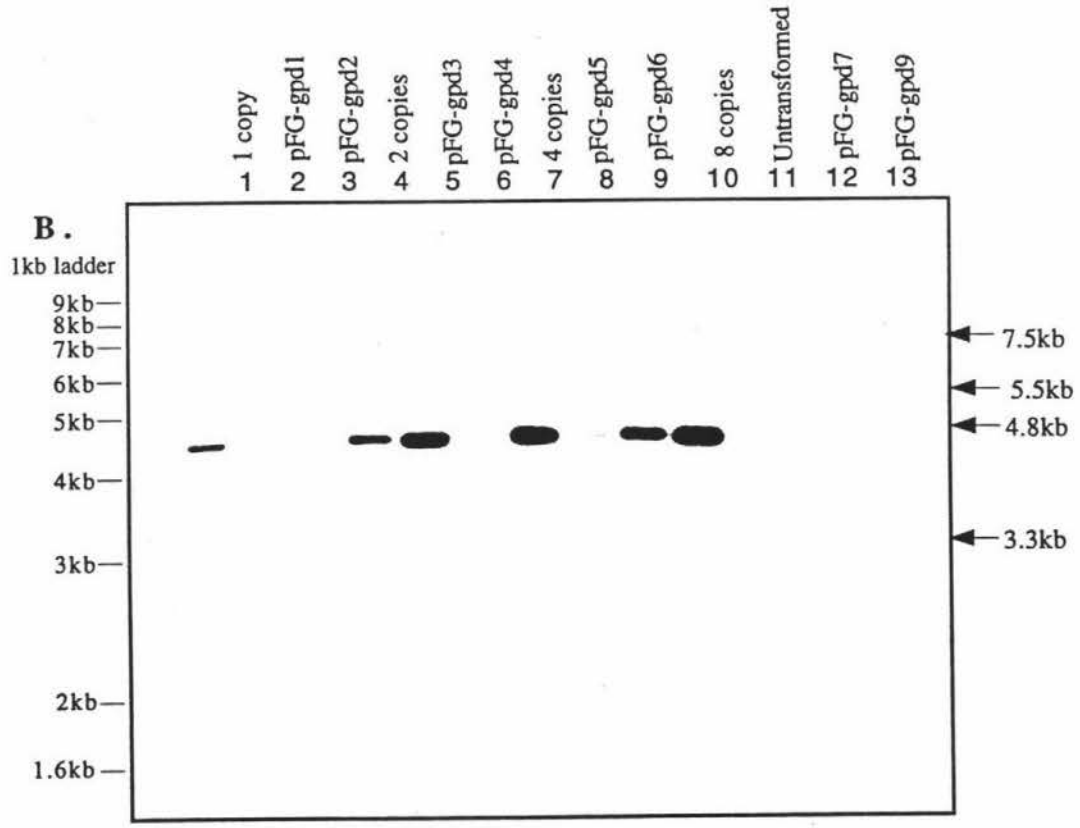

B.
1kb ladder
1 1 copy
2 pFG-gpd1
3 pFG-gpd2
4 2 copies
5 pFG-gpd3
6 pFG-gpd4
7 4 copies
8 pFG-gpd5
9 pFG-gpd6
10 8 copies
11 Untransformed
12 pFG-gpd7
13 pFG-gpd9
9kb
8kb
7kb
6kb
5kb
4kb
3kb
2kb
1.6kb
7.5kb
5.5kb
4.8kb
3.3kb

Table 8 Summary of the types of integrations.

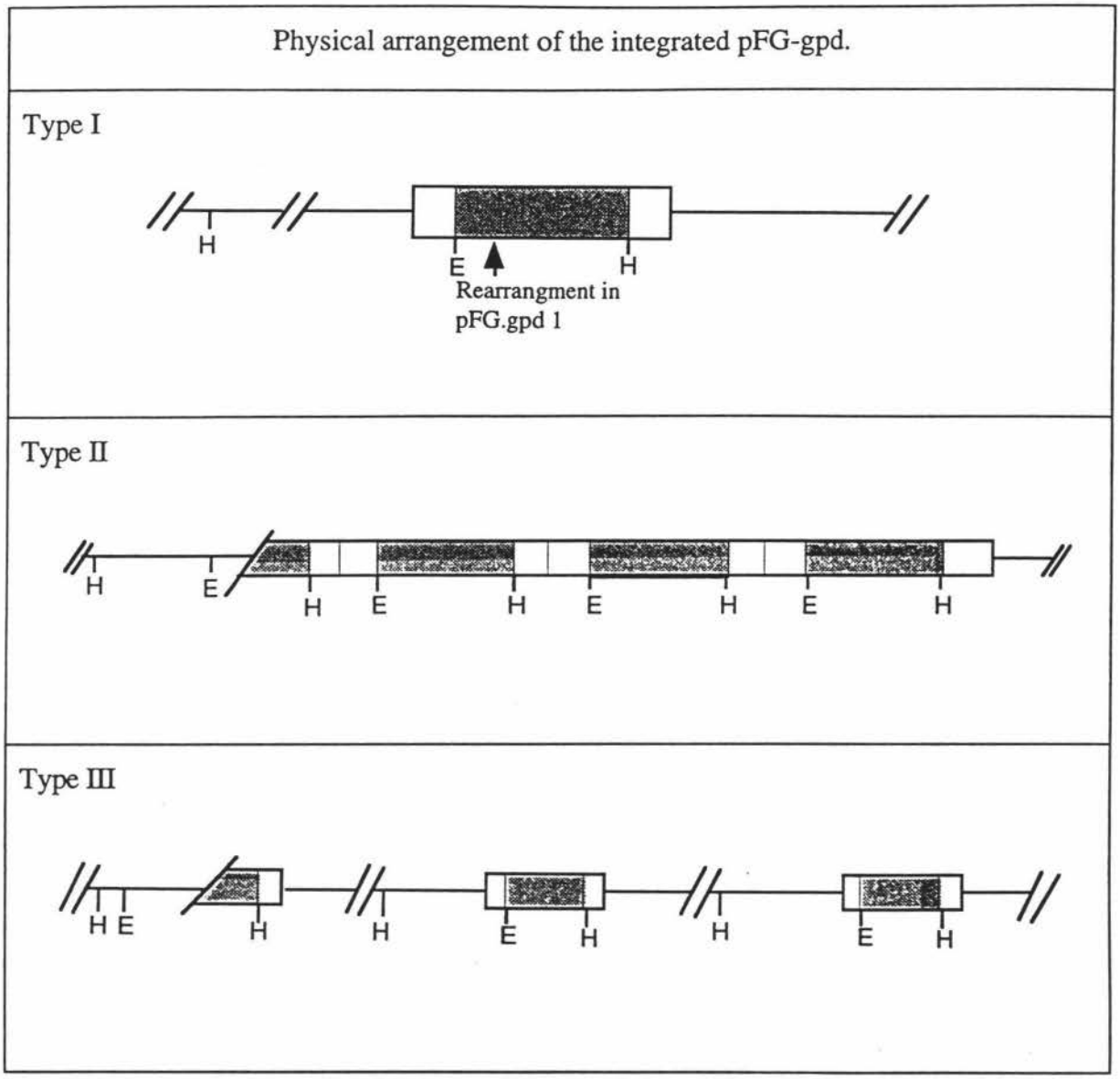
Type of Transformation event	pFG.gpd Transformant no.	No. of integrated copies	No. of intact copies	No. of integration sites
Type I	4	1	1	1
	5	1	1	1
	7	1	1	1
	9	1	1	1
Type II	3	4	3	1
Type III	6	3	2	3
Exceptions[a]	1[b]	1	0	1
	2[c]	0	0	0

[a] These transformants cannot be defined in the above categories.

[b] Contains an active rearrangement of the GUS cassette.

[c] The pFG-gpd DNA was lost in this transformant correlating with a loss of the GUS positive phenotype.

Figure 11 Diagrammatic representation of the types of integrations observed.



Type I integration was single copy, and it was observed in transformants, pFG-gpd 4, 5, 7, and 9. Type II integration was integration as tandem repeats, and it was observed in transformant pFG-gpd 3. Finally, type III integration was multi-copy integration at separate sites in the genome and was observed in transformant pFG-gpd 6.

The exceptions are transformants pFG-gpd 1,and pFG-gpd 2. Transformant pFG-gpd 2 initially showed GUS activity, however lost the activity when the transformant was transferred to fresh media. No hybridisation on both Southern autoradiographs (Figure 10; lane 3) shows that GUS activity was lost by the loss of pFG-gpd DNA rather than inactivation of the *gusA* gene. The pFG-gpd 1 DNA produced a 5.8 kb band in both Southern autoradiographs (Figure 10; lane 2). This may represent a single copy of pFG-gpd that has under gone rearrangement by deletion in the transformation procedure. It seems to have lost a section containing the *Eco*RI site, however it has retained the GUS positive phenotype.

The pattern seen for each 'type I' transformant was a single band which varied in size in Southern A and a single band of 4.8 kb representing the intact GUS cassette in Southern B. The transformants, pFG-gpd 4, 5, 7, and 9, were type 1 transformants. When *Hin*dIII digested DNA was probed the DNAs produced single bands (Figure 10A) of 14-15 kb (pFG-gpd 4; lane 6), 10-11 kb (pFG-gpd 5; lane 8), 20-30 kb (pFG-gpd 7; lane 11), and 7.7kb (pFG-gpd 9; lane 12). When *Eco*RI/*Hin*dIII digested DNA was probed the DNAs all produced a 4.8 kb band representing the intact GUS cassette (Figure 10B). The patterns produced by the pFG-gpd 9 DNA (lane 12) are consistent with an integration event occurring as a cointegrate with pAN8-1. The 7.7 kb band represents a complete plasmid as would be observed for tandem integration but the flanking copy of pFG-gpd is not observed (the significance of this is discussed in Section 4.2.1).

A 'type II' transformant has pFG-gpd integrated as tandem repeats. In Southern autoradiograph A (Figure 10A) the pFG-gpd 3 DNA produced a 7.7 kb band with equivalent intensity to 3 copies of pFG-gpd per genome when compared to the concentration standards (lane 5). There was also an additional 12-13 kb band. In Southern autoradiograph B (Figure 10B) a 4.8 kb band with an intensity equivalent to 3 copies of the plasmid was produced (lane 5). A second smaller band of 3.3 kb was also produced. The band 7.7 kb band was the size of the plasmid and represented pFG-gpd integrated as tandem repeats. The additional 12 -13 kb band represented a fourth copy within the tandem repeat region with flanking DNA sequences attached. The second Southern autoradiograph showed that 3 copies of the 4.8kb GUS cassette were intact

and the additional 3.3 kb band represented the fourth interrupted GUS cassette (see Figure 11 for diagrammatic representation of this arrangement).

In 'type III' transformants multiple copies of pFG-gpd have integrated into separate parts of the genome. The pFG-gpd 6 DNA showed three bands of approximately 11 kb, 14 kb and 18 kb in Southern autoradiograph A (Figure 10A; lane 9). Southern autoradiograph B (Figure 10B) showed a 4.8 kb band with equivalent intensity to 2 copies of the plasmid and a second band of 7.5 kb. These show 3 separate integration events have occurred in the genome. Also the 7.5 kb band in Southern autoradiograph B shows that one of these copies has an interrupted GUS cassette (see Figure 11 for diagrammatic representation of this arrangement).

3.5.2 Physiological Characteristics of *A. nidulans* Transformants.

If studies are to be carried out using the *gusA* reporter gene the physiology of the transformants must resemble that of the WT strain as closely as possible. The physiological characteristics of colony extension, dry weight increase, and protein concentration of the transformed *A. nidulans* clones were determined to resolve whether the transformants were compromised by the presence of the transforming DNA.

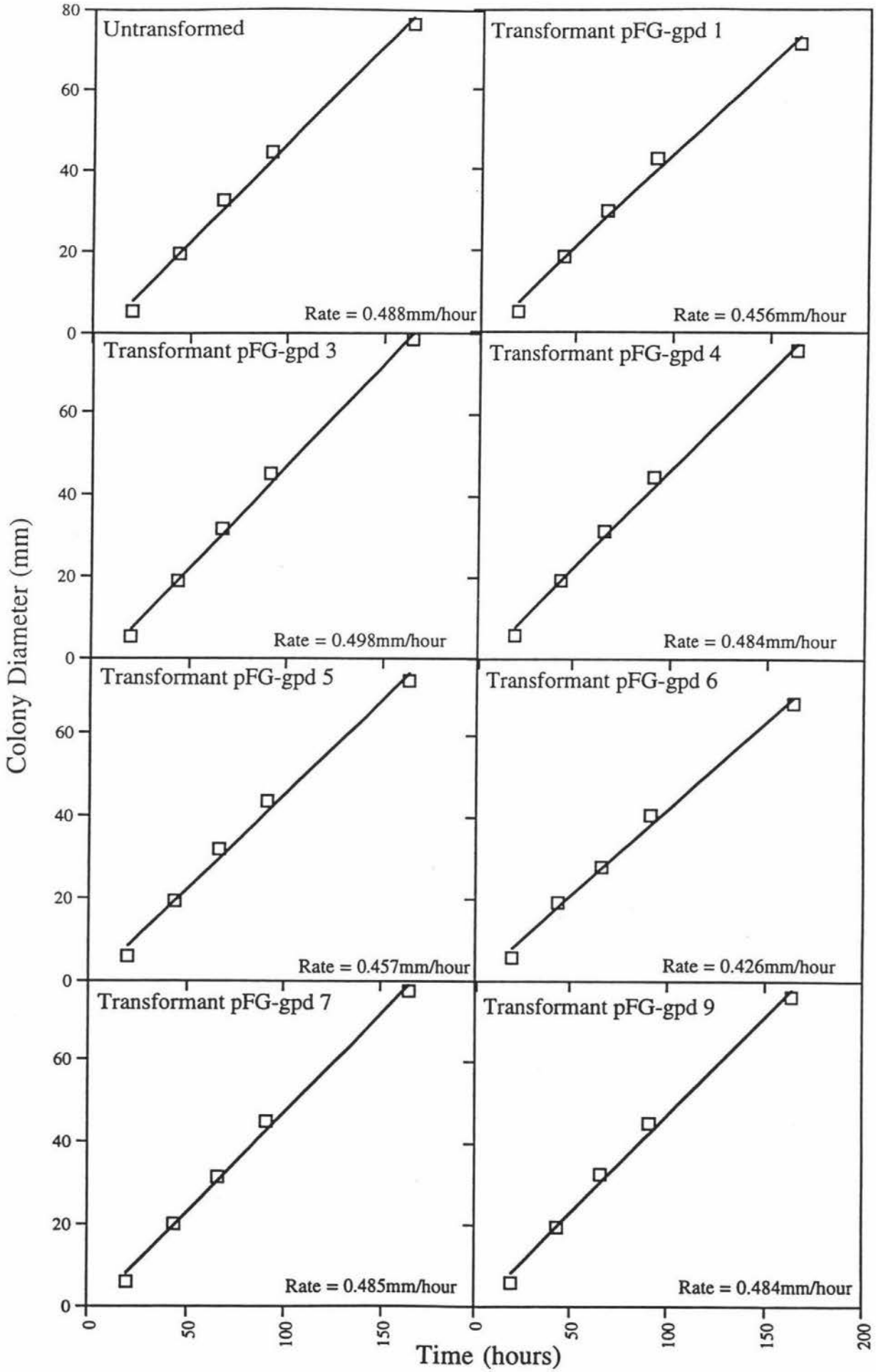
3.5.2.1 Analysis of Growth Rates by Colony Extension.

Growth rate was measured by colony extension over time by following the increase in diameter of colonies of the transformants and comparing to the increase in diameter of the untransformed *A. nidulans* WT. (Section 2.12.1).

Graphs in Figure 12 demonstrate that there was no significant difference in growth between transformants and the untransformed control. Also, no positive or negative correlation was observed between copy number and colony extension. The transformed colonies diameter increased by between 0.426 and 0.498 mm/hour, with the untransformed colony increasing by 0.488 mm/hour. These results indicate that the transformation process had not affected the growth of the transformants. However this was tested again by an alternative method of dry weight determination (Section 3.5.2.2).

Figure 12 Graphs of colony extension versus time.

Graphs represent data of two separate colony extension experiments, with duplicates of each strain in each experiment. All growth rates showed linear correlation coefficients of between 0.99 and 1.0 (Section 2.14.1).


Untransformed
Rate = 0.488mm/hour
Transformant pFG-gpd 1
Rate = 0.456mm/hour
Transformant pFG-gpd 3
Rate = 0.498mm/hour
Transformant pFG-gpd 4
Rate = 0.484mm/hour
Transformant pFG-gpd 5
Rate = 0.457mm/hour
Transformant pFG-gpd 6
Rate = 0.426mm/hour
Transformant pFG-gpd 7
Rate = 0.485mm/hour
Transformant pFG-gpd 9
Rate = 0.484mm/hour
Colony Diameter (mm)
Time (hours)
0
20
40
60
80
0
50
100
150
200

3.5.2.2 Analysis of Growth Rates by Dry Weight Determination.

The rate of growth was determined by a second method of dry weight increase over time for three of the transformants as well as the untransformed *A. nidulans* WT control (Section 2.12.2). The linear portion of the growth curve produced represents the log phase of the growth, and exponential regression analysis on this portion gives the culture doubling time (Section 2.14.1). This experiment also gave information about the timing of the log phase of growth so that samples could be obtained from the mid-log phase to be used in the GUS assays (Section 3.5.4).

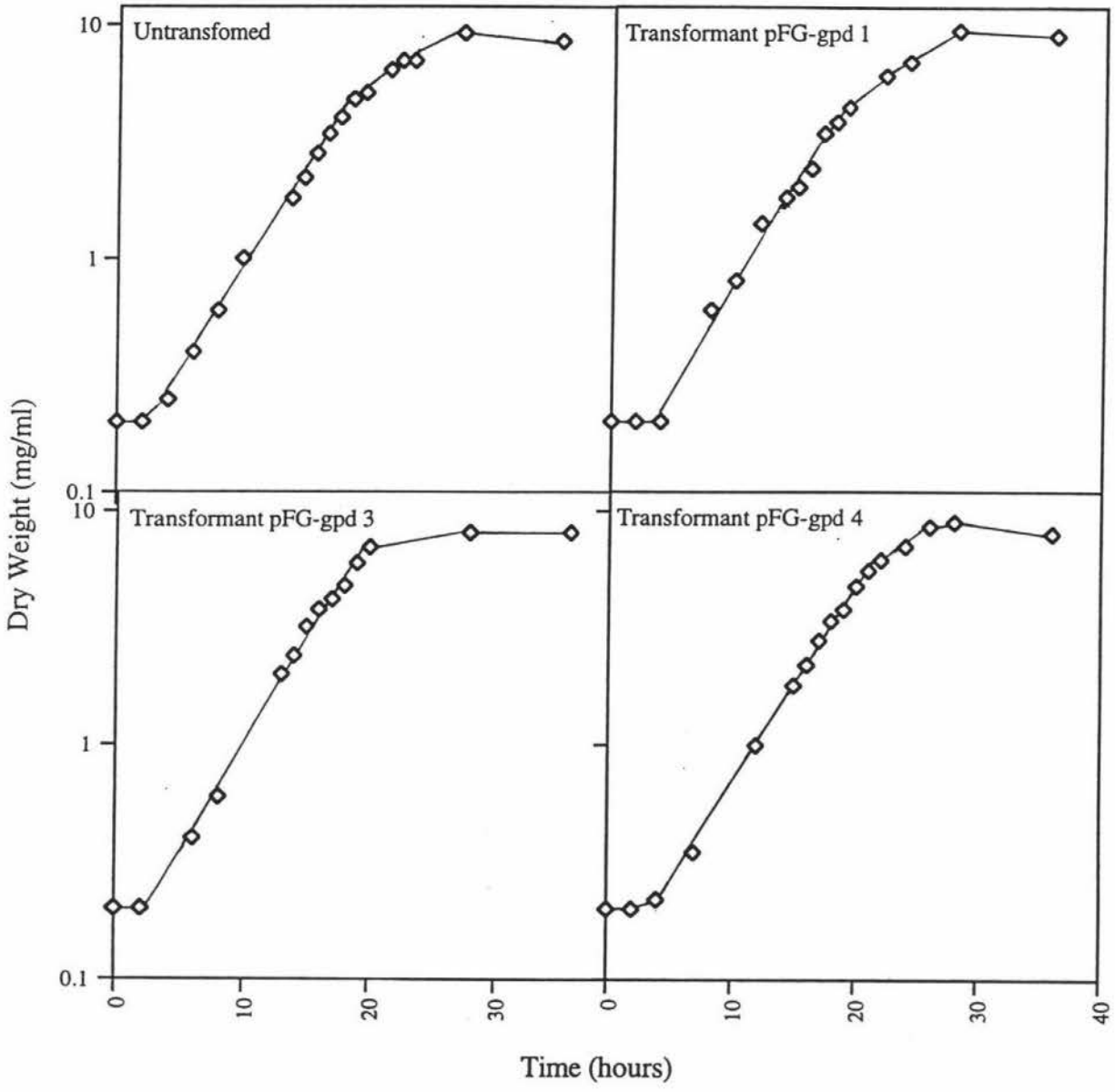
The growth curves obtained are shown Figure 13 and the culture doubling times are shown in Table 9.

As well as giving very similar growth rates during the log phase, the total dry weight yields from all transformants tested were identical. The remaining transformants (pFG-gpd 5, 6, 7 and 9), although not as well characterised, also gave similar growth rates and yields (data not shown). In summary, the results confirm findings of colony extension experiments that no apparent differences in growth were observed that could be attributed to the transformed DNA.

3.5.3 Analysis of Total Protein Levels in Transformants at Mid Log Phase.

The amount of total protein content during the log phase of growth is another physiological feature that should be conserved between *A. nidulans* transformants and wild type if the transformants have not been compromised by the transforming DNA. Samples were taken of each transformant at mid log phase of growth (as determined in Section 3.5.2.2) and the total protein was determined by the Bradford method of protein determination (Section 2.13.1). It was shown that there were no significant differences in the protein levels between all assays. The protein concentrations are shown in Table 10. Samples varied between 78 and 101 μg protein / mg dry weight. Variation occurred because of inaccuracy in weighing rather than differences in protein content, as it was

Figure 13 Growth of untransformed and transformed *A. nidulans* in liquid media defined as dry weight.



The growth rates of untransformed *A. nidulans,* transformants pFG-gpd 1, pFG-gpd 3, and pFG-gpd 4 were assessed by dry weight determination. At set time intervals samples were taken from a liquid culture. These were freeze dried and weighed. Growth curves shown are obtained from the composite data of 2 separate dry weight determinations. The cultures doubling times are given in Table 9.

Table 9 Growth rates of transformants determined by dry weight versus time.

Transformant	Dry weight doubling time (hours)[a]
Untransformed	3.8 ± 0.1
Transformant pFG-gpd 1	4.0 ± 0.3
Transformant pFG-gpd 3	3.4 ± 0.3
Transformant pFG-gpd 4	3.5 ± 0.2

[a] Determined from exponential regression analysis on dry weight growth curves (Section 2.14.1), standard errors are shown.

Table 10 Protein concentrations in GUS extracts of transformants in mid-log phase of growth.

GUS assays[a]	Protein concentration of GUS extract (μg/ml)[b]	Protein content (μg protein / μg dry weight)
Untransformed	133.7	0.200
	134.9	0.201
pFG-gpd 1	116.9	0.174
	135.7	0.203
pFG-gpd 3	138.4	0.207
	127.5	0.177
pFG-gpd 4	140.8	0.210
	118.8	0.177
pFG-gpd 5	131.8	0.197
	151.8	0.227
pFG-gpd 6	140.0	0.209
	141.2	0.211
pFG-gpd 7	138.8	0.207
	139.2	0.208
pFG-gpd 9	128.6	0.192
	148.2	0.221

[a] Assays relate to the duplicate GUS extracts made for each transformant (Section 3.5.4).

[b] Protein concentrations determined by reading from a Bovine Serum Albumin standard curve created from spectrophotometer absorbance readings at 595nm.(Section 2.13.1). The data represents the average of duplicate readings of each GUS extract.

observed that differences between duplicates of the same transformants were as great as differences between the transformants. [The protein concentrations obtained from this experiment were used as standards when determining the relationship between the GUS activity and pFG-gpd copy number (Section 3.5.4).]

3.5.4 Analysis of GUS Expression in pFG-gpd Transformants.

It was important for future studies to known whether GUS activity levels were affected by the site (or sites) of integration and whether these levels were related to copy number. Therefore the GUS activity in the transformants was determined and compared to the number of intact copies of the GUS cassette determined in Section 3.6.1. Any variations in *gusA* gene expression between transformants containing a single transformed gene was also analysed. Assays for total protein performed on the GUS extracts (Section 3.5.3), allowed the specific activity of the GUS enzyme to be determined for protein concentration as well as dry weight.

The results of the GUS assays are shown in Table 11. The rate of the enzyme reactions, and enzyme specific activities were determined. The specific enzyme activity was compared to the number of active copies of the GUS cassette, and these results are also shown in Table 11.

The results of the GUS assays show that untransformed *A. nidulans* has negligible endogenous GUS activity. The rate of enzyme activity was consistent between duplicate samples from the same transformant. The specific activity of the GUS enzyme for 'type 1' or single copy transformants gave an average of 29.4±2.68 nmol MU/min/ng protein. Transformant pFG-gpd 6 containing 2 intact copies of the GUS cassette has a mean GUS specific activity of 64.4 nmol MU/min/ng protein, approximately twice that of the single copy transformants. Transformant pFG-gpd 3 that contains 3 intact copies of the GUS cassette in tandem repeats has a mean GUS specific activity of 119.2 nmol MU/min/ng protein, a rate approximately 3-5 times that of single copy transformants. This transformant has a fourth copy of the GUS cassette that, although not complete, may be active as indicated by the GUS enzyme specific activities per copy of the GUS expression cassette (Table 11; discussed further in Section 4.2.3). Overall, in the

transformants analysed there was a coefficient of linear correlation of 0.97 between GUS enzyme specific activity and the number of intact copies of the GUS cassette determined in Section 3.5.1. These results show that transformed gene copy number is more important than position effects in transformation with the *gusA* gene in *A. nidulans* (discussed further in Section 4.3.4).

Table 11 Specific activities of GUS Enzyme in pFG-gpd transformants showing the correlation with copy number.

pFG-gpd transformants.[a]	No. of intact GUS cassettes[b]	GUS Specific Activity (nmol MU /min/ng dry weight)[c]	GUS Specific Activity (nmol MU /min/ng dry weight) / intact copy[d]	GUS Specific Activity (nmol MU /min/ng protein)[c]	GUS Specific Activity (nmol MU /min/ng protein) / intact copy[d]
Untrans-	0	0.07	N/A[e]	0.35	N/A[e]
formed		-0.08		-0.37	
1	1	4.69	5.1±0.5	26.1	26.1±0.1
		5.43		26.0	
4	1	6.90	5.9±1.4	31.9	29.3±3.7
		4.89		26.7	
5	1	6.19	7.4±1.7	30.5	33.6±4.3
		8.54		36.6	
7	1	6.12	6.2±0.09	28.7	28.9±0.3
		6.24		29.1	
9	1	6.17	6.2±0.01	31.2	29.2±2.8
		6.19		27.2	
6	2	13.95	7.0±0.01	64.7	32.2±0.2
		13.93		64.1	
3	3 - 4[f]	23.92	6.0±0 to 8.0±0	116.4	29.8±1.0 to
		23.92		121.9	39.7±1.2

[a] Duplicate extracts were assayed for each transformant as well as the untransformed control

[b] As determined in Section 3.5.1

[c] Calculations for the determination of GUS specific activities are shown in Section 2.13.2

[d] Duplicate GUS assay specific enzyme activities were averaged to determine the GUS specific activity per intact copy and standard errors are shown.

[e] Not Applicable.

[f] Although there are only 3 intact copies, the 4th disrupted copy may be expressing the GUS gene.

4. DISCUSSION.

4.1 OVERVIEW.

In this thesis a promoter-less GUS expression plasmid, pFunGus, containing a multi-cloning site was constructed. To test it, a promoter containing derivative, pFG-gpd, was also constructed. The plasmid pFG-gpd was integrated into *A. nidulans* via cotransformation, however, attempts to obtain the high cotransformation frequencies reported in literature proved unsuccessful and only very low cotransformation frequencies were obtained. The GUS expression plasmid pNOM102 also cotransformed at a very low frequency, but at a higher frequency than pFG-gpd. The pFG-gpd cotransformants containing between one and four copies of the *gusA* gene gave growth rates similar to that of untransformed *A. nidulans.* GUS expression levels showed a linear correlation with the number of copies (~30nmols MU/min/µg protein/copy of the *gusA* gene).

4.2 EVALUATION OF COTRANSFORMATION.

4.2.1 Possible Reasons for Low Levels of Cotransformation Frequencies of pFG-gpd.

Cotransformation of pFG-gpd (the promoter containing derivative of pFunGus), with pAN8-1, occurred at a frequency of <0.1%. Only eight stable cotransformants were found and these cotransformants were the result of large scale screening of transformants for cotransformation. Initially, it was attempted to increase the cotransformation frequencies by testing the parameters of the transformation system. When this was unsuccessful the transformation method used in Punt *et al.* (1992) was recreated as closely as possible; including using the WT strain of *A. nidulans* rather than R21, changing the media used and the transformation method. However, statistical analysis on the results showed that these changes did not significantly raise the cotransformation frequency (99% confidence; Section 2.14.2). Instead, the eight cotransformants obtained were the consequence of large scale screening of pAN8-1 transformants. [These results can be compared to other studies that show

cotransformation frequencies, in experiments using *gusA* and selecting first for the dominant selectable marker, of above 38%, and generally significantly higher (Roberts *et al.*, 1989; Section 1.4.3)].

There are several stages at which cotransformation may have failed: (i) inactivity of pFG-gpd plasmid through incorrect construction, (ii) the incorrect processing of the *gusA* gene product, (iii) the integration event resulting in gene inactivation, (iv) the compete failure to integrate the cotransforming plasmid or (v) failure of the transformation process. These possibilities are discussed below with comments and experimental evidence pertaining to their relative significance.

Two possible explanations can be easily discarded. A fault in construction of the plasmid pFG-gpd that was not detected in a number of analytical digests. Initially the transient expression evidence, and later the stable transformants obtained, demonstrated that the plasmid contained a fully functional GUS expression cassette. Secondly, when expressing a heterologous prokaryotic gene in a eukaryotic host, incorrect cell processing of the gene product eg. gycosylation, may occur resulting in an inactive product (Saunders *et al.*, 1989). However, this study and previous studies have shown the *gusA* gene product to be active in eukaryotic hosts including *A. nidulans* (Roberts *et al.*, 1989).

The GUS cassette could be inactivated during integration through one of the following mechanisms; 'hot-spot' integration of *gusA* into a point in the genome, a predominance of type III integration events or inactivation of the *gusA* gene by a gene inactivation mechanism of the cell.

Where there is sequence homology within the transformed sequence and the genome, integration will often occur at that site (type I integration). If the *gusA* gene had a degree of homology to a 'spot' in the *A. nidulans* genome, the result may be integration and inactivation of the *gusA* gene. Reported cotransformation frequencies with the *gusA* gene of up to 80% would tend to discard this possibility (Flaherty *et al.*, 1995).

Type III integration results from a double cross-over event between the homologous sequences in the genome and the introduced plasmid (Figure 3). The outcome is sequence replacement, and the resulting transformant would not show GUS expression

because the only integration of plasmid DNA would occur within the *gpdA* promoter. Heterologous gene expression controlled by *gpdA* expression signals has been demonstrated in *A. nidulans* a number of times, and studies have shown a predominance of type II integration events (Punt *et al.*, 1987; Roberts *et al.*, 1989).

Meiotic instability of transformed phenotypes has been observed in Ascomycetes (Section 1.3.5). The genes are inactivated through cell mechanisms, e.g. recombination and methylation. However, spontaneous meiotic divisions are rare and mitotic instability is not commonly observed in *A. nidulans* (Yelton *et al.*, 1985; Turner, 1994).

By following successful, previously described protocols for cotransformation in *A. nidulans*, we can discard all of the previous possible explanations. Integration of transforming DNA has been hypothesised to be the limiting event in transformation (Mönke and Schäfer, 1993; Groteueschen and Metzenberg, 1995). In creating GUS cotransformants, this may be compounded by the fact that *gusA* is selectively negative (ie. there is no advantage to the fungi to integrate the DNA, and there may be negative effect of integration). Consequently, there is no pressure for the integration of the *gusA* DNA to occur. This is unlike dominant or auxotropic selectable markers, in that unless the phenotype conferred by the transforming DNA is present, the fungi are not viable. This was demonstrated in a filamentous fungus transformed with the *gusA* gene linked in the same vector to the dominant selectable marker, hygromycin (Mönke and Schäfer, 1993). GUS positive transformants were obtained when transformants were first selected for hygromycin resistance. However, no stable transformants were obtained when GUS selection was first carried out. A second study by Grotelueschen and Metzenberg (1995), also showed that the competence for transformation is at the level of the nucleus. They hypothesised that because nuclei in a protoplast population divide asynchronously, there is only a narrow window in the nuclear cycle in which the genome is available to be transformed (see Section 1.3.3). Therefore the limiting event in transformation is the availability of suitable chromosome sites for integration. These studies suggest that the most likely explanation for the low cotransformation of the GUS plasmid is that an aspect of this system is preventing integration.

However, literature has also reported successes in cotransformation using the GUS expression plasmid, pNOM102. Therefore, the lack of integration of the GUS

expression plasmids may be the result of a technical failure. For example, scoring of non-transformants as transformants, or poor DNA quality.

Low transformation frequencies can be obscured by scoring non-transformants as transformants. In cotransformation experiments this would result in misleading, low cotransformation frequencies. Transformation frequencies of pAN8-1 into *A. nidulans* WT were between 3-250 transformants/μg pAN8-1 in this study. Some of this variation can be attributed to protoplast viability; the frequencies compared to those in other studies which reported transformation frequencies of up to 300 transformants/μg DNA (Yelton *et al.*, 1984). Investigations into the identification of abortive transformants allowed these to be recognised, and not scored as transformants (Section 3.4.2.1). Also colonies on negative control plates (untransformed protoplasts plated onto phleomycin containing plates) established a background of phleomycin resistant colonies of less than 1.5 per μg of pAN8-1. Therefore because the transformation frequencies were as expected, and abortive transformants and phleomycin resistant colonies were factored in, it can be accepted that the colonies scored for cotransformation were pAN8-1 transformants.

Another explanation is that differences in the DNA quality were a factor in preventing cotransformation. This has been shown to be a parameter affecting frequencies of transformation (Fincham, 1989). This was investigated by using both PEG purified DNA and DNA purified on a CsCl gradient; then assessed for its purity by OD_{260}/OD_{280} ratio readings in the spectrophotometer. Also, all transforming plasmids (i.e. pAN8-1, pFG-gpd and pNOM102) were prepared at the same time from the same *E. coli* host strain (initially DHI and then XL1; Section 2.1). No significant differences were observed between the two preparations of DNA in either the transformation or cotransformation frequencies.

In conclusion the very low cotransformation frequencies, in direct contradiction to those reported in the literature, cannot be easily explained. As discussed above, this is hypothesised to be due to failure of the GUS expression plasmid to integrate into the genome because of a technical problem with the cotransformation system that could not be determined in the course of this study.

4.2.2 Possible Explanations for Higher Cotransformation Frequencies Observed with pNOM102 than with pFG-gpd.

Analysis of all cotransformation experiments with pFG-gpd and pNOM102 showed that the cotransformation frequencies of pNOM102 were significantly higher (a mean of 2.2%) than those for pFG-gpd (a mean of 0.45%) with 99% confidence (shown by the z-test; Section 2.14.2). For cotransformations containing pNOM102 mixed with pFG-gpd, cotransformation frequencies were also significantly lower than for pNOM102 alone (a mean of <1.4%) with 99% confidence (shown by the z-test; Section 2.14.2). Therefore we can hypothesise that a characteristic of plasmid, pFG-gpd, contributes to the lower cotransformation frequencies. Also, when combined in a cotransformation with pNOM102, pFG-gpd causes inhibition of the pNOM102 cotransformation process.

Characteristics of the pFG-gpd plasmid that could contribute to its' lower cotransformation frequencies could be either the DNA quality of pFG-gpd or the sequence differences between pFG-gpd and pNOM102.

As discussed previously (Section 4.2.1) all plasmid DNA was prepared to be of equal quality (determined by OD_{260}/OD_{280} ratio readings measured in the spectrophotometer; Section 2.6.1).

The sequence differences between pFG-gpd and pNOM102 are minor but may be significant. The plasmids, pNOM102 and pAN8-1, are based on the same plasmid, pAN52-1 (which in turn is based on pUC118). Therefore 76% of pNOM102 is homologous with pAN8-1. The plasmid pFG-gpd is based on a different backbone plasmid, pGEM-1, therefore, the percentage of pFG-gpd similar to pAN8-1 is lower (62%), with the identical sequences split by two non-homologous regions. These differences may result in pNOM102 and pAN8-1 forming a cointegrate at a higher frequency than pFG-gpd and pAN8-1. A cointegrate is formed by the covalent linkage of the cotransforming plasmids, through recombination of homologous sequences during the process of transformation. The two plasmids then integrate as one. If, contrary to prior reports (Wernars *et al.*, 1987), only single or low copy integrations are possible, there would be a higher possibility of pNOM102 being carried into the genome with pAN8-1 than pFG-gpd.

Southern hybridisation analysis of pFG-gpd cotransformants showed that only two out of the eight transformants could have been created by integration of a pFG-gpd-pAN8-1 cointegrate. This is because *Hin*dIII cuts pAN8-1 and thus if a cointegrate was formed we would expect to see a band of 7.7 kb when digested with *Hin*dIII, as in the cases of transformants pFG-gpd 3 and 9. Detailed analysis of these and pNOM102 transformants would be required to test this hypothesis.

In conclusion, pFG-gpd cotransforms poorly in this system. This may be due to technical problems with this cotransformation system, shown by low cotransformation frequencies of pNOM102 and pFG-gpd (Section 4.2.1). The differences between pNOM102 and pFG-gpd may also result in lower cotransformation of pFG-gpd, when compared to the GUS expression plasmid pNOM102 [within this system].

4.3 EVALUATION OF INTEGRATION EVENTS IN TRANSFORMANTS.

4.3.1 A Predominance of Single Copy Integrations with Intact GUS Expression Cassettes Observed in pFG-gpd Transformants.

Analysis of the eight pFG-gpd transformants by Southern hybridisation (Section 3.5.1) revealed three classes of transformation events: single copy integration (6 transformants), multi-copy integration as tandem repeats at the same site (1 transformant), and multi-copy integration at separate sites (1 transformant). In each of the two multi-copy transformants, one GUS cassette out of three or four cassettes was disrupted during the process of integration. Transformant 1, a single copy transformant, produced a 5.5kb band whether digested with *Hin*dIII, or *Hin*dIII and *Eco*RI, therefore containing a disrupted GUS expression cassette. Hence, out of a total number of thirteen integration events, three resulted in disrupted GUS cassettes. This demonstrates that it can not be assumed that all integration events result in intact copies of the expression cassette, however as will be discussed (Section 4.3.4), neither can it be assumed that these disrupted copies do not express the *gusA* gene. Another transformant, pFG-gpd 2 initially displayed the GUS positive phenotype but later lost this activity. Southern hybridisation analysis showed the absence of integrating DNA. Providing that this is not the result of switching of stocks, this implies that gene inactivation mechanisms, such as methylation, do not play a role in transformed

phenotype instability. Finally, transformant 9 gives a 7.7 kbband the size of the pFG-gpd plasmid upon digestion with *Hin*dIII only. This cannot be due to integration as tandem repeats, as a second band corresponding to the flanking pFG-gpd DNA was not observed. An explanation for this is the integration of pFG-gpd as a cointegrate with pAN8-1 (Section 4.2.2), thus *Hin*dIII cleaves once within pFG-gpd, and once within pAN8-1, yielding the 7.7 kb fragment that was detected when probed with the *gusA* gene.

These results show that pFG-gpd seems to predominantly integrate the GUS expression cassette intact, in single copy integration events. Overall, this demonstrates the possibility of creating and evaluating transformants with a variety of different copies of the pFG-gpd vector.

4.3.2 Analysis of Types Integration Events Shows a Predominance of Type II (Ectopic) Integration Events.

The results of the Southern hybridisation analysis enable the transformants to be described in terms of the types of integration, as described by Hinnen *et al.* (1978; Section 1.3.4). Type III integration, or gene replacements, would not display GUS expression, and are therefore not selected for in these experiments. Type I integration, or homologous integration into the *gpdA* promoter, seemed to be a rare event as the length of flanking DNA was not identical in any of the eight transformants. Therefore it was assumed that the most prominent type of integration was type II, or ectopic integration. This is reinforced by relevant studies that show almost exclusively type II transformants when *A. nidulans* was transformed with the dominant selectable markers *hph* (hygromycin resistance; Punt *et al.*, 1987; Cullen *et al.*, 1987) or *Bm* (bleomycin resistance; Van Engenelenburg *et al.*, 1989), fused to either *trpC* or *gpdA* expression signals. Therefore, pFG-gpd does not appear to target integration to specific sites in the genome. The significance of this is discussed in Sections 4.3.4 and 4.4.

4.3.3 Physiological Response of *A. nidulans* to the Presence of pFunGus Derivatives.

Gene expression studies become more credible the closer the system reflects that of the wild type organism. This study showed that the physiology of *A. nidulans* transformed with pFG-gpd was not significantly compromised when it was compared to untransformed *A. nidulans*. The physiological indicators that showed no difference were: (i) the rate of growth, (ii) the total yield of mycelia, (iii) the length of the lag phase of growth, and (iv) the total protein content. Differences were not observed regardless of the number of copies integrated (up to 4 copies), and the number of integration sites (up to 3 events in the genome). Therefore, pFunGus was shown to be a good candidate for studying gene expression without significantly effecting the biology of transformed fungus.

4.3.4 Correlation Between GUS Expression Levels and Transformed Gene Copy Number.

For the study of gene expression, as well as for the commercial production of gene products, it is ideal to have the ability to customise the levels of transformed gene expression without the added complication of position effects (Section 1.3.6). When transforming with a gene, several factors contribute to the level of expression including; the 5′ and 3′ sequences used, the position the sequence is integrated into the genome and the number of copies of the sequence in the genome. In these experiments, the 5′ and 3′ sequences were constant, therefore the important factors to be considered were position effects and copy number (position effects are described in detail in Section 1.3.6). In the analysis of the transformants GUS expression levels, the relative importance of position and copy number were determined (Section 3.5.4).

The *gusA* gene expression was quantified for each of the transformants. The experiments showed that the transformants have high levels of expression of the *gusA* gene, compared to the expected (Roberts *et al.*, 1989) negligible background expression seen in non-transformed *A. nidulans*. They also exhibited replication of GUS expression levels, between transformants with a single copy of the gene. The rate of *gusA* gene expression increased linearly with copy number up to three or four copies

(the maximum number tested in this study). This relationship predicted that although transformant 3 appeared to have three intact copies and one disrupted copy of the GUS cassette, this disrupted copy was also expressing the *gusA* gene. A second transformant, pFG-gpd 1, also contained a disrupted GUS expression cassette, however, the GUS activity was retained and was not significantly lower than the other single copy transformants (Section 3.5.4). These disrupted cassettes were missing the *Eco*RI site at the 5′ terminal end of the cassette. The essential promoter elements for transcription of *gpd* are at positions, -30 nucleotides (nt), -250nt, and -650nt from the transcription start site. The essential elements may have been retained in the disrupted GUS cassettes of transformants pFG-gpd 1 and pFG-gpd 3, as the *Eco*RI site is at the approximate position -2200nt (Punt *et al.*, 1990). This shows that recombination and rearrangements can occur within the integrating DNA without effecting the gene activity. Therefore, although it can not be assumed that all copies are actively integrated, neither can it be assumed that these disrupted copies are inactive.

The evidence for the apparent absence of position effects in the determination of levels of transformed *gusA* expression has been described above. Firstly that GUS expression increases linearly with copy number (up to 4 copies), and secondly that all the single copy transformants show very little variation in GUS expression between each transformant. Most of the studies in literature back up this finding (Section 1.3.6), as position effects seem to play an increasingly higher importance as the complexity of the genome and the amount of repetitive DNA increases. Ascomycetes have a relatively simple genome organisation therefore this result is consistent with the previous findings.

There is a second explanation for the apparent absence of position effects. This is the targeting of transforming DNA into a specific area of the genome that is transcriptionally active. As discussed previously (Section 4.3.2), homologous (type I) integration into the *gpdA* promoter does not appear to be occurring, however the DNA could still be integrating into a specific part of the genome. If the transformed DNA is integrating into the same general area it would have similar activity. It has been previously hypothesised that ectopic integration is not completely random, and this is one theory for the determination of nuclear competence (Section 1.3.3). To resolve this possibility, further investigations could be carried out involving CHEF gel analysis to

determine whether copies are integrated onto the same or different chromosomes followed by restriction enzyme analysis to determine the general area on the chromosome where integration had occurred.

These results demonstrate that transformed copy number is directly proportional to GUS expression levels. This is partly due to the absence of position effects when transforming the vector pFG-gpd. Therefore, copy number can be estimated from the GUS expression levels which is a technical advantage when screening transformants, or inversely, copy number can be used to tailor the amount of gene product that is required for a particular application.

4.4 SUMMARY AND FUTURE PROSPECTS.

The results presented show the potential of the plasmid, pFunGus, for the study of gene expression in *A. nidulans* using the *gusA* reporter gene. The expression of the *gusA* gene product will usually reflect the expression of the gene that the promoter is fused to the *gusA* gene originates from. However, if the regulation of this gene is at a level other than transcription, this may not occur. Fortunately, for the use of reporter genes in fungi the predominate regulation is at the level of transcription (Caddick and Turner, 1993).

The plasmid, pFunGus, constructed in this study was designed with a small size and a multi-cloning site to enable flexibility when inserting different regulatory elements. The system studied could be used for the investigation of many different cell processes as there were no apparent physiological effects of the transformed vector on *A. nidulans*. Position effects were not evident in this system, therefore levels of transformed gene expression are predicable. Gene copy numbers would still need to be determined if quantitative measurements were to be made between transformants. For example, if regulatory elements from a single promoter were to be analysed for their quantitative contribution to gene expression. Although position effects were not observed in the 8 transformants tested in this study, they cannot be ruled out. Therefore, type I transformants would be the most desirable as the position in genome would always be constant and thus variation in gene expression level attributable to the position in the genome would be completely avoided.

If this system was to be used, the frequency of obtaining *gusA* transformants would have to increase as it is impractical to have to screen thousands of transformants. The simplest way to achieve this would be by covalently linking the phleomycin and GUS cassettes. In bacteria, transformation frequencies have been shown to be inversely proportional to the size of the transforming DNA (Ausubel *et al.*, 1994). However, this and previous studies have shown DNA integration, rather than uptake, to be the limiting event in fungal transformation. Therefore, we would expect a large increase in the frequency of *gusA* transformants.

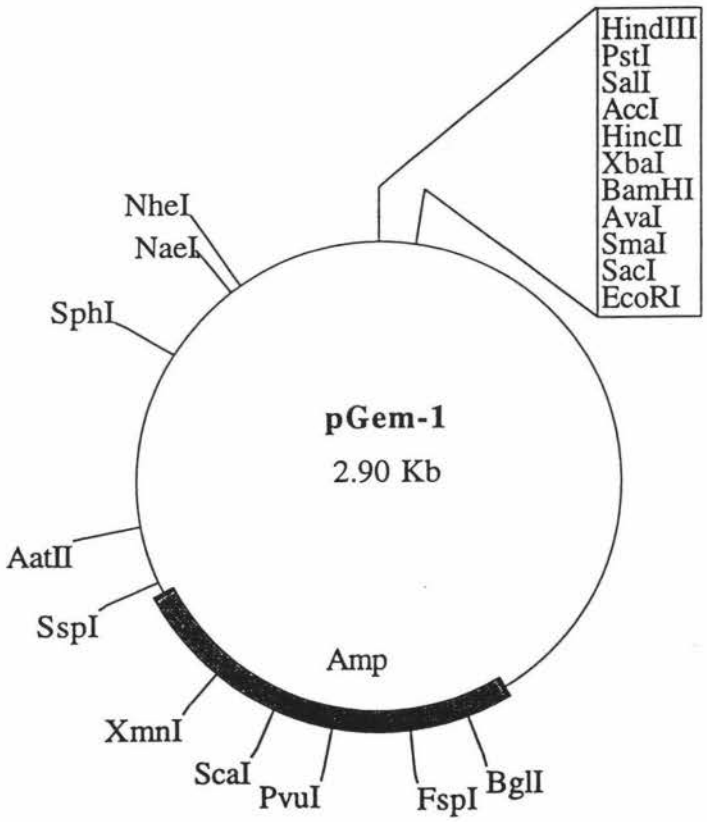
Also demonstrated in this study was the potential use of transient expression studies as a rapid method for the evaluation and possible quantification of GUS expression. This avoids the need for a fungal selectable marker, and also negates possible problems associated with cotransformation, position effects or gene copy number.

This system may be used for quantitative studies of promoters and promoter elements. The presence of GUS expression may be used to observe metabolic processes *in situ* by fluorescence microscopy. Insertion of inducible elements would allow the induction conditions to be studied, which could be applied in industry to maximise yields. Transitional fusions of secretion signals to the *gusA* gene could allow the study of the secretion of products.

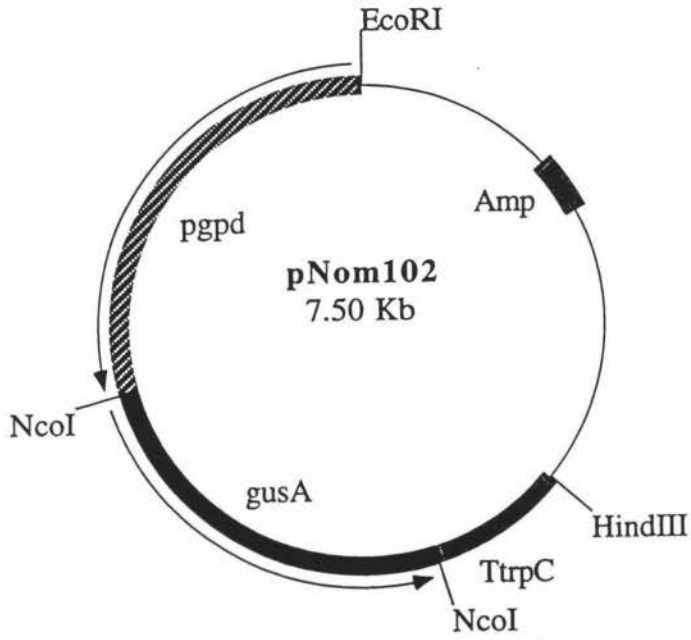
Reporter gene studies using the *gusA* gene are not limited to *A. nidulans*. The *gusA* gene has already been used to study economically important and scientifically interesting processes in other fungi. Examples of processes that could be studied in the future are: those involved in growth and cell wall synthesis, lytic enzymes, regulatory genes (Davies and Hynes, 1989), proteases (Thompson, 1991), developmental genes (Timberlake, 1990), and industrial metabolite production.

APPENDIX 1 Plasmids Maps

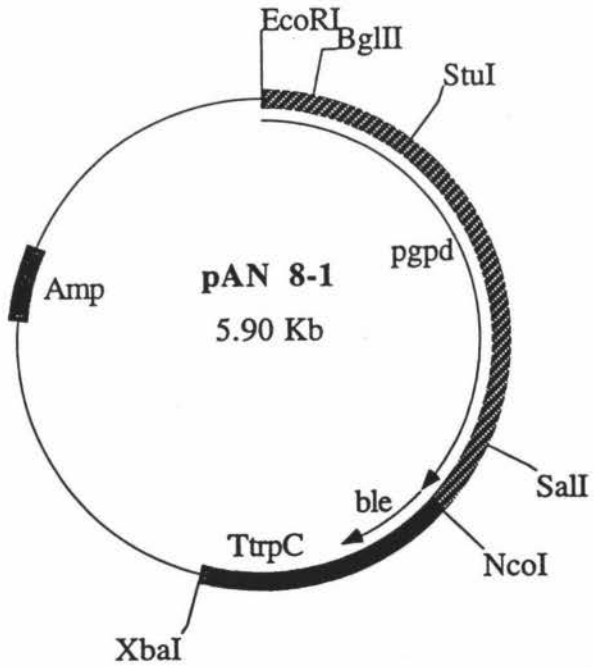
Restriction map of pGEM-1 showing sites for: *Hin*dIII, *Pst*I, *Sal*I, *Acc*I, *Hinc*II, *Xba*I, *Bam*HI, *Ava*I, *Sma*I, *Sac*I, *Eco*RI, *Bgl*I, *Fsp*I, *Pvu*I, *Sca*I, *Xmn*I, *Ssp*I, *Aat*II, *Sph*I, *Nae*I, and *Nhe*I.



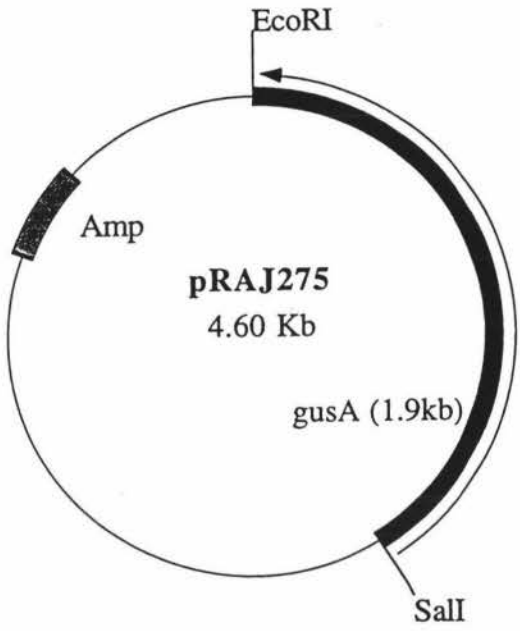
Restriction map of pNOM102 showing sites for: *Eco*RI, *Hin*dIII, and *Nco*I.



Restriction map of pAN8-1 showing sites for: *Eco*RI, *Bgl*II, *Stu*I, *Sal*I, *Nco*I and *Xba*I.



Restriction map of pRAJ275 showing sites for: *Eco*RI and *Sal*I.



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