Chapter 1

INTRODUCTION

1.1. General Introduction

For many centuries, humans have been using fermented food and beverages. The knowledge about fermentation by bacteria and fungi in the recent century, together with the increasing interest in more production at a cheaper expense, has opened a new prospect in the science of biotechnology. Nowadays modern industrial fermentation with genetically improved micro-organisms yields in production of many useful substances such as primary and secondary metabolites and also enzymes at a relatively cheap price. The commercial use of several fungal and bacterial species is not limited to only fermentation. Using improved micro-organisms, now, humans are able to produce a range of products with medical, agricultural and nutritional applications. For instance, a number of *Aspergilli* are widely used in production of organic acids, amino acids, single-cell proteins (used in human nutrition), lipids and several extracellular enzymes (Bigelis, 1985).

Undoubtedly a comprehensive knowledge of the biology, the biochemistry of metabolic pathways and also the molecular biology of these organisms is of a fundamental importance. However, it is not possible to study

every individual species and therefore, it is desirable to establish proper models for major biological phenomena in a way that they can explain homologous phenomena in other related organisms.

Accordingly, certain fungal species including *Saccharomyces cerevisiae*, *Neurospora crassa* and *Aspergillus nidulans* have been found as convenient representatives in basic genetics and molecular biology of either fungal or eukaryotic systems.

Although *A. nidulans* has not been significantly used for commercial purposes, it has a number of characteristics which are appealing to employ it as a model organism. Knowledge and methodologies in formal genetics and molecular biology in such a model then, may have at least three major benefits:

- contribution to the whole body of pure sciences,
- applications in improvement of other industrial species,
- extension to other homologous/analogous systems in other eukaryotes.

In this project, the secretion of extracellular proteases in *A. nidulans* was used to study a model fungal regulatory system for gene expression.

A putative regulatory mutation affecting the extracellular protease production, at the first step, was genetically characterised. Then, its functional interactions with some other regulatory mutations were investigated. Finally, to facilitate its characterisation at the molecular level, a 35-50 kb DNA fragment of the wild-type genomic DNA carrying the corresponding gene was isolated.

1.2. *Aspergillus nidulans*

A number of characteristics in *A. nidulans* are of special interest in genetic research (Arst and Scazzocchio, 1985):

a) *A. nidulans* is a homothallic fungus. That is, due to the ability to self-fertilise, the concept of mating types is irrelevant and the individual can undergo karyogamy. A great advantage is the possibility of crossing any pair of strains (section 1.2.1).

b) The parasexual cycle which (fig. 1.1) allows easy mapping of new mutations to chromosomes. According to Fincham *et al.* (1979), the parasexual cycle is very rare and is an alternative way to the sexual reproduction. In nature, it consists of three stages: fusion of haploid nuclei, mitotic cross-over and haploidisation. In the laboratory, however, diploid strains are readily obtained and by subjecting diploid individuals to certain chemicals such as benomyl, haploidisation is induced (Clutterbuck, 1974). Benomyl interrupts the function of spindle and impairs the normal segregation of chromosomes (section 1.2.1).

c) Haploid, diploid and heterokaryon states are all possible. Haploidy is especially valuable in the study of mutations as recessive mutations are easily scored. Diploids and heterokaryons are of importance in mitochondrial genetics, the study of the cytoplasm's effect on expression of nuclear genes and the study of allelic interactions in complementation tests (section 1.2.1).

d) *A. nidulans* grows on simple-defined media and utilises a variety of nutrients. Therefore, handling the organism and studying metabolic mutations are facilitated (section 1.2.2).

e) Nutritional screening is readily available to investigate the control of gene expression (section 1.2.2).

1.2.1. Biology of *A. nidulans*

1.2.1.1. Systematics and Description

A. nidulans belongs to the genus *Aspergillus*, order Plectomycetes, sub-class Euascomycetes of class Ascomycetes in the phylum Fungi (Fincham *et al.*, 1979). The ascospore, *i.e.*, the product of meiosis is surrounded in a special sporangium called an ascus. Asci are formed in the fruit body or cleistothecium and are released subsequent to its rupture.

According to Fincham *et al.* (1979), *A. nidulans* is a mycelial ascomycete and grows as branching septate filaments called hyphae. In each compartment (hyphal cell) there are a number of haploid nuclei. Due to a central pore in each septa, cytoplasm is continuous, therefore the term coenocytic is applied in reference to the cell organisation.

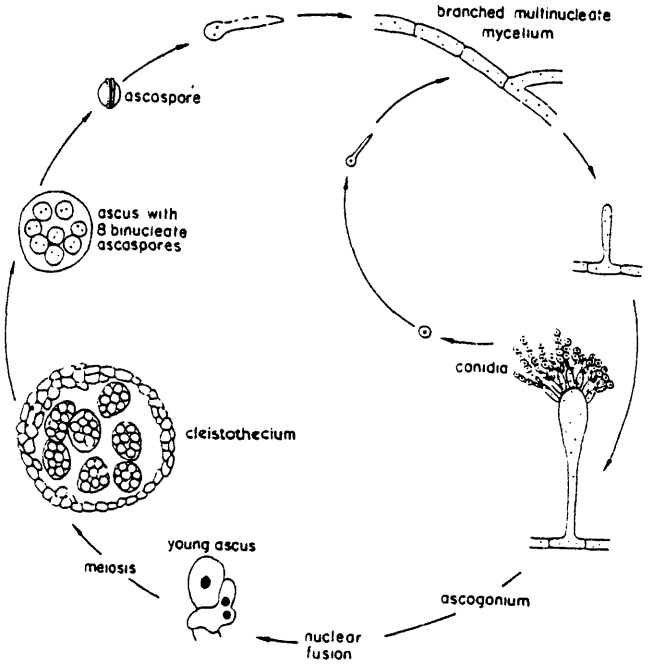

Figure 1.1. The life cycle of *A. nidulans*.
(after Fincham *et al.*, 1979)

There are eight chromosomes present in each haploid nucleus carrying the genetic information. Segregation of alleles undergoes the normal principles of

Mendelian inheritance. Meiotic crossing-over normally happens when the diploid nuclei develop haploid spores in sexual life-cycle.

1.2.1.2 Asexual Development

In asexual development, according to Smith *et al.* (1977), the first step is transformation of a hyphal cell into a foot cell which bears conidiophore. The latter has a globose multinucleate vesicle at the end where primary finger-like sterigmata and then secondary sterigmata protrude. Each secondary sterigmata is uninucleate and buds off a chain of uninucleate conidia (fig. 1.1). In wild-type strains conidia are green to dull-grey-green in colour. Each conidium is capable of developing a new colony under appropriate circumstances.

Another form of asexual development is hyphal fusion. Fincham *et al.* (1979) stated that fusion can be forced leading to formation of heterokaryons (n+n) in order to get new combinations for the genes under study. Induction occurs as forcing hyphae to grow very close elevates the probability of hyphal fusion. According to Cove (1977), nuclear fusion leading to formation of diploid (2n) mycelia is likely to occur after the cell fusion. Diploid nuclei can then, undergo conidiation (2n conidia).

1.2.1.3. Sexual Reproduction

In contrast to asexual reproduction, fusion of two gamete nuclei is required for the sexual development. As *A. nidulans* is homothallic, nuclei from the same individual can fuse. Meiosis results in development of four spores which increase in number to eight with a further mitotic division. Every group of eight spores is surrounded by the ascus in the cleistothecium. Fincham *et al.* (1979) claimed that a mature cleistothecium contains up to 100 000 asci. Figure 1.2 summarises the complete life cycle in *A. nidulans*.

Given sexual and parasexual life-cycles, *A. nidulans* appears as a versatile tool for genetic studies such as complementation tests (allelic interactions) and haploidisation studies (mapping mutations to chromosomes):

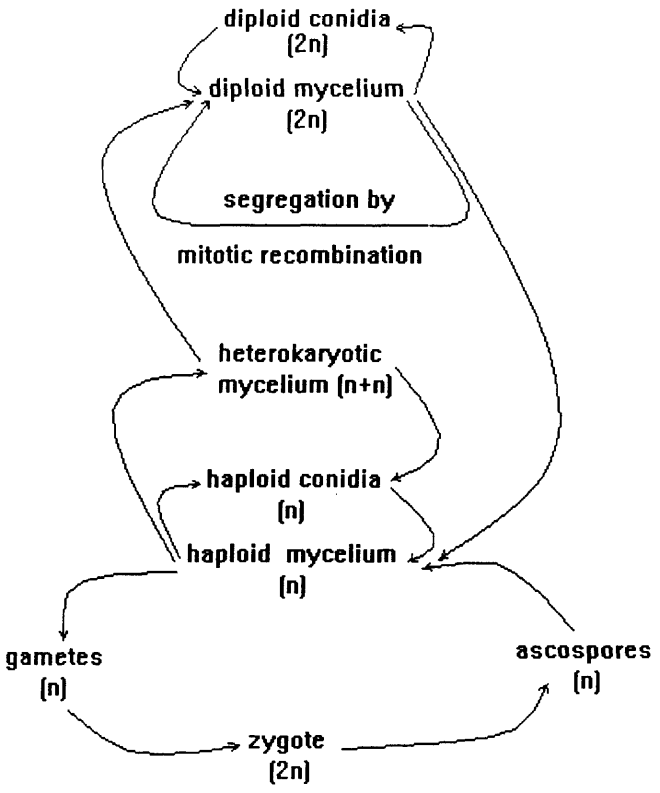

Figure 1.2. Haploidy/diploidy in *A. nidulans*.
(after Cove, 1977)

Complementation Tests

Complementation tests are used whenever it is suspected that two mutations are alleles of the same gene. These studies are applicable if mutations under study have clear functional deficiencies compared to the wild-type strain Fincham *et al.* (1979). Since most laboratory strains of *A. nidulans* are haploid, recessive mutations have the opportunity to express themselves. In heterokaryons and diploids where two sets of each chromosome are present, the expression of mutated/wild-type phenotypes is used to study the allelic interactions. If wild-type phenotypes for both mutations are observed, two mutations are in two different loci as each of them is complemented by the respective wild-type allele. In contrast, observation of a mutated phenotype means that two mutations under study are alleles of the same gene.

Haploidisation

Fusion of two haploid nuclei in a hyphal cell, normally is a rare phenomenon and results in a diploid nucleus. A diploid strain, then, may either reproduce as a diploid individual or develop haploidy. Käfer has made extensive studies on mechanisms leading to haploidisation (Käfer, 1960, 1961 and 1975).

Haploidisation is the basis for mutation mapping and results in aneuploidy. Non-disjunction segregation of chromosomes during mitosis, according to Käfer (1975), occurs at a relatively high rate (1%). As a result, aneuploids [hyper-haploids (n+x) and hyper-diploids (2n+x); x ranging between 1 and n-1] are produced.

Käfer (1960) stated that aneuploids appear unstable in respect to markers used in the study. When conidial head from an unstable colony is transferred onto a selection plate, it develops only haploid sectors. She furtherly concluded that haploidisation is brought up through several steps where more and more chromosomes are lost until stable haploid is formed.

Käfer (1960) also showed that aneuploidy in *A. nidulans*, similar to that of *Penicillium chrysogenum*, could be induced by physical (*e.g.* X-rays) and chemical (*e.g.* nitrogen mustard) stimulants. However, other chemicals like *p*-fluorophenylalanine (Fincham *et al.*, 1979) and benomyl (Clutterbuck, 1974) are also recommended.

By forcing haploid strains to form diploids and then inducing the haploidisation different genetic combinations are obtained. Using a mapping strain with appropriate (necessarily recessive) markers on each chromosome, the pattern of combination between the mutation under study and given markers can help to figure out the chromosome carrying the mutation. Since mitotic crossing-over does not occur during haploidisation, 100% association between the presence of the mutant phenotype and the presence of the wild-type phenotype for any one of markers indicates that the same chromosome carrying the marker also carries the wild-type allele of the mutation under study. At the next stage, sexual crosses using recombinaion frequency analysis might be applied to localise the mutated gene on the chromosome.

1.2.2. Molecular Biology in *A. nidulans*

During past few decades, a number of aspects of the genetics and physiology of *A. nidulans* have been studied. According to Turner and Ballance (1985), these mostly cover areas in regulation of carbon and nitrogen source utilisation, mitochondrial genetics, mitosis and differentiation.

A basic understanding of gene expression and regulation at the molecular level is one of the latest aspects of interest in fungal studies. During 1980's with development of techniques for gene isolation and analysis, a progressive improvement was attained in this field. In order to develop an independent and efficient system, different approaches have been made for cloning genes and transformation in *A. nidulans*. The following is a brief review of some typical approaches.

1.2.2.1. Cloning Genes

No major progress in this field was achieved until the early 1980's due to the lack of an efficient transformation system in this organism (Green and Scazzocchio,1985). Fincham (1989) and Timberlake (1991) noted works of Tilburn *et al.* (1983) and Yelton *et al.* (1984) as the most important contributions in the establishment of a reliable transformation system in *A. nidulans*. Since then, different strategies have been employed in cloning and analysis of genes. Table 1.1 summarises a number of typical examples.

1.2.2.2. Transformation

The wider application of transformation in fungi not only has lead to more information about gene expression and regulation, but also according to Timberlake (1991), now we have enough observations to make generalisations about the fate of transforming DNA. This can be used to develop new strategies for manipulation of fungal genome for scientific and commercial purposes.

Table 1.1. Cloning strategies in *A. nidulans*.

gene	function	cloning strategy
trpC[1]	*trpC* codes for a trifunctional polypeptide which together with the product of *trpA* are involved in biosynthesis of tryptophan.	**complementation in *E. coli***: λ library of wild-type *A. nidulans* was used to complement *trpC*⁻ strain of *E. coli*.
amdS[2]	*amdS* codes for an acetamidase enzyme.	**differential screening**: labelled cDNA from a high-level-producing diploid strain and a non-producing strain of *A. nidulans* were used to screen a wild-type λ-genomic library
alcAalcR-cluster[3]	the cluster includes the genes required for utilisation of ethanol.	**brute force***: clones from λ genomic library were used one by one to probe digested genomic DNAs from wild type and mutated strains carrying detectable chromosomal aberrations in Southern transfers. Clone detecting a different pattern in two strains carried the gene.
yA[4]	*yA* codes for a developmentally regulated enzyme which causes the green colour of conidia.	**complementation in *A. nidulans***: a cosmid containing both *E. coli* and *A. nidulans* selectable markers was used for construction of wild-type genomic library. This was used to transform *yA*⁻ strain to wild type.
brlA[5]	*brlA* is a developmental gene involved in conidiation	**complementation in *A. nidulans***: a plasmid library of wild-type DNA was used to transform *brlA42* strain to wild type.
areA[6]	*areA* codes for an activator protein and mediates nitrogen metabolite repression.	**Screening**: λ clones of the genomic library were probed with the total labelled genomic DNA from the wild type strain and an *xprD-1*-derived duplication-deficiency strain. Clones hybridising with wild type but not with the other carried the gene.
pkiA[7]	*pkiA* codes for the pyruvate kinase enzyme	**heterologous hybridisation and complementation in *A. nidulans***: DNA fragments hybridising exclusively to the corresponding gene in yeast was cloned in pBR322 and used to transform *pkiA*⁻ strain to wild type.

1 Yelton *et al.* (1983)
2 Hynes *et al.* (1983)
3 Green and Scazzocchio (1985)
4 Yelton *et al.* (1985)
5 Johnstone *et al.* (1985)
6 Caddick *et al.* (1986b)
7 de Graaff *et al.* (1988)

* the same method was used by Hull *et al.* (1989) to clone *prnADBC* cluster

As Timberlake (1991) reviewed, different vectors are available for fungal transformation. Since *E. coli* is usually used for propagation of the recombinant DNA, vectors normally carry bacterial sequences for selection and propagation in *E. coli*. Also it is desirable to have sequences in the vectors which can be used for selection of fungal transformants. In practice whenever the selectable marker is not available, co-transformation with another recombinant DNA containing a selectable marker is necessary.

According to Timberlake (1991), selectable markers fall into three categories:

- markers which complement pre-existing mutations such as auxotrophic markers;
- dominant or semi-dominant genes that confer a new function or lead to drug resistance which are called as drug resistance or added-function markers;
- sequences that cause selectable mutations after integration into the genome of recipient cell.

In *A. nidulans* a number of genes have been used as markers: *amdS* (Tilburn *et al.*, 1983 an Wernars *et al.*, 1987), *trpC* (Yelton *et al.*, 1984 and Wernars *et al.*, 1987), *argB* (Johnstone *et al.*, 1985 and Richey *et al.*, 1989) and *prn* genes (Hull *et al.*, 1989). As an example for the second category $BenA^R$ confers resistance against benomyl which normally disrupts the function of micro-tubules (Dunne and Oakley, 1988, cited in Timberlake, 1991). Disruption of the *yA* and *wA* genes required for development of conidia has been used as visual markers (Mayorga and Timberlake, 1990).

1.2.2.2.1. Procedures in Transformation of *A. nidulans*

Although different protocols are used for this purpose (table 1.2), transformation consists of three major steps. Different protocols mostly vary in details in one or more of these steps. Description of Timberlake and Marshall (1989) of transformation events is as follows:

a) **Preparation of protoplasts**

Cell walls are removed from conidia or hyphal cells with the aid of cell wall-degrading enzymes. This results in formation of osmotically-sensitive cells known as protoplasts. The most widely used substance for perforation or digestion of cell walls is a commercial product, Novozyme 234, which is a mixture of hydrolytic enzymes notably 1,3-glucanases and chitinase. These are secreted by the filamentous fungi *Trichoderma harzianum* (Timberlake and Marshall, 1989) and *Trichoderma viride* (Fincham, 1989). Preparation of

protoplasts must be supported by an osmotic stabiliser. In *A. nidulans* KCl (0.6-0.7 M) and $MgSO_4$ (1.2 M) are used exclusively (Fincham, 1989).

Table 1.2. Transformation in *A. nidulans*

method	protoplast prep.	DNA uptake*	regeneration*
1	Novozyme 234 + helicase in 1.2 M $MgSO_4$ buffered with 10 mM NaOP	buffer: 1.2 M (1 M) sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl incubation: 1. DNA + protoplasts 20 min. RT† 2. 10 vol. PEG[a] 20 min. RT	centrifugation then resuspended in the same buffer then transferred to ANM+1 M sucrose
2	Novozyme 234 + β-glucoronidase in 1.2 M $MgSO_4$ + 10 mM NaOP	buffer: 1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl incubation: 1. DNA + protoplasts 20 min. RT 2. 3.88 vol. PEG[b] 20 min. RT	1. centrifugation then resuspended in 0.5% yeast extract, 2% D-glucose, 1.2 M sorbitol 2. centrifugation then resuspended in the same buffer then transferred to 1.2 M sorbitol + 1.5% agar
3	Novozyme 234 in 0.6 M KCl	buffer: 0.6 M KCl, 50 mM $CaCl_2$ incubation: 1. DNA + protoplasts + 0.25 vol. PEG 20 min. on ice 2. 10 vol. PEG[c] 20 min. RT	20 vol. of the same buffer then transferred to 2% agar medium
4	not mentioned	buffer: 1 M sorbitol, 50 mM $CaCl_2$ incubation: 1. DNA + protoplasts + 0.25 vol. PEG 20 min. on ice 2. 10 vol. PEG[c] 5 min. RT	20 vol. of the same buffer then transferred to osmotically stabilised medium (1.3% agar)

1 Tilburn *et al.* (1983), *amdS*, and Johnstone *et al.* (1985) *brlA* (but PEG similar to *b*)
2 Yelton *et al.* (1984), *trpC* plasmid, and Yelton *et al.* (1985) *trpC* cosmid
3 Turner and Ballance (1985), *pyrG*
4 Wernars *et al.* (1987), co-transformations

* the term vol. refers to the initial volume of buffer used for DNA uptake
† RT refers to the room temperature
a 25% polyethylene glycol 4000, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH7.5
b 60% polyethylene glycol 4000, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH7.5
c 25% polyethylene glycol 6000, 50 mM $CaCl_2$, 10 mM Tris-HCl, pH7.5

b) **DNA uptake**

When cell walls are removed or perforated, transforming DNA(s) is added in the presence of Ca^{2+}. Then with addition of polyethylene glycol (PEG) protoplasts are forced to fuse. According to Fincham (1989), either linear or circular double-stranded DNA can be used for transformation.

Most of protocols ask for application of a high concentration of PEG after a certain incubation period (table 1.3). This presumably facilitates trapping DNA by treated cells as it forces them to clump. For more information about a modified transformation method based on that of Yelton *et al.* (1984) please see the section co-transformation in Chapter 2 "*Materials and Methods*".

Electroporation has been used, as an alternative way, for introducing new DNA to protoplasts (Richey *et al.*, 1989). Nevertheless, this method did not appear to be very efficient as the transformation frequency did not exceed 8.4 transformants per 1 μg of DNA whereas in some conventional transformation experiments the frequency of 500 transformant/μg has been reported (Johnstone *et al.*, 1985).

c) **Regeneration of transformed protoplasts**

After DNA uptake, fused protoplasts are plated on an osmotically-balanced medium which is selective for the marker on the introduced DNA. Therefore, the new DNA allows transformants to grow and regenerate on the plate.

1.2.2.3. The Fate of Transforming DNA

Before the work of Gems *et al.* (1991), no *A. nidulans* autonomously replicating vectors were used in transformation studies. Therefore, transforming DNAs could not be perpetuated extrachromosomally. In a review, Timberlake and Marshall (1989) confirmed that in filamentous fungi it is very hard to maintain the new DNA in the extrachromosomal state. Addition of

centromeres to the vector might help prevent integration if it increases mitotic stability. However, there had been no report on doing this in filamentous fungi.

Transforming DNA can integrate into the recipient cell's genome at either homologous or heterologous sites. In *A. nidulans* this is shown by Tilburn *et al.* (1983) and Yelton *et al.* (1985). The linkage or non-linkage of integrated DNA to the homologous chromosomal locus can be checked by outcrossing the transformed strain. Absence or very low frequency of segregants with the mutated phenotype confirms the homologous integration event, and vice versa (Fincham, 1989).

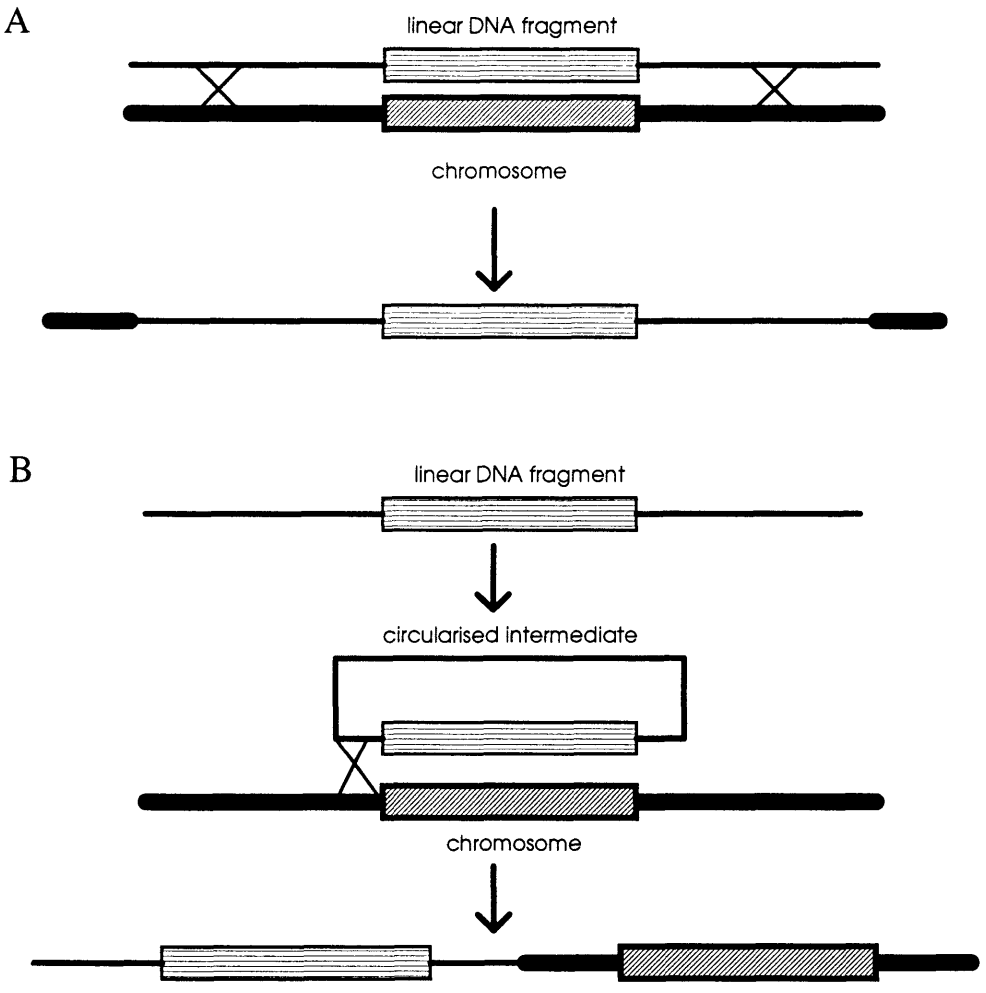

Figure 1.3. Integration at homologous site. Integration may occur to both linear (A) and circular DNA (B). If the introduced DNA is circular, it integrates according to (B).
(after Timberlake, 1991)

As Timberlake and Marshall (1989) stated, integration of linear DNA is also possible. Circularisation of these molecules prior to integration can result in tandem duplications. The presence of multiple copies of the integrated DNA is observed in circular transforming DNAs. Mechanisms for different integration events are shown in figures 1.3 and 1.4.

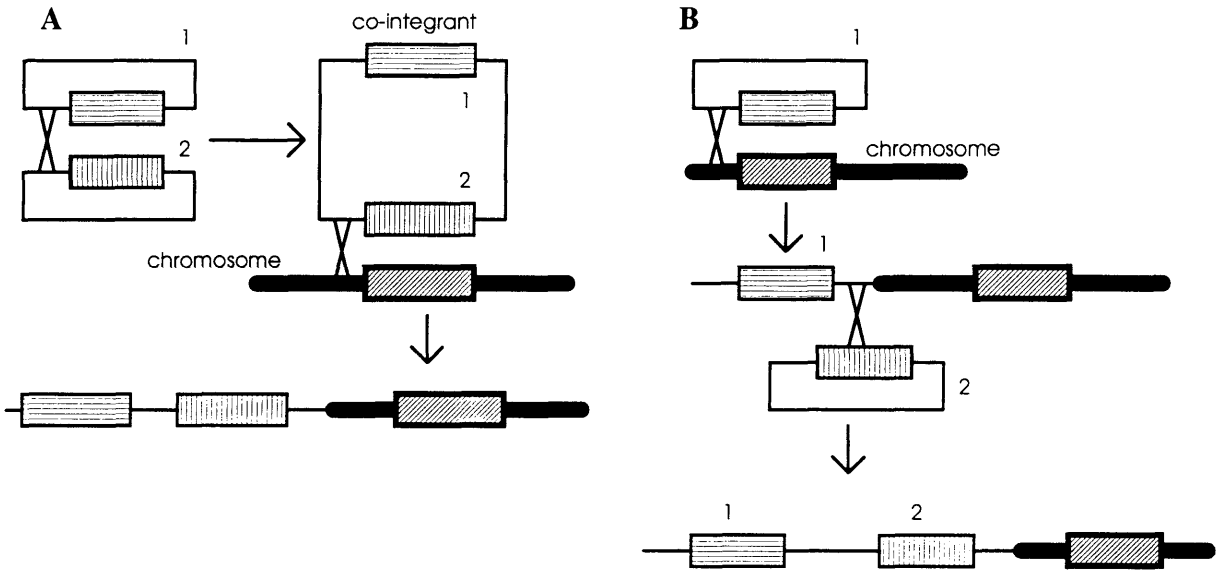

Figure 1.4. Multiple integration at homologous site. Tranforming DNA may produce dimers or multimers prior to integration (A) or individual DNAs integrate one after another (B).
(after Timberlake, 1991)

However, Gems *et al.* (1991) reported on isolation of a sequence from *A. nidulans* genomic DNA which once inserted into a plasmid vector (pILJ16, containing *argB*$^+$ gene) could increase the transformation frequency by 250 folds. According to their data, the new plasmid, ARp1, behaved like a replicon as the transformant phenotype remaind unstable through the asexual progeny. ARp1 was also able to be transmitted through the sexual cross. Gems *et al.* (1991) reported that ARp1 also appeared to replicate autonomously in *A. oryzae* and *A. niger*, resulting in 25 and 30-fold increase in transformation frequencies, respectively.

1.2.2.4. Co-transformation

When the transforming DNA is neither carrying a selectable marker nor confers a selectable trait, co-transformation might be considered as an useful alternative. That is, the main transforming DNA is taken up simultaneously with another DNA which confers a selectable phenotype after being transformed. According to Fincham (1989), when the protoplast is exposed to more than one kind of DNAs, it is possible that the cell taking up one also takes up the other one.

The work of Wernars *et al.* (1987) provides a good example where different combinations of selected and non-selected DNAs were used for co-transformation. They showed that when a strain carrying non-functional *amdS* gene was co-transformed with a mixture of *amdS* and *A. nidulans trpC-E. coli lacZ* plasmids, around 85% of *amdS*$^+$ transformants expressed functional β - galactosidase activity on X-gal medium. In another part of research two different plasmids each carrying either *amdS* or *trpC* genes were used to transform a double mutant strain carrying non-functional forms of the respective genes. Two approaches were employed where first one of the plasmids was used as the prime transforming DNA and the other as the co-transforming DNA. Then, the first plasmid was used as the co-transforming DNA while the second was used as the transforming DNA. The result showed that the absolute concentration of co-transforming DNA as well as its nature have the most important role in determination of co-transformation frequency.

1.2.3. Metabolism in *A. nidulans*

1.2.3.1 Metabolism of Carbon Sources

As shown in table 1.3, *A. nidulans* utilises a variety of organic compounds as carbon sources. The rate of growth on different carbon sources indicates that the organism prefers to utilise certain carbon sources rather than others. In other words, there is an hierarchal preference in utilisation of different carbon sources. A qualitative, yet useful, comparison was made on the amount of mycelia and degree of conidiation in wild-type *A. nidulans* individuals when

grown on different carbon sources (Cochrane 1958, cited in McCullough *et al.*, 1977).

In a review, McCullough (1977) stated that the utilisation of carbon sources in wild-type strain is a little affected by the nature of nitrogen source whereas the regulation of nitrogen utilisation is more affected by the nature of carbon source. Moreover, when grown on media containing different carbon sources, certain responses involved in both inhibition of uptake and repression occur for certain carbon sources. That is, the presence of certain carbon sources represses activities related in utilisation of other carbon sources.

Table 1.3. Level of growth on different carbon sources in *A. nidulans*.

good	moderate	poor	no growth
sucrose	cellobiose, lactose, maltose, melibiose, raffinose, trehalose		
D-glucose	D-galactose	L-rhamnose	L-sorbose D-fucose
D fructose	D-mannose		
	D-mannitol D-sorbitol		
	D-glucoronate D-glucoronate-δ-lactone	D-gluconate D-gluconat-δ-lactone	D-galacturonate
L-arabinose D-lyxose D-xylose	D & L-arabitol D ribitol xylitol	D-arabinose D-ribose L-xylose	
		D-erythrose erythritol	
	glycerol acetate ethanol	D&L-lactate succinate tartrate	citrate
	butyrate	oleate palmitate	

(after McCullough *et al.*, 1977)

Davis and Hynes (1991) also refered to some effects of carbon regulation on certain activities which are involved in nitrogen metabolism [*e.g.*, inhibition

of NADP-dependent glutamate dehydrogenase at transcriptional level under the effect of carbon catabolite repression (Hawkins *et al.*, 1989, cited in Davis and Hynes, 1991)].

McCullough (1977) reviewed different uptake systems in *A. nidulans* about which a little is known (due to few uptake mutations available). However, it is known that there is a constitutive active uptake for galactose, glucose and fructose. The other kind of uptake is inducible, *e.g.* for lactose which is readily induced by lactose but poorly by galactose.

The tricarboxylic Acid (TCA) Cycle is the metabolic heart of energy supply in *A. nidulans,* as it provides ATP and a number of biosynthetic intermediates. A wide range of enzymatic reactions are required for catabolism of different carbon sources to supply acetyl-CoA, the substrate of TCA cycle. Most of these, according to McCullough (1977), are included in Embden-Meyerhoff-Parnas and Pentose Phosphate pathways.

1.2.3.2 Metabolism of Nitrogen Sources

According to Kinghorn and Pateman (1977), ammonium has the central role in anabolic pathways in *A. nidulans*. The organism produces many organic compounds including amino acids, pyrimidines, purines, amines and polyamines in which ammonium and L-glutamine are used for nitrogen incorporation. The literature shows that these two are, therefore, the most preferred nutrients for nitrogen supply. As alternate sources, *A. nidulans* utilises nitrate, nitrite, urea, amides, amines, polyamines, purines and amino acids which all release ammonium through different catabolic pathways. For example (Kinghorn and Pateman, 1977):

- Nitrate is reduced to nitrite which is then reduced to ammonium.
- γ-amino-N-butyric acid (GABA) is transaminated to glutamate and releases ammonium.
- Purines such as xanthine and hypoxanthine are converted to uric acid which later produces urea through several steps. Then, ammonium is obtained from urea.

1.2.3.3. Metabolism of Sulphur Sources

Sulphur can be obtained from either inorganic or organic compounds. The metabolism of inorganic sulphur sources involves catabolic and anabolic pathways of different sulphur-containing amino acids. As Arst (1968) explained extracellular sulphate is reduced to sulphite via a number of enzymatic reactions after being taken up. This can be furtherly reduced to sulfide ion (S^{2-}). According to Kinghorn and Pateman (1977), S^{2-} is then, used for synthesis of amino acids such as methionine, cysteine and homocysteine which can be inter-converted under various conditions.

1.3. Genetic Control of Metabolism

Regulation of gene expression in eukaryotes, in general, takes place at different stages including epigenetic changes and DNA rearrangements, transcriptional and post-transcriptional controls. Epigenetic control monitors transcription events without changing the nucleotide sequence of the DNA (*e.g.*, DNA methylation). The information available about the genetic regulation of metabolic pathways in *A. nidulans* are mainly involved in transcriptional control of gene expression particularly transcription on/off switches (Davis and Hynes, 1991).

According to the consensus definition of eukaryotic genes in literature, a gene is a combination of DNA sequences which when function together form an expressible unit. This combination consists of two major parts: the transcribed region which codes the information for synthesis of the gene product, and the promoter which flanks the transcribed region. The latter is identified by DNA-binding proteins involved in transcription (transcription factors). This region in most of higher eukaryotes consists of AT-rich sequences carrying consensus sequences known as TATA box. The latter is recognised by the transcriptional factor TFIID. There are also short DNA sequences (motifs) upstream the transcribed region which are the binding sites for DNA-binding regulatory proteins. After binding to their target DNA site, these proteins can interact in initiation of transcription (Davis and Hynes, 1991)

Once TFIID binds to the target DNA, TFIIB is attached by protein-protein to the TATA-binding protein (TFIID). Aggregation of other general transcription factors (TFIIA-TFIIJ) as well as the RNApol forms the transcription apparatus (Hahn, 1993b). This machinery is able to transcribe the gene at basal levels (Ha *et al.*, 1993, cited in Hahn, 1993, and Hisatake *et al.*, 1993). As Hahn (1993a) states, chromatin-associated proteins such as nucleosomes and histone H1 can inhibit basal transcription as they do not permit the transcription apparatus form at the promoter. However, in order to obtain higher levels of transcription, it has to be activated by transcription activators. According to Hahn (1993a) three types of activators exist in metazoans. These consists of L-proline-rich, L-glutamine-rich and acidic activators. Acidic activators are present in all eukaryotes from yeasts to human. Acidic activators are able to interact with chromatin-associated proteins. According to Ptashne (1988) and Ptashne and Gann (1990), regulatory proteins have two major components. The first is the DNA-binding motif which is responsible to bind the protein to the target site. The specificity, then, is defined by the Van der Waals and hydrogen bond contacts between the amino acid sequence in binding motif and the edge of base-pairs in the target DNA site. The second component is the regulatory site which may or may not be on the same protein. If not, another protein/polypeptide chain with the regulatory motif is to bind to the binding protein before or after attachment to the target DNA. The regulatory protein then, may interact with the transcription complex to repress or activate transcription initiation. Catabolite repression, according to Johnston (1987) can either operate directly on the target sequence or interact with the activator protein.

Two models have been proposed for the action of acidic activators. The first model implies that acidic activators facilitate the binding of general transcription factors TFIID and TFIIB to the promoter. Available data do not favour this assumption as they indicate that TFIID and TFIIB attach to the promoter with high affinity in absence of any activator. According to the second model, TFIID and TFIIB bind to promoter and attain a non-productive conformation. The rest of sub-units in transcription complex can bind to this non-productive structure and initiate transcription, but it would be at very low efficiency. Acidic activators may bind either to the TFIID-TFIIB non-productive dimer or to a specific DNA site close to it and convert the conformation to a productive form. Therefore, transcription is brought about at high levels (Hahn, 1993b). So it is expected that repressor proteins bind to either DNA target sites for activators or TFIID and TFIIB factors and prevent

the conversion of non-productive form to the productive one. In fact, according to Hahn (1993b), the presence of transcription inhibitors that bind to TFIID and others that bind to TFIIB has been shown. However, Davis and Hynes (1991) specified that in most of fungal genes, regulatory proteins bind to specific sites upstream to the coding region and interact with transcription apparatus.

According to Ptashne (1988), regulatory proteins are not highly-specific for their targets. This permits a number of genes being co-regulated by the same regulatory protein. In *A. nidulans*, for instance, the *alcA* gene coding for alcohol dehydrogenase I and the *aldA* gene coding for the aldehyde dehydrogenase are regulated by the product of the *alcR* gene. Not all the target sites in regulated genes bear complete homology between themselves. This can be one possibility to explain diverse pleiotropic effects caused by mutations changing the binding sequences in wide-domain acting regulatory proteins (such as *areA102*, described later).

Mutations in regulatory regions, also known as *cis* elements may change the transcription level. Sometimes mutations in regulatory proteins (*trans* acting) can change the affinity/compatibility of the binding motif to the target sequence resulting in loss of transcription or enhanced levels of transcription. In *A. nidulans*, many mutations in *cis* elements suppress the loss-of-function mutations of the latter type so that the altered protein can bind to the target sequence and resume the normal activity (Davis and Hynes, 1991). Namely, *gabI*, *nis-5*, *prn*^{d} and *uap-100* mutations in *A. nidulans* are of this type which result in a bypass of the need for *areA*-mediated activation. The *areA* gene product is believed to activate most of activities in nitrogen metabolism (discussed in wide-domain regulation).

Regulatory Genes in *A. nidulans*

Arst and Scazzocchio (1985) categorised *trans*-acting regulatory genes in *A. nidulans* in three groups: integrator genes, pathway-specific regulators and wide-domain acting regulators.

1.3.1. Integrator Genes

The concept of integrator genes was first described by Britten and Davidson (1973, cited in Arst, 1976). According to their model, integrator genes are parallel-acting positive regulatory genes which integrate the expression of a number of structural genes.

The *amdR* gene in *A. nidulans* (Hynes and Pateman, 1970a) also known as *intA* (Arst, 1976) provides a well-studied example. The expression of this gene is induced by ω amino acid inducers and in turn integrates the expression of five unlinked structural genes. They are *amdS*, the structural gene for acetamidase (Hynes and Pateman, 1970a,b), *gatA*, encoding γ-amino-butyric-acid (GABA) transaminase and *gabA*, encoding the GABA permease (Arst, 1976), *lamA* and *lamB* structural genes for lactamase and lactam permease (Arst *et al.*, 1978 and Katz and Hynes, 1989). The latters are needed for the utilisation of 2-pyrrolidinone, the lactam form of GABA. Arst (1976) explained that mutations in *intA* have effects on the above-mentioned structural genes: Partially dominant $intA^{c}$ can lead to constitutive levels of acetamidase production whilst recessive $intA^{-}$ halves acetamidase level. None of *intA* mutations change the structure of acetamidase.

The *amdR* gene has been cloned (Andrianopoulos and Hynes, 1988). Early molecular characterisation of the gene has shown that *amdR* is transcribed at a low constitutive levels. Multiple copies of this gene obtained by transformation studies result in elevated levels of expression for *amdS* and *lamA* structural genes (Andrianopoulos and Hynes, 1988). Andrianopoulos and Hynes (1990) sequenced and characterised the gene. The predicted protein has a cysteine-rich zinc-finger DNA-binding motif at the N terminal. Their study showed that there must be at least two activation regions in the C terminal half which are both required for wild-type activities of the gene product. Littlejohn and Hynes (1992) identified a sequence upstream to the *amdS* coding region corresponding to the site of *amdR* action. This region shows identity with sequences related to *amdR*-regulation of *gatA* and *lam* genes. Homology between *amdR* genes in *A. nidulans* and *A. oryzae* has been reported by Wang *et al.* (1992) extending to most of the coding regions. This homology includes the regions corresponding to DNA-binding motifs and putative activation domains. According to authors, *amdR* genes from *A. nidulans* and *A. oryzae* are functionally interchangable between these species.

1.3.2. Pathway-Specific Regulation

This is characterised by the action of genes mediating induction or repression of the synthesis of the enzymes and permeases for an individual metabolic pathway. A good example, in this case, is induction by nitrate and nitrite via the regulatory gene *nirA*.

In nitrate assimilation, both the nitrate and nitrite reductase enzymes are of major importance. These enzymes are encoded by *niaD* and *niiA* genes, respectively. According to Arst and Scazzocchio (1985), *nirA* mediates nitrate induction of *niaD*, *niiA* and several other enzymes in the pentose phosphate pathway (presumably to ensure sufficient NADPH supply required for assimilation). Cove (1979, cited in Arst and Scazzocchio, 1985) explained that the loss-of-function mutation $nirA^-$ leads to non-inducibility of all these activities by nitrate. Arst and Scazzocchio (1985) have mentioned two other mutations in *nirA*: a gain-of-function $nirA^d$ which bypasses the need for *areA* gene product (discussed later) and causes derepressed expression of genes under *nirA* control, and second, a rare constitutive mutation, $nirA^c$ which lessens the need for co-inducer.

1.3.3. Wide-Domain Regulation

Regulatory genes of this kind control the expression of structural genes from different pathways. Nitrogen metabolite repression and carbon catabolite repression are examples of this type of regulation.

1.3.3.1. Nitrogen Metabolite Repression

There are different enzymes and permeases involved in uptake and catabolism of a wide range of substances that are utilised as nitrogen sources in *A. nidulans*. Utilisation of some of these nutrients are preferred to others and therefore, in their presence production of enzymes and permeases needed for other nutrients are subjected to a regulatory circuit known as nitrogen

metabolite repression. Nitrogen repression is mediated by a wide-domain positively-acting regulatory gene designated as *areA*.

Pateman *et al.* (1973) observed systems including nitrate reductase, xanthine dehydrogenase, acetamidase and formamidase, L-glutamate uptake, urea uptake and extracellular protease secretion were inactive in the presence of ammonium. In addition to ammonium, according to Hynes (1974), L-glutamine and L-glutamate are involved in nitrogen metabolite repression. According to Arst and Scazzocchio (1985), L-glutamine seems to be the effector for the *areA* gene. Intracellular level of glutamine (derived from ammonium assimilation) seems to be the key signal in nitrogen repression as mutants lacking a functional NADP-dependent glutamate dehydrogenase (*gdhA*) or glutamine synthetase (*glnA*) are insensitive to ammonium repression.

Many mutations in the *areA* locus have been subjected to genetic analysis. In a review, Arst and Scazzocchio (1985) categorised *areA* mutations in two groups. Mutations in the first group, $areA^r$ (also known as $areA^-$), are of loss-of-function nature. These mutations lead to full time repression of activities under *areA* control. $areA^r$ mutants grow very poorly on all nitrogen sources except ammonium. The second category comprises $areA^d$ mutations which have derepressed levels of some activities under the control of *areA* gene. One significant $areA^d$ mutation is *xprD-1* which leads to derepression of almost all metabolic pathways subjected to nitrogen repression (Cohen, 1972).

Repressed levels of activities in $areA^-$ mutants, which are recessive to the wild-type allele, indicate the putative positively-acting nature of the *areA* gene product. Different *areA* mutants exhibit a variety of phenotypes and this heterogeneity suggests that the *areA* product is a polypeptide chain which is involved directly in repression rather than coding for an enzyme that catalyses a secondary product involved in repression (Marzluf, 1981). On the other hand, according to Arst and Scazzocchio (1985), the non-hierarchal heterogeneity of phenotypes of *areA* alleles not only indicates that the a*reA* product is directly involved in the regulation of expression, but also suggests that the receptor sites for *areA* protein differ in their structure. Some *areA* mutants have different, even opposing, effects on expression of structural genes under *areA* control causing reduced (or no altered) expression of some and enhanced (derepressed) expression of others.

Kudla *et al.* (1990) presented the sequence of the *areA* gene and showed that the *areA* protein is coded by a single open reading frame (ORF) in the

gene. They also characterised different regions of the protein as essential and non-essential by transformation analysis of different deletions from different parts of the gene. The *areA* gene product contains 719 amino acids of which 342 N-terminal residues are inessential for activity. Also 124 C-terminal residues are not essential for activation function of the *areA* protein. A 52 residue region which forms a zinc-finger is, on the other hand, essential. While the zinc-finger and adjacent C-terminal sequences are highly basic, the N-terminal side of the zinc-finger is acidic and required for gene activation. Kudla *et al.* also showed that the C-terminal residues are essential in nitrogen repression. They showed that chromosomal rearrangements or artificial deletions removing 55 or more residues from the C-terminal lead to loss of nitrogen repressibility.

The *xprD-1* phenotype was first speculated to be due to the overproduction of wild type *areA* product. Arst (1982) showed *xprD-1* is associated with a near terminal pericentric inversion in chromosome which he hypothesised fused the coding region of *areA* to a more efficient promoter. However, after molecular analysis of the gene (Kudla *et al.*, 1990), it was shown that the *xprD-1* mutation is an inversion truncating 124 N-terminal residues of the *areA* gene product (fig. 1.5). This truncation leads to loss of nitrogen repressibility. Therefore, in *xprD-1* mutants, *areA* is transcribed at wild-type levels, but the product is insensitive to nitrogen status.

Kudla *et al.* (1990) showed that changes in the zinc-finger have different consequences. While some of them including *areA19*, *areA209* and *areA217* lead to loss-of-function mutations due to single amino acid substitution, others including *areA102*, *areA30* and *areA31*, also due to single amino acid substitution, change the specificity of the protein for different receptor sites. For instance, the *areA102* allele differentially affects activation from different promoters, leading to elevated levels of growth on some nitrogen sources such as urea, L- glutamate and L-citrulline and reduced levels on other nitrogen sources such as formamide and uric acid. This is due to a conservative leucine-valine substitution in zinc-finger. *areA30* and *areA31* are revertants of *areA102* and exhibit mirror phenotype of *areA102*. That is, they grow strongly on nitrogen sources where *areA102* has a poor growth, and vice versa.

More recently, Stankovich *et al.* (1993), observed that in agreement with Kudla *et al.* (1990), C-terminal truncations in the *areA* gene have only minor effects on activation function of the product. In fact, according to their

findings, *areA* proteins with up to 153 deleted residues at their C-terminals (*e.g., areA1540, areA1600* and *areA1601*) still retained most of the activities under *areA* control since strains carrying these mutations utilised a vast majority of nitrogen sources. However, Stankovich *et al.* (1993) affirmed that different regions in *areA* protein may have different functions depending on the target gene. They observed that activation of few structural genes including *uapA, ureA, lamA* and/or *lamB* were impaired in *areA1600* truncating 153 C-terminal residues.

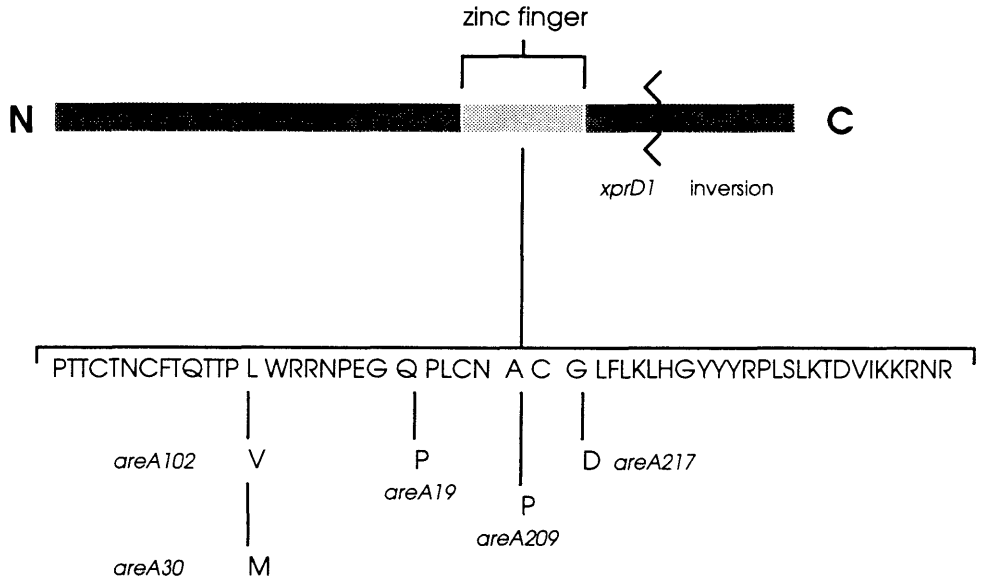

Figure 1.5. Sequence changes in the *areA* protein. Changes in the DNA sequence coding for the zinc finger may cause different mutations with different effects.
(after Davis and Hynes, 1991).

A similar system for nitrogen catabolite repression exists in *Neurospora crassa*. The repression is mediated by a major gene, *nit-2*, with wide-domain positively acting nature and regulates expression of genes related to utilisation of nitrate and nitrite, amino acids purines and a number of other nitrogen sources as well as extracellular proteases (Reinert and Marzluf, 1975, cited in Hanson and Marzluf, 1975, and Grove and Marzluf, 1981). According to Dunn-Coleman *et al.* (1979, cited in Premakumar *et al.*, 1980) ammonium is the repressing nutrient in nitrogen repression which dictates its effect through L-glutamine. The *nit-2* gene product was reported by Grove and Marzluf (1981) as a non-histone DNA-binding protein. This protein consists of 1036 amino

acids and contains a single zinc-finger domain (Fu and Marzluf, 1990). According to Fu and Marzluf, gel retardation and DNA-footprint studies supported that the *nit-2* protein binds specifically to its recognition sites upstream from the transcribed region of nitrogen-regulated structural genes. The consensus sequence for recognition sites is TATCTA. The similarity between the *nit-2* gene in *Neurospora crassa* and the *areA* gene in *A. nidulans* was investigated by Davis and Hynes (1987). In their experiments, these authors observed that the *N. crassa nit-2* gene complemented *areA* mutations in *A. nidulans* individuals and restored the utilisation of various nitrogen sources.

1.3.3.2. Carbon Catabolite Repression

As mentioned before, *A. nidulans* utilises a variety of carbon sources. However, according to availability and economy of the carbon source present in the medium, the organism prefers to keep production of enzymes and permeases involved in utilisation of non-preferred carbon sources at basal levels. This phenomenon, called carbon catabolite repression, is mediated by a wide-domain acting regulatory gene designated as *creA* (Arst and Cove, 1973). Early studies of Arst and Baily (1977) showed that cyclic-AMP does not play a major role in carbon repression.

According to genetic data (Baily and Arst, 1975 and Hynes and Kelly, 1977), mutations in *creA* gene are associated with abnormal morphology and derepression of many catabolite-repressible activities. These mutations are recessive to wild type, hence, are supposed to be of a loss-of-function type. Dowzer and Kelly (1989) cloned the *creA* gene and provided the first evidence for the negatively-acting nature of its product. They showed that transformants containing multiple copies of wild-type *creA* express tighter carbon catabolite repression. In addition, they showed that the *creA* gene encodes a transcript approximately 1.8 kb long. They showed also that the amount of *creA* transcript can be raised up to three folds depending on the carbon source. Sequence analysis of the *creA* gene by Dowzer and Kelly (1991) indicated that the gene codes for a 415-aa protein and does not contain any introns. The derived amino acid sequence featured zinc finger DNA-binding motifs which resembled to C_2H_2 fingers of the transcription factor TFIIIA of *Xenopus laevis*. The finger regions are similar to those of the *MIG1* gene product which is

involved in carbon catabolite repression in *Saccharomyces cerevisiae*. The authors also reported on presence of a sequence of nine alanine residues starting at the position 131. Since alanine-rich regions are present in a number of repressor proteins, Dowzer and Kelly (1991) suggested that their data supported the repressor DNA-binding function of the *creA* gene product. However, in their study total deletion of the *creA* gene function appeared to be deleterious which may imply that repression is not the only function of the gene product. Kulmburg *et al.* (1993) used gel mobility shift experiments to show the specific DNA-binding identity of the *creA* protein. Their results indicated the specific binding of the *creA* protein to promoters upstream to two genes in ethanol regulon: *alcA*, the gene coding for the alcohol dehydrogenase-I and *alcR*, a *trans*-acting gene required for induction of *alcA*. Cubero and Scazzocchio (1994) performed DNase I and methylation protection studies on putative *creA* binding sites between *prnD* and *prnB* genes in proline gene cluster (Hull *et al.*, 1989). They found the consensus sequence for *creA* binding sites to be 5'-SYGGRG-3'. They mentioned that some of possible sites derived from the consensus 5'-SYGGAG-3' to be context-dependant. Two different sites separated by a single base, 5'-GCGGAGACCCCAG-3' appeared to be essential for carbon catabolite repression of *prnB* transcription. In addition to the binding of *creA* protein to certain consensus sites in a context-dependent manner, Espeso and Penalva (1994) reported on five non-consensus 6-base-pair sequences in *ipnA* gene (coding for isopenicillin N synthetase) recognised by the *creA* protein. Since the presence of these sequences correlated with the presence of a second neighbouring *creA* binding site, Espeso and Penalva suggested that this pattern might be for stabilising the *creA* binding to the target DNA.

Loss-of-function mutations in *creA* gene (*creA*^{d} mutants) have been described by Baily and Arst (1975) and Hynes and Kelly (1977). Most of these have been isolated in *areA*$^{-}$ strains by allowing the growth on nutrients which can be utilised as both nitrogen and carbon sources in the presence of carbon repression. That is, nutrients such as acetamide, L-proline, L-glutamine and GABA can not be used by *areA*$^{-}$ strains as sole nitrogen sources, but can be used as carbon sources if carbon catabolite repression is not operating. *creA*^{d} mutants were isolated as they allowed *areA*$^{-}$ individuals utilise above nitrogen nutrients in the presence of a strong repressive carbon source (*e.g.* sucrose). The *creA204* mutation was isolated and characterised by Hynes and Kelly (1977) and is very similar to *creA*^{d} mutations. Arst *et al.* (1990) identified the nature of the most extreme *creA*^{d} mutation, *creA*^{d}*30*. The latter reduces the

radial growth rate drastically resulting in an ultracompact colony morphology. According to Arst *et al.* (1990), given the *creA* locus is located near the left end of the chromosome I, tight linkage between *creA^{d}30* and *yA2* mutation located on the other end of the chromosome suggests that the *creA^{d}30* mutation is the result of a pericentric inversion in the chromosome I. It is noteworthy that none of the above mutations have been reported to affect the production of extracellular protease in *A. nidulans*.

The carbon catabolite repressor coding gene in *A. niger* was isolated by Drysdale *et al.* (1993). The amino acid sequence of *creA* gene products in *A. niger* and *A. nidulans* exhibited 90% similarity (82% identity). Zinc finger regions in these two proteins showed 96% similarity (84% identity) to those of *MIG1* gene product in *S. cerevisiae*. Drysdale *et al.* (1993) also reported on functional interchangability of *creA* gene products between the two *Aspergillus* species.

Hynes and Kelly (1977) reported two other genes, *creB* and *creC*, presumably involved in the carbon catabolite repression. Two mutations were isolated in these genes designated as *creB15* and *creC27* with a similar approach for isolation of *creA* mutations. Interestingly, these mutations allowed the *areA*$^{-}$ strain to grow only on acetamide. They did not suppress *areA*$^{-}$ on L-proline, L-glutamine and GABA. They also adversely affected the utilisation of some nitrogen sources. The effect on utilisation of various carbon sources was diverse. That is, while the utilisation of some nutrients such as lactose, D-quinate and amino acids was highly reduced, mutants showed slight effects on D-fructose, L-rhamnose and D-mannose or no effect on D-glucose, glycerol and ethanol.

In addition to carbon metabolism, carbon catabolite repression seems to have effects on other metabolic pathways, as well. For example, Espeso and Penalva (1992) reported that presence of repressing carbon sources in growth media caused low levels of penicillin production by repressing isopenicillin-N-synthetase structural gene, *ipnA*, whereas growth on media with non-repressive carbon sources resulted in derepressed levels of penicillin production. Molecular data presented by Espeso and Penalva (1994) confirmed the interaction of the *creA* protein with *ipnA* gene. Transcription of *ipnA* gene is also under pH control (explained in section 1.4.2). Based on their observations on isopenicillin biosynthesis, Espeso *et al.* (1993) proposed that a second *creA*-independent mechanism of carbon regulation controls the secondary

metabolism genes. This mechanism is responsive to external pH and can override carbon catabolite repression. The evidence for existance of the second independent mechanism relies on derepressed levels of *ipnA* transcription even in presence of repressing carbon sources when external pH is high. In contrast, the effect of growth on derepressing carbon sources could not be prevented by acidic external pH. Espeso *et al.* (1993) mention that these two regulatory mechanism work in concert as mycelia grown on derepressing carbon sources tend to alkalinise the medium whereas growth on repressing carbon sources results in acidification of external pH.

1.3.3.3. Sulphur Metabolite Repression

Exogenous methionine represses a number of metabolic pathways for utilisation of less preferred sulphur sources such as sulphate (Natorff *et al.*, 1993). There are mutated genes that abolish methionine-mediated sulphur repression which have been categorised in four groups by Natorff *et al.* (1993). Previously-studied *suA25meth* and *mapB1* mutations were reported to be similar to scon mutations in n. crassa. Natorff *et al.* (1993), inspired by the *scon* mutations of *N. crassa*, have changed the names of these mutations and assigned them to *sconA* and *sconB* genes. They also isolated mutations in two further regulatory genes *sconC* and *sconD*. Strains carrying *scon* mutations are insensitive to sulphur repression and constitutively uptake sulphate and through sulphate assimilation, increase intracellular sulphur-containing amino acid pools such as homocystein. As a secondary consequence, the level of folate metabolising enzymes is increased. Cysteine which is synthesised by the reverse trans-sulphuration of methionine seems to be the effector for the methionine mediated repression Natorff *et al.*, 1993).

1.4. The Study of Extracellular Proteases in *A. nidulans*

Amino acid residues are the sub-units in the construction of polypeptide chains. Regardless of the biochemical properties of different amino acids, they all contain carbon and nitrogen as major components. There are also amino acids which carry sulphur atom(s) as a part of their structure. Catabolic pathways for metabolism of most of amino acids are available in many organisms. Therefore, amino acids and accordingly, polypeptides and proteins, may be considered as potential carbon, nitrogen and sulphur sources. If so, their peptide chain is to be hydrolysed to obtain individual amino acids. This can be done inside the cell but large molecular size of proteins is a problem for their uptake. Therefore, extracellular proteolytic enzymes are secreted to at least partially digest them.

1.4.1. Extracellular Protease Enzymes in *A. nidulans*

According to Matsubara and Feder (1971), many microbial fungi utilise protein molecules to get carbon, nitrogen and sulphur nutrients. Extracellular proteases (mostly of endopeptidase type) are secreted into the medium in order to break down the polypeptide chains and release amino acid residues. Cohen (1973a) reported on detection of different proteases in *A. nidulans*. Electrophoresis on cellulose acetate sheet was used to separate different species of enzymes. Then the sheet was incubated in contact with substrate film (10% milk) and at the end of incubation period the pattern of milk clearing was studied. In addition to molecular size, pH and inhibitor sensitivity were employed as extra properties to characterise enzymes.

Proteases in *Aspergilli* were already reviewed by Matsubara and Feder (1971). They stated that in most fungi three type of proteolytic enzymes (proteases) are produced: a) pepsin-like acid proteases, b) di-isopropyl-phosphofluoridate (DIFP)-sensitive neutral and alkaline proteases and c) metal-ion-dependent neutral and acid proteases.

Cohen (1973a) showed that in *A. nidulans* when protein serves as the sole nitrogen source, three neutral or alkaline extracellular proteases (α, γ and ε) and one intracellular protease (β) are synthesised. Based upon his findings, Cohen (1973a) suggested that proteases in *A. nidulans* are broadly similar to those of

related fungi. The presence of proteases of neutral and alkaline types were conclusively detected, but no acid protease was identified. This, according to Cohen (1973a) was probably due to the method employed for detection which was not appropriate for identification of acid protease enzyme(s). However, to date, no detailed investigation about individual types of extracellular proteases and their synthesis in response to different nutritional/environmental conditions has been reported in *A. nidulans*.

1.4.2. Regulation of Extracellular Protease Production in *A. nidulans*

The production of extracellular proteases is selective and the level of production varies under different conditions (Cohen, 1972). Given the utilisation of proteins as sources for carbon, nitrogen and sulphur nutrients, the selective production of extracellular proteases must be responsive to limiting nutritional conditions for either of these sources. Cohen (1973b) stated that the production of extracellular proteases is not induced by exogenous proteins and speculated that carbon, nitrogen and sulphur repression regulate the production level. The mode of regulation of protease production on *N. crassa* is different from that of *A. nidulans* as, according to Drucker (1972) and Cohen *et al.* (1975), extracellular proteases are produced under inducing growth conditions. That is, when protein is used as the major carbon, nitrogen and/or sulphur source(s), the presence of adequate amounts of preferred carbon, nitrogen and sulphur nutrients represses the protease production, but with lowering the amount of preferred nutrients, the exogenous protein induces the protease production. The effect of ammonium repression on protease production in *A. nidulans* has been already shown as in the presence of ammonium and two other low-molecular weight nitrogen sources (*i.e.*, L-glutamine and L-glutamate), production and release of these enzymes is repressed whereas starvation for nitrogen triggers their synthesis and subsequent release (Cohen, 1972, 1973a and 1973b). According to Cohen (1973b), if any one of nitrogen, carbon and sulphur repressions is lifted, the production of extracellular proteases is activated. Despite few regulatory mutations, effects of carbon and sulphur regulatory systems on protease production have not been studied in details.

The pH of the growth medium could affect the secretion of extracellular acid and alkaline proteases. Caddick *et al.* (1986a) showed that in *A. nidulans*, production of some enzymes which are at least in part extracellular such as acid phosphatase and alkaline phosphatase were affected by the pH of growth medium. A similar effect was observed for certain permeases (*e.g.*, that for GABA). They speculated a wide-domain acting regulator, *pacC*, responsive to growth medium pH with a positive effect on some activities (*e.g.*, acid phosphatase) and a negative effect on the others (*e.g.*, alkaline phosphatase). Other genes studied, *palA*, *B*, *C*, *E* and *F*) were suggested to be involved in production of an effector molecule which inactivates the *pacC* gene product. This effector, according to their model is presumed to be present and effective at alkaline pH. These genes, designated by Caddick *et al.* (1986a), are believed to confer pH homeostasis in *A. nidulans* (Rossi and Arst, 1990, Shah *et al.*, 1991 and Espeso *et al.*, 1993). More recent work by Tilburn and Arst (unpublished, cited in Espeso *et al.*, 1993) show that the *pacC* protein contains three putative C_2H_2 zinc fingers supporting the proposed transcription factor nature of the gene product. Findings of Tilburn *et al.* (unpublished, cited in Espeso *et al.*, 1993) contradicts the proposed model for the repressor nature of the *pacC* gene product. According to authors, mutations in *pacC* mimic alkalinity resulting in transcription of alkaline pH-specific genes. These mutations comprise gain-of-function alleles which support the *pacC* protein as a transcription activator. Hence, the modified model speculates that the *pacC* protein is an activator for transcription at alkaline pH and *pal* gene products catalyse an effector which potentiates its function.

It seems that in *A. nidulans,* the function of pH regulatory circuit is not limited to the intracellular pH homeostasis. The organism seems to modify the medium pH to obtain optimal pH, too. Rossi and Arst (1990) isolated four genes, designated as *phrA, B, C* and *D*, which are also involved in pH regulation circuit in *A. nidulans.* The *phrA-4, phrB-8, phrC-8* and *phrD-13* mutations in these genes conferred resistance to acidic pH down to 2.1-2.2 (compared to pH2.5 in wild type). These mutations appeared to be unlinked to *pacC* and *palA, B, C, E* and *F* loci. When grown on media with moderate pH, strains carrying *phr* mutations expressed reduced acidification of the external pH. Two *phrB-8* and *phrC-9* strains were sensitive to low external pH in media with high buffering capacity.

Shah *et al.* (1991) found that the biosynthesis of β -lactam antibiotics (*e.g.*, penicillin G) in *pacC* mutants which mimic alkalinity is higher than in the

wild type whereas in strains carrying *pal* mutations the penicillin production is lost. They found that penicillin non-producing mutations *npeC007* and *npeD0045* are alleles to *palA*$^-$ and *palF*$^-$, respectively. In addition to carbon catabolite repression (as explained in section 1.3.3.2), the penicillin biosynthesis appears to be under control of pH regulation as well. According to Espeso and Penalva (1992), at moderate external pH and in the presence of repressive carbon sources such as sucrose, the penicillin production is low. However, as Espeso *et al.* (1993) found, external alkaline pH and also mutations in *pacC* override the carbon regulation and lead to derepressed levels of isopenicillin biosynthesis even in the presence of sucrose.

Neutral, acid and alkaline extracellular proteases are active in different pH ranges. It may be predicted that the production of different extracellular proteases can be responsive to the external pH. That is, for instance, when growing in an acidic medium where proteins are to be utilised, only acid protease is produces whereas alkaline protease which is not functioning in the acid pH range is not. Although no work has been reported on the effect of pH regulatory system in *A. nidulans* on extracellular protease production, information presented above suggest the possibility of pH control.

A number of mutations in the following loci affecting the production of proteases in *A. nidulans* have been reported:

xprC. *xprC-1* was initially reported to be a recessive single mutated gene resulting in simultaneous loss of all extracellular proteases (Cohen, 1973a). In fact, according to the report, 48 hour derepressed cultures of *xprC-1* strain contained no protease of any kind except intracellular type β. *xprC-1* strains grow and differentiate normally on media containing nitrogen sources other than proteins. Nevertheless, later the author noted that there had been a mistake and in fact, two mutations were involved in *xprC-1* phenotype (M.E. Katz, personal communications). Therefore the concept of *xprC* as a single gene involved in the production of extracellular proteases is no longer valid.

areA. The *xprD-1* (*areA^{d}-1*) strain was isolated by Cohen (1972) and showed derepressed levels not only for proteases but also for nitrate reductase, xanthine dehydrogenase and glutamate uptake activities. Hynes (1974), in addition, reported on elevated levels of milk clearing in *areA102* mutants. *areA*$^{-}$ mutations result in the loss of all activities under *areA* control including the production of extracellular proteases. The *areA* gene is already described in nitrogen metabolite repression (section 1.3.3.2).

creB and ***creC***. Hynes and Kelly (1977) reported on two mutations in *creB* and *creC* genes with diverse pleiotropic effects on a number of activities. *creB15* and *creC27* mutations expressed elevated levels of milk clearing on plates where milk served as either carbon or nitrogen source. The effect was greater when milk was used as the sole carbon source. These mutations appeared as poor suppressors of *areA*$^{-}$ (*areA217*) on milk plates either in presence or absence of glucose. They exhibited great additive effects on milk clearing when present with *areA102*.

xprE. The other putative regulatory gene involved in production of extracellular proteases is *xprE* (M.E. Katz, unpublished data). Studies showed that the *xprE1* mutant did not produce any milk clearing on -N milk plates. Extracellular protease activities in assays under nitrogen and/or carbon limiting conditions also remained at low levels. The *xprE1* strain still produced extracellular proteases (although at altered levels) under sulphur limiting conditions (M.E. Katz *et al.*, unpublished data). This indicates that *xprE1* is not a mutation in one of structural genes for extracellular proteases as activity was retained in sulphur starvation. Moreover, *xprE* gene is possibly not involved in sulphur regulation of extracellular protease production. The *xprE1* mutated gene segregates as a single gene and is recessive to *xprE*$^{+}$. *xprE1* is not a secretory mutation due to normal extracellular activities of invertase under the same circumstances where extracellular protease activity was not detected (P. Flynn and M.E. Katz, unpublished data). RNA dot-blot studies are suggestive that *xprE* is involved in transcription of extracellular protease structural genes (R.N. Rice and M.E. Katz, unpublished data). If so, the *xprE* product is of a putative positive-acting nature. At this stage, no further evidence is available to show whether *xprE1* is a missense mutation altering the function/biochemical

properties of the *xprE* protein or if it affects the transcription rate of the *xprE* gene.

Three revertants of the *xprE1* strain had been already isolated with UV-irradiation experiments (M.E. Katz, unpublished data). While the *xprE1* strain showed no milk clearing activity on -N milk plates, revertant strains carrying suppressor mutations showed high levels of extracellular protease activities on the same type of plates. These suppressors were to be studied in detail and this project was focused on genetic characterisation and molecular isolation of one of them, designated later as *xprF1*.

Studies performed by M.E. Katz (unpublished data) on the other two suppressors, called as *xprF2* and *xprG*, show that they are very similar in behaviour to *xprF1*. *xprF2* is an allele to *xprF1*, although its effect on milk clearing is not as extreme as *xprF1*. *xprG* is located on the chromosome VII but is unlinked to the *xprF* locus.

1.5. Aims of Study

This study was focused on the following subjects in order to give an overall image about *xprF1*, and introduce it as a new regulatory mutation:

Genetic Characterisations:

- the number of genes affected and the mode of segregation
- pleiotropy and dominancy properties of *xprF1*
- location of the *xprF* locus

Functional Characterisations:

- prove the *xprF1* as a regulatory mutation
- interaction between *xprF1* and other regulatory mutations affecting the extracellular protease production in *A. nidulans*
- the mode of action of the *xprF* gene

Molecular Isolation of the *xprF* Gene:

- isolation of the cosmid clone carrying the wild-type *xprF* allele from an available chromosome-specific genomic library of wild-type *A. nidulans* (Brody *et al.*, 1991).

Chapter 2

MATERIALS and METHODS

2.1. Strains

Strains of *Aspergillus nidulans* used for genetic and molecular experiments are listed in table 2.1. Strains ac102, H17A12, HNA795, J3, J9, *niiA4*, SA4B17, SA15B12, SA27D8 and *y paba mas* were provided by Prof. Michael J. Hynes. The strain *sconD6* was provided by Prof. Andrezj Paszewski. Strains MEK1, MEK3-R4, R4-11 and R4J3#43 were provided by Dr. Margaret E. Katz. The MEK1 strain carried the *xprE1* mutation and was isolated as a mutated strain (UV irradiation mutagenesis) which did not produce any milk clearing on medium where milk served as the sole nitrogen source. MEK3-R4 was a revertant of the MEK3 (another *xprE1* strain) also isolated by UV mutagenesis. MEK3-R4 was isolated as it expressed a stronger growth on medium containing dialysed milk as the nitrogen source. R4-11 was a segregant from a cross between MEK3-R4 and J5 (*prnAΔ 457 fwA1; niaD500*). R4J3#43 was a segregant from a cross between AM1 and J3.

Strains AM1-AM6 were isolated in this study. AM1, AM2 and AM3 were segregants from the cross "R4-11 × *y paba mas*". AM4 was a segregant from a cross between AM2 and *niiA4*. AM5 and AM6 were segregants from the cross "AM4 × J9".

Table 2.1. *A. nidulans* Strains

strain	Genotype
ac102	*biA1; areA102; niiA4*
AM1	*pabaA1, yA1, acuE; xprF1*
AM2	*pabaA1, yA1, acuE; xprF1; riboB2*
AM3*	*yA1, acuE; riboB2*
AM4	*yA1, acuE; xprF1; niiA4, riboB2*
AM5	*yA1; acuE; prnΔ 309, xprF1; niiA4, riboB2*
AM6	*pabaA1; prnΔ 309, xprF1; niiA4*
H17A12	*suA-adE20, yA1, adE20; areA217; riboB2*
HNA795	*pabaA1; gabA2*
J3	*acrA1; amdSΔ 368, galA1; pyroA4; hxA; sB1; amdA7, nicB8; riboB2*
J9	*pabaA1; prnΔ 309*
MEK1	*biA1; xprE1; niiA4*
MEK3-R4*	*suA-adE20, yA1, adE20; pyroA1;xprE1; xprF1*
niiA4	*biA1; niiA4*
R4-11*	*prnA457, xprF1; niaD, riboB2*
R4J3 #43	*pyroA4; amdA7, nicB8*
SA4B17	*creA204, biA1; niiA4*
SA15B12	*biA1; creB15*
SA27D8	*biA1; creC27; niiA4*
sconD6	*yA1; pyroA4; sconD6*
y paba mas	*pabaA1, yA1, acuE*

* These strains carried a new uncharacterised mutation discussed later.
- The meanings of the gene symbols are given in Clutterbuck (1993).
- The *sconD6* mutation is described in Natorff *et al.* (1993).

2.2. Growth Media and Solutions

The standard minimal medium (-N) was that of Cove (1966). Wherever required, one or more components such as the carbon source, individual vitamins or mineral components were omitted. -N minimal medium containing 1% (w/v) skim milk as the nitrogen (-N milk) source was that of Cohen (1972). The sulphur-free medium was the modified -N minimal medium where sulphur-containing salt ingredients were excluded.

-CN medium with milk as both carbon and nitrogen source (-CN milk) and -CN medium with 10 mM ammonium tartrate containing skim milk as the carbon source (-C milk) were also used. Modified -N milk medium with 1% dialysed milk was another form of milk plate employed. Except the latter, 0.08% (w/v) sodium deoxycholate was added to all milk media. Ingredients and recipes for growth media and other solutions are in appendices 1 and 2.

The following points were considered in preparation of media:

- Solid media contained 1.2% agar [(w/v) Oxoid Agar No.1], except in Protoplast Regeneration Medium where 1% agar was used.

- A concentration of 10 mM was used for all nitrogen sources in different media. However, milk and dialysed milk, when used as nitrogen source, were at 1% (w/v) level.

- Alternative carbon sources for the standard minimal medium were generally used at 50 mM concentration. Exceptions included: glucose: 1% (w/v); sucrose: 1% (w/v); glycerol: 0.5% (v/v); milk/dialysed milk: 1%(w/v).

- Media included 1% (v/v) vitamin solution (appendix 1). There were, however, particular selective media which contained all vitamins except a specific one. For instance, "-ribo medium" contained every ingredient of vitamin solution except riboflavin.

2.3. Genetics

Methods used in formal genetics followed those of Clutterbuck (1974).

2.3.1. Crosses

Conidia from strains to be crossed were stabbed on a complete medium plate a few millimetres apart. After a 48-hour incubation at 37 °C, pieces of agar from the areas between two colonies containing heterokaryons were transferred to a small (5.5 cm) plate with the appropriate selective (unsupplemented) medium. The selective medium for each cross was determined in order to select only heterokaryons. After 48-hour incubation at 37 °C, the air was excluded by taping the small plate and incubation was carried on for seven days. Then, four cleistothecia from each cross were rolled on an agar plate to remove parental mycelia and conidia. Each cleistothecium was disrupted and the spores were dispersed in 1 ml distilled sterile water separately.

100 µl of diluted spore suspensions was spread on complete plates and after 48 hours of incubation, colonies were picked up to make master plates. Each master plate contained 24 segregants plus parental colonies as controls. Normally two master plates were set up for each cross. After 48 hours, colonies were replicated on different test plates with a 26-pronged replicator. Test plates were scored after 2 days. Table 2.2 lists crosses carried out.

2.3.2. Diploid Strain

A diploid strain was obtained using *xprF1* strain (AM1) and a mapping strain (J3). The procedure was started as for a cross. However, heterokaryons were transferred to a 9 cm plate containing the selective medium (ANM, 10 mM sodium nitrate) followed by an incubation for 48 hours at 37 °C. Mycelia with vigorous growth were transferred to a new selective plate and incubated at similar conditions. Transfers were continued until balanced heterokaryons were obtained.

Table 2.2. Genetic Crosses

cross	selective plate	test plates*
R4-11 × *y paba mas*	nitrate	Milk plates (a, b, c and d†),-paba, -ribo, acetate, nitrate, sucrose + acetamide, proline, alanine, uric - acid, hypoxanthine, GABA
AM2 × *niiA4*	nitrate	milk plate (c), -paba, -ribo, nitrate, sucrose + acetamide (N source), proline, uric acid, hypoxanthine, acetamide (C source), proline (C source), sorbitol, glycerol
AM3 × *niiA4*	nitrate	milk plates (a and c), sucrose + acetamide, proline, uric - acid, hypoxanthine
AM4 × J9	nitrate	milk plate (c), -paba, -ribo, acetate, nitrate proline
AM1 × R4J3		milk plate (c), sucrose + acetamide
AM6 × *sconD6*	nitrate	milk plates (a, b and c) ‡
AM1 × ac102	nitrate	milk plates (a, b and c), sucrose + acetamide
AM5 × SA4B17	ANM plus ammonium	milk plates (a, b and c), sucrose + allyl alcohol
AM5 × SA15B12	nitrate	milk plates (a, b and c), sucrose + allyl alcohol
AM5 × SA27D8	ANM plus ammonium	milk plates (a, b and c), sucrose + allyl alcohol
AM6 × H17A12	nitrate	milk plates (a, b and c), alanine
AM5-1 × J9	nitrate	milk plates (a and c), -paba, -ribo, acetate, nitrate, proline, hypoxanthine
AM5-3 × HNA795	nitrate	milk plates (a, b and c), proline, hypoxanthine, GABA
AM5-5 × HNA795	nitrate	milk plates (a, b and c), proline, hypoxanthine, GABA

* General recipe for all plates described in appendix 1 (Selective Media)

† different roles of milk in media: a. sole nitrogen source (-N milk); b. both nitrogen and carbon source (-CN milk); c. sole carbon source (-N milk); d. dialyses milk as the sole nitrogen source.

‡ Two methods were used for identification of *sconD6* mutants: a. 24-hr colonies on complete plates were flooded with 30 mM indoxyl sulphate in 0.5 M Tris-HCl; pH8.0; b. segregants were tested on sulphur-free minimal medium containing 1 mM sodium selenate and 5 mM methionine.

Then, conidia were scraped and dispersed in Tween solution (~0.02% Tween 80). Aliquots of conidial suspension were spread on selective plate where only diploid conidia were able to grow.

Genetic purification of the diploid colony was carried out by streaking one of the colonies on selective plate.

2.3.3. Mapping

The AM1/J3 diploid was forced to undergo haploidisation by growing on benomyl plate (0.001 mg/ml, appendix 2). After six days green and yellow haploid sectors were picked up and used for making three master plates. Green haploid sectors were identified as they had a deeper green colour than diploid conidia.

Haploids were checked for all markers carried by AM1 and J3 and also for the effect of *xprF1* on milk clearing. This included testing on -paba, acetate, acriflavine, galactose, -pyro, hypoxanthine, -thio, -nico, -ribo and different milk plates.

2.4. Plate Tests

In experiments such as genetic crosses, mapping and examination of dominancy properties, strains and/or segregants of crosses were tested on solid media. Depending on the nature of the mutation(s) under study and regarding the remarks in section 2.2, appropriate combinations of different carbon and nitrogen sources together with appropriate vitamins were used.

Conidia were transferred to plates and incubated at 37 °C for 2 days. Then, each individual colony was scored for a number of aspects:

Growth. The level of growth ability was indicated by scoring the colony in a range between '-' to '+++' while comparing with that of wild-type control(s). This helped to study several factors such as: what were priorities in utilisation of different carbon and/or nitrogen sources; was milk a preferred carbon and/or nitrogen source; how utilisation of different nutrients were

affected by mutations under study; which individuals expressed derepressed levels of particular activities under certain metabolic conditions and which expressed normal or repressed levels, etc..

Morphology. The appearance of each colony was also considered and compared with those of controls. Any additional feature associated with the absence/presence of genes under study were recorded. For instance, a brown colour in the medium right underneath the colony was observed to be associated with growth of *xprF1* individuals on media containing different alternative nitrogen sources.

Milk Clearing. Milk proteins give an opaque appearance to milk plates. Since these proteins can be utilised as carbon, nitrogen and sulphur sources, biochemical break-down of protein chains, due to hydrolytic activity of secreted proteases, leaves a clear spot on the plate encompassing the colony. The size of milk clearing (halo) is proportional to the level of protease activity and therefore, by scoring the size of halo on milk plate using "+/-" system, protease activity is indirectly scored.

N.B. In each case a number of controls were employed to make the scoring relative to standard growth, morphology or milk clearing of wild-type (otherwise controls are specified). In genetic crosses, parental strains were used as references.

2.5. Extracellular Protease Assay

Assays followed the procedure of Hynes (1974). Each assay was carried out twice and every time results were compared with those of wild-type control. Different protease assays varied in their preparations and the procedure for the assay, itself, was the same for all.

a) preparation

Preparations for different protease assays are summarised in table 2.3. At the end, mycelia were filtered using Whatman No.1 filter papers. Volumes of broths and also dry weight of mycelia (dried at 65 °C overnight) were recorded.

Table 2.3. Preparation for assays under different conditions

assay	strains	preparation
nitrogen starvation (-N)*	- wild type, - *xprF1*, - *xprF1 xprF*$^+$ transformants	1. overnight culture† of conidial suspension‡ in 50 ml ANM; 10 mM ammonium tartrate; 1% vitamin solution 2. filtration and wash with cold sterile distilled water 3. transfer of mycelia to 25 ml of ANM; 1% vitamin solution 4. incubation for 4 hours
carbon starvation (-C)*	- wild type, - *xprF1*, - *xprF1 xprF*$^+$ transformants	similar to that of (-N), but mycelia were transferred to 25 ml Carbon-free broth; 10 mM ammonium - chloride; 1% vitamin solution
nitrogen and carbon starvation (-N-C)*	- wild type, - *xprF1*, - *xprF1 xprF*$^+$ transformants	similar to that of (-N), but mycelia were transferred to 25 ml Carbon-free broth; 1% vitamin solution
growth under sulphur limiting conditions	- wild type, - *xprF1*	20-hr culture of conidial suspension in 25 ml Sulphur-free ANM; 10 mM ammonium tartrate; 1% vitamin solution
growth with different nitrogen sources	- wild type, - *xprF1*, - *xprF1 areA*$^-$	a) ammonia 20-hr culture of conidial suspension in 25 ml ANM; 10 mM ammonium tartrate; 1% vitamin solution b) alanine 20-hr culture of conidial suspension in 20 ml ANM; 10 mM alanine; 1% vitamin solution
growth with different carbon sources	- wild type, - *xprF1*	a) glucose 20-hr culture of conidial suspension in 25 ml Carbon-free broth; 1% glucose; 10 mM ammonium chloride; 1% vitamin solution b) glycerol 20-hr culture of conidial suspension in 25 ml Carbon-free broth; 0.5% glycerol; 10 mM ammonium chloride; 1% vitamin solution

N.B. For each case, the assay was carried out also under non-limiting conditions (C+N+S) for control.

(*) also carried out for 16 hours of starvation

(†) all incubations were carried out at 37 °C in a rotary shaking incubator.

(‡) conidial suspension was prepared by scraping conidia from growth plates (-N minimal medium supplemented with ammonium and containing 2.2% agar)and dispersion in 10 ml of sterile Tween (4 drops of Tween 80 in 1 litre of distilled water). Suspension was added to broth up to 1:10 ratio.

b) Assay

The substrate solution used for detection of protease activity contained 0.5% azocasein in 100 mM sodium orthophosphate buffer; pH7.2. Azocasein, when hydrolysed by proteases, releases a coloured substance which is soluble in trichloro acetic acid (5% TCA). Therefore, the colour of reaction medium at the end is proportional to the level of protease activity. Concentration of the coloured substance is measured by reading the OD at 440 nm.

For each growth medium prepared in "a" (containing extracellular proteases) two 10 ml plastic tubes were marked as 0 hr (blank) and 4 hr. 1 ml of each medium was added to each tube preceded by addition of 1 ml azocasein solution. 5 ml of cold 5% trichloroacetic acid (TCA) was added to 0 hr tubes followed by incubation on ice for 4 hours. 4 hr tubes were incubated at 37 °C in a linear shaking water bath. After 4 hours, 5 ml of cold 5% TCA was added to stop the reaction. Non-dissolved substances were pelleted at 1200 g (room temperature; 10 minutes) in a swinging bucket centrifuge. The OD of each 4 hr supernatant was read against the 0 hr blank at 440 nm. Extracellular protease activity for each sample was represented in arbitrary units and was calculated as follows:

activity = absorbency(per ml) × [volume of culture (ml) ÷ mycelial weight (gm)]

2.6. Isolation of the *xprF* Gene

2.6.1. Transforming DNA

Cosmid clones from the wild-type *Aspergillus nidulans* genomic DNA (Brody *et al.*, 1991) were used as transforming DNA. Random libraries of the 2.6-3.1 × 10^7 bp genomic DNA had been constructed in two different cosmid

vectors Lorist2 and pWE15 (Evans *et al.*, 1987, cited in Brody *et al.*, 1991, Gibson *et al.*, 1987). Lorist2 is a 5.6 kb vector constructed by Gibson *et al.*, (1987) and contains bacteriophage λ origin of replication. It carries two terminator sequences from the *trp a* (tryptophan operon) and *rrnC* (ribosomal operon) terminators of *E. coli*. The authors reported that these terminators prevent vector genes from interference by promoters within the insert. According to Gibson *et al.* (1987), inserts with size of 35 to 50 kb can be inserted in Lorist2. pWE15 is an 8.8 kb vector carrying ColE1 origin of replication and can take sequences with size of 32-42 kb (Sambrook *et al.*, 1989).

Brody *et al.* (1991) obtained eight chromosomes of the organism separated by pulse-field-gel-electrophoresis. Each chromosome was radio-labelled and used to probe the genomic library of each type. This resulted in isolation of clones within the library which were specific to the labelled chromosome.

Studies had shown when preparing libraries using the above vectors, DNA sequences unstable in one vector were often stable in the other (Evans *et al.*, 1987, cited in Brody *et al.*, 1991). Brody *et al.*, (1991) mention this as the reason for carrying out the procedure for two different libraries.

a) Preparation of Glycerol Stocks of Cosmid Pools

There were 468 individual clones representing the chromosome VII. To save time, every 24 clones were pooled together and 20 cosmid pools were obtained among which 9 contained Lorist2 (kanamycin-resistant) clones and the rest were pWE15 (ampicillin-resistant) clones. *E. coli* cells carrying individual cosmid clones in each pool were patched on 2× YT plates (1% agar) containing 50 μ-g/ml of appropriate antibiotic (ampicillin or kanamycin) and incubated overnight at 37 °C. The surface of each plate was flooded with 1.5 ml of 40% glycerol in 2× YT broth and colonies were resuspended and removed to 1.5 ml microfuge tubes. Stocks were kept at -20 °C.

Preparation of cosmid DNAs from individual clones and pooling equal amounts of them prior to transformation would have been more advisable. That is because it would assure that all cosmid DNAs were represented equally. Considering the number of individual clones and the time limitations, however, this method did not seem practical.

b) Preparation of Cosmid DNA

The procedure followed the modified form of Birnboim and Doly (Sambrook *et al.*, 1989) for small-scale alkaline lysis. 50 µl of each pooled glycerol stock was transferred to 10 ml of 2× YT broth containing 50 µg/ml of appropriate antibiotic and cultured for 16 hours at 37 °C (shaking incubator). Bacterial cells were removed to a 10 ml plastic tube and pelleted at 1200 g in a swinging bucket centrifuge at the room temperature for 10 minutes. The pellet was resuspended in 300 µl of lysozyme/RNase solution (50 mM glucose; 25 mM Tris-HCl; pH8.0; 10 mM EDTA; 2 mg/ml lysozyme; 40 mg/ml RNase) and kept on ice for 30 minutes. Precipitation of lysed cell particles was completed by addition of 600 µl alkaline SDS (0.2 M sodium hydroxide; 1% sodium dodecyl sulphate) and incubation on ice for 5 minutes followed by addition of 450 µl ice-chilled 5 M potassium acetate; pH4.8 and incubation on ice for another 5 minutes. Cell components were pelleted at 12000 g at 4 °C for 10 minutes. The supernatant was re-centrifuged for 10 minutes and then, DNA was precipitated by addition of 2 vol. cold absolute ethanol. DNA was pelleted at 12000 g at room temperature for 15 minutes and then, vacuum dried and resuspended in 100 µl TE buffer; pH8.0 (1 M Tris; pH8.0; 0.5 M EDTA; pH8.0). Extraction was carried out twice with equal volumes of phenol/chloroform/isoamyl alcohol (50:49:1) and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated with 0.1 vol. 3 M sodium acetate; pH5.8 and 2.5 vol. cold absolute ethanol and left on ice for 20 minutes. This was followed by centrifugation at 12000 g at 4 °C for 15 minutes and then, washing with cold 70% (v/v) ethanol. Pellet was vacuum dried and resuspended in 20 µl TE buffer; pH8.0. Aliquot's of DNA were resolved on agarose gel [0.8% agarose (Promega); 1× TAE (Tris-Acetate-Gel Buffer: 0.04 M Tris; 2 mM ethylenediamine acetic acid di-sodium salt; pH7.6)] to estimate the DNA concentration and general situation of prepared DNA in respect to degradation or RNA contamination.

N.B. The procedure for preparation of individual cosmids was slightly different since no glycerol stock was prepared. Instead, single colonies carrying each cosmid clone were used directly to inoculate 10 ml 2× YT broths. The rest of procedure was exactly the same.

2.6.2. Preparation of pAN222

pAN222 (Hull *et al.*, 1989) was used in co-transformations with cosmid DNA. This recombinant plasmid consists of a 12.6 kb fragment from chromosome VII of *A. nidulans* inserted in pBR322 vector. Because it carries the wild-type *prnD* and *prnB* genes, pAN222 is able to transform *prnΔ 309* mutants to *prn*$^+$. pAN222 was prepared in large scale following the protocol of alkaline lysis method (Sambrook *et al.*, 1989).

A single colony of *E. coli* carrying the plasmid was cultured overnight (at 37 °C in shaking incubator) in 10 ml 2× YT broth containing 100 µg/ml ampicillin. 2.5 ml of this was used to inoculate 250 ml 2× YT broth with ampicillin for 16 hours. Cells were harvested and pelleted for 10 minutes at 1200 g (4 °C). The pellet was resuspended in 6 ml of Solution I (50 mM glucose; 25 mM Tris-HCl; pH8.0; 10 mM EDTA; pH8.0) and transferred to a 50 ml plastic tube containing 12 mg (2 mg/ml) lysozyme. After 10 minute incubation at room temperature, 12 ml of Solution II (0.2 M sodium hydroxide) was added and the tube was left on ice for 5 minutes. This was followed with addition of 9 ml of Solution III (5 M potassium acetate; pH4.8) and 10 minute incubation on ice. Tube contents were centrifuged at 12000 g for 15 minutes (4 °C) and the supernatant was removed to a new tube and re-centrifuged for 15 minutes. 0.6 vol. isopropanol was added to the supernatant to precipitate DNA (5 minutes at room temperature). DNA was pelleted at 12000 g for 10 minutes (4 °C) and washed with cold 70% ethanol. Then it was vacuum-dried and resuspended in 2 ml of TE buffer; pH8.0 and removed to a 10 ml plastic tube and incubated with 20 µl RNase (10 mg/ml) at 37 °C for 1 hour.

Extraction was carried out once with 4 ml of phenol/chloroform/isoamyl alcohol (50:49:1) and once with chloroform/isoamyl alcohol (24:1). The aqueous phase was collected in a siliconised 15 ml corex tube. After addition of 0.1 vol. 3 M sodium acetate; pH5.8 and 2.5 vol. cold absolute ethanol, DNA

was pelleted at 8000 g for 30 minutes (4 °C). DNA was vacuum-dried and resuspended in 2 ml of TE buffer; pH8.0. 2 µl of DNA solution was run on 0.8% agarose; 1× TAE to estimate DNA concentration, the plasmid size and also for checking the general situation of prepared DNA.

Purification of pAN222

Promega's Magic Maxi-Preps Kit (appendix 3) and instructions were used for plasmid purification. 2 ml of prepared plasmid solution was removed to a sterile container (*e.g.*, a MacCartney) followed by addition of 10 ml of purification resin. After mixing, contents were transferred to a Maxicolumn and vacuum was applied to pull the resin/DNA into the column. The column was washed twice with 13 ml and 12 ml of Wash Solution, respectively. Every time, vacuum was used to pull liquids down to column. The resin was rinsed with 5 ml of 80% ethanol and was left to dry for 10 minutes while the air was sucked through the column. 1.5 ml preheated (65-70 °C) TE buffer; pH8.0 was added to elute DNA (1 minute). Dissolved DNA was collected by centrifugation at 1300 g for 5 minutes.

The OD of 500 µl of 1:100 diluted DNA solution was read at 260 nm (UV) against the blank (500 µl distilled water). Given the reading value of 1.00 was equal to 50 µg/ml DNA, the concentration of sample was calculated in µg/µl. Also the purity of DNA preparation was checked by reading the OD at 280 nm: the $OD_{260\ nm}/OD_{280\ nm}$ ratio represented the level of purity as the ratio of 2.00 was known for 100% purity (Sambrook *et al.*, 1989).

2.6.3. Transformation

a) Preparation of Protoplasts

Conidia from the strain to be transformed (AM5) were suspended in 10 ml Tween solution and transferred to a conical flask containing 200 ml ANM broth containing 10 mM ammonium tartrate and 1% vitamin solution. Conidia were incubated at 23 °C for about 14 hours in a linear shaking water bath. The

growth had to be monitored at this stage as the young mycelia were used in transformations and cells had to be harvested before growing further.

Cells were filtered with Mira-Cloth (Calbiochem) and washed thoroughly with 300 ml of cold sterile 0.6 M magnesium sulphate. The excessive liquid was removed with the aid of pieces of sterile blotting papers and the weight of wet cells was measured. At the next stage, cells were removed into a 125 ml pre-chilled sterile flask and resuspended in 5 ml of osmotic medium (1.2 M magnesium sulphate; 10 mM sodium orthophosphate buffer; pH5.8) per gm of wet weight. From this stage on, cells had to be kept on ice all the time and all tubes and solutions used (except PEG, described below) had to be ice-chilled prior to use. 1 ml of Novozyme Solution [2 mg/ml Novozyme 234 (NovoBiolab) in osmotic medium] per gm of wet weight was added and cells were left on ice for 5 minutes. After addition of 250 µl bovine serum albumin (12 mg/ml BSA in osmotic medium) per gm of wet weight, the flask was incubated at 30 °C for 2 hours (in a rotary shaker).

Cells were cooled on ice and swirled vigorously to free protoplasts from mycelia debris. Cells were then, removed to a 30 ml corex tube and overlayed with equal amount of trapping buffer (0.6 M sorbitol; 10 mM Tris-HCl; pH7.0) and centrifuged in a swinging-bucket centrifuge at 4000 g at 4 °C for 20 minutes. Protoplasts were collected from interface with a hook-tip Pasteur pipette and transferred to a new tube. 20 ml of 1× STC (1.2 M sorbitol; 10 mM Tris-HCl; 10 mM calcium chloride; pH7.5) was added to the tube followed by centrifugation at 7000 g for 5 minutes (4 °C). Protoplasts were washed twice with 10 ml 1× STC and pelleted at the same speed for 5 minutes and finally resuspended in just enough amount of 1× STC (50 µl of protoplast suspension was used for each transformation treatment) and kept on ice.

b) Transformation

The procedure was adapted from Yelton *et al.* (1983) with some modifications. In each round of transformation, a number of treatments were set up in 10 ml plastic tubes. These included a -DNA and a pAN222 control and several "pAN222+cosmid" pool treatments. 5 µl of pAN222 stock (~4.4 µ g DNA) and 15 µl of cosmid stock (~4 µg DNA) were added to tubes. Equal amount of 2× STC (2.4 M sorbitol; 20 mM Tris-HCl; 20 mM calcium chloride; pH7.5) was added to keep the concentration equal to 1× STC. Treatments were

brought to volume (50 µl) with 1× STC. 50 µl of protoplast was added and transformation was immediately started by addition of 25 µl of PEG solution [60% (w/v) polyethylene glycol 4000; 10 mM Tris-HCl; 10 mM calcium chloride; pH 7.5]. After 30 minutes incubation on ice, 1 ml PEG was added to each tube and tubes were incubated at room temperature for 30 minutes. Finally, 5 ml cold 1× STC was added and cells were pelleted at 8000 g for 5 minutes (4 °C). At the next step, cells were resuspended in 200 µl 1× STC.

In order to figure out the number of protoplasts, non-protoplasts and contaminants (if any), a number of control plates were used:

H2: 100 µl of 1:100 diluted -DNA treatment (10 µl of -DNA treatment in 990 µl distilled water) was spread on a non-selective (supplemented) Protoplast Regeneration Medium plate (1% glucose; 1.2 M sucrose; pH 7.0; 1% agar, supplemented with 10 mM ammonium tartrate; 1% vitamin solution).

S2, S3, S4: 1:100 (S2), 1:1000 (S3) and 1:10000 (S4) dilutions of -DNA were prepared with 1X STC. 100 µl of each dilution was plated on the regeneration medium plates containing 10 mM ammonoium as a nitrogen source.

-DNA: the remaining of -DNA treatment was spread on two selective plates (*i.e.*, Protoplast Regeneration Medium containing L-proline as a nitrogen source).

pAN222: pAN222 treatment was also spread on two selective plates.

At the end, contents of each "**pAN222+cosmid**" treatment were spread on selective regeneration plates. All plates were incubated at 37 °C for 5 days.

2.6.4. Screening Transformants

Transformants from each "pAN222+cosmid" treatment were picked up and used to set up master plates on complete plates. Master plates were replicated on two types of milk plates where milk was serving as the sole nitrogen and carbon sources, respectively.

2.6.5. Confirmation of Genetic Markers in Transformants

Transformants suspected of carrying the wild type *xprF* allele had to be checked to make sure there was no contaminant. At the first step, such colonies were streaked out on complete plates to get genetically-pure colonies. Pure colonies were tested on acetate, nitrate, -paba and -ribo to check whether all markers are the same as the untransformed strain (AM5).

2.6.6. Confirmation of Integration Events

Preparation of Genomic DNA from Transformants

Genomic DNA was prepared from six *xprF*$^+$ transformants for DNA dot blot and Southern blot analyses. For each transformant, conidia were dispersed in 10 ml Tween Solution and the suspension was transferred to 2 conical flasks containing 50 ml ANM broth; 10 mM ammonium tartrate; 1% vitamin solution. After 16 hours of incubation at 37 °C with shaker, mycelia were filtered and dried by blotting papers. At next stage, mycelia were snap-frozen in liquid air and vacuum dried at -55 °C in a freeze-drier overnight. On the following day, dried mycelia were pulverised and removed to a 10 ml plastic tube. 3 ml of 50 mM EDTA pH8.0; 0.2% (w/v) SDS was added and the tube was incubated in 65 °C water bath for 15 minutes. This was followed with centrifugation at 1200 g (room temperature) for 20 minutes. The supernatant was removed to a new tube and after addition of 0.6 ml ice-chilled 5 M potassium acetate; pH<6, the tube was kept on ice for one hour. The tube was centrifuged at 1200 g (room temperature) for 20 minutes and supernatant was carefully removed to a new tube. After this, the genomic DNA was precipitated by addition of equal volume of isopropanol and gentle swirling the tube. Using a glass hook, genomic DNA was removed to a microcentrifuge tube containing 0.5 ml cold 70% ethanol. DNA was washed for several times with 70% ethanol. After this step, DNA was vacuum dried and resuspended in 400 µl TE Buffer; pH8.0. After addition of 10 µg/ml RNase, the tube was left on bench overnight to let DNA pellet dissolve. Extraction was carried out three times with phenol/chloroform/isoamyl alcohol (50:49:1) and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated by 0.1 volume 3 M

sodium acetate; pH5.8 and 2.5 volume cold absolute ethanol. Centrifugation at 12000 g (4 °C) for 5 minutes pelleted DNA which was then washed once with cold 70% ethanol. At the end, it was vacuum dried and resuspended in 100 µl TE Buffer; pH8.0 overnight. Aliquots of prepared genomic DNAs were tested on 0.8% agarose; 1× TAE gel to estimate the concentration prior to use.

Preparation of Probes

pBR322. This DNA had been used in construction of pAN222 (Hull *et al.*, 1989) and was purchased from Promega.

wild-type λ DNA. The *cos* sites of λ DNA were used in construction of pWE15 and Lorist2 vectors. This DNA was purchased from Promega.

L32F12. The L32F12 cosmid clone containing wild-type *xprF* allele was prepared following the procedure discussed before and digested with *Eco*RI.

***argB* gene.** As the control probe in the DNA dot blot the *argB* gene was isolated and labelled. pMOO6 carried the *argB*$^+$ gene (Upshall, 1986). pMOO6 was the pUC19 (Perron *et al.*, 1985) with a 3.3 kb *Xba*I insert ligated into its polylinker site. The *Xba*I fragment was a sub-clone of a 6.2 kb *Bam*HI fragment isolated by Berse *et al.* (1983) from *A. nidulans* genomic DNA. The 3.3 kb *Xba*I insert was excised by digesting recombinant DNA with *Xba*I and resolving fragments on 1% agarose; 1× TAE. The 3.3 kb fragment was then, purified with Progenius DNA Purification Kit (Progen; appendix 3).

Pieces of agarose gel containing the DNA fragment were cut and removed to 1.5 ml microcentrifuge tubes. Enough sodium iodide binding solution (6 M NaI) equal to at least 2.5 folds of gel volume was added to the tubes. Tubes were left in 55 °C water bath to let the gel dissolve. 5 µl of silica matrix was added to each tube and tubes were incubated at room temperature for 5 minutes while being shaken gently. Silica matrix was pelleted at 12000 g for 30 seconds; supernatant was removed and the silica was washed twice with ethanol wash solution [100 mM sodium chloride; 1 mM EDTA; 10 mM Tris-HCl; pH7.5 with 50% (v/v) ethanol] and once with 80% (v/v) ethanol. Ethanol was removed with vacuum drying and DNA was eluted into 10 µl TE buffer; pH8.0 at 55 °C for 5 minutes. After brief centrifugation, supernatant containing DNA was collected. The second elution was carried out. 2 µl of purified DNA was run on gel to check the concentration and fragment purity.

DNA Labelling

Boehringer Mannheim's Digoxigenin-dUTP (DIG) Kit (appendix 3) and instructions were employed for non-radioactive labelling of above-mentioned probes. For circular DNAs, prior to labelling DNAs were linearised. The digest was subjected to extraction with phenol as described before.

Each DNA to be labelled was denatured by heating in boiling water for 10 minutes and chilling immediately on ice. Enough amounts of each DNA was transferred to a microfuge tube together with 2 µl Hexanucleotide Mixture and 2 -µl dNTP Labelling Mixture. The volume was brought up to 19 µl with distilled water. 1 µl Klenow Enzyme was added and tubes were incubated at 37 °C overnight. The reaction was stopped by addition of 2 µl of 0.2 M EDTA solution pH8.0. Labelled DNA was precipitated with 2 µl 4 M LiCl and 60 µl cold absolute ethanol and left at -70 °C for 2 hours. DNA was pelleted at 12000 g for 15 minutes and washed with cold 70% ethanol, then, vacuum dried and resuspended in 50 µl TE Buffer; pH8.0.

2.6.6.1. DNA Dot Blot Analysis

After isolation of the first two transformants, in order to prove the presence of transforming DNAs, genomic DNAs from AM5-1 and AM5-2 were probed with pBR322 and λ DNA through DNA dot blot. Genomic DNA from *niiA4* was used as the negative control. Later, in order to study the number of integrated copies of the transforming cosmid clone, genomic DNAs from AM5-1, AM5-3, AM5-4, AM5-5 and AM5-6 transformed strains and also the wild-type control were blotted on two nitrocellulose membranes. One membrane was probed with the labelled *argB* gene (as control) while the second one was probed with labelled *Eco*RI fragments from L32F12 (cosmid clone carrying the *xprF*$^+$).

With Dot Blot Apparatus (Schleicher & Schuell SRC96D Minifold-I) genomic DNAs were blotted on two pieces of nitrocellulose filter (Schleicher & Schuell). A set of dilutions (1 µg, 500 ng, 250 ng and 125 ng of DNA) of each freshly denatured DNA were loaded on the corresponding wells. Each dilution was set up using equal volume of 60% 20× SSC (3 M NaCl; 0.3 M Na-citrate; pH7.0); 40% formaldehyde. Wells were filled with 20× SSC and vacuum was

applied to pull down the liquid through the membrane. To obtain even blots on the surface of filter, prior to transfer of DNAs to wells, 100 µl of 20× SSC was added to each wet well. After addition of DNA, vacuum was applied and then wells were filled again with 20× SSC. After taking the liquid out, apparatus was disassembled and excessive moisture was removed from filters with 3MM papers. Filters were baked overnight at 65 °C.

Prehybridisation was carried out for 8 hours at 37 °C using Hybridisation Solution (described in 2.6.6.2). The rest of steps for hybridisation and detection are similar to those of Southern blot analysis and are described in the following section.

2.6.6.2. Southern Blot Analysis

Genomic DNAs from AM5-1 and AM5-3 were used in this experiment. Genomic DNA from *niiA4* and *Eco*RI fragments from L32F12 were used as controls.

Resolving Genomic DNAs

2 µg of each prepared genomic DNA was digested overnight with *Eco*RI. After addition of 3 µl of gel loading dye, genomic DNAs were resolved on 0.8% agarose; 1× TAE gel for 16 hours.

Gel Pretreatment and Southern Transfer

All steps at this stage were performed at the room temperature. Gels were acid-hydrolysed in 0.25 M HCl for 10 minutes. Gels were then, rinsed with distilled water and denatured twice with 1.5 M NaCl; 0.5 M NaOH each time for 20 minutes. After rinsing with distilled water, neutralisation of gels was carried out twice with 1 M Tris; 1.5 M NaCl; pH8.0, each time for 30 minutes. Southern transfer was carried out for 18 hours. After disassembling the apparatus, nitrocellulose filters were soaked in 6× SSC for 5 minutes and then. dried at room temperature. Filters were baked at 65 °C for 4 hours.

Hybridisation and Detection

Instructions and chemicals followed Boehringer Mannheim's AMPPD (Lumigen) Kit. This substance can be used as a substrate for alkaline phosphatase activity. DIG-labelled probes conjugate with DIG-Alkaline Phosphatase which is able to catalyse the chemiluminescence reaction.

Filters from Southern blot and DNA dot blot were prehybridised for 4 hours at 37 °C in the hybridisation solution. Hybridisation with probes was carried out overnight at 37 °C.

Buffer 1	0.1 M maleic acid; 0.15 M NaCl; pH7.5 (autoclaved)
Blocking Solution	10% (w/v) Blocking Reagent in Buffer 1
Buffer 2	1% (w/v) Blocking Reagent in Buffer 1
Buffer 3	0.1 M Tris-HCl; 0.1 M NaCl; 0.05 M $MgCl_2$; pH9.5
Hybridisation Solution	50% (v/v) Formamide; 5× SSC; 2% (w/v) Blocking Reagent (in Buffer 2); 0.1% (w/v) N-Lauryl Sarcosine; 0.02% (w/v) Sodium Dodecyl Sulphate
Washing Buffer	0.3% (v/v) Tween 20 in Buffer 1

Stringency washes were performed: 2× 5 minutes at room temperature in 2× SSC; 0.1% (w/v) SDS and 2× 15 minutes at 65 °C in 0.1× SSC; 0.1% (w/v) SDS.

Filters were washed in washing buffer and were incubated for 30 minutes in buffer 2. Then filters were incubated under similar conditions with anti-DIG-AP-conjugate (75 mU/ml, diluted in buffer 2). Washing was performed for 2× 15 minuted in washing buffer. Filters were left in buffer 3 for 5 minutes to equilibrate the pH. At next stage, they were incubated in dark with enough amount of Lumigen substrate (10 mg/ml, diluted in buffer 3) for 5 minutes. The excessive moisture was removed and damp filters were sealed in hybridisation begs.

N.B. All steps which followed stringency washes were performed at room temperature and on a rocking platform.

Exposure to X-ray films was carried out overnight at room temperature.